EXPERIMENTAL STUDIES

ON

NEWCASTLE DISEASE VACCINE

The Effect of Vaccine Prepared from the Virus

Inactivated with Beta-Propiolactone

and Adsorbed on Aluminium Phosphate Gel

by

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Introduction

Ever since 1926 when Newcastle disease was recognized as an independent disease of the chicken distinguishable from fowl plague[23], researches on the development of vaccines to combat this chicken epidemic have progressed at a remarkable pace the world over.

Today, research reports on such vaccines are legion. The first live- and inactivated-virus types of these vaccines were developed almost simultaneously, and both have since been remarkably improved in their efficacy. Now available are numerous commercial or non-commercial varieties of whichever type of such vaccines desired.

Notwithstanding this spectacular progress of research on vaccination against the endemic and the resultant profusion of supply of such vaccines, there still remain not a few difficulties in attempting to compare the live- and inactivated-virus types of these vaccines by their merits and demrits and determine which is preferable.

It is claimed that in the Island of Cyprus[20] and in some parts of Canada[4], an extensive application of some vaccine of the live-virus type have proved clearly effective in each region. It is also claimed that in some other countries, the use of vaccines of the live-virus type has been justified by their

proven efficacy[10, 76, 85] in places where the epidemic was prevalent. But, there are some skeptics who place little hope in the capacity of the live-virus type of such vaccines to attain complete control of the diseases[56, 76, 85].

The inactivated-virus type of such vaccines, on the other hand, is considered by many researchers[8,12,26,41,76,81,89] to impart only a temporary or short-lived immunity to chickens against the epidemic. But it is also claimed by some researchers[5,10, 97] that some vaccines of the inactivated-virus type are good enough to impart a long-range immunity in case they are derived from a local virus strain[98] or an antigenically suitable virus strain, or one each of two such kinds of vaccine is used in a proper combination for double vaccination.

In producing vaccines of the inactivated-virus type, the initial practice was to use, as the virus material, a virus-containing organ emulsion taken from some viscera of chickens killed by Newcastle disease.

But this material has been generally replaced by chicken embryos infected with the Newcastle disease virus (NDV) since 1934 when Burnet[13] came up with his monumental discovery that this particular virus grows very well in developing chicken eggs, and thus provides a virus material of an almost always stabilized titer.

As for methods of inactivation of the virus, all sort of physical or chemical processes have been devised and tried over a long period of time. They include such methods as the crystal violet[3, 6, 24, 45, 46, 54, 55, 68, 96], urethan[11], ultraviolet irradiation[9, 12, 54], heating[25], sodium salt of 8 hydroxy-7-iodoquinoline-5-sulphonic acid[53], and formalin[16, 21, 34, 40, 47, 48, 53, 68-72, 75, 82, 92, 95].

However, except only for the formalin method, no known process has ever fully come up to the rigid standard of research requirements.

Today, formalin is most widely recognized and used as an inactivator of the particular virus. Numerous researchers have reported their confirmation of generally good immunizing effect of the vaccines made from the formalin-inactivated virus[16,41, 57,83].

But even this outstanding inactivator, formalin, is not entirely free from criticism as to its efficacy. Some researchers have reported having obtained not very satisfactory results after their trials with formalin[16, 41, 57, 83]. Meanwhile, Brandly et al.[12] and Hanson et al.[34] have pointed out what they considered to be certain differences in antigenicity to occur among various formalin inactivated-virus vaccines depending on virus strains to be used.

Now, there are two notable identical reports by Hartman

et al.[37] and Mangun et al.[64] that they have found beta-propiolactone(BPL) to be the most promising and dependentable virucidal agent ever known. They have invariably arrived at the conclusion after a series of laboratory screenings of a total of 140 varieties of virucidal agents to pick one with the least adverse effects on the plasma protein in the course of their studies on the problem of serum hepatitis transmission through blood transfusions or administration of infusion solutions. Their common research purpose was to the virus of serum hepatitis found in human blood or its products.

In fact, BPL is a remarkable organic compound that is now attracting increasingly wider attention among researchers as a virus inactivator of for their apparent superiority to formalin in many respects.

BPL is now generally known to have little ill effect on plasma protein because it is very quick in its inactivating action and moreover, all suplus amount of a given dose of it left after inactivation turns into acid end-products chiefly made up a hydroacrylic acid (beta-oxy-propionic acid) due to hydrolysis. It is also known that BPL may be freely applied without regard to pH, and unlike formalin, it works very well even with virus materials which are not very fit for purifying.

Smolens & Stokes[87] have reported that after an ultra-violet irradiation of human serum specimen heavily infected

with T_4 bacteriophage and then a treatment of the irradiated serum with BPL of 1.5 g per liter in concentration they discovered that BPL, used at least up to that much concentration, had no effect on the electrophoretic pattern of the serum protein.

Masu et al.[67] have also reported that they used BPL for inactivating the infectious canine hepatitis virus and obtained similarly favorable results during the course of their study on the disposal of microorganisms contaminating an immune serum of canine distemper. After applying BPL to a canine serum preparation artificially infected with the infectious canine hepatitis virus to a very heavy degree, they have claimed that BPL attains complete inactivation of the virus in 30 minutes when used in a concentration of 0.3% at 37°C, in six hours when used in the concentration of 0.2% at 4°C. Moreover, they have reported that BPL, when applied in such concentration, does not have any effect on the neutralizing antibodies of the serum.

LoGrippo[58] has also announced that BPL, when employed in concentration's ranging between 0.05 and 0.7%, achieved complete inactivation of viruses, fungi and bacteria after his study on the necessary concentrations of this organic compound for inactivating such microorganisms.

On top of this, LoGrippo & Hartman[59, 60] have come up with a very simple and rapid method of producing a BPL-inactivated vaccine of virus. They reported their discovery that BPL is capable of inactivating the virus within 10 to 15 minutes at 37°C. They also reported their success in producing such a vaccine of a far higher antigenic potency than those inactivated with formalin or phenol. The process they have developed has since come to be applied to inactivation of many different kinds of virus, including arboviruses[28, 58, 60] and rabies virus[58, 60 77, 79].

This process was first applied to the production of a Newcastle disease vaccine by Mack & Chotisen[62, 63]. Reporting on the results of their experiment with the vaccine, they said a group of chickens vaccinated with a BPL-inactivated virus vaccine registered a 100% rate of survival after a challenge infection with a virulent Newcastle disease virus 16 days after vaccination compared with a 97.4% mortality of the same challenge infection, among a control group of chickens.

Superiorities of the vaccines made from BPL-inactivated viruses to other types of vaccine inactivated with formalin or other agents have subsequently been recognized and reported by such researchers as Winmill & Weddell[96], Simmins & Baldwin[86], Haig et al.[33], Sullivan et al.[88], Gill et al.[31], Keeble et al.[49], Keeble & Wade[51], Keeble & Coid[50],

Piercy et al.[78], Pini et al.[79], Christie et al.[15], Akat[2], Cooper[17], Hemsly[39], Cherby & Vallette[14] and Hofstad et al.[42].

Nevertheless, Appleton et al.[5] have reported negatively, saying they failed to find any marked differences in efficacy between BPL and formalin inactivating virus.

Hofstad et al.[42] compared the efficiencies of three different types of inactivated-virus vaccines--- 1) γ ray-irradiated, 2) BPL-inactivated, and 3) formalin-inactivated--- and reported that the BPL-inactivated type has proved to retain the highest antigenicity of the three. They thus concluded that the BPL-inactivated virus type of vaccine should be the best of all sorts of vaccines against the Newcastle disease in general use today.

Haig et al.[33] and Keeble & Coid[50] have reported that the BPL-inactivated virus type of vaccine, even when applied to one-day-old chicks without any maternal antibody, does not deter their growth. Cooper[17] tried various commercial BPL-inactivated virus vaccines on hens in their egg-laying season and reported that none of the vaccines had any ill effect on the chickens' egg production.

According to a report by Keeble & Wade[51], other types of inactivating agents than BPL, such as formalin and phenol,

which also work as protein coagulants, affect both the nucleic acid and coating protein of the virus in their inactivating actions, and, therefore, are apt to cause a change in the antigenicity of the virus.

In contrast, they claimed, BPL, which is a sort of alkylate, may work a change in the nucleic acid of the virus and thus cause the virus to loss its infectivity, but will hardly affect the antigenic protein of the virus in its inactivating action, thus making it possible to produce a vaccine of a high antigenicity.

