ANTIBODY RESPONSE OF TURKEYS VACCINATED WITH FORMALIN INACTIVATED NEWCASTLE DISEASE VIRUS

By

Shamseldin Halfawi Rached

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HISTORICAL REVIEW

Newcastle Disease

What is now generally known as Newcastle disease was first reported by Kraneveld[1,2] in 1926 as a highly diffusible and fatal infection of poultry prevalent in the Dutch East Indies. Kraneveld demonstrated that the causative agent was present in the secretions from the respiratory tract, and that it was absent from the blood and internal organs. Although Berkefeld filtrates were innocuous, Kraneveld eliminated the possibility that the causative agent was of a bacterial nature. The same disease was also seen in 1926 at Newcastle-on-the-Tyne in England and it was reported by Doyle[3] in 1927. Doyle was successful in demonstrating that the causative agent was a filterable virus and that this virus was distributed in the various internal organs rather consistently. He could reproduce the disease by inoculation but not with equal facility in all cases as regards the type of the inoculum and mode of inoculation. He also found that the virus was completely neutralized by the homologous immune serum. Doyle proposed the name Newcastle disease and reported that there was no cross-immunity between this disease and fowl plague. In the immediately following years a similar disease appeared in India, in Ceylon, in Korea, in the Phillipines, and in various other places. In each instance a new name was applied so that there appeared in the literature a variety of names describing a disease that had many characteristics in common. Investigators soon suspected that perhaps these diseases might be identical. This suspicion was confirmed after an exchange of viruses among the

workers of the Dutch East Indies, India, England, and the Phillipines, who made cross-immunity and neutralization tests. Thereafter most of the various names under which the disease had been described were dropped from the literature.

It may be well to review the geographical[4] distribution of this disease. It was reported from the Dutch East Indies (1926) and England (1927), India and the Phillipines (1928), Korea and Japan (1929), Ceylon (1930), Australia (1931), Kenya (1937) the middle Congo (1939), Palestine and Italy (1940), Germany (1941) and its occurrence was recorded in other parts of Europe during 1942-1944. Newcastle disease was recognized for the first time in South Africa during 1944-1945. In the United States, the disease was described in the report of the California State Department of Agriculture of 1940[5] as a nervous disorder occurring after or towards the end of outbreaks of a disease which was thought to be chicken bronchitis Further observations on symptoms and lesions were published by Beach[6], who suggested that the symptoms might be due to invasion of the central nervous system by chick bronchitis virus, or to some dietary disturbance. Additional work by Stover[7], who described the disease under "respiratory nervous disorder of chickens," demonstrated that the disease was transmissible and was independent of any of the other known diseases of chickens. He reported that the causative agent was filterable. Later Stover[8] observed the disease in poults and succeeded in reproducing typical symptoms in chicks by the injection of unfiltered air sac and lung extract from affected poults.

Beach[9] in 1942, named the disease "avian pneumoencephalitis." He stated that the disease had been present in California as early as 1935 and perhaps earlier.

The unusually mild character and low mortality rate which characterized the malady upon its initial appearance in the United States allayed the suspicion that it might be Newcastle disease and delayed perception of a possible relationship to the typical, fulminating and highly fatal Newcastle disease, as previously seen in other parts of the world. The identity of several strains of so-called "avian pneumoencephalitis" virus with typical Newcastle disease virus, in this instance the Hertfordshire strain, was established on the basis of initial in vitro neutralization tests with Newcastle disease immune serum from England by Beach[10] in 1944. The virus found in this country appears to be identical immunologically with that encountered abroad, although the disease caused by the respective agents differs somewhat. In this country the disease is associated with a comparatively low mortality rate in adult birds and with a high mortality in chicks. In other countries the disease is characterised by a uniformly high mortality rate.

The disease was known to have reached the east coast by 1944, when the virus was isolated by Beaudette[11] in New Jersey. At the present time the disease has been diagnosed in 42 of the States and the District of Columbia.[12]

An outbreak in adult turkeys was described by Hoffman,[13] who succeeded in transmitting the disease to chickens and turkeys by inoculation of filtered or unfiltered material.

The disease in turkeys was described by Jungherr and Terrel in Connecticut[14] in 1945 and the virus was isolated from turkeys in New York state by Levine[15] in 1946 and from 10-day-old poults by Fenstermacher et al.[16] in Minnesota in the same year. The disease was reported from California as affecting poults[8] and adult turkeys[13] in much the same way as it affected young chickens. The disease in turkeys as reported from New York[15] caused more than 50 per cent mortality. Symptoms were mild and were not as evident as in young chicks. The air sacs and their membranes were cloudy and the virus isolated from turkeys was neutralized by known Newcastle disease immune serum.

The symptoms in turkeys as reported from Minnesota[16] were not as prominent as in chickens. Nearly mature turkeys seldom showed nervous symptoms. The commonest symptom was a snuffing or sneezing sound. Poults, 2 to 3 days old, showed evidence of respiratory trouble but they did not show nervous symptoms.

The mortality among poults was 60 per cent and losses did occur among mature birds. Serum collected from the survivors 4 months after infection had no inhibitory substances against Newcastle disease virus as demonstrated by the hemagglutination-inhibition test.

Immunization

Earlier reports of the use of physical or chemical agents in the process of attenuation or modification of the Newcastle disease

virus to develop a satisfactory method of active immunization of chickens against the disease showed negative, irregular and generally unsatisfactory results.[2]

Encouraging results were produced by the use of tissue-virus vaccines, inactivated by formalin.[2]

Topacio and Coronel[17] in 1939 failed to immunize test birds by the use of tissue or "crop"-virus vaccine treated with chloroform, formalin, lactic acid, or merthiolate.

Haddow and Idnani[18] in 1941 reported encouraging results with the use of a formalin treated virus adsorbed on aluminum hydroxide gel and "matured" for 8 to 10 days at 14 C to 16 C or for 1 to 4 days at 37 C before use.

Iyer and Dobson[19] in 1941 reported irregular and unsatisfactory results from the use of an infected embryo tissue emulsion treated with crystal violet as a vaccine against Newcastle disease. In a later report, Iyer[20] presented promising results obtained by the use of this same vaccine. At the same time, he reported that there was no protection following injection of a formalinized embryo tissue vaccine.

Hoffman[13] in 1941 attempted the use of a mixture of the virus and its homologous immune serum for immunization of chicks against the "respiratory nervous disorder." This neutralized virus gave no protection when the birds were infected 30 days after vaccination. The use of the lung and air sac materials from infected turkeys as a source of antigen treated with chloroform or formalin produced irregular results.

Beach[9] in 1942 reported encouraging results obtained in vaccination of chicks against "avian pneumoencephalitis" using a whole embryo vaccine which contained 0.1 or 0.2 per cent formalin. Two doses of the vaccine were slightly more effective than one dose in vaccination of chickens more than 7 days old while chicks vaccinated at an age below 7 days were very inadequately protected.

Beach[21] in 1944 reported on the use of formalinized embryo emulsion vaccine in the field. The vaccine, although not preventing the occurrence of infection, enabled the chickens to withstand a severe natural infection with a relatively low mortality and a slight decrease in egg production.

Brandly et al.[22] in 1946 found that vaccine prepared by the application of short ultraviolet rays (1600 to 1800 A) to inactivate the Newcastle disease virus had no advantages over the formalin inactivated preparation. Under practical conditions, pH 5 to pH 7 was found to be without an appreciable effect on the potency of the formalin inactivated vaccine stored at 6 C for a period of 471 days. The use of amnio allantoic fluids, of embryo tissue, or of the whole egg without albumen, as vaccines indicated that the tissues were equally as suitable as the fluids provided that a minimal titer of 10^7 embryo lethal doses per 0.05 cc. inoculum was required for any material used in the vaccine preparation. Inactivated vaccines produced a transient immunity. A substantial immunity was present up to 4 months in some cases. In other cases immunity was not detectable 3 weeks after vaccination.

The addition of adjuvants such as lanolin, mineral oils, and alumina gel caused an increase in the degree and duration of the immunity response. The presence of killed Mycobacterium butyricum as an adjuvant definitely augmented the immunity response. Unfortunately, this agent in combination with the oily adjuvants, produced marked tissue reactions as well as tuberculin sensitivity, thus eliminating it from use in vaccines designed for field use. The use of certain virus strains from the eastern United States which had been rendered apathogenic for chickens through propagation in serial passages in embryonated eggs produced an earlier and m substantial immunity to challenge infection and an earlier appearance of serum antibody titer than did the formalin inactivated material. Serum antibody titers used as a means of evaluation of the vaccinal response showed a fair degree of correlation with refractivity to challenge infection except during the period when active immunity was apparently waning. The authors suggested the initial use of an inactivated vaccine followed after 1 or 2 weeks by a living, modified, i.e. chicken-apathogenic, virus.

Brandly et al.[23] reported that the development of an active immunity in response to vaccination was inhibited by congenital passive immunity present in young chicks conferred by Newcastle disease immune dams. Protection afforded by vaccines prepared from the entire content, exclusive of albumen, of eggs from Newcastle disease immune hens appeared to be inferior to that afforded by vaccines prepared from normal hen eggs.

Acevedo and Mendoza[24] in 1947, reported encouraging results with the use of 2 vaccines prepared from a mixture of infected amniotic fluid, chorioallantoic membrane, and the embryo proper emulsified in an equal volume of glycerinated phosphate buffer and inactivated by the addition of 0.05 per cent crystal violet and 0.05 per cent formalin, or 0.7 per cent sodium borate and 0.1 per cent formalin. The addition of 0.5 per cent potassium alum to the vaccine treated with sodium borate and formalin enhanced the antibody forming properties of the vaccine. Vaccines prepared the same way from duck embryos gave variable results and the protection derived was of short duration.

Satisfactory results were reported in 1947 by Coronel[25] who used an "adsorbate" vaccine. This vaccine contained equal parts of ground infected embryonic tissue and fluids from the same harvest which were suspended in an equal quantity of alumina gel emulsified in phosphate buffer or in distilled water of pH 7 to pH 7.4. The preparation was treated with 0.1 per cent formalin.

Van Roekel et al.[26] in 1948 reported the use of a strain of Newcastle disease virus of low virulence applied by the cutaneous "stock" method in the web of the wing to immunize chickens without producing an active outbreak of the disease.

Iyer and Dobson[27] in 1940 reported that continued passage of the Hertfordshire strain of Newcastle disease virus through embryonated chicken eggs resulted in the recovery of a variant apathogenic for chickens. At the 33rd and subsequent passages up to the 56th,

the strain of virus ceased to produce fatal results in chicken but its immunizing properties had been retained. Inoculation of chickens with this virus protected them against a challenge with 10^8 chicken lethal doses. In another test, only 14 passages of the same strain were enough to modify the virus. Iyer[20] in 1943 confirmed these findings and stated that this modified virus produced a "silent" infection in inoculated chickens followed by refractivity to challenge with active virus.

Brandly et al.[22] found that 3 strains, one isolated in New Jersey, the second in New York, and the third from an infection of stock chickens of the Huntington laboratory at Harvard University, although retaining a high and persistent degree of pathogenicity for chickens embryos tended to acquire a low pathogenicity for chickens after one or a few egg passages.

Acevedo and Mendoza[24] reported that the virulence of Newcastle disease virus was not changed after 50 passages in chicken, duck, and turkey embryos. A hundred or more serial passages of the virus in chick embryos by Coronel[25] did not attenuate the virus sufficiently to make it a safe vaccine for adult birds.

Topacio and Coronel[17] reported that after several intracerebral passages in ducks, the virulence of the virus was not altered when tested on chickens.

Komarov and Goldsmit,[28] lead by Doyle's[3] report that he was unable to infect chickens with the mouth exudate of a sick duck, thought of the possibility of a modification of the virus through intracerbral passage in ducklings. Chickens inoculated intradermally with virus modified by 10 intracerebral passages in ducklings did not develop symptoms and were subsequently found to be immune.

A strain of the virus which was serially passed by Komarov and Goldsmit[29] through embryonated duck eggs lost its ability to produce a systemic reaction when injected either subcutaneously or intramuscularly into susceptible birds and produced a substantial immunity against the disease for at least 3 months.

Efforts by Brandly et al.[22] to attenuate two Newcastle disease virus strains for chickens through "zigzag" passages from young mice by the intracerebral route to embryonated eggs via the allantoic chamber route resulted in no evidence of any significant modification in chicken pathogenicity.

Attempts were made by Reagan et al.[30, 31] to adapt a California strain of the Newcastle disease virus to the Syrian hamster. The virus was successfully carried by the intracerebral route in this animal in more than 50 serial passages. This Newcastle-hamster-virus failed to infect rabbits but induced infection in white Swiss mice and guinea pigs when injected intracerebrally. The virus was not successfully passed beyond the first passage in guinea pigs and the first 4 successive passages in mice. A monkey, Macaca rhesus, was infected

intracerebrally with the hamster adapted virus but not with the egg adapted virus. The same authors reported later[32] that the hamster brain virus of the 49th passage when injected into the base of the wattle in young chickens revealed little pathogenicity and a satisfactory immunity upon pen exposure challenge at the end of 30 days. A suspension of the brain of a sheep, which had succumbed to the intracerebral injection of hamster brain virus, when injected into chickens showed no pathogenicity, but the immunity response was not fully satisfactory upon pen exposure challenge at the end of 30 days.