Now, it has also been reported[35, 36, 58, 61, 87] that a consecutive treatment of a virus with ultraviolet irradiation and BPL by applying BPL either before or after ultraviolet irradiation is more effective in inactivating the virus than using only ultraviolet irradiation or BPL, whichever singly[35, 36, 58, 61, 67, 87]. This seems to imply that both of these two inactivating agents work on the same component of the virus. It has also been reported[60] that a virus inactivated by BPL has a very high antigenic potency. This seems to prove, just as described above, that the BPL-inactivated virus loses infectivity by a change in its nucleic acid, but retains its antigenic protein with relatively little changes.

Apparently such action of BPL compares sharply with those of formalin and phenol which chiefly work on the protein of

the virus and thus causes a decided decrease in the antigenic property of the virus.

There is some variance of opinion as to the reactivating phenomenon of the particles of the virus treated with some protein coagulating agent like formalin or phenol. In fact, there does exist a possibility that these inactivating agents, because of their action to cause aphysical change in the proteinous outer coating of the virus, often cannot penetrate deep enough to reach the nucleic acid of the virus. Thus, it is beliebed[38] that these virus particles develop a reactivating phenomenon in case the nucleic acid of the virus is exposed to the danger of being released.

In contrast, no such reactivating phenomenon is believed to occur in BPL-inactivated virus vaccines because BPL easily penetrates the protein coating of the virus without hardly affecting it physically and reaches the nucleic acid to destroy the latter's infectivity. It has been reported that in the virus of murine encephalomyocarditis inactivated with BPL, no reactivation was observed.

It has been well known for a long time already that some kinds of oil and metallic ion have an adjuvant action to help the antibody production. The problem of adding with some adjuvant to the Newcastle disease virus has also been studied

by many researchers in the past. Now in common use as this kind of adjuvant-added virus of the kind is an aluminagel-added type[12, 19, 31, 32, 74, 90, 91] and an oil-added type[12, 65, 66].

Brandly et al.[12] has reported that Falba oil is superior to aluminagel as such adjuvant. Tsubaki & Masu[94], who compared several kinds of oil adjuvant they mixed in a formalin-inactivated Newcastle disease vaccine, have reported that the Arlacel A- Bayol F type adjuvant was the most effective.

But, there is also a report[84] criticizing the use of oil adjuvants for vaccines applied to broiler chickens for the reason such adjuvants stay long in the chickens' muscles.

Besides, there is a report[29] that a certain kind of oil, when used as an adjuvant, caused an undesirable post-vaccination reaction of granuloma type.

Experiments to add adjuvants to the BPL-inactivated virus vaccines have been reported by Gill et al.[31] and Haig et al.[33] who invariably preferred Aluminium hydrogel as the best adjuvant.

In Japan, there are still very few reports on the use of BPL as an inactivator of vaccines of whatever kind. Now available are only two reports of the kind worth notice--- that of Yoshino & Saito[99] on their attempt to apply BPL to

the production of a rabies vaccine and that of Nakamura et al.[73] to their try to inactivate the Japanese B encephalitis vaccine. Neither of these BPL-inactivated vaccines has yet reached the stage of clinical application.

The present report represents the findings obtained from author's experiment to produce a Newcastle disease vaccine by using BPL as a virus inactivator and an aluminium phosphate gel as an adjuvant. Here are the findings.

Materials and Methods

Virus used: Egg-passage virus of the Sato strain of the Newcastle disease virus (NDV) group was employed. As the seed virus specimen, the infected allantoic fluid of such eggs was used without dilution after preserving it at a temperature of -70°C.

Beta-Propiolactone (BPL): Choosing a commercial BPL produced by Tokyo Kasei Kogyo K. K. (Tokyo Chemical Industry Co.), the agent at a temperature of -20°C was preserved. Every time it was used, the preserved agent was diluted tenfold by a sterilized distilled water refrigerated to 4°C and immediately applied it to the virus specimen.

Aluminium Phosphate Gel: Following the method of Holt[43], 560 ml of a 10% solution of Aluminium chloride was added to 2,790 ml of distilled water. While stirring the mixture, 560 ml of a 15.75 % solution of sodium phosphate (tribasic) was added to it gradually. Then the triple mixture was washed by centrifugation was washed with 560 ml of an Aluminium phosphate solution, and next the Aluminium phosphate content in the whole mixture was calculated, finally obtaining a gel suspension fluid of between 30 and 40 mg/ml.

Preparation of the Vaccine: A number of 11-day-old developing chicken egg was inoculated by planting 0.2 ml of the Sato strain of NDV of 10,000 EID_{50} into the allantoic cavity of each egg. The eggs which died after 48 to 72 hours of subsequent incubation were opened and their embryos and allantoic fluids were harvested immediately following death or after one night's preservation after death at 4°C. After preserving the embryo and allantoic fluid specimens overnight in a frozen condition, the embryo was processed into a 30% emulsion and the allantoic fluids was added to the emulsion. This preparation was then cleared of large crude tissue pieces by centrifugation.

The supernatant fluid of this centrifuged liquid was extracted and the viruses in it were inactivated at 4°C by the addition of BPL. The preparation was next turned into a vaccine by adding an Aluminium phosphate to a 50% concentration.

NDV Calculations: The effective dosages of the above preparation was determined by planting intramuscularly 1.0 ml of its 10-fold serial dilution into each of number of three-month-old white leghorns and keeping the chickens under observation for 10 days to register their death or survival.

The infectivity titration in the developing chicken eggs was measured by plating 0.2 ml of a 10-fold solution of the preparation into each allantoic cavity of a number of 11-day-old

developing chicken eggs and then incubating the eggs at 36°C for 48 hours. After the incubation, the allantoic fluid of each eggs was harvested and the fluid's hemagglutinating activity was measured to see if it is positive or not, that is, whether infected or not. In this measurement, the Reed & Muench method was followed to work out a 50 % infectious dose.

For determination of whether the NDV inactivation has been completed or not, 0.5 ml of the preparation was planted in the allantoic cavity of each egg. After the egg was then incubated for 72 hours at 36°C, the inactivation was determined by the hemagglutinating activity of the allantoic fluid. But, in some cases, the preparation was planted on a ehick embryo cell culture, to find out the degree of infection by the resultant CPE (cyto-pathogenic effect) or by the hemagglutinating activity of the culture fluid.

Chick Embryo Cell Culture: The embryo of each of a number of 9-to 10-day-old developing eggs was taken out and a cell solution was made from it by means of trypsin digestion. The specimen was then incubated for 24 hours at 37°C feeding, through a roller tube, a growth medium (Earle's balanced salt solution, which has a 0.5 % content of lactalbumin hydrolysate, a 0.1 % content of an yeast extract and a 5 % content of horse serum).

On this embryonic specimen the virus specimen was planted.

The embryonic specimen was then reincubated after being transferred to a maintenance medium (Earle's B.S.S., which has a 0.5% content of lactalbumin hydrolysate, a 0.1% content of an yeast extract and a 1% content of horse serum).

Potency Test for Vaccine: For this test a number of white leghorns of three age groups ----- two-week, three-month and five-month was used. One groupe consisted of five to 10 birds. Ten to 14 days after vaccination, the chickens were given a challenge infected with NDV of 1,000 to 10,000 CID_{50} and placed under 10 to 14 days of observation to determine the efficacy of the vaccine by the chicken's death or survival.

Hemagglutination-Inhibition Test (HI Test): 0.25 ml of an antigen of four units was added to 0.25 ml of the inactivated serum diluted two-fold by stages. After incubating the mixture for 10 minutes, a 0.5 % chick red blood cell suspension fluid was added to it. Stirring it throughly and then leaving it standing still for 60 minutes, the test tube bottom pattern of the mixture was read to determine the test results.

Experimental Results

I. NDV Inactivation Test with Beta-Propiolactone:

1) Inactivation of NDV with various concentrations of BPL: As a preliminary trial to find out the NDV-inactivating

actions of BPL of different concentrations, first the BPL of varying concentrations at 37°C was tried.

Thus NDV-infected and killed chick embryos was processed into a 30% emulsion by means of a phosphate-buffered saline solution (PBS) of pH 7.2. This solution, after the addition of an allantoic fluid, was centrifuged for 30 minutes at 3,000 rpm.

The supernatant fluid of this centrifuged solution was extracted and five specimens were made from the fluid by adding BPL in five different concentrations of 0.5, 0.25, 0.1, 0.05 and 0.025%. These specimens were incubated in a 37°C water-bath.

During this water-bath process, samples were taken from the specimens at certain intervals of time. A 0.2 ml dose of each sample taken was planted into the allantoic cavity of a number of 11-day-old growing chicken eggs. After the eggs so processed were cultured for 72 hours, the hemagglutination activity of their allantoic fluids was examined. All negative results were deemed to represent successful inactivation. The results are given on Table 1.

Table 1

Inactivation of NDV at 37°C with various concentrations of BPL

BPL % / Time	0.5	0.25	0.1	0.05	0.025	Cont.
10 min.	0/5	0/5	5/5	5/5	5/5	5/5
20 min.	0/5	0/5	1/5	5/5	5/5	5/5
60 min.	0/5	0/5	0/5	0/5	5/5	5/5
120 min.	0/5	0/5	0/5	0/5	5/5	5/5

Virus material: 30% embryo suspension plus allantoic fluid.

BPL: β - propiolactone ; NDV: Newcastle Disease Virus.

Numerator: Number of eggs infected.

Denominator: Number of eggs tested.

As the table illustrates, inactivation was attained in 10 minutes when the BPL concentration in the supernatant fluid was either 0.5 or 0.25%, and in 60 minutes when that concentration was either 0.1 or 0.05%. But no inactivation was attained even in 120 minutes when that concentration was 0.025%.

Next, similar trials were held to compare BPL's inactivating capacities at two different temperatures of 4°C and 37°C, obtaining results as given on Table 2.

Table 2

Inactivation of NDV at 4°C and 37°C with various concentrations of BPL

Temperature	4°C						37°C					
BPL % / Time (min)	0.5	0.25	0.1	0.05	0.025	Cont.	0.5	0.25	0.1	0.05	0.025	Cont.
2	0/5	0/5	5/5	5/5	5/5	5/5	0/5	0/5	0/5	0/5	5/5	5/5
5	0/5	0/5	5/5	5/5	5/5	5/5	0/5	0/5	0/5	0/5	5/5	5/5
10	0/5	0/5	0/5	5/5	5/5	5/5	0/5	0/5	0/5	0/5	5/5	5/5
24	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	5/5
48	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	5/5
72	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	5/5
96	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	5/5

BPL: β -propiolactone ; NDV: Newcastle Disease Virus

Numerator: Number of eggs infected

Denominator: Number of eggs tested

These comparative studies were conducted as follows: BPL was added to a virus fluid obtained by the same process as described above in different concentrations also as given above. One half of the samples were incubated in a refrigerator kept at 4°C and the other half in a water-bath of 37°C and samples were taken from each half at different intervals of time.

A 0.2 ml dose of each sample was planted in the allantoic cavity of a number of 11-day-old developing chicken eggs. The processed eggs were incubated for 72 hours and the results were determined by the hemagglutination activity of the egg's allantoic fluids, also in the same way as mentioned above.

It was discovered that inactivation was attained in 120 minutes when the BPL concentration was 0.05% or larger, and in 24 hours when the BPL concentration was 0.025% both in case the incubation temperature was 37°C.

In contrast, it was found that inactivation generally progressed slowly when that temperature was 4°C, that is, in 120 minutes when the BPL concentration was either 0.5 or 0.25%, in 10 hours when the BPL concentration was 0.1%, and in 24 hours when the BPL concentration either 0.05 or 0.025%.

It was thus understood that a long period of incubation after the BPL addition makes possible the attainment of the same degree of inactivation as in the case of the higher incu-

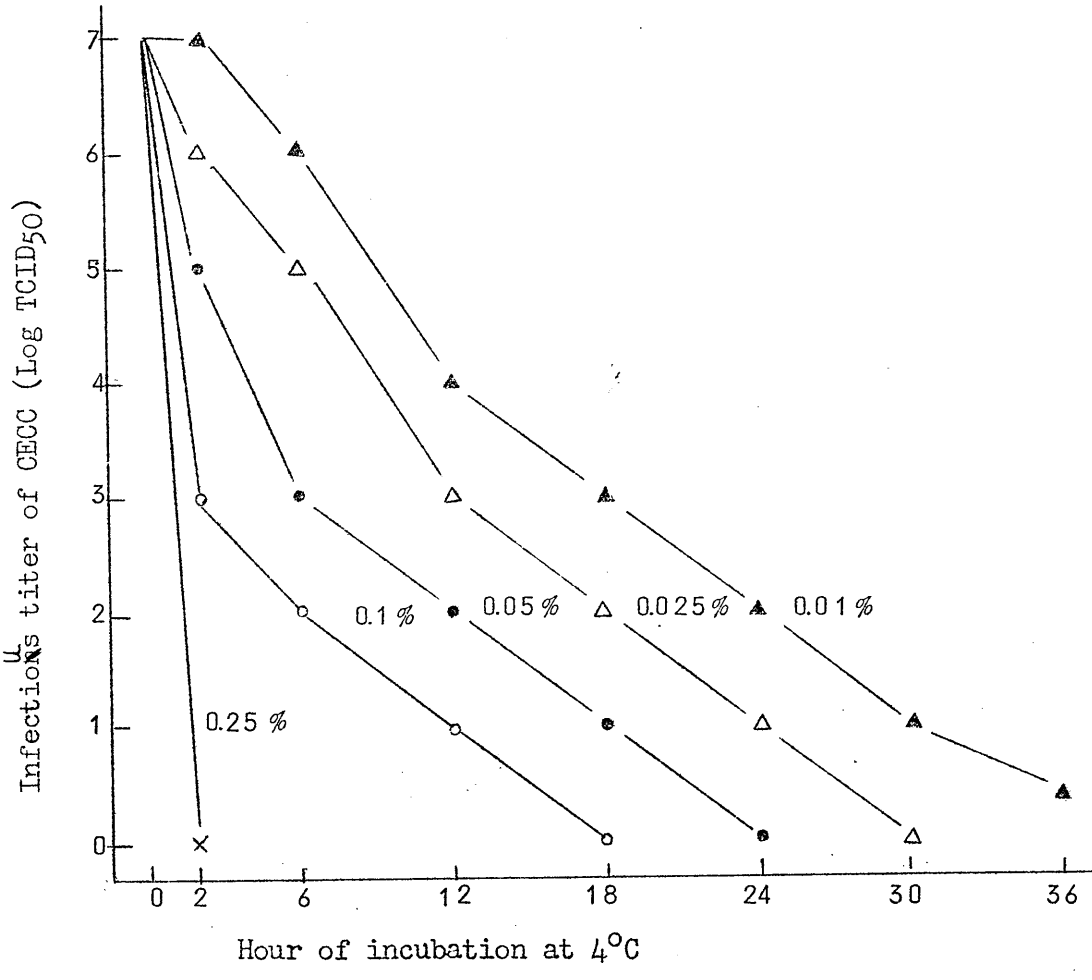
bation temperature of 37°C ~~even~~ when that temperature is only 4°C.

Consequently, it was attempted to obtain a certain inactivating curve of BPL in different concentrations at 4°C by planing BPL-added virus samples obtained by the same process as given above on chick embryo cell cultures to determine the results, using CPE as the indicator. The results are shown on Fig. 1. The figures given on Fig. 1 represent averages obtained from a series of two trials.

As illustrated on Fig. 1, it took 120 minutes (two hours) to attain inactivation when the BPL concentration was 0.25%, and 18, 24 and 30 hours respectively when the BPL concentration was 0.1, 0.5 and 0.025%. But it was impossible to achieve inactivation even in 36 hours when the BPL concentration was 0.01 %.

Fig. 1

Inactivation of NDV at 4°C with various concentrations of BPL



NDV: Newcastle Disease Virus ; BPL: β -propiolactone

CECC: Chick embryo cell culture

2) Effect of pH on BPL's Inactivating Action:

To find out the effect of pH on BPL's inactivating action, the virus fluid, prepared in the same may as described above, was processed into three kinds of a 30% emulsion that is, of 6.7, 7.0 and 8.0 pH by means of a phosphate-buffered saline solution. Then BPL was added to the supernatant fluid of each preparation in a 0.1% concentration. Half of these BPL-added preparation were incubated at 4°C, and the other half at 37°C. During these incubation periods, samples were taken from each group at certain intervals of time.