Experimental Procedures

The object of this work was to study the antibody response of turkeys through serological tests at certain time intervals after vaccination with formalin inactivated Newcastle disease virus followed by refractivity tests to challenge with virulent virus at the termination of the study.

Two different strains of the virus were used: Nc 46-967, chicken origin, and TD, turkey origin. Strain Nc 46-967 was isolated in the Department of Bacteriology and Public Health, Michigan State College, in May, 1946 from a naturally infected chicken. Strain TD (Jones strain) was isolated in the New York State Veterinary College, Cornell University, from naturally infected turkeys and was received through the courtesies of Drs. P. P. Levine and J. Fabricant.

Preparation of vaccines.

Strain Nc 46-967 was isolated originally from infected lung and tracheal material by inoculation of embryonated chicken eggs via the allantoic sac route. This virus was capable of killing all inoculated embryos within 72 hours in the first and all subsequent passages. Allantoic fluid was harvested from embryos dead on the second post inoculation day and used for inoculum for the next passage. Vaccine was prepared from the 15th passage of this strain (Nc 46-967$\frac{15}{--}$) which contained $10^{8.5}$ embryo lethal doses per 0.1 cc. inoculum via the allantoic cavity in 9- to 10-day-old embryos.

Strain TD was received as an allantoic fluid preparation of the virus. Other than that the virus was of turkey origin, no history or protocol was available. Vaccine was prepared from allantoic fluid harvested from the 3rd passage of the virus in this laboratory. This passage ($TD\frac{3}{-}$) contained $10^{9.3}$ embryo lethal doses per 0.1 cc. inoculum via the allantoic cavity route.

The two vaccines were prepared by the addition of formalin to a final concentration of 0.1 per cent of the allantoic fluid preparations of Nc 46-967$\frac{15}{-}$ and $TD\frac{3}{-}$. The formalinized allantoic fluids were stored at 5 C for 72 hours at which time the virus was found to be completely inactivated as indicated by its inability to produce mortality when injected into 10-day-old embryonated chicken eggs via the allantoic sac route.

Vaccination.

Seven turkey hens about 6 months old were used for the experimental work. On March 3, 1947, blood was collected from all turkeys for determination of prevaccination antibody levels against Newcastle disease virus by both serum neutralization and hemagglutination-inhibition tests with the results as shown in Table 1.

Table 1--Prevaccination Antibody Levels

Turkey No.	Serum Neutralization N I *	Hemagglutination-Inhibition I P **
33	50	5
34	16	5
36	10	5
40	10	5
42	10	5
48	2	5
85	8	5

* Neutralization index
** Inhibitory power

The turkeys were divided into 3 groups of 2 turkeys each, one bird for the Nc strain and the other bird for the TD strain. The 7th turkey served as a control. The turkeys in Group 1 received a single injection of the vaccine and the turkeys in Groups 2 and 3 received 2 injections of the vaccine with an interim of one and 2 weeks, respectively. On March 10, 1947, 1.0 cc. of the vaccine was injected intramuscularly into the pectoral muscles of each turkey. On March 17, turkeys 34 and 42 of Group 2 were revaccinated and two weeks after the original vaccination turkeys 36 and 48 of Group 3 were revaccinated. See Table 2. All birds were kept in one enclosure under the same conditions of management.

Table 2--Vaccination of Turkeys

Turkey No.	Vaccine	Dose	Inoculation Date	Route
33	Nc 46-967[15]	1.occ.	3-10-1947	im.*
34	"	"	3-10-1947	"
	"	"	3-17-1947	"
36	"	"	3-10-1947	"
	"	"	3-24-1947	"
40	TD[3]	"	3-10-1947	"
42	"	"	3-10-1947	"
	"	"	3-17-1947	"
48	"	"	3-10-1947	"
	"	"	3-24-1947	"
85 (Control)	---	---	---	---

* Intramuscularly into the pectoral muscles.

Blood was collected under aseptic conditions from each turkey at the following periods after the original vaccination: 1, 2, and 3 weeks; 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, 4, and 9 months. The samples were collected immediately prior to the injection of the second dose of vaccine in turkeys revaccinated one or two weeks after the initial vaccination. The serum was tested for bacterial sterility prior to storage at - 35 C for subsequent tests of its antibody content.

The various samples were stored for the following periods: prevaccination samples, 12 days; one week samples, 106 days; 2 weeks samples, 113 days; 3 week samples, 120 days; one month samples, 113 days; $1\frac{1}{2}$ months samples, 176 days; 2 months samples, 169 days; $2\frac{1}{2}$ months samples, 168 days; 3 months samples, 162 days; 4 months samples, 134 days; 9 months samples, 4 days and post challenge samples, 4 days.

In a few cases the serum was found to be contaminated with bacteria. Due to the small amount of serum and to obviate the possibility of loss of serum by filtration and decrease of antibody by adsorption resort was had to the use of penicillin. To each 1.0 cc. of serum, 10,000 Oxford units of penicillin were added in sufficient diluent to give a 10^{-1} dilution of the serum. In every case penicillin was effective in rendering the serum bacteria free.

Brandly et al.[33] reported that the addition of 1,000 Oxford units of penicillin per 0.1 cc. dose of Newcastle disease virus showed no appreciable alterations in titer or infectivity for eggs or for chickens. Moses[34] reported that in serum neutralization tests on Newcastle disease virus and in testing of materials for isolation of the virus, penicillin in concentrations as high as 25,000 Oxford units per 1.0 cc. had no particular effect on the virus, the antibody or the viability of the embryos.

Turkey No. 40 died on May 12, 1947, from infectious enterohepatitis. The control turkey No. 85 died on July 7, 1947, as a result of suppurative sinusitis and subsequent septicemia. Turkey No. 34 died on October 10, 1947 from infectious enterohepatitis. Due to the death of these turkeys only 5 serum samples were collected from No. 40, 8 samples from No. 85 and 9 samples from No. 34.

Turkeys Nos. 33, 36, 42, and 48 were challenged on January 7, 1948, 10 months after vaccination, with 10^6 embryo lethal doses in 1.0 cc. of inoculum which was injected intramuscularly into each bird. Turkeys 33, 36, and two control turkeys Nos. 29 and 58 were injected with the 19th passage of Nc 46-967 while turkeys 42 and 48 and a control turkey No. 28 were injected with the 5th passage of TD.

Serological tests.

The serum samples were tested for antibody content by:

(1) Their capacity to inhibit hemagglutination of turkey red blood cells by Nc 46-967. Sera from turkeys Nos. 42, 48, and 85 at $2\frac{1}{2}$, 3, 4 and 9 months after vaccination and the post challenge sera from turkeys 42 and 48 were tested for their capacity to inhibit hemagglutination by both Nc 46-967 and TD virus strains.

(2) Their capacity to neutralize Nc 46-967. Sera from turkeys Nos. 42, 48, and 85 at $2\frac{1}{2}$, 3, 4, and 9 months after vaccination and the post challenge sera from turkeys 42 and 48 were tested for their capacity to neutralize both Nc 46-967 and TD virus strains.

Hemagglutination-inhibition and serum neutralization tests of each serum were conducted under the same conditions with no more than one or two days between both tests.

Hemagglutination (H) and hemagglutination-inhibition (HI) tests.

Hirst[35] in 1941 reported that when infected allantoic fluid with influenza virus was mixed in a test tube with washed normal chicken red blood cells, a visible agglutination of the cells occurred after 5 to 20 minutes. The cells sedimented rapidly and formed a characteristic ragged granular pattern on the bottom of the tube. When red blood cells were added to normal allantoic fluid, the cells sedimented slowly without aggregation and formed a sharp round disc on the bottom of the tube. Hirst showed that this hemagglutination phenomenon was inhibited with influenza antiserum in high dilutions and that such inhibition was specific with regard to the strain of the virus used.

The same phenomenon was discovered independently by McClelland and Hare[36] who showed, in addition, that human and guinea pig red blood cells were also susceptible to hemagglutination and that the virus was adsorbed on the sedimented cells.

Hirst[37] demonstrated later that this phenomenon constituted a valuable basis for the quantitative estimation of influenza virus hemagglutinin and its corresponding antibody. The rise of influenza virus antibody in human serum after an infection with the virus was as readily demonstrated in vitro with the hemagglutination-inhibition test as with the mouse serum neutralization tests. Normal ferret, rabbit, mouse, guinea pig and human serum would also inhibit the agglutination of red blood cells but in much lower dilutions than would a specific immune serum. Hirst used 0.5 cc. volumes of various serum dilutions with 0.5 cc. of virus (4 times the desired final concentration to produce the end point agglutination) and 1.0 cc. of a 2.0 per cent red blood cell suspension. Readings were made after incubation of the test for one hour at room temperature at 22 C to 25 C. The end point was considered to be the serum dilution in the tube in which the supernatant fluid would compare to standard red blood cell suspensions containing 0.75 per cent to 0.5 per cent red blood cell suspension in saline, i.e., the serum dilution which would inhibit hemagglutination by the virus of about one-half of the final concentration of red blood cell suspension (1.0 per cent) used in the test. Hirst[37] had pointed out that virus preparations inactivated at 56 C for 15 minutes or by storage at 23 C for several days would retain their hemagglutination properties. Incubation of the serum-virus mixture was unnecessary as the antigen-antibody reaction seemed to be an immediate one.

In a later contribution, Hirst and Pickels[38] described the use of a photo-electric densitometer to measure the optical densities of the middle layer of the red blood cell suspension in the titration tubes. Galvanometer readings were converted into values expressing the percentage of red blood cells which remained in suspension after 75 minutes at room temperature.

Hirst[39] described the effect of 0.75 per cent, 1.5 percent, and 3.0 per cent suspensions of red blood cells at 23 C and the adsorption and elution of influenza hemagglutinins at temperatures of 4 C, 27 C, and 37 C. With high concentrations of cells at 23 C, the maximum adsorption of influenza hemagglutinins was more complete than with low concentrations of cells. Correspondingly, the subsequent elution of hemagglutinins was less complete in 6 hours when high concentrations of cells were used. Maximum adsorption of influenza hemagglutinins took place in 5 hours at 4 C, 25 minutes at 27 C and 3 to 5 minutes at 37 C. Elution was less than 1.0 per cent in 18 hours at 4 C, 25 per cent in 6 hours at 27 C and nearly 100 per cent in 6 hours at 37 C. The addition of formalin to a final concentration of 0.1 per cent did not affect the hemagglutinative capacity while the infectivity of the virus was destroyed.

Stuart-Harris[40] reported that the end point of hemagglutination was difficult to detect by incubation at room temperature and that better readings were produced by incubation for 2 hours at 4 C. He found that hemagglutination of cells by some normal sera

was more obvious at 4 C than at room temperature and for this reason he preferred to incubate the test at room temperature. He stated that the use of red blood cells from different chickens or even the cells of the same bird at different times of the year gave significant differences in hemagglutination-inhibition tests with influenza virus. The same passage of the virus in amniotic and allantoic fluids from different eggs gave variations in results with the same serum. This was later described by Hirst[41] as due to a different degree of aggregation among different virus preparations. In the same report, Hirst described variations in influenza virus titers due to aging of red blood cells at 4 C.

Miller and Stanley[42] found that the pH of the medium had only a minor effect on the hemagglutination test in a range of pH 6 to pH 8 and that errors with the use of unbuffered saline were unlikely. Miller and Stanley reported that the temperature exerted a direct influence on the titer but that the most reproducible results were obtained at about 24 C. A 50 per cent loss in the ability of red blood cells to be agglutinated by the virus occurred during 7 days storage at 4 C. The virus hemagglutinin was less stable when the virus was stored at -70 C than at 4 C. These authors designated the test as the CCA test (chicken cell agglutination).

Miller[43] reported that the hemagglutinin activity was more stable in concentrated than in dilute virus preparations, more stable at 4 C than at room temperature, and at pH 7 than in the acid range.

Other investigators[44-47] have employed a variation of the method of reading the test by utilizing the pattern of the sedimented cells for estimating the degree of hemagglutination or hemagglutination-inhibition.

Salk[45] used a 0.25 per cent suspension of chicken red blood cells and recorded the results as: +(complete agglutination), 0 (no agglutination), and ±(partial or slight agglutination as seen in the intermediate reactions). The end point was considered to be the highest dilution of the virus producing maximal hemagglutination and the titer was expressed as the reciprocal of the final dilution of virus after the addition of the red blood cell suspension. In the hemagglutination-inhibition test the serum titer was the highest dilution of serum causing complete hemagglutination-inhibition.