A 0.2 ml dose each of these samples was planted into the allantioc cavity of each of a number of 11-day-old developing chicken eggs. After 72 hours of incubation of these eggs, the hemagglutination activity of the allantoic fluids of the eggs was checked to ascertain inactivation by negative findings. The results are illustrated on Table 3.

As is clear from the table, when the pH was within the range of 6.0 to 7.0 or to 8.0, there was no effect of pH on BPL's inactivating action, whether the incubating temperature was 4°C or 37°C.

Table 3

Effect of various pH value on the BPL inactivation of NDV at 4°C and 37°C

Inactivation at 4°C

Time / pH	2 hrs.	5 hrs.	10 hrs.	24 hrs.	48 hrs.	72 hrs.
6.0	0/5	0/5	0/5	0/5	0/5	0/5
7.0	0/5	0/5	0/5	0/5	0/5	0/5
8.0	0/5	0/5	0/5	0/5	0/5	0/5
control (BPL free)	5/5	5/5	5/5	5/5	5/5	5/5

Inactivation at 37°C

Time / pH	10 min.	20 min.	30 min.	1 hr.	2 hrs.	5 hrs.
6.0	0/5	0/5	0/5	0/5	0/5	0/5
7.0	0/5	0/5	0/5	0/5	0/5	0/5
8.0	0/5	0/5	0/5	0/5	0/5	0/5
control (BPL free)	5/5	5/5	5/5	5/5	5/5	5/5

Concentration of BPL: 0.1%

Numerator: Number of eggs infected

Denominator: Number of eggs tested

3) Effect of BPL on Hemagglutination Titer:

The effect of BPL on the hemagglutination titer was studied by the same processes as in the aforesaid trials to see the difference in inactivation between the temperatures of 4°C and 37°C.

Samples taken at certain intervals of time were diluted to two-fold, and to a 0.5 ml of each was added a 0.5 ml of a 0.5% chicken erythrocyte suspension fluid. The mixtures, after stirring, was left standing in a test tube for 60 minutes at 4°C. The test tube bottom patterns were then read to determine the results which are given on Table 4.

As Table 4 shows, there was no appreciable difference in the hemagglutination titer between the processed and the unprocessed groups when the BPL concentration was 0.1% or less and the incubation temperatures was 4°C.

But when the BPL concentration was either 0.5% or 0.25% and the incubation temperature was the same, the processed samples registered a sharp decrease in the hemagglutination titer to 1/4th of the control group both in 10 and 24 hours of incubation.

This seems to indicate that when the incubation temperature is 4°C and the BPL concentration is 0.1% or less, BPL has hardly any effect on the hemagglutinin.

But it was confirmed that when the incubation temperature

Table 4

Effect of various concentrations of BPL on the hemagglutination titer of NDV

Temperature	4°C						37°C					
BPL % / Time (hours)	0.5	0.25	0.1	0.05	0.025	Cont.	0.5	0.25	0.1	0.05	0.025	Cont.
2	640	640	640	640	640	640	80	320	320	320	320	640
5	640	640	640	640	640	640	40	320	160	320	320	640
10	160	640	640	640	640	640	40	160	80	80	160	320
24	160	160	640	640	640	640	20	80	80	80	160	320
48	80	160	640	640	640	640	40	80	80	80	160	320
72	80	160	640	640	640	640	20	40	40	80	80	160
96	80	160	640	640	640	640	10	40	80	80	80	160

BPL: β -propiolactone ; NDV: Newcastle Disease Virus

is 37°C, BPL has remarkable effects on the hemagglutinin. At this high temperature, the hemagglutination titer dropped to only 1/8th of the control group in two hours of incubation, and 1/16th in 96 hours in case the BPL concentration was 0.5%. At the same temperature, the hemagglutination titer also decreased to between 1/2 and 1/4th of the control group in the case the BPL concentration was 0.25, 0.1, 0.5 or 0.025%.

4) Effects of Different Virus Samples on BPL's Inactivating Action:

To find out what effects different varieties of virus samples will have on BPL's inactivating action, four kinds of test fluid was prepared. They were:

1) A 30% emulsion of NDV-infected chick embryo made by means of a phosphate-buffered saline solution (PBS) of pH 7.2;

2) The same emulsion to which a NDV-infected allantoic fluid of chicken embryo was added;

3) An NDV-infected allantoic fluid of chick embryos only; and 4) Viruses taken from a NDV-infected allantoic fluid of chick embryos after the fluid was centrifuged at 59,000 G, and then resuspended in a phosphate-buffered saline solution (PBS) of pH 7.2.

To these fluids, BPL was added in five different concentration of 0.5, 0.25, 0.1, 0.05 and 0.025%. These processed samples

were left standing at 4°C and samples were taken them at certain intervals of time.

The results are shown on Table 5.

As indicated on Table 5, complete inactivation was attained in two hours when the BPL concentration was 0.025% in case of the simple 30% emulsion.

In the case of the 30% emulsion to which a NDV-infected allantoic fluid was added, inactivation was completed in two hours the BPL concentration was 0.25%, in two hours when that concentration was 0.1%, and in 24 hours when that concentration was 0.025%.

In the case of a NDV-infected allantoic fluid alone, inactivation was attained in two hours when the BPL concentration was 0.25%. But no inactivation was attained with the fluid when the BPL concentration was less than that percentage as far as the time range of the experiment was concerned.

Table 5

Inactivating effect of BPL on different types of virus materials

30% chicken embryo suspension

BPL % / Time (hours)	0.5	0.25	0.1	0.05	0.025	Cont.
2	0/4	0/4	0/4	0/4	0/4	4/4
5	0/4	0/4	0/4	0/4	0/4	4/4
10	0/4	0/4	0/4	0/4	0/4	4/4
24	0/4	0/4	0/4	0/4	0/4	4/4
48	0/4	0/4	0/4	0/4	0/4	4/4
72	0/4	0/4	0/4	0/4	0/4	4/4

30% chicken embryo suspension plus allantoic fluid

BPL % / Time (hours)	0.5	0.25	0.1	0.05	0.025	Cont.
2	0/4	0/4	4/4	4/4	4/4	4/4
5	0/4	0/4	2/4	4/4	4/4	4/4
10	0/4	0/4	0/4	4/4	4/4	4/4
24	0/4	0/4	0/4	0/4	0/4	4/4
48	0/4	0/4	0/4	0/4	0/4	4/4
72	0/4	0/4	0/4	0/4	0/4	4/4

Allantoic fluid

BPL % / Time (hours)	0.5	0.25	0.1	0.05	0.025	Cont.
2	0/4	0/4	2/4	2/4	4/4	4/4
5	0/4	0/4	2/4	2/4	4/4	4/4
10	0/4	0/4	2/4	3/4	4/4	4/4
24	0/4	0/4	2/4	2/4	4/4	4/4
48	0/4	0/4	2/4	4/4	4/4	4/4
72	0/4	0/4	2/4	2/4	4/4	4/4

Purified virus

BPL % / Time (hours)	0.5	0.25	0.1	0.05	0.025	Cont.
2	0/4	0/4	0/4	0/4	0/4	4/4
5	0/4	0/4	0/4	0/4	0/4	4/4
10	0/4	0/4	0/4	0/4	0/4	4/4
24	0/4	0/4	0/4	0/4	0/4	4/4
48	0/4	0/4	0/4	0/4	0/4	4/4
72	0/4	0/4	0/4	0/4	0/4	4/4

Numerator: Number of eggs infected ; Denominator: Number of eggs tested

In the case of the material resuspended in the PBS solution, complete inactivation was achieved, just as in the case of the 30% emulsion, in two hours when the BPL concentration was 0.025%.

As the result of these trial with the four different finds of NDV materials, it was confirmed that:

1) Inactivation may be attained more easily in case of a 30% emulsion of NDV-infected chick embryos and a purified virus sample (resuspended in a PBS solution);

2) Inactivation may be also attained in case of a 30% emulsion of NDV-infected chick embryo, to which a NDV-infected allantoic fluid of NDV-infected chicken eggs was added, although there was some variation in the time required depending on the BPL concentrations; but

3) Inactivation was difficult to attain in case of an NDV-infected allantoic fluid of chicken eggs alone when the BPL concentration was 0.1% or less.

II. NDV Absorption and Adjuvant Action of Aluminium Phosphate Gel:

Many studies have been reported on attempts to boost the immunization action of the inactivation action of the inactivated-virus vaccines of different varieties by the addition of some kind of adjuvant or other.