Burnet[47] tested the ability of Newcastle disease virus infected allantoic and amniotic fluids to agglutinate chicken red blood cells in a manner similar to that affected by the influenza virus. A modification of Hirst's test as suggested by Lush[46] was employed to permit reading of the test according to the pattern of the sedimented cells rather than according to the rate and degree of clearing of the red blood cell suspension.[35,36,38,39]

Lush[46] pointed out that there was definite hemagglutination with Newcastle disease virus which affected only a small proportion of the cells so that the density of the suspension was not greatly reduced and made the modification of Hirst's technic necessary.

The modification by Lush[46] as used by Burnet[47] depended on the different patterns of agglutinated or unagglutinated cells at 4 C to 6 C. The volume of the reacting materials was reduced to 0.25 cc. and a 2.0 per cent red blood cell suspension was used. Readings were made after 2 hours. Maximal agglutination was designated as + + + and the end point as + where there was partial agglutination of cells peripherally with a definite lentiform centre of sedimented cells. Burnet[47] demonstrated differential hemagglutinative activity of the Newcastle disease virus among red blood cells of various species of animals (guinea pig, monkey, horse, mouse, sheep, rabbit, ferret) and man.

Hemagglutination-inhibition by specific Newcastle disease immune serum was also demonstrated by Burnet[47] and Lush.[46] Lush, through the use of the hemagglutination-inhibition test, verified the absence of a serological relationship between fowl plague and Newcastle disease viruses.

Burnet et al.[48] pointed out that the hemagglutinative activity and the infectivity of Newcastle disease virus were not affected at 56 C for 30 minutes whereas at 60 C for 15 minutes a complete destruction of the infectivity and a reduction of the hemagglutinative titer by 50.0 per cent was observed. The hemagglutinative activity was completely destroyed at 65 C for 15 minutes. The addition of from 0.1 per cent to 0.25 per cent formalin destroyed the infectivity of the virus but the hemagglutinative titer was unaltered. The phenomenon was described

as a complex reversible reaction in which both the virus-antibody and virus-red cell unions were reversible and that a final concentration of 0.125 per cent of red blood cells was about the practical limit for the test to be easily read.

Brandly et al.[33] found that the application of Lush's[46] modification to demonstrate Newcastle disease virus in infected allantoic fluids yielded indifferent results. This problem was overcome by a reduction of the red cell suspension from 2.0 per cent to 1.0 per cent or less; incubation of the test at 25 C rather than at 6 C and reading the test at intervals of 15, 30, and 45 minutes instead of 2 hours. Turkey red blood cells were also agglutinated in typical manner. The hemagglutinative titer of Newcastle disease virus was retained well after 60 C for 30 minutes, ultra-violet light irradiation at 1600 A to 1800 A for 0.3 to 1.08 seconds and the addition of formalin to a final concentration of from 0.2 to 0.4 per cent. These treatments were sufficient to destroy the infectivity of the virus. Specific Newcastle disease immune serum inhibited hemagglutination by several strains of Newcastle disease virus. Some evidence of strain specifity was seen with this test.

Cunha et al.[49] reported on the use of the hemagglutination test in estimating the distribution of Newcastle disease virus in purified fractions of infected allantoic fluid preparations. They found that the results with Lush's[46] modification of the test were inconsistent and that the intensity of the reaction was frequently too low for use.

They obtained satisfactory results when the tests were conducted at 23 C to 27 C and pH 7.5 with a 1.0 per cent chicken red blood cell final concentration. The end point was the virus dilution in which the supernatant fluid matched a standard suspension of 0.63 per cent red blood cells. A factor present in normal allantoic fluid was found to have an inhibitory action on the hemagglutination test. The hemagglutinin of Newcastle disease virus was associated only with the viral agent and variations in the ratio of hemagglutinative activity and infectivity with the purified virus were greater than those seen with influenza viruses. These variations were due to the relatively low capacity[46,47,49] of the Newcastle disease virus to agglutinate red blood cells and also to the greater difficulty in controlling the conditions of the reaction.

Florman[50] in a later report pointed out that at room temperature there was less hemagglutination with virus dilutions than with high virus dilutions, i.e., prozone reaction. With chicken cell suspensions of 1.0 per cent or less and with a shorter reaction period as recommended by Brandly et al.[33] this disturbing effect persisted and the reactions were difficult to read and reproduce. Florman carried out the reactions as originally modified by Lush[46] and Burnet[47]. He reported that the red blood cells of an occasional individual chicken were markedly insensitive to agglutination by the virus at 4 C, and that the end point obtained with the virus titration at 4 C was usually 2 to 4 times lower than when the test was performed

at room temperature. His data, however, failed to show significant differences in the titers obtained at the two temperatures.

Hanson et al.[51] used 4 routes to inject embryonated eggs with Newcastle disease virus and found that the order and time of appearance of demonstrable hemagglutinins was distinctive for each route. Hemagglutinins developed most rapidly in eggs inoculated by the intravenous, yolk sac and the allantoic sac routes, respectively. Embryos inoculated on the chorioallantoic membrane failed to show detectable hemagglutinins in the allantoic fluid, and in chorioallantoic and amnionic membrane suspensions until 24 hours after inoculation. Hemagglutination titers showed a progressive increase from the first appearance of the hemagglutinins until the death of the embryo. The hemagglutination titers at the time of death confirmed the findings of Burnet[47] and of Brandly et al.[33] on the effect of the route of the virus inoculation on the development of the hemagglutinins in the infected embryo fluids. The presence of a normal hemagglutinin in both normal and Newcastle disease virus infected yolk sac membranes was revealed. This normal hemagglutinin was inhibited by the addition of either normal serum or Newcastle disease immune serum. The embryo LD_{50} titration was much more sensitive as an indicator of the presence of Newcastle disease virus than was the hemagglutination test. The LD_{50} embryo titer was produced by inoculation of one millionth of the amount of virus demonstrable by the hemagglutination test.

Brandly et al.[52] confirmed the variations in agglutinability of chicken red blood cells from different individuals although they had a negligible variation when they used turkey cells from eight different turkeys. Attenuation or modification of 2 Newcastle disease virus strains on serial transfers up to 60 passages resulted in no change of hemagglutinative activity. Variations within the range of pH 6 to pH 8 did not cause an alteration in the hemagglutination velocity, pattern or titer.[42] Isoagglutinins were demonstrated in only 1.0 per cent of 23 chickens of 3 different breeds. Heteroagglutinins for turkey red blood cells were present in 75.0 per cent of the chicken serum samples tested whereas heteroagglutinins for chicken red blood cells were present in only 5.0 per cent of the turkey serum samples tested. Comparisons were made of "alpha" or conventional procedure for the hemagglutination-inhibition test wherein serial dilutions of the virus are admixed with a fixed amount of serum to the "beta" procedure wherein an amount of virus solution containing 10 hemagglutinin units is admixed with an equal quantity of serial serum dilutions. Using the "beta" procedure as a standard the authors pointed out that there was a 93.0 per cent agreement of the "alpha" and the "beta" procedures. Investigations of the relationship between hemagglutination-inhibition and serum neutralization titers lead to the observations that during the period of ascending titers following infection or vaccination a good agreement of both titers might be obtained. During the descending phase, these titers

might show either correlation or divergence. The hemagglutination-inhibition bodies were observed to disappear first. Revaccination or reinfection caused an increase in titer if present, or if absent, the appearance of both serum neutralizing and hemagglutination-inhibition titers.

Beach[53] reported on the application of the hemagglutination-inhibition test in the diagnosis of "avian pneumoencephalitis." In the hemagglutination test, 0.5 cc. of 2-fold dilutions of the virus were admixed with an equal quantity of a 1.0 per cent red blood cell suspension. Reading was made by viewing the pattern of sedimented cells after 25 to 35 minutes at 21 C to 24 C. The end point was taken as the formation of a small central disc of unagglutinated cells surrounded by a granular area of agglutinated cells. The end point was designated as ++.

In the hemagglutination-inhibition test, 0.25 cc. of serial 2-fold dilutions of the serum was admixed with an equal quantity of virus dilution containing 2 to 4 hemagglutinin units. After 5 to 10 minutes incubation at 21 C to 24 C, 0.5 cc. of a 1.0 per cent chicken red blood cell suspension was added and readings were made at 20 to 35 minutes. The highest dilution of serum which inhibited hemagglutination, i. e., ++, was termed the hemagglutination-inhibition titer of the serum. Beach reported that the hemagglutination-inhibition titer was not always correlated with the severity of exposure to the virus or with the degree of resistance of the donor to the challenge.

He suggested that infection with active virus might be required for the production of hemagglutination-inhibition antibodies in the blood. A serum titer as low as 1:10 which corresponds to an inhibitory power of 20 to 40 using the "alpha" or convential method of the hemagglutination-inhibition test was found to be of diagnostic significance.

Burnet et al.[54,55] reported that type O human red blood cells[54] and chicken red blood cells treated and stabilized with Newcastle disease virus at 37 C for 2 hours, i. e. subjected to agglutination followed by elution, were specifically agglutinated by high dilutions of either Newcastle disease immune sera from laboratory animals or sera from humans recovered from infectious mononucleosis. They related the changed character of the cells to the adsorption to their surface of a cell sensitizing agent (CSA) in the Newcastle disease virus preparation. It was suggested that the "antigen" concerned was a modified cell constituent and not a direct product of the virus.

Anderson[56] found that Newcastle disease virus stabilized cells when mixed with an excess of normal cells had the capacity to cause specific agglutination of the mixture. He ascribed this phenomenon to an agglutinin on stable cells (ASC). Agglutination by stable cells was found to be due to a firm adsorption of Newcastle disease virus particles on the surface of the cells. The agglutinin on stable cells was neutralized by immune Newcastle disease sera. The serum titers corresponded closely with the direct hemagglutination-inhibition titers. It appeared that in Newcastle disease immune serum, the same antibody molecule

might function either as an anti-hemagglutinin or as an agglutinin for stabilized cells. Infectious mononucleosis immune sera which caused agglutination of human cells stabilized with Newcastle disease virus had no action as an anti-hemagglutinin.

Anderson found that all of the manifestations of Newcastle disease virus action on red cells were essentially due to the virus particles as such and that the CSA, ASC and the single particles of virus hemagglutinin are identical. The Newcastle disease virus in infected embryo fluids was not found to be homogenous and large aggregates of the virus could be segregated. After hemagglutination, elution occurred by the partial disruption of the aggregates and one virus particle remained attached to the red blood cell. The presence of this particle on the red cell rendered the cell susceptible to agglutination (CSA) by Newcastle disease immune serum and it functioned as the retained hemagglutinin (ASC).

A higher hemagglutination titer of Newcastle disease virus was observed at 37 C than at 4 C. The higher titer was considered to be due to the disruption of virus aggregates in contact with red blood cells with the ultimate liberation of single virus particles.

Procedure

Preliminary tests using chicken red blood cells with turkey sera confirmed [52,57] the presence of an heteroagglutinin in normal turkey sera for chicken red blood cells.

Materials

(1) Red blood cells: Turkey blood was collected in chemically clean sterile vials containing 1.0 cc. of 2.0 per cent sodium citrate in saline solution[57] for each 9.0 cc. of blood. The cells were washed 3 times in 10 or more parts of 0.85 per cent saline solution, centrifuging twice at 1600 r.p.m. for 8 minutes and then once at 1000 r.p.m. for 10 minutes. After the third washing, the saline was removed and the packed cells were stored in the refrigerator for as long as 6 days[41,57] before being used. A 0.5 per cent suspension of red blood cells in saline was made just prior to use with the test. Throughout the entire work the red blood cells were obtained from 2 disease-free turkeys. This was done to limit variations[40,41,42] resulting from the use of cells from different donors.

(2) Virus: Blood-free allantoic fluid from embryonated chicken eggs killed by Newcastle disease virus on the 2nd day following inoculation via the allantoic cavity. The virus was inactivated by the addition of formalin to a final concentration of 0.1 per cent.[33,48,57] and was stored at 4 C to 6 C.[42,57]

(3) Saline solution: 0.85 per cent sodium chloride in distilled water.

(4) Serum: Serum diluted 1 in 5 with saline. Certain sera were prepared in higher dilutions as the occasion demanded.

Method

Chemically clean round bottom glass tubes (75 mm. by 12 mm. inside diameter) were used.[58] Progressive 2-fold dilutions of the formalized virus from 1 in 5 through 1 in 5120 were prepared in 0.85 per cent saline. To 0.25 cc. of each virus dilution was added 0.25 cc. of the serum to be tested except in the virus titration tubes where 0.25 cc. of saline solution was added. The tubes were shaken well and 0.25 cc. of the 0.5 per cent red cell suspension was added and the tubes were shaken again. The control tubes contained either saline and cells or serum and cells for the respective tests. The tests were incubated at a room temperature of from 22 C to 27 C. Readings of the virus titration tubes were made at 15, 30, and 45 minute intervals, but with the tubes containing serum, readings were made at 15, 25, and 35 minute intervals. The results were read[45] by viewing the tubes from the bottom of the rack through a mirror fixed at a proper angle.