It is now generally believed that some kind of mineral

oil[94] or alum[44] makes an effective adjuvant. It is also known that a very good immunizing effect can be obtained when an aluminium phosphate gel is made to absorb the virus.

These discoveries are now widely applied to development of, and studies on, Newcastle disease vaccines.

As the adjuvant it was tried a kind of aluminium phosphate gel which is frequently used for purifying the diphtheria toxoid.

1) NDV Absorption Testing on Aluminium Phosphate Gel:

The undiluted aluminium phosphate gel was obtained by adjusting the original preparation of the compound chemical to 30 to 40 mg/ml in quantum.

Then this preparation was added in different concentrations of between 10 and 80%, to a 30% emulsion of NDV-infected chick embryos, to which an NDV-infected allantoic fluid of chicken eggs had been previously added.

After stirring the varying mixture throughly, these were left standing for three different periods of time---24, 48 and 72 hours.

Thereafter, these preparations were subjected to five minutes of centrifugation at 3,000 rpm and then the HA (hemag glutination) titers of their supernatant fluids were measured, obtaining results as given on Table 6.

Table 6

Adsorption of NDV on Aluminium phosphate ($AlPO_4$)

No.	Final conc. of $AlPO_4$ gel %	Hemagglutination titers 24 hrs. after	48 hrs. after	72 hrs. after	Adsorption rate of HA nin. (%)	Infectivity titer of 72 hours after
1	10	80	80	80	50	$10^{6.25}$
2	20	80	80	80	50	$10^{6.5}$
3	30	40	40	40	75	$10^{5.5}$
4	40	40	40	20	87.5	$10^{5.75}$
5	50	20	20	20	87.5	$10^{5.0}$
6	60	20	20	20	87.5	$10^{5.25}$
7	70	10	10	0	100	$10^{4.25}$
8	80	0	0	0	100	$10^{4.5}$
9	0	160	160	160	0	$10^{7.25}$
10	100	0	0	0	0	0

Now, a control group separately prepared by adding PBS instead of the aluminium phosphate gel registered 160 times in HA titer.

In contrast, the aluminium phosphate gel added preparations with 10 to 20% concentrations of the gel reached 80 times or half as much as the control group in the HA titer in 72 hours.

In case of a 30% gel concentration, the preparation reached

40 times in HA titer, and the preparations of 40 to 60% gel concentrations attained 20 times during the same period of time.

But the preparations of 70% or larger gel concentrations gave no HA titer.

The phenomenon of NDV absorption by the aluminium phosphate gel was found to occur with the preparations within 24 hours after the final mixtures were stirred and left standing. Hardly any difference was seen in respect to time.

Similar results were obtained from the preparations when they were applied to inactivated viruses.

The infectivity titer of the preparations when applied to developing chicken eggs was found to rise or fall according to the ratio of HA, that is, the less the gel concentration was, the higher was the titer, and the more the gel concentration, the lower the titer.

Specifically, it was found that the absorption of NDV by an aluminium phosphate gel increased in percentage according to the growth in the gel concentration, and that when the gel concentration was 50%, the amount of viruses decreased to less than 1/100th of the original level.

2) Adjuvant Action of aluminium Phosphate Gel:

In order to see if the addition of an aluminium phosphate

gel to the NDV vaccines will have any adjuvant effect, a number of chickens was immunized with the BPL-inactivated vaccines, to which an aluminium phosphate gel was either added or not added.

Specifically, this protection test against infection was conducted as follows;

First four different kinds of vaccine was prepared:

1) A BPL-inactivated vaccine, to which an aluminium phosphate gel was added to 50% in concentration;

2) A vaccine of the same kind, to which PBS instead of an aluminium phosphate gel was added to 50% in concentration;

3) A formalin-inactivated vaccine, to which an aluminium phosphate gel was added to 50% in concentration; and

4) A vaccine of the same kind, to which PBS was added to 50% in concentration.

Two different doses---1.0 ml and 0.1 ml---- of each vaccine were injected into many groups of 3-day-old chickens of 15 birds per group, through the thigh muscles.

After the lapse of 14 days from the injections, the chickens were given challenge infections with a NDV virus of 1,000 CID_{50}. They were then kept under observation for two weeks to determine the test results as given on Table 7.

Table 7

Adjuvant action of Aluminium phosphate ($AlPO_4$) gel

Type of vaccine \ Dose and route / Chicken No.	1.0 ml., i.m.					0.1 ml., i.m.				
	1	2	3	4	5	1	2	3	4	5
BPL inactivated NDV	S	S	S	S	S	D_5	S	S	D_6	S
BPL inactivated NDV plus 50% $AlPO_4$ gel	S	S	S	S	S	S	S	S	S	S
Formalin inactivated NDV	S	S	S	S	S	D_6	D_5	S	S	S
Formalin inactivated NDV plus 50% $AlPO_4$ gel	S	S	S	S	S	S	S	S	S	D6
Control (Unvaccinated)	D	D	D	D	D	D	D	D	D	D

Challenge: 14 days after vaccination
Challenge virus: 1,000 CID_{50}
S: surviving; D: dead (D_5 or D_6 means the date of death: days after challenge)

As illustrated above, all the four varieties of vaccine registered some protective action when the 1.0 ml dose had been used. But in case the 0.1 ml was used, only the BPL-inactivated vaccine, to which an aluminium phosphate gel was added, attained the complete 100% rate of protection.

The formalin-inactivated vaccine, to which the aluminium phosphate gel was added, showed only 80% in protection rate.

The BPL-inactivated and the formalin-inactivated vaccines, to which PBS was added instead of the aluminium phosphate gel, both attained a much lower protection rate of 60%.

It was thus confirmed that whether the vaccine is inactivated by BPL or formalin, an aluminium phosphate gel added to the vaccine has a decided efficacy as an adjuvant. It was also discovered that this is more clearly true with the BPL-inactivated type of vaccine than the formalin-inactivated type.

It was also studied about the neutralizing antibody titer of each of four different types of vaccine----a mineral oil adjuvant type, an aluminium phosphate gel type, an aluminium hydroxide gel type, and, as a control material, a centrifuged supernatant fluid of an emulsion of NDV-infected chick embryo---by immunizing chickens with these vaccines and holding a neutralizing test of the chickens' serum on developing chicken eggs.

The mineral oil adjuvant type vaccine was produced by mixing Bayol F and Arlacel A at the rate of 9 : 1, and, after sterilizing the mixture, adding to it an equal amount of a supernatant fluid of a 30% emulsion of NDV-infected chick embryos, and stirring the preparation by a homogenizer for five minutes into a complete vaccine.

The aluminium phosphate gel type vaccine and the aluminium

hydroxide gel type vaccine were produced by adjusting their original preparations to 30 to 40 mg/ml in quantum, and then adding an equal volume of a virus fluid to each preparation and stirring each preparation by a homogenizer for five minutes into a complete vaccine.

The control vaccine was produced by adding a 30% emulsion of NDV-infected chick embryos to an equal volume of PBS and adjusting the virus content in the preparation to the same level as the adjuvant vaccine.

For immunization, two 3-month-old cocks were used for each of the four kinds of vaccine after a 1.0 ml dose of each vaccine was given the cocks intramuscularly, serum samples were taken from the chickens at certain intervals of time. The serum samples were then inactivated for 30 minutes at 56°C and then challenged with a virus of 1,000 EID_{50}. The results are given on Table 8.

One week after vaccination, none of the vaccinetreated serums showed any neutralizing antibody. But two weeks after, all the serum, except the control vaccine group, registered some neutralizing antibodies.

Such neutralizing antibodies were recognized in serum even two months after vaccination in case of the oil adjuvant vaccine and the aluminum phosphate gel vaccine.