Distinct patterns of red blood cells were formed depending upon the presence, absence, or inhibition of the hemagglutination of the cells by the virus. Where hemagglutination was maximal, a uniform film of agglutinated red blood cells covered the entire bottom of the tube. Where there was no agglutination, the cells sedimented into a disc shape at the lowest point of the curvature of the bottom of the tube. Reactions intermediate between complete

agglutination, inhibition, or absence of agglutination, were seen as irregular clumps of cells associated with a ring of finely aggregated or unagglutinated cells. Results were recorded as + (complete agglutination, - (no agglutination or complete inhibition of the agglutination by the serum) and ± (partial or slight agglutination or partial agglutination-inhibition). In the control tubes, the cells sedimented to the bottom of the tube and formed a central, sharply demarcated disc.

The virus titer was the highest dilution of the virus in which complete hemagglutination was present. This dilution was considered to contain 1 hemagglutinin unit (HA) which was capable of producing complete hemagglutination of the 0.165 per cent final red blood cell suspension within 45 minutes at a room temperature of from 22 C to 27 C.

The serum titer was the lowest dilution of the virus in which complete hemagglutination-inhibition was present. This dilution was considered to contain a concentration of hemagglutination-inhibition units equal to the number of hemagglutinin units present. The inhibitory power (IP) of the serum was considered to be the number of the hemagglutination-inhibition units present in 0.25 cc. of the undiluted serum. An inhibitory power of 80 or more was considered to be diagnostic of either previous vaccination against or infection with Newcastle disease virus.[12,52,57]

Table 3 is a representative protocol of hemagglutination and hemagglutination-inhibition tests utilizing as samples the titration of sera collected 1[illegible] months after vaccination of the turkeys.

Table 3

Hemagglutination and Hemagglutination-Inhibition Tests.

A. Procedure

Material	Virus dilution											Control
	u.d.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	
H test:													
Virus dil. (cc.)	----------					0.25 cc. per tube						----------	0
0.85% Na Cl (cc.)	----------					0.25 cc. per tube						----------	0.5
0.5% suspension r.b.c. (cc.)	----------					0.25 cc. per tube						----------	0.25
HI test:													
Virus dil. (cc.)	----------					0.25 cc. per tube						----------	0
Serum dil. (cc.)	----------					0.25 cc. per tube						----------	0.5
0.5% suspension r.b.c. (cc.)	----------					0.25 cc. per tube						----------	0.25

B. Results

Material	Virus dilution												Control
	u.d.	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	
H test:													
Nc 46-967[15]	+	+	+	+	+	+	+	+	±	-	-	-	-
HI tests:													
33 (dil 1:20)	+	+	+	±	-	-	-	-	-	-	-	-	-
34 (" ")	+	+	+	-	-	-	-	-	-	-	-	-	-
36 (" ")	+	+	+	+	-	-	-	-	-	-	-	-	-
40 (dil 1:10)	+	+	+	+	+	+	+	-	-	-	-	-	-
42 (" ")	+	+	+	+	+	+	-	-	-	-	-	-	-
48 (" ")	+	+	+	+	±	±	-	-	-	-	-	-	-
85 (dil 1:5)	+	+	+	+	+	+	±	-	-	-	-	-	-

Interpretation:
H titer of virus 320
HI titer of sera

Serum	Titer
33	320
34	320
36	160
40	10
42	20
48	80
85	10

Serum neutralization tests

Doyle[3] reported that Newcastle disease virus was completely neutralized by specific immune serum obtained from chickens that had survived inoculation of Newcastle disease virus attenuated by storage and had subsequently resisted contact infection as well as 2 infections with the active virus. Immune serum and virus infected mouth exudate were mixed in equal parts, kept at 14 C for 4 days in the dark, and then injected intravenously in 1.0 cc. doses into suceptible chickens which remained healthy.

Burnet and Ferry[59] were the first to show that Newcastle disease virus multiplied rapidly in the embryonated chicken egg. After inoculation on the chorioallantoic membrane, the virus caused death of 10-day-old embryos within 30 to 48 hours.

Keog[60] immunized chickens with an Australian strain of Newcastle disease virus. He found that when undiluted immune serum was admixed with the virus in vitro and inoculated upon the chorio-allantoic membrane, the serum protected against 10^3 embryo lethal doses of the virus. He reported that no time effect could be demonstrated in neutralization of the virus by specific immune serum. On dilution, the virus was reisolated from the virus-serum mixtures, indicating that the neutralization reaction was completely reversible.

Beach[9] reported that 100 minimum infecting doses of "avian pneumoencephalitis" virus in lung tissue dessicated in vacuo and suspended in 0.05 cc. of saline or broth when mixed with an equal quantity

of homologous immune serum and held at 6 C for 4 hours failed to infect chicks inoculated intramuscularly with 0.1 cc. of the mixture.

Burnet[47] injected Newcastle disease virus into the allantoic cavity of embryonated chicken eggs which resulted in free multiplication of the virus and specific death of the embryo. He pointed out that there was no serological relationship between Newcastle disease virus and the different types of influenza virus.

Minard and Jungherr[61] inoculated 8-to-10-day-old embryonated chicken eggs via the allantoic cavity route with 0.1 cc. of a mixture of equal volumes of decimal dilutions of "avian pneumoencephalitis" virus and undiluted suspected chicken serum which had been incubated at 37 C for one hour. Embryos were re-incubated for 4 days. The dilution at which about one-half of the inoculated embryos survived was taken as the critical titer. The neutralizing doses were expressed as the reciprocal of the difference between the titer of the virus and the titer of the serum-virus mixture. They pointed out that in two instances parallel serum titrations by intramuscular injection in chicks and allantoic cavity injection in embryos gave similar results: one negative serum and the other had 10^4 neutralizing doses per 0.1 cc.

Beaudette and Black[11] utilized the technic of Minard and Jungherr[61] for serum neutralization tests with the modification of the test by incubating the embryos until hatching. They found that serum from recovered birds neutralized from 10^2 to 10^6 embryo

lethal doses of the virus.

In 1945 the Committee on Transmissible Diseases of Poultry of the U.S. Livestock Sanitary Association[62] considered the serum neutralization test to be diagnostic if the serum tested neutralized at least 10^3 embryo lethal doses of Newcastle disease virus.

Brandly et al.[33,63] reported that in the determination of the neutralization capacity of serum against Newcastle disease virus the serum titer is reproducible within 10-fold limits.[12] Their standard procedure was to add undiluted serum to an equal quantity of various decimal dilutions of the virus up to the estimated titer of the virus. Control titrations of the virus were always made in parallel with the serum titrations. The mixtures were incubated from 30 to 90 minutes at room temperature prior to injection of series of at least 4 eggs per dilution. The eggs were reincubated for 4 days. They found that there were no significant differences in titers of the serum-virus mixtures within the limits of 24 hours incubation from 22 C to 38 C before inoculation.[60] Virus titers in chicken and in eggs were virtually identical.[61] The highest values in serum neutralization tests were obtained when chicks were used for titration. The next highest values were obtained when the allantoic chamber route was used in embryonated eggs.

A neutralizing capacity of at least 10^3 embryo lethal doses,[12] preferably 10^4 embryo lethal doses[33,63] when the allantoic cavity

route was used[58] was considered as a diagnostic titer indicating prior Newcastle disease virus infection or vaccination against the disease.

Procedure

Embryonated eggs 9-or 10-days old at 37.3 C in an electric, forced-draft incubator were used for virus titrations and serum neutralization tests. Five eggs were used per dilution. The site for injection into the allantoic sac was determined by trans-illumination of the egg and selection of an area of the chorioallantoic membrane free of large blood vessels about 3 mm. below the base of the air cell. A small hole was drilled through the shell, without piercing the shell membrane, by means of a small metal drill attached to the chuck of an electric motor. Another hole was made at the upper extremity of the shell over the air cell to serve as an air vent and to allow equalization of the pressure produced by the inoculum within the egg. Tincture of metaphen was painted over the holes in the shell and allowed to dry before the eggs were inoculated.

Decimal dilutions of Newcastle disease virus infected allantoic fluid were prepared in sterile nutrient broth which contained no salt. Serum-virus mixtures were prepared by mixing equal quantities of the decimal dilutions of the virus with undiluted serum which were then incubated at room temperature from 22 C to 27 C for 30 to 60 minutes. Quantitative determinations of virus infectivity were made by mixing equal parts of each decimal dilution of the virus

with an equal quantity of the nutrient broth diluent.

Inoculations of 0.1 cc. per egg were made with 1.0 cc. tuberculin syringes fitted with a 27 gauge, $\frac{1}{2}$ inch needle. The virus titration series were inoculated last in order to take into consideration any possible destruction of the virus during the incubation period. Bacterial sterility tests were made of each preparation. After the eggs were inoculated, the holes in the shells were sealed with melted paraffin and the eggs were reincubated for 6 days.[64,65] The eggs were candled daily and embryos found dead during the first 24 hours were not included in the final results as the mortality was considered to be due to nonspecific causes.

The results of the serum and virus titrations were evaluated according to the 50 per cent end point formula of Reed and Muench.[6]

The titer of the virus was the highest dilution of the virus in which the 50 per cent end point mortality rate occurred and there was considered to be one embryo lethal dose in the inoculum. The number of the embryo lethal doses in 0.05 cc. of undiluted inoculum was the reciprocal of the logarithm of the titer of the virus.

The titer of the serum was the lowest dilution of the virus in which the 50 per cent end point survival rate occurred. The difference between the virus titer and the serum titer was the neutralizing index (NI), the antilog of which was the number of neutralizing doses in 0.05 cc. of the undiluted serum.

Evaluation of the antibody response in turkeys

A) Neutralization and hemagglutination-inhibition of the Nc strain of virus.

Table 4--Turkey 33

Serum	Virus Titer*	Virus dilutions 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
Pre-Vacc.	9.2							5	4	1		7.5	50	5
1 week+	8.5				5	5	5	5	2			6.8	500	320
2 weeks	8.3		5	5	5	0	0					3.5	63,100	320
3 weeks	8.2	5	5	5	3	2						3.5	50,120	640
1 month	7.6		5	5	4	4	0					4.3	1,995	160
1½ month	8.3		5	5	5	4	3	0				5.0	1,995	320
2 months	8.5					5	4	0	0	0		5.4	1,259	80
2½ months+	8.3					5	5	5	5	0		7.5	63	80
3 months	8.3				5	3	2	0				4.5	6,310	80
4 months+	8.3						5	5	5	2		7.8	32	80
9 months+	8.8		5	2	1	1	0					2.2	39,810,000	1,600
Post Challenge	8.8	4	3	1	1	0						1.3	31,620,000	1,600

(This footnote applies also to tables 5 to 15)
* Negative logarithm of the titer of the virus used in each titration.
** Embryos dead out of 5 inoculated per dilution.
+ Serum diluted 1 in 10.
++ Neutralizing index
# Inhibitory power

Table 5--Turkey 34

Serum	Virus Titer*	Virus dilutions										Serum		
		10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer*	NI++	IP#
Pre-Vacc.	9.2							5	5	3		8.2	10	5
1 week+	8.5				5	5	5	5	4			7.4	126	160
2 weeks+	8.3		5	5	5	3	1					4.3	100,000	320
3 weeks	7.6		5	4	2	1	1					3.0	39,810	800
1 month	7.6		5	5	3	2	1					3.4	15,850	200
$1\frac{1}{2}$ months	8.5				5	5	2	0	0			4.8	5,012	320
2 months	8.3				5	1	0	0	0			3.6	50,120	320
$2\frac{1}{2}$ months+	8.3				5	5	5	1	0			5.6	5,012	160
3 months	8.2			5	5	3	0	0				4.2	10,000	160
4 months+	8.2						5	4	2	0		6.7	316	160

Table 6--Turkey 36

Serum	Virus Titer*	Virus dilutions										Serum		
		10^{0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer*	NI++	IP#
Pre-Vacc.	9.2							5	5	5		8.5	10	5
1 week	8.5				5	3	2	2	1			5.0	3,162	320
2 weeks+	8.5					5	5	2	0	0		5.8	5,012	640
3 weeks+	8.2			5	5	5	1	1				4.6	31,620	800
1 month	7.6	5	5	5	4	1						3.1	12,590	160
$1\frac{1}{2}$ months	8.5				5	5	4	2	0			5.8	501	80
2 months	8.5					3	1	0	0			4.3	15,850	80
$2\frac{1}{2}$ months+	8.3					5	5	5	5	1		7.6	50	80
3 months	8.3			5	5	5	2	2				5.2	1,259	80
4 months+	8.3						5	5	4	2		7.6	40	80
9 months+	8.8		5	3	2	1	0					2.7	12,590,000	1,600
Post-Challenge	8.8	5	5	4	1	0						2.5	1,995,000	1,600

Table 7--Turkey 40

Serum	Virus Titer*	Virus dilutions										Serum		
		10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer*	NI++	IP#
Pre-Vacc.	9.2							5	5	5		8.5	10	5
1 week	8.5				5	5	5	5	5			7.5	250	25
2 weeks+	8.3					5	5	5	5	1		7.6	50	25
3 weeks+	8.3					5	5	5	3	1		7.3	100	25
1 month	8.2						5	5	5	2	0	7.8	3	10
$1\frac{1}{2}$ months	8.5						5	5	5	3	1	8.3	2	10

1 week serum sample diluted 1 in 25.

Table 8--Turkey 42

Serum	Virus Titer*	Virus dilutions 10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
Pre-vacc.	9.2							5	5	5		8.5	50	5
1 week	8.5						5	4	3	0		7.0	32	40
2 weeks	8.3					5	5	4	1	1		6.5	63	40
3 weeks+	8.3					5	5	4	4	2		7.5	59	20
1 month	8.5							5	4	1	0	7.5	10	40
1½ months	8.5						5	5	2	0	0	6.8	50	20
2 months	8.5							4	1	0	0	6.5	100	20
2½ months+	8.5							5	5	1	1	7.7	63	20
3 months+	8.2							5	5	2	2	8.8	0	20
4 months+	8.3						5	5	5	2	0	7.8	32	20
9 months+	8.8		5	2	2	1	1	1				2.8	10,000,000	800
Post-Challenge	8.6	5	5	5	4	1						3.5	125,900	6,400

Table 9--Turkey 48

Serum	Virus Titer*	Virus dilutions 10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
Pre-vacc.	8.7							5	5	4		8.4	2	5
1 week	8.5						5	3	1	0		6.3	159	160
2 weeks+	8.2					5	5	5	5	0		7.5	50	80
3 weeks	7.6					5	2	0	0	0		4.8	631	320
1 month	8.5					5	4	2	0	0		5.7	631	80
$1\frac{1}{2}$ months	8.5					5	3	2	1	0		5.6	794	80
2 months	8.3					5	5	3	1			6.3	100	20
$2\frac{1}{2}$ months+	8.5							5	5	4	0	8.4	14	20
3 months	8.2							5	2	1	1	7.2	10	20
4 months+	8.3						5	5	5	1	0	7.6	50	40
9 months	8.8		5	3	3	3	2	1				4.0	2,524,000	800
Post Challenge	8.6	5	5	1	0	0						1.6	10,000,000	12,800

9 months serum sample diluted 1 in 40.

Table 10--Turkey 85 (control)

Serum	Virus Titer*	Virus dilutions										Serum		
		10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer*	NI++	IP#
Pre-vacc.	8.7								5	2	0	7.8	8	5
1 week	8.5								5	3	0	8.2	2	5
2 weeks	8.5								5	1	0	7.6	8	5
3 weeks	8.3								5	2	0	7.8	3	5
1 month	8.2								5	2	0	7.8	3	10
$1\frac{1}{2}$ months	8.3								5	2	0	7.8	3	10
2 months	8.5								5	3	1	8.3	2	10
$2\frac{1}{2}$ months	8.3								5	3	1	8.3	1	10
3 months	8.2								5	2	0	7.8	3	10
4 months+	9.0								5	4	2	8.6	25	5

B) Neutralization and hemagglutination-inhibition of the TD strain of virus.

Table 11--Turkey 42

Serum	Virus Titer*	Virus dilutions										Serum		
		10^{0} **	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer*	NI++	IP#
$2\frac{1}{2}$ months+	8.7								5	5	2	8.8	10	20
3 months+	8.5								5	1	1	7.7	63	20
4 months+	8.7								5	5	2	8.8	10	20
9 months+	8.7		5	4	0	0	0					2.4	19,950,000	12,800
Post Challenge	8.8	5	0	0	0	0						0.5	199,500,000	12,800

Table 12--Turkey 48

Serum	Virus Titer*	Virus dilutions 10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
$2\frac{1}{2}$ months+	8.1							5	5	2	0	7.8	20	20
3 months	8.7							5	4	1	0	7.5	16	20
4 months+	8.7							5	5	5	2	8.8	10	20
9 months+	8.7	5	3	1	0	0	0					1.3	251,200,000	12,800
Post Challenge	8.8	0	0	0	0	0						0	631,000,000	12,800

Table 13--Turkey 85 (control)

Serum	Virus Titer*	Virus dilutions 10^{0}_{**}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
$2\frac{1}{2}$ months	8.1								5	5	1	8.6	0	10
3 months	8.5								5	4	0	8.4	1	10
4 months+	8.7								5	5	2	8.8	0	10

Antibody response in challenge-infection controlls=

A) Neutralization and hemagglutination-inhibition of the Nc strain of virus.

Table 14

Turkey	Serum	Virus Titer*	Virus dilutions 10^{0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
28	Pre-Chal.+	8.6					0	0	0	0	0		3.5	1,259,000	640
	Post-Chal.	8.6	5	0	0	0	0						8.1	125,900,000	51,200
29	Pre-Chal.+	8.6					2	1	1	0	0		4.2	251,200	640
	Post-Chal.	8.8	5	1	1	1	0						0.9	79,430,000	51,200
58	Pre-Chal.+	8.8	5	5	5	3	1						3.3	3,162,000	1,600
	Post-Chal.	8.8	5	4	0	0	0						1.4	25,120,000	12,800

B) Neutralization and hemagglutination-inhibition of the TD strain of virus.

Table 15

Turkey	Serum	Virus Titer*	Virus dilutions 10^{0}**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Serum Titer*	NI++	IP#
28	Pre-Chal.+	8.8		5	5	5	1	0					3.6	1,585,000	640
	Post-Chal.	8.8	3	2	0	0	0						0.5	199,500,000	1,280

= Turkey 28 was challenged with 10^{6} embryo lethal doses of the NC 46-967 $\frac{19}{-}$ intramuscularly.
Turkeys 29 and 58 were challenged with 10^{6} embryo lethal doses of the TD $\frac{5}{-}$ intramuscularly.
Post-Challenge sera were collected 12 days after inoculation.

Discussion

Turkeys 33, 36, 42, and 48 survived to the end of the experiment. Tables 4, 6, 8, 9, 11, and 12 show the results of titrations of the sera from these turkeys during a period of 10 months after vaccination. Blood collected at the 9th month showed a high neutralizing capacity and inhibitory power. This lead to the testing of several unvaccinated turkeys (about 18 months to 2 years old) which had been housed in the same poultry building, but in different pens, during the experimental period. Serum from these turkeys also had a high neutralizing capacity and inhibitory power.

Since there was no history of Newcastle disease in the poultry building and the experimental control turkey No. 85 showed no serological evidence of exposure to the virus during the first 4 months of the experiment, the conclusion was drawn that all of the turkeys in the building had been exposed to a subclinical infection sometime between the 4th and 8th month after vaccination.

As further evidence of the presence of Newcastle disease in the poultry building during this period, serum from a turkey which had been removed from the building at about the 4th month and housed in a different building failed to reveal the presence of neutralizing and inhibitory agents at any time during the course of the experiment. This turkey was used as a donor of red blood cells for the hemagglutination tests and as a source of negative serum.

The control turkey No. 85 died from suppurative sinusitis and septicemia 4 months and 2 weeks after the beginning of the experiment. Isolation of the virus from turkey 85 was not attempted. Tables 10 and 13 show the results of titration of the serum of this turkey with both Nc and TD strains of the virus for the 4 month period.

Turkeys 34 and 40 died from infectious enterohepatitis 7 and 2 months, respectively, after the beginning of the experiment. Tables 5 and 7 show the results of titrations of their sera with the Nc strain of the virus during the period of their participation in the experiment.

Ten months after vaccination, turkeys 33 and 36, together with turkeys 29 and 58 which were chosen as controls, were challenged with 10^6 embryo lethal doses of the 19th embryo passage of the Nc strain. Turkeys 42, 48, together with turkey 28, which was chosen as a control, were challenged with 10^6 embryo lethal doses of the 5th passage of the TD strain. The virus was injected in 1.0 cc. of nutrient broth intramuscularly into each of the turkeys. Blood was collected 12 days after challenge and the turkeys were kept for 5 weeks under observation.

Turkey 28, had a serum neutralizing index of $10^{6.2}$ prior to challenge but showed extensive diarrhea for 10 days after inoculation. Tables 14 and 15 show the serum neutralizing capacity and inhibitory power of turkeys 28, 29, and 58 prior to and after challenge.

The vaccinated turkeys withstood the challenge infection with no apparent systemic disturbance although there was a cessation of egg production. Egg production was resumed by turkeys 36 and 29 three weeks after challenge but the other turkeys had not resumed egg production at the termination of the experiment 5 weeks after the challenge infection.

After 5 weeks of observation, the turkeys were killed and postmortem examinations were made. The air sac membranes were clear and all of the internal organs appeared to be normal on gross examination. Inoculation of embryonated chicken eggs with yolk material from an infertile egg, spleen and air sac membranes from turkeys 33, 36, 28, and 29 failed to reveal the presence of Newcastle disease virus.

Response to vaccination.

Turkeys 33, 34 and 36 showed a significant serological response to vaccination with the Nc strain of the virus. Turkeys 34 and 36 which received 2 injections of the Nc strain of the virus vaccine at intervals of 1 and 2 weeks, respectively, showed a slightly higher but significant serological response to vaccination than did turkey 33 which received only 1 injection of the vaccine. The response of turkeys 34 and 36 was of a longer duration than that of turkey 33. Turkey 34 showed a decidedly better response than did turkey 36. The maximum neutralizing capacity was attained in turkeys 33 and 34 the 2nd week after vaccination and in turkey 36 the 3rd week after

vaccination. The sera of these turkeys showed a significant neutralizing capacity for 3 months while the hemagglutination-inhibitory power of the same sera remained at the positive level or above for at least 4 months. See Tables 4, 5, 6, and Figures 1, 2, 3.

Turkeys 40 and 42 showed only a slight serological response to vaccination with the TD strain of the virus vaccine. The neutralizing capacity and inhibitory power of the sera of these turkeys was always below the positive level. Turkey 48 which received 2 injections of the TD strain of the virus vaccine 2 weeks apart showed a better serological response in inhibitory power than in neutralizing capacity. The inhibitory power remained at or above the positive level for $1\frac{1}{2}$ months and then began to wane. In contrast, the neutralizing capacity of the serum was consistently below the positive level for $1\frac{1}{2}$ months and then began to wane. See Table 7, 8, 9, and Figures 4, 5, 6.

Brandly et al.[22] observed differences in antigenic and/or immunogenic properties among the RO, C, E and 11914 strains of Newcastle disease virus. Strain RO gave the highest protection rate among chickens vaccinated with the 4 strains, and strain 11914 showed the lowest immunogenicity and provided only feeble protection. Unlike the others, strain 11914 did not protect best against homologous strain challenge.

The low serological response observed in turkeys vaccinated with the TD strain of the virus might have been due to a low antigenicity and/or immunogenicity of this strain.

The presence of low antigenic strains of Newcastle disease virus emphasizes the importance of analysis of strains for vaccine production.

The control turkey 85 (Table 10 and 13) showed a low serum neutralizing capacity and inhibitory power for the 4 months of participation in the experiment and subsequent termination by death.

The exposure of turkeys 33, 36, 42 and 48 to the Newcastle disease infection which was suspected of having occurred sometime between the 4th and 8th months after vaccination resulted in the appearance of very high neutralizing capacities and inhibitory powers of the sera of these turkeys. A similar serological response was noted in turkeys 42 and 48 although prior to the infection they showed a low serological response to vaccination with the TD strain of the virus vaccine.

Turkeys 33 and 36 showed no increase in either the neutralizing capacity or inhibitory power of their sera in response to the challenge infection. This suggests that they were hyperimmune to Newcastle disease infection as a result of vaccination with the inactivated virus and/or exposure to a natural infection.

In turkey 42 (Table 11) there was a 10-fold increase in the neutralizing capacity against the TD strain between the pre and postchallenge sera but in turkey 48 (Table 12) there was less than a 3-fold increase in neutralizing capacity against the same strain.

The heterogeneity of the Nc and TD virus strains was observed in neutralization tests of the postchallenge serum samples. Sera from

turkeys 42 and 48 had a neutralization capacity 1500 and 60 times greater, respectively, with the homologous TD virus strain than with the heterologous Nc virus strain. This variation was not conspicuous in the inhibitory power of the sera. In turkey 48 the inhibitory power was the same for the Nc and TD strain but in turkey 42, the inhibitory power was a 2-fold greater with the homologous strain than with the heterologous strain. The prechallenge serum of turkeys 42 and 48 showed a higher neutralizing capacity and inhibitory power against the TD strain than against the Nc strain.

The response of turkeys 28, 29, and 58 (Tables 14 and 15) to challenge infection was manifested by an 89-to 135-fold increase in neutralizing capacity and an 8-to 80-fold increase in inhibitory power of sera obtained 12 days after challenge.

Beach[53] reported that chickens immunized against "avian pneumoencephalitis" with formalin inactivated virus were negative to the hemagglutination-inhibition test and that infection with active virus might be required for the production of hemagglutination-inhibitory antibodies. The "beta" method of the hemagglutination-inhibition test was used. Beach stated that refractivity to challenge with an inhibitory titer as low as 1:10 when 2 to 4 hemagglutinative units of the virus were used in the test appeared to be of diagnostic significance.