Table 8

Comparison of adjuvant action of oil adjuvant, aluminium phosphate gel, and aluminium hydroxide gel

Type of adjuvant	Collection of sample: Egg No. / Chick No.	Pre. 1	Pre. 2	1 week 1	1 week 2	2 weeks 1	2 weeks 2	3 weeks 1	3 weeks 2	1 month 1	1 month 2	2 months 1	2 months 2
Oil adjuvant *	1	+	+	+	+	-	-	-	-	-	-	-	-
	2	+	+	+	+	-	-	-	-	-	-	-	-
	3	+	+	+	+	-	-	-	-	-	-	-	-
	4	+	+	+	+	-	-	-	-	-	-	-	-
$AlPO_4$ gel	1	+	+	+	+	-	-	-	-	-	-	-	-
	2	+	+	+	+	-	-	-	-	-	-	-	-
	3	+	+	+	+	-	-	-	-	-	-	-	-
	4	+	+	+	+	-	-	-	-	-	-	-	-
$Al(OH)_3$ gel	1	+	+	+	+	-	-	-	-	+	+	+	+
	2	+	+	+	+	-	-	-	-	+	+	+	+
	3	+	+	+	+	-	-	-	-	+	+	+	+
	4	+	+	+	+	-	-	-	-	+	+	+	+
Control **	1	+	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+	+

* Freund incomplete adjuvant, Bayol F 9 : Arlacel A 1

** Adjuvant free

+: HA positive ; -: HA negative

But, in case of the aluminium hydroxide gel vaccine, neutralizing antibodies were observed in serums only as far as the end of the third week after vaccination.

This testing served to confirm that the aluminium phosphate

gel has a sufficient efficacy as an adjuvant.

III. Test on Efficacies of BPL-Inactivated Vaccine and Aluminum Phosphate Gel-Added Vaccine:

Having confirmed BPL's capacity to inactivate NDV and the adjuvant effect of the aluminium phosphate gel, now the preparation of the NDV vaccine was proceeded and the determine of its antigenicity, presevability, immunological durability and safety were tried.

1) BPL Contents relative to NDV's Antigenicity:

In order to find out the loss of the antigenicity of the vaccine due to BPL, the virus samples with BPL were inactivated by adding BPL to the samples in different concentrations, and then an aluminium phosphate gel was added to each preparation to 50% in concentration to the complete vaccine samples.

Each of these vaccine samples was planted on chickens to study its protective action against infection.

Specially, the original virus samples were inactivated at 4°C by BPL added to the samples in four different concentrations of 0.25, 0.1, 0.05 and 0.025%. After the disappearance of the infectivity in all the samples, an aluminium phosphate gel was added to each sample to make the complete vaccine preparations.

In immunizing chickens with these preparations, two groups of eight chickens was used each. One group was intramuscularly

given a 1.0 ml dose of the vaccine preparations per bird, and the other a 0.1 ml dose of the preparations per bird.

Ten days after vaccination, all the chickens were given a challenge infection with an NDV of 1,000 CID_{50}. The results determined by the birds' death or survival during a subsequent two week period of observation are given on Table 9.

Table 9

Antigenicity of vaccines inactivated with BPL of various concentrations

Vaccine dose and route / BPL %	1.0 ml., i.m.	0.1 ml., i.m.
0.25	0/8	2/8
0.1	0/8	0/8
0.05	0/8	0/8
0.025	0/8	0/8
Control	8/8	

Challenge: 10 days after vaccination

Challenge virus: 1,000 CID_{50}

Numerator: Number of chickens infected and died

Denominator: Number of chickens challenged

As is clear from the table, all the vaccines attained the 100% rate of protection against infection in case of the

chickens given a 1.0 ml dose injection.

The chickens given a 0.1 ml dose injection showed no difference in the rate of protection attained among different concentration of BPL between 0.1 and 0.025%.

But two of eight chickens given the vaccine with a 0.25% concentration of BPL died due to infection.

This results seemed to suggest that the BPL concentration of 0.25% or larger causes the loss of NDV's antigenicity.

Considering the fact that inactivation is difficult to attain with BPL of 0.01% content even in 36 hours, as indicated on Fig. 1, the safest BPL contents to use seem to lie in the 0.05 to 0.01% range.

Therefore a trial vaccine in 11 different lots was produced by inactivating the virus specimens with BPL at its apparently maximum safety concentration of 0.05% to experiment with the vaccine's protective against infection.

Using five 3-month-old chickens for each lot of the vaccine, a 1.0 ml dose of each vaccine preparation was planted on the chickens and 10 days thereafter, the birds were challenged with an NDV of 10,000 CID_{50}. While keeping these vaccinated birds under observation for two weeks, the results were determined by their survival or death as listed on Table 10.

Table 10

Preventative effect to infection of BPL vaccine inoculated into chickens

Group / Chicken No.	Vaccinated group					Unvaccinated control group				
Vaccine No.	1	2	3	4	5	1	2	3	4	5
1	N	N	N	N	N	D	D	D	D	D
2	N	N	N	N	N	D	D	D	D	D
3	N	N	N	N	N					
4	N	N	N	N	N	D	D	D	D	D
5	N	N	N	N	D					
6	N	N	N	N	N					
7	N	N	N	N	N					
8	N	N	N	N	N					
9	N	N	N	N	N	D	D	D	D	D
10	N	N	N	N	N					
11	N	N	N	N	N					

Challenge: 10 days after vaccination; challenge virus: 10,000 CID_{50}; N: Normal (Survived); D: Dead.

As is evident from the table, all but one of chickens given the Lot No.5 vaccine survived.

2) Test on Relations Between Virus Volumes and Immunogenicity in vaccines:

Up to now, as the principal vaccine preparation, a super-

natant fluid was used, with a 50% content of an aluminium phosphate gel, of a 30% emulsion of NDV-infected chick embryos, to which an NDV-infected allantoic fluid of chicken eggs was added, i.e., a 15% emulsion of the virus (chick embryo) emulsion.

Now, for this new test, the percentage of this virus emulsion was modified into four densities of 15, 10, 5 and 2.5%, and all the samples were inactivated by adding BPL to 0.1% in concentration.

The protective capacity of each of these preparations against infection and its HI (hemagglutination-inhibition) titer were studied by planting a 1.0 ml dose of each of these preparations on 6-week-old chickens.

Ten days after vaccination, the chickens were challenged with a virus of 10,000 CID_{50}.

The serums used in this HI test were extracted immediately before the challenge infections. The results of this test are given on Table 11.

As the table shows, there was no appreciable difference among emulsions of 15 to 25% in density as far as the vaccine's protective action against infection was concerned.

But, in respect to the HI titer, there was wide variation between 15 and 2.5% in emulsion density.

Table 11

Comparison between the Hemagglutination-inhibition (HI) antibody titer and protective action in chickens inoculated with various dosis of vaccine

% of infected chicken embryo suspension in vaccine	HI titer immediately before challenge Chick No.						Results of challenge Chick No.					
	1	2	3	4	5	6	1	2	3	4	5	6
15	32	64	32	32	64	64	S	S	S	S	S	S
10	64	64	64	32	32	64	S	S	S	S	S	S
5	32	16	16	16	16	16	S	S	S	S	S	S
2.5	16	16	16	16	16	8	S	S	S	S	S	S
Control	N. D.						D_4	D_3	D_4	D_3	D_4	D_4

Challenge: 10 days after vaccination; Challenge virus: 10,000 CID_{50} ; S: Surviving ; D: Dead (D_4 or D_3 means the date of death: days after challenge)

Among emulsion of 15 to 10% in density, there was still scarcely any difference, as they invariably registered 32 times or more of HI titer. But emulsions of 5 to 2.5% in density showed only 8 to 16 times of HI titer, or decidedly lower than the 15 to 10% group.

This results apparently suggested that emulsions of 10% or more in density are ideal for making good vaccines.

3) Test on Times Required for Immunization and Periods of Immunological Duration:

A number of chickens vaccinated by the Lot No.1, 9 and 11 of the vaccines were tested to find out the times required for immunization and the periods of immunological duration with the vaccines.

Some 5-month-old hens and some two-week-old cocks were used for testing the vaccines' protective action against infection, neutralization of the chickens' serums in developing chicken eggs and their HI titers.

Results of this test on the vaccines' protective action against infection with the 5-month-old hens are listed on Table 12.

After the lapse of different periods, as given on the table, from vaccination of the chickens with a 1.0 ml dose each of the three different lots of the vaccine, the birds were challenged with a virus of 1,000 CID50.

During a subsequent 10-day period of observation, the chickens' death or survival was registered to determine the results of this testing. It was thus discovered that none of the vaccines developed any protective action at least seven days after vaccination.

Table 12

Preventative effect to infection of BPL vaccine inoculated into 5-month-old chicken

Test lot No.	Immunization period 7 days	14 days	1 month	2 months	3 months	4 months	7 months
1	5/5	0/5	0/5	0/5	0/5	0/5	0/5
9	5/5	4/5	0/5	0/5	0/5	0/5	0/5
11	5/5	0/5	0/5	0/5	0/5	0/5	0/5
Control	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Challenge virus: 1,000 CID_{50}

Numerator: Number of chickens infected and dead

Denominator: Number of chicken challenged

In contrast, all the vaccines but only Lot No.9 were found to have developed a sufficient protective action to make the chickens survive a challenge infection 14 days after vaccination.