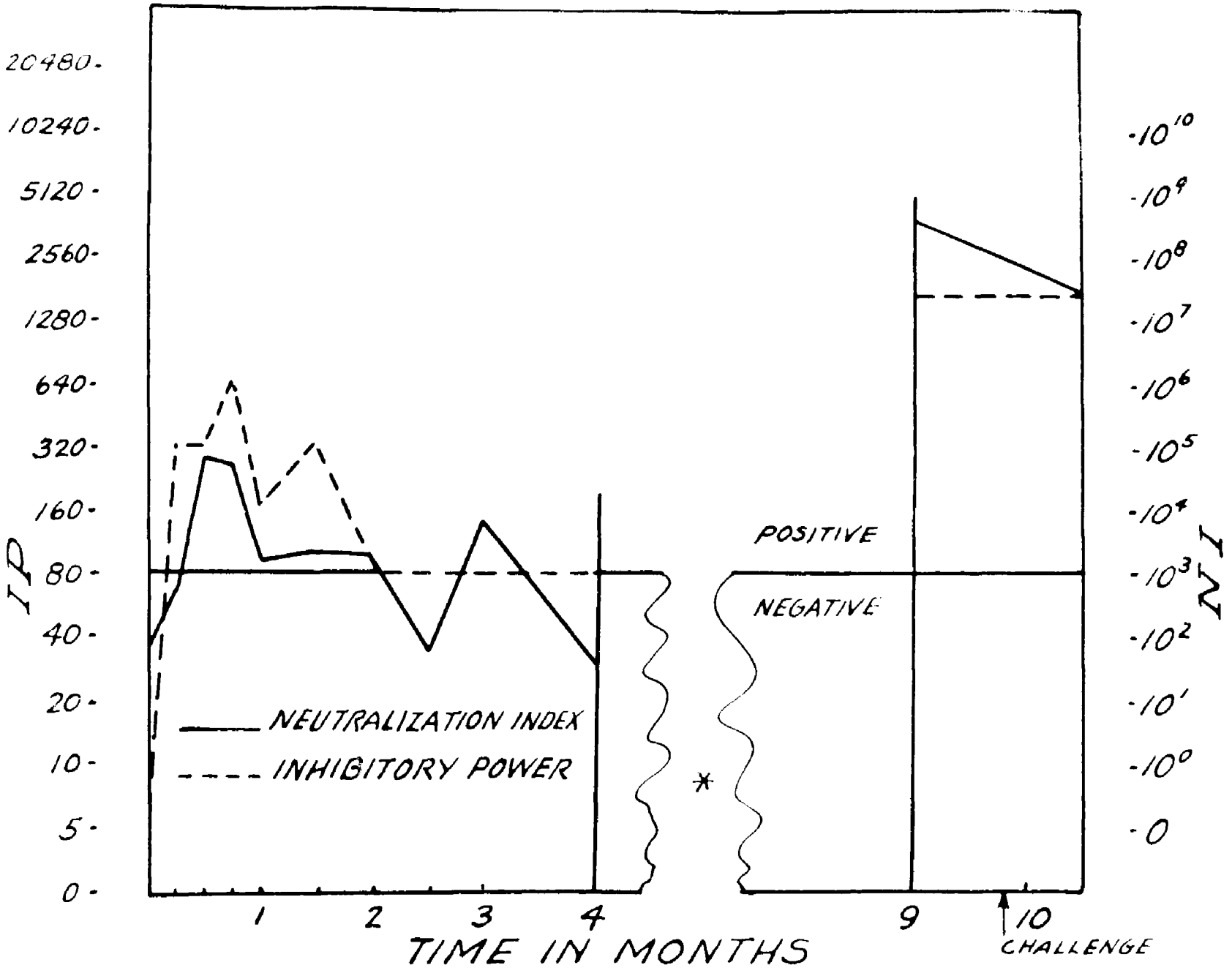
Formalin inactivated vaccine prepared from the Nc strain of Newcastle disease virus used in the present study produced a high inhibitory power in turkeys for at least 4 months after vaccination.

The "beta" method titer of 1:10 reported by Beach[53] corresponded to an inhibitory power of from 20 to 40 in the "alpha" method used in the present study. An inhibitory power of 20 to 40 is extremely low and unsafe to correlate with a neutralizing capacity of 10^3 embryo lethal doses or more which has been generally considered as diagnostic of previous infection or of vaccination against Newcastle disease.

Correlation of neutralizing capacity and hemagglutination-inhibitory power of serum.

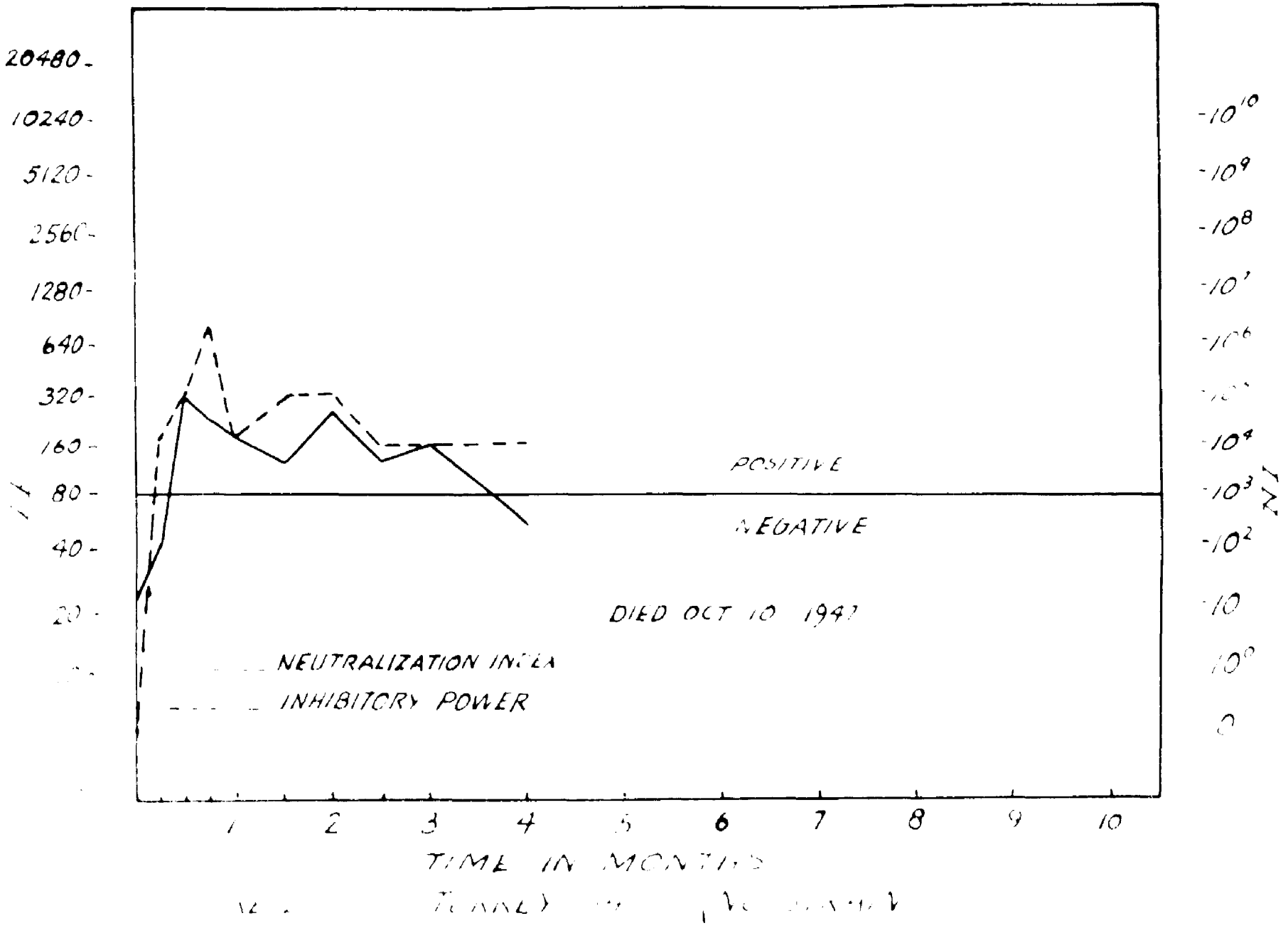
Figures 1 to 7 show the neutralizing capacity and inhibitory power of the various sera at different intervals during the experiment. The inhibitory power correlated well with the neutralizing capacity particularly during the ascending titer period. The inhibitory power increased more rapidly and decreased later than the neutralizing capacity. The neutralizing capacity might drop below the positive level 3 months after vaccination. The inhibitory power might remain at the positive level for at least 4 months.

The inhibitory power alone might indicate a previous infection or vaccination while the neutralizing capacity of the same serum might fail to give positive results. See Figure 1, turkey 33 at 2 and 4 months; Figure 2, turkey 34 at 4 months; Figure 3, turkey 36 at 4 months and Figure 6, turkey 48 from the 1st week to $1\frac{1}{2}$ months, inclusive. This would suggest that the inhibitory power of serum should not be considered as the sole criterion for the diagnosis of Newcastle disease in turkeys.



* SERUM SAMPLES NOT COLLECTED DURING THIS PERIOD

FIGURE 1 TURKEY 33 (NC STRAIN)


20480
10240
5120
2560
1280
640
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160
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40
20
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10
1
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POSITIVE
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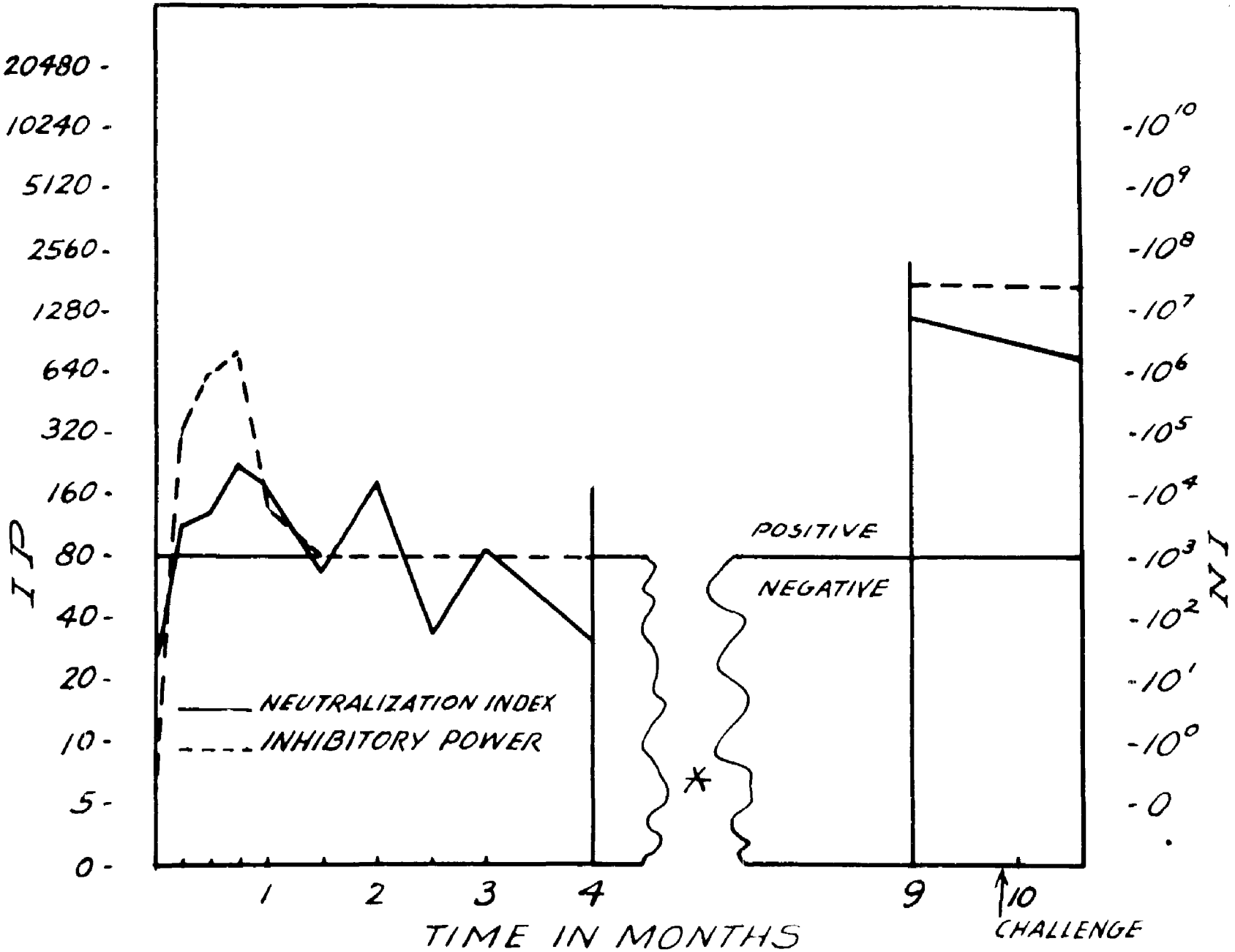


*SERUM SAMPLES NOT COLLECTED DURING THIS PERIOD

FIGURE 3 TURKEY 36 (NC STRAIN)

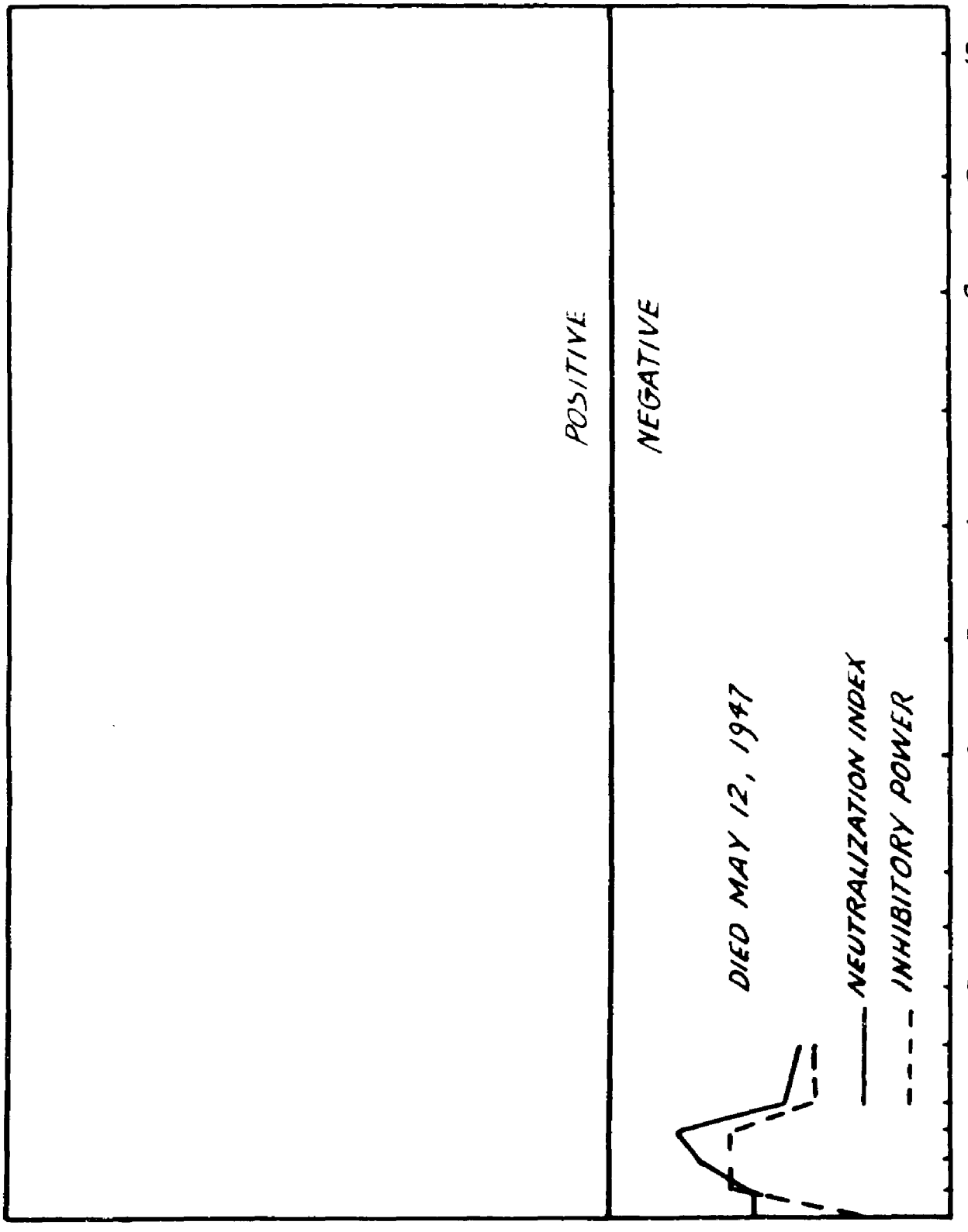
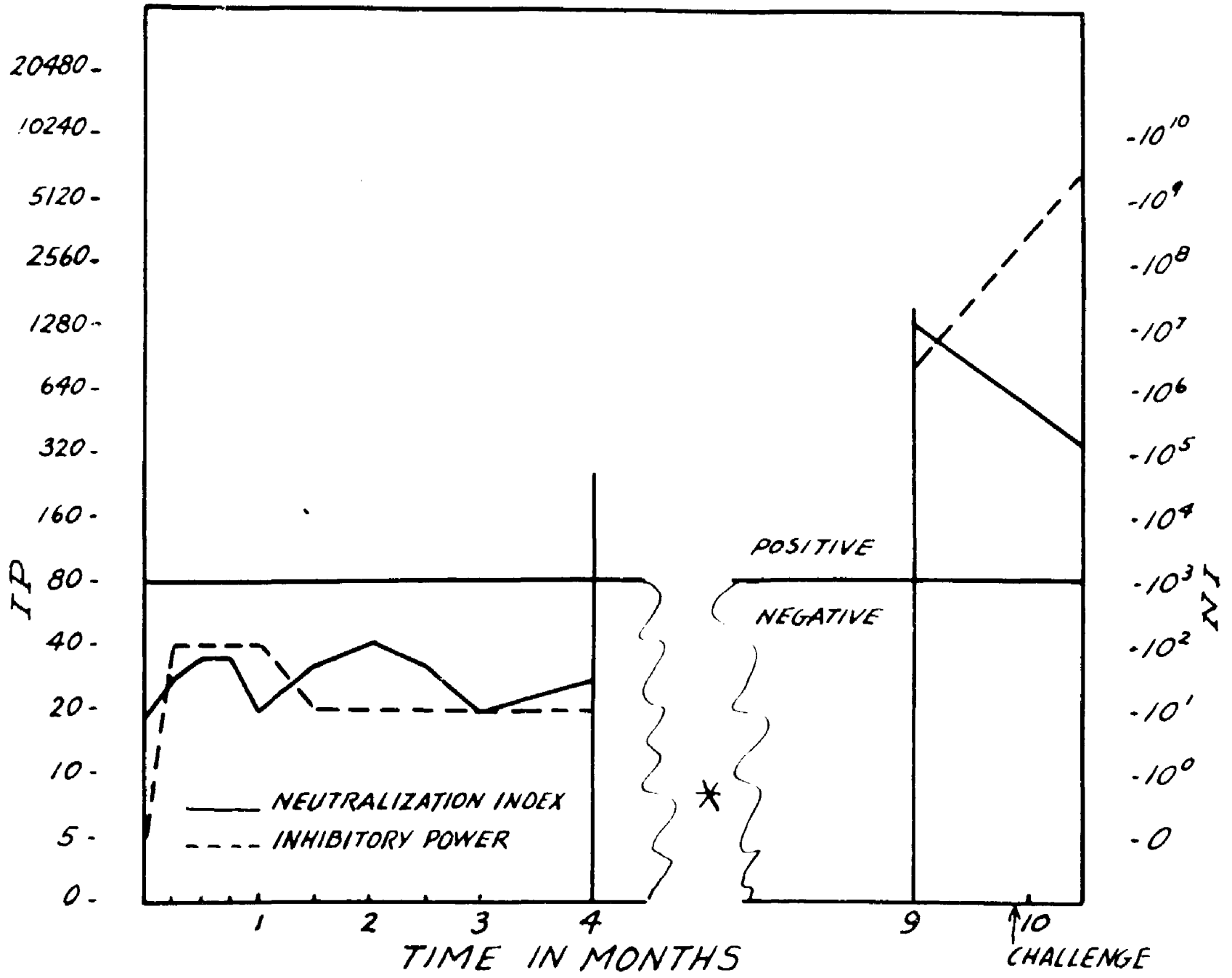
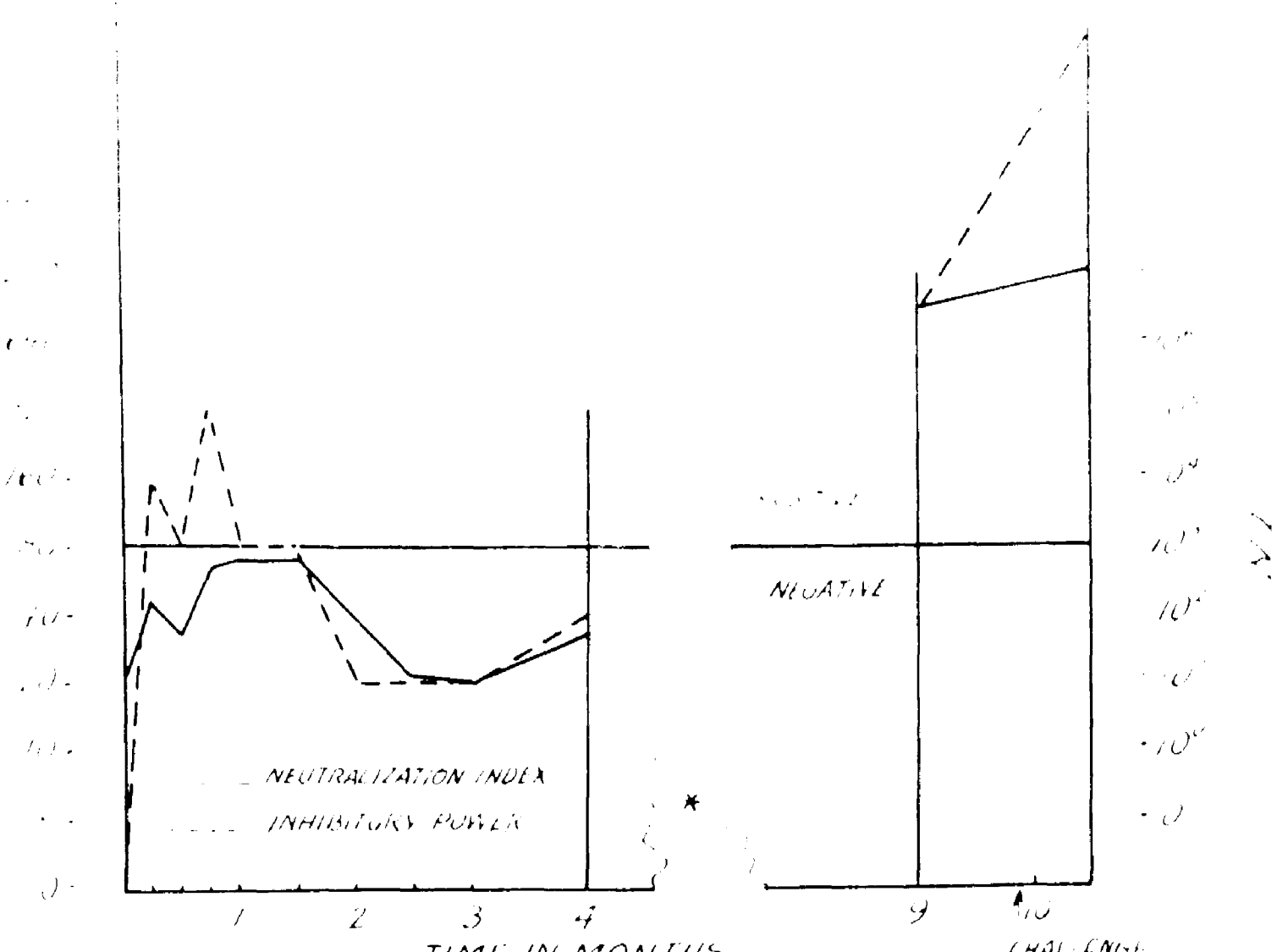


FIGURE 4 TURKEY 40 (TD STRAIN)



*SERUM SAMPLES NOT COLLECTED IN THIS PERIOD

FIGURE 5 TURKEY 42 (TD STRAIN)



* SERUM SAMPLES NOT COLLECTED DURING THIS PERIOD

FIGURE 6 TURKEY 48 (TD STRAIN)