One month after vaccination, all the vaccines without a single exception proved to have attained enough protective action to save the chickens.

Furthermore, even after the lapse of seven months from vaccination, the vaccines were found to be still effective enough to make the chickens survive a challenge infection.

It was tried a neutralization test on serums of vaccinated chickens in developing chicken eggs by injecting a 1.0 ml dose

of the Lot No.1 of the vaccine into muscles of five-month-old chickens.

Serum samples were taken from the chickens during a period of nine months thereafter. Each serum sample collected was inactivated for 30 minutes and then was mixed with an equal volume of a virus solution of 1,000 EID_{50}. The mixture, after being left standing for 30 minutes at 37°C to allow the virus to work on the serum, was planted into the allantoic cavity of 11-day-old developing chicken eggs.

The eggs were then incubated for 72 hours and the HA titers shown by their allantoic fluids were studied to determine the final results, which are shown on Table 13.

As illustrated on the table, no neutralizing action was seen in any mixed serum sample one week after injection. But all serum samples showed a neutralizing action at all times from two weeks after to nine months after injection.

Table 13

Neutralization test in embryonated eggs, inoculating with virus and immune serum of vaccinated chickens

Group	Chicken No.	Immunization Period												
		Pre.	1W	2W	3W	1M	2M	3M	4M	5M	6M	7M	8M	9M
Vaccinated group	A-1521	4/4	4/4	3/4	0/4	0/4	4/4	3/3	4/4	3/3	4/4	4/4	3/3	4/4
	A-1522	4/4	4/4	0/4	0/4	0/4	0/4	0/3	0/4	0/3	0/4	0/4	0/4	0/4
	A-1525	4/4	4/4	0/4	0/3	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/3	0/4
	A-1528	4/4	4/4	0/3	0/4	0/4	0/3	0/3	0/4	0/4	0/4	0/3	0/3	0/4
	A-1529	4/4	4/4	0/3	0/4	0/4	0/3	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	A-1531	4/4	4/4	0/4	0/3	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Unvaccinated control group	A-1526	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
	A-1532	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4

Numerator: Number of eggs infected

Denominator: Number of eggs tested

Challenge virus: 1,000 EID_{50}

Pre: Pre-vaccination; W: week; M: month

It was also assembled an equal volume taken from each of these serum samples at each of five different times, one week, two weeks, one month, three months and five months after injection. Each sample of this pool was divided into five dilutions of 1 : 10, 1 : 50, 1 : 100, 1 : 250 and 1 : 1,000. All these dilutions were used for a series of neutralizing tests as described above. Table 14 below represents the results.

Table 14

Neutralization test in embryonated eggs, inoculating with virus and immune serum of vaccinated chickens

Serum dilution	Immunization Period				
	1W	2W	1M	3M	5M
1 : 10	1/5	0/5	0/5	0/5	0/5
1 : 50	5/5	0/5	0/5	0/5	0/5
1 : 100	5/5	0/5	0/5	0/5	0/5
1 : 250	5/5	2/5	5/5	5/5	5/5
1 : 1,000	5/5	5/5	5/5	5/5	5/5

Numerator: Number of eggs infected

Denominator: Number of eggs tested

Challenge virus: 10,000 EID_{50}

W: week ; M: month

As the table shows, none of the diluted serum samples registered any neutralizing action one week after planting in allantoic cavity.

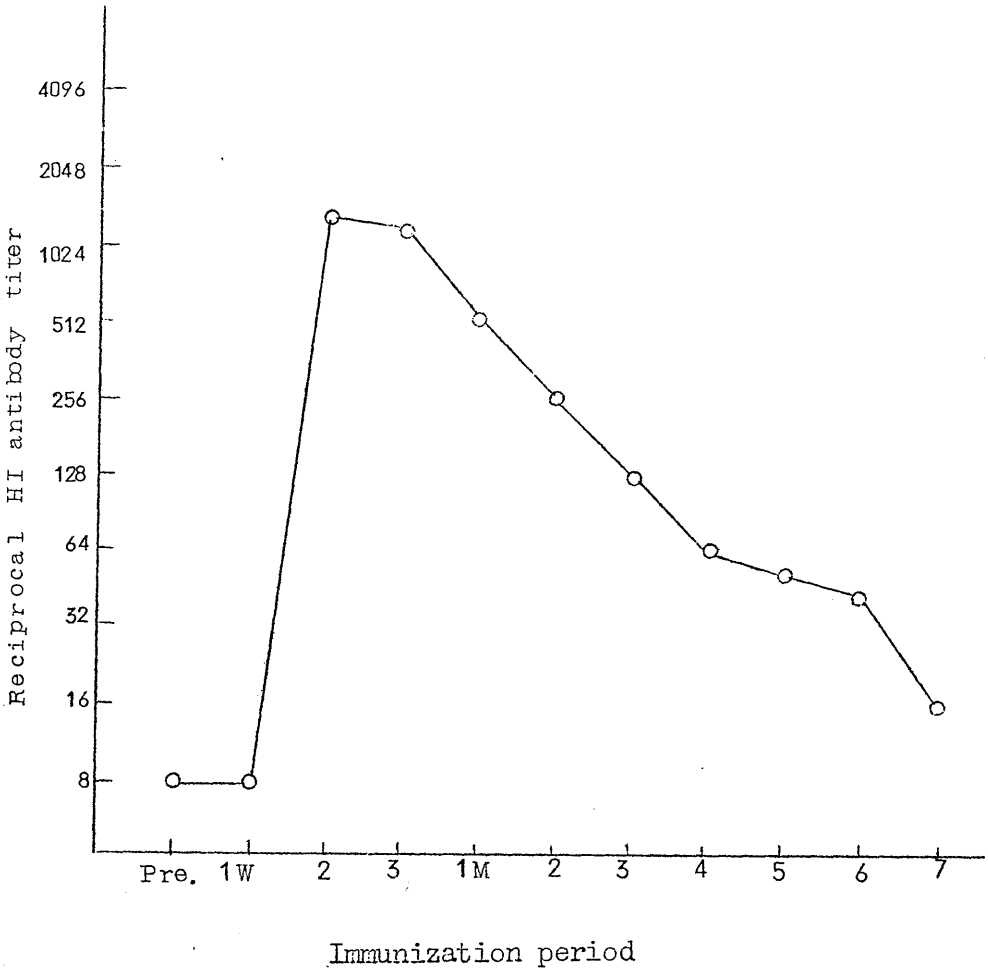
But, all serum samples collected between two weeks and five months after planting had more or less neutralizing in case of 1 : 10, 1 : 50, 1 : 100 ratios.

The HI titers of these serums were measured at different times after planting and obtained results by their average titers as illustrated on Fig. 2.

As evident from the figure, the HI titers of the one-week immunological group showed no development. But in the case of the two-week group, the titer was found to have developed to 1,024 times. From one month after planting or thereabout, however, the titer was found to start declining until it was down to almost the same level as before vaccination 7 months after.

Fig. 2

Development and decline of Hemagglutination inhibition antibody titer in chickens after vaccination



In the case of two-week-old chicks, the study was made on serum samples taken at different times after intramuscular injections of the vaccine.

Table 15

Development of immunity in 2-week-old chickens inoculated with BPL vaccine

Test Vaccine Lot No.	Immunization period (days) 5	7	10	14	21	28
9	10/10	10/10	0/10	0/10	0/10	0/10
10	10/10	9/10	0/10	0/10	0/10	0/10
11	10/10	10/10	0/10	0/10	0/10	0/10
Control	10/10	10/10	10/10	10/10	10/10	10/10

Challenge virus: 1,000 CID_{50}

Numerator: Number of chickens infected and dead

Denominator: Number of chickens challenged

The table above represents the results of a protection test against infection when immunized chickens were challenged with a virus of 1,000 CID_{50}. As the table shows, no protective action against infection was observed seven days after vaccination. But in case of 10 days or longer after vaccination, all remaining chickens survived a challenge infection.

Incidentally, a similar protection test was held by changing the volume of the challenge viruses, obtaining results as shown on Table 16.