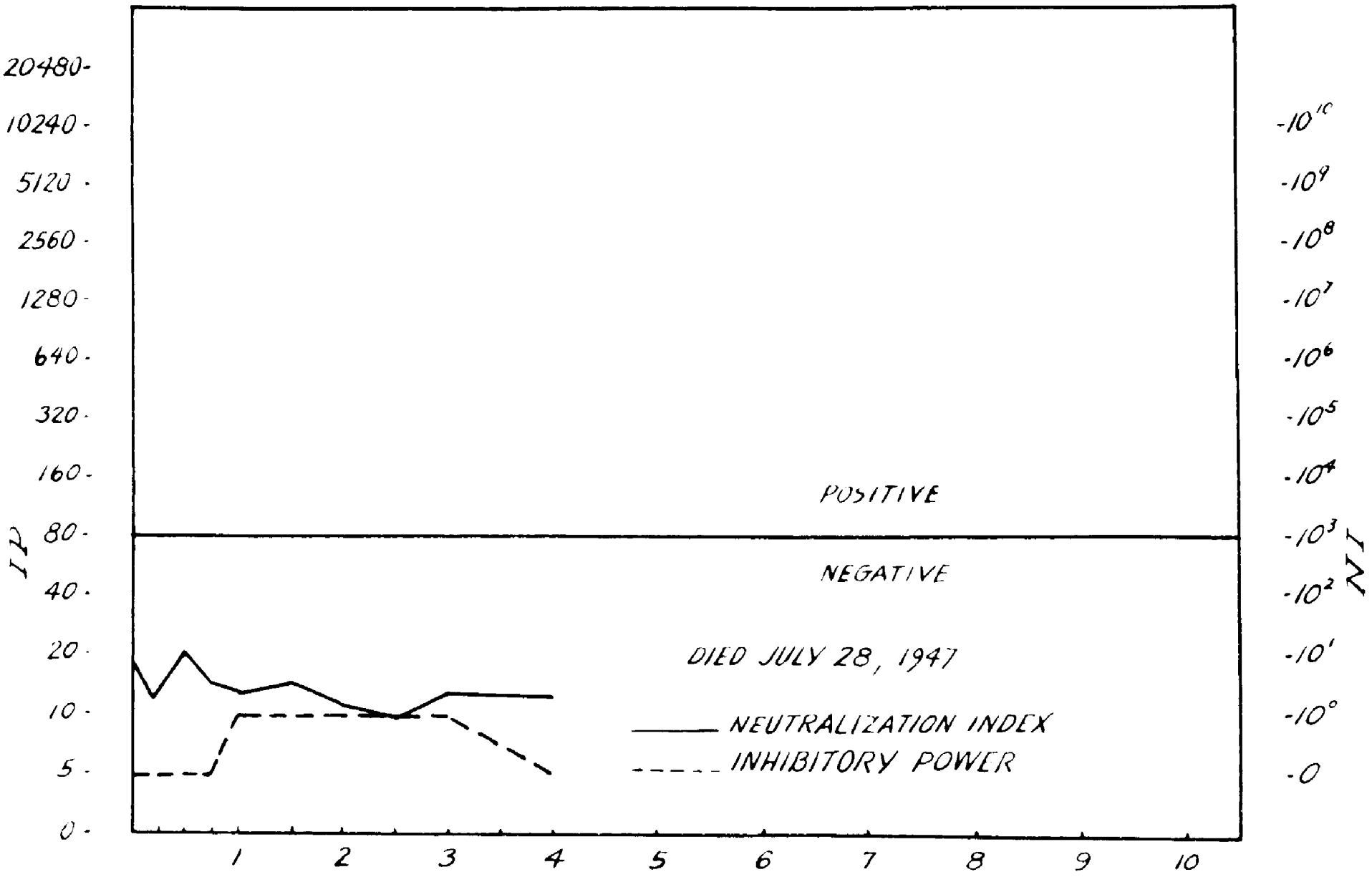


FIGURE 7 TURKEY 85 (CONTROL)

An inhibitory power of 80 was probably too low to correlate with a neutralizing capacity of 10^3 embryo lethal doses or more. An inhibitory power of 80 correlated with a serum neutralization capacity range from $10^{1.5}$ to 10^4 embryo lethal doses but coincided best with the lower part of this range. These variations might have been due to:

1) The use of decimal dilutions of the virus in serum neutralization tests and 2-fold dilutions of the virus in the hemagglutination-inhibition test.

2) Newcastle disease virus has a relatively low capacity to produce hemagglutination of turkey red blood cells, as compared, for example, with influenza and fowl plague viruses which have a high capacity to produce hemagglutination of chicken red blood cells.

3) The difficulties in controlling the hemagglutination-inhibition test with Newcastle disease virus.

In hemagglutination-inhibition tests, variations from 2-to 4-fold in the hemagglutinative activity of different virus preparations usually were without effect on the computation of the inhibitory power of a serum. In a few tests using a serum of constant dilution and 2 virus preparations with 2-or 4-fold differences in hemagglutinative activity, the end points of hemagglutination-inhibition were the same for the serum. Due to the difference in the virus hemagglutinative activity, 2 different inhibitory powers were obtained for the same serum.

The following points are suggested for standardization of hemagglutination and hemagglutination-inhibition tests:

1) The virus should be a selected strain of standard hemagglutinative activity.

2) Dilution of serum to be used in the hemagglutination-inhibition test should not be greater than the minimum required for a satisfactory interpretation of the hemagglutination-inhibitory power of the serum. In several tests it was observed that a serum diluted 1 in 20 and 1 in 25 inhibited hemagglutination with the same dilution of virus. When the inhibitory power of the serum was computed a 25.0 per cent variation was obtained.

3) A standard red blood cell suspension should be used in the tests. A 0.5 per cent turkey red blood cell suspension proved to be satisfactory in the present studies.

4) The tubes used in the test should be uniform in size, diameter and curvature of the bottom. A 12 x 75 mm. agglutination tube was found to be satisfactory. Difference in the curvature of the bottom of the tubes might produce irregularities in the aggregation of red blood cells and difficulties of interpretation of the tests.

5) The test should be conducted at a standard temperature[42] and time interval. Incubation within the range of 22 C to 27 C was found to be satisfactory.. Reading of the hemagglutination test at 15, 30 and 45 minutes was satisfactory. With hemagglutination-inhibition test

readings at 15, 25 and 35 minutes were necessary because of early elution of the virus.

6) Interpretation of the hemagglutination end point should be constant in all laboratories. It is justifiable to consider partial hemagglutination (±) as the end point, but due to the possibility of partial hemagglutination occurring in successive virus dilutions it is preferred to use as the end point the highest dilution of the virus which shows complete hemagglutination.

Newcastle disease virus

50 per cent embryo lethal dose (LD_{50}), and hemagglutinative activity.*

Table 16

Virus	Titration date	LD_{50} (0.05cc of virus)	Titration date	HA (0.25cc of formalinized virus)
Nc 46-967[16]	7- 3-47	$10^{-8.2}$	6-23-47	320
harvested on 6-21-47	7-10-47	$10^{-8.2}$	6-26-47	160
	7-17-47	$10^{-8.3}$	7- 1-47	160
	7-24-47	$10^{-8.2}$	7- 2-47	160
	7-31-47	$10^{-8.3}$	7- 9-47	160
	8- 7-47	$10^{-7.6}$	7-16-47	160
	10- 2-47	$10^{-8.2}$		
Nc 46-967[17]				
harvested on 7-19-47	10-10-47	$10^{-8.3}$	7-22-47	160
			8-25-47	160
			10- 7-47	160
			10- 9-47	320
			10-27-47	160
			11- 4-47	160
			12-15-47	320
			12-17-47	80
			1-16-48	80
			2-13-48	40
Nc 46-967[18]	10-24-47	$10^{-8.5}$	11-11-47	640
harvested on 10-23-47	10-31-47	$10^{-8.5}$	11-25-47	5120
	11- 7-47	$10^{-8.3}$	11-26-47	5120
	11-14-47	$10^{-8.5}$		
	11-21-47	$10^{-8.2}$		
	11-28-47	10^{-9}		
	12- 5-47	$10^{-8.3}$		
Nc 46-967[19]	12-12-47	$10^{-9.2}$	1-28-48	2560
harvested on 12-8-48	1- 1-48	$10^{-8.8}$	1-29-48	2560
	1- 8-48	$10^{-8.6}$		
	1-22-48	$10^{-8.8}$		
	1-30-48	$10^{-8.6}$		
Nc 46-967[20]				
harvested on 2-2-48	2-21-48	$10^{-8.6}$	2- 5-48	640
TD[4]	11-14-47	$10^{-8.1}$	11-22147	320
harvested on 10-11-47	11-21-47	$10^{-8.5}$	11-24-47	640
	12-12-47	$10^{-8.3}$	12-17-47	640
			1-27-48	160
			2- 6-48	160
TD[5]	1- 1-48	$10^{-8.7}$	1-27-48	640
harvested on 12-15-47	1- 8-48	$10^{-8.7}$		
	1-22-48	$10^{-8.8}$		

* The active virus was stored at -35 C and the formalinized virus at 4 C to 6 C.

The data in Table 16 indicates that infected Newcastle disease virus (Nc 46-967[16]) allantoic fluid stored at -35 C maintained a stable 50 per cent end point titer for at least 3 months. The hemagglutination activity of formalinized allantoic fluid infected with the same strain of virus (Nc 46-967[17]) and stored at 4 C to 6 C was stable for about 5 months after which there was a decline in the hemagglutinative activity.

A virus titer of 10^{-8} corresponded generally to an hemagglutination titer of from 160 to 320 while a virus titer of 10^{-9} corresponded to an hemagglutination titer of from 320 to 640 or higher.

Cunha et al.[49] worked with purified Newcastle disease virus and reported that the hemagglutinative unit was of an average of $10^{-6.34}$ gm. of virus and that $10^{-13.72}$ gm. of virus constituted the 50 per cent unit of infectivity for chick embryos. These data would indicate that one embryo lethal dose would correspond to about 24 million hemagglutinative units.

Hanson et al.[51] reported that a 50 per cent mortality rate of embryonated eggs was induced by one millionth of the amount of Newcastle disease virus demonstrable by the hemagglutination test.

From Table 16, it was concluded that one hemagglutinin unit would correspond to from 2 to 10 million embryo lethal doses of virus having a titer of from 10^{-8} to 10^{-9} embryo lethal doses.

The effect of dilution of serum on neutralization test.

Hirst[37] reported that a dilution of 1 in 5 of influenza immune serum resulted in a 10-fold decrease in neutralizing capacity when tested in mice.

Brandly[67] cited some results obtained with neutralization of fowl plague virus by specific immune serum in which a dilution of 1 in 10 of immune serum resulted in a 100-fold decrease in titer.

The protocols of Brandly et. al.[33,23] showed that a dilution of 1 in 10 of Newcastle disease immune serum resulted in a 100-fold decrease in neutralization capacity but a dilution of 1 in 5 resulted in a 10-to 100-fold decrease in neutralizing capacity.

Osteen and Anderson[12] reported that a dilution of 1 in 10 of Newcastle disease immune serum with negative chicken serum failed to decrease the neutralizing capacity, but a dilution of 1 in 20 resulted in a 10-fold decrease in the neutralizing capacity of the undiluted serum.

Table 17

Turkey	Serum	LD_{50} Newcastle disease virus neutralized by 0.05 cc. of serum	
		undiluted	1 in 10
33	3 weeks	$10^{4.7}$	$10^{2.7}$
34	2 weeks	$10^{5.6}$	10^{4}
36	2½ months	10^{2}	$10^{0.7}$
48	2 weeks	$10^{1.7}$	$10^{1.7}$
48	1 month	$10^{2.8}$	$10^{0.1}$

Table 17 shows the results of 5 neutralization tests using undiluted serum and the same serum diluted 1 in 10 in nutrient broth, Dilution of immune serum 1 in 10 decreased the neutralizing capacity from 20-to 100-fold.

Summary

1) Two strains of Newcastle disease virus (Nc 46-967, chicken origin, and TD, turkey origin) inactivated with formalin were used for experimental vaccination of turkeys. The serological response of the turkeys at certain time intervals was evaluated by serum neutralization and hemagglutination-inhibition tests.

2) A fair degree of correlation of the neutralizing capacity and the inhibitory power of the serum was observed particularly during the ascending titer period.

3) One hemagglutinin unit was found to correspond to from 2 to 10 million embryo lethal doses of a virus having a titer of from 10^{-8} to 10^{-9} embryo lethal doses.

4) Dilution of immune serum 1 in 10 with nutrient broth decreased its neutralizing capacity from 20-to 100-fold.

5) Turkeys vaccinated with Nc 46-967 strain showed significant neutralizing capacity and inhibitory power for 3 and 4 months, respectively, after vaccination.

6) Strain TD was either of low antigenicity or very sensitive to 0.1 per cent formalin inactivation as the turkeys vaccinated with this strain showed only a negligible serological response.

7) A subclinical Newcastle disease infection of the turkeys from 4 to 8 months after vaccination produced a marked serological response.

8) The turkeys were challenged with an intramuscular injection of 10^6 embryo lethal doses of the active virus 10 months after vaccination. Clinical symptoms of Newcastle disease were not observed in the vaccinated turkeys which had been exposed to the sub-clinical infection.

9) Sera collected 12 days after challenge revealed that 2 turkeys were hyperimmune to Newcastle disease as there was no increase in neutralizing or inhibitory agents. In the other turkeys there was a significant response to challenge as revealed by an increase of the neutralizing capacity and inhibitory power of the serum.

10) Efforts to isolate the virus from turkeys killed 5 weeks after challenge failed to reveal the presence of the virus in the spleen, the air sac membranes, and the yolk material of an unfertile egg.

11) Vaccine prepared from formalinized Newcastle disease virus infected allantoic fluid of high antigenicity would be effective for immunization of turkeys against a potential infection with the disease.

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