Table 16

Development of immunity in 2-week-old chickens inoculated with BPL vaccine; challenge with different dosis of virus

Test vaccine lot No.	Dosis of challenge virus	Immunization period (days) 5	7	10	14	21	28
10	10^3	5/5	5/5	0/5	0/5	0/5	0/5
	10^4	5/5	5/5	0/5	0/5	0/5	0/5
11	10^3	5/5	5/5	0/5	0/5	0/5	0/5
	10^4	5/5	5/5	0/5	0/5	0/5	0/5
Control	10^3	5/5	5/5	5/5	5/5	5/5	5/5
	10^4	5/5	5/5	5/5	5/5	5/5	5/5

Dosis of vaccine inoculated: 0.2 ml/chick, intramusculary

Numerator: Number of chickens infected and dead

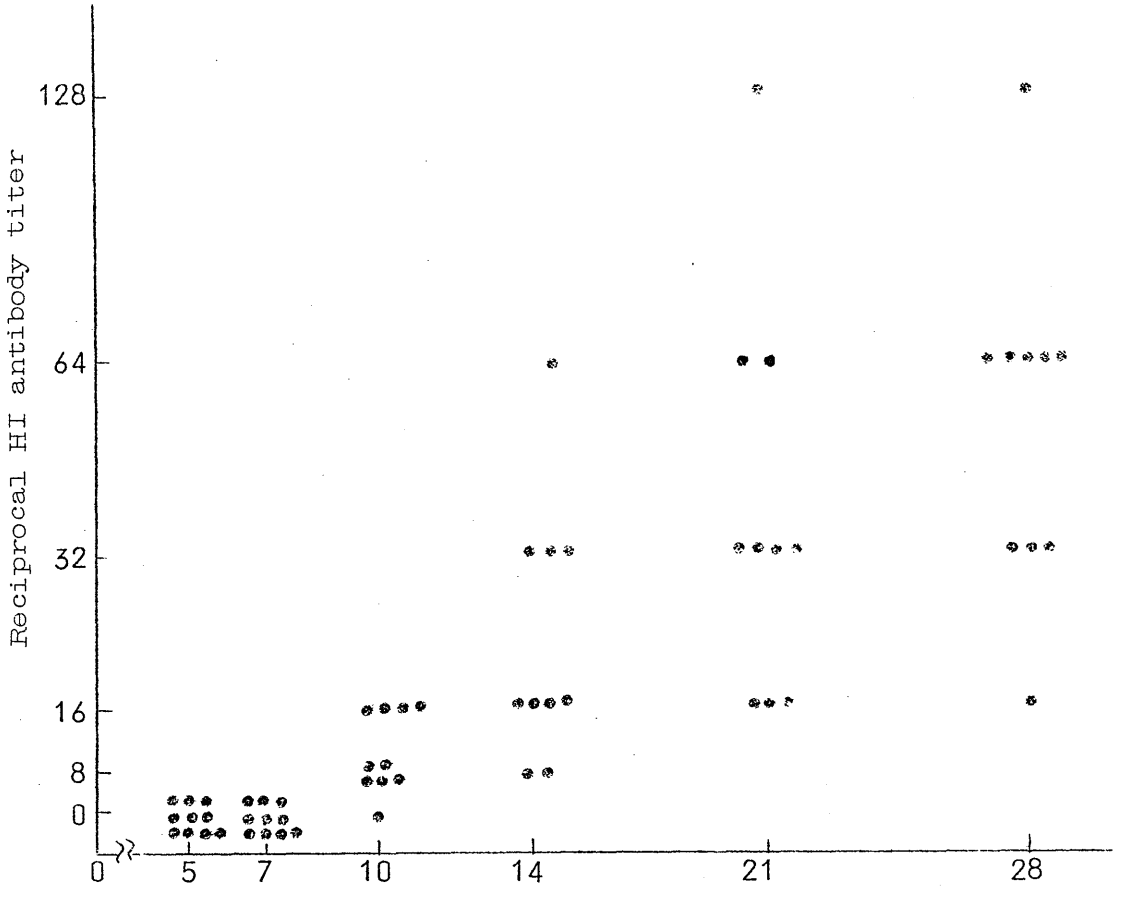
Denominator: Number of chickens challenged

As indicated by the table, all chickens were found to have survived a challenge infection with viruses whether or 1,000 CID_{50} or 10,000 CID_{50} in case of 10 days or longer after vaccination.

Rates of development of the HI titer also studied are given on Fig. 3.

Fig. 3

Hemagglutination inhibition antibody response in chickens inoculated with BPL vaccine (Test Lot No.9)



As is clear from the figure, hardly any development of the HI titer was observed up to seven days after vaccination, but development was seen from 10 days after vaccination.

However, the HI titer was found to hit its peak two weeks after vaccination in case of five-month-old chickens and then start to decline, and to keep developing gradually in case of two-week-old chicks.

These results apparently indicated that immunization usually starts about 15 days after vaccination.

As for the period of the immunological duration of the vaccines, the protective activity was found to remain at least as far as seven months after vaccination, although the study did not go longer as far as the immunological duration is concerned. The neutralizing test of vaccines in developing chicken eggs shows that the antibodies remained in serum until nine months after vaccination.

4) Test on Vaccine Preservability:

The vaccine preparation was preserved, dividing into a number of 100 ml portions for different periods of time at $4^{\circ}C$, and then a 1.0 ml dose of each portion was planted into the muscles of some 3-month-old chickens.

Fourteen days after vaccination, all the chickens were challenged with a virus of 10,000 CID_{50}.

During a subsequent 10-day period of observation, the results were determined by the chickens' death or survival as shown on Table 17.

Table 17

Immunogenicity of BPL vaccine stored at 4°C

Test Vaccine Lot No.	Time stored at 4°C (months)					
	3	5	7	10	14	17
1	0/6		0/6		0/6	0/6
2		0/6		0/6		
3	0/6		0/6			
4		0/6		0/6	0/6	1/6
5	0/6		0/6			
6		0/6		0/6	0/6	
7	0/6		0/6			
8		0/6		0/6	0/6	0/6
9			0/6			1/6
10	0/6			0/6		
11		0/6			0/6	

Numerator: Number of chickens infected and dead

Denominator: Number of chickens challenged

As the table shows, the chickens acquired the 100% immunity in case of the vaccine sample preserved for 14 months. But, even the sample preserved for 17 months still retained sufficient immunizing capacity, although there was some decline in its antigenicity.

A similar test at a preservation temperature of 37°C brought results as given on Table 18.

Table 18

Immunogenicity of BPL vaccine stored at 37°C

Test vaccine lot No.	Time stored at 37°C (days)	
	7	21
7	0/5	0/5
8	0/5	0/5
11	0/5	0/5
11 $AlPO_4$ gel free	5/5	5/5

Numerator: Number of chickens infected and dead

Denominator: Number of chickens challenged

Specifically, the vaccine samples were preserved in two groups for one week and for three weeks respectively both at 37°C. Then, 1.0 ml dose each of the samples were planted into the muscles of chickens.

Fourteen days thereafter, the chickens were challenged with a virus of 10,000 CID_{50}. and their death or survival during a subsequent 10-day period of observation was registered as the test results.

A similar test was tried with the vaccine samples, to which no aluminium phosphate gel was added.

It was found that all the vaccine preparations proved effective after preservation both for one week and for three weeks at 37°C. But, in case no aluminium phosphate gel was added, the vaccine samples were found to have lost their efficacy even after only one week of preservation.

This finding showed that the aluminium phosphate gel not only is effective as an adjuvant, but has a clear protective action in preservation of vaccines.

5) Test on Vaccine Safety:

The observation continued for a period of seven days on the clinical reaction of the chickens to vaccinations with various lots of the vaccine brought no abnormal finding.

Lastly, the effects of the vaccinations was studied on the laying eggs. The chickens used in this testing were white leghorns which had been used for assaying egg—laying capacities of chickens at a certain Japanese chicken farm.

Two groups of 18 of these chickens were prepared each,

and kept them under one month of oviparous observations.

To each of the 18 chickens of one group a 1.0 ml dose of the vaccine was given intramuscularly. The other group was kept as a control group.

After another month of observation, it was found that the vaccinations had no effect whatever on the chickens' oviposition as shown by the results given on Table 19.

Table 19

Effect on egg production of hens inoculated with BPL vaccine

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Vaccinated group	11	10	11	9	13	13	8	13	15	12	13	13	15	9	14
Invaccinated Control group	11	15	12	11	12	15	14	11	10	11	11	7	13	12	7

Days	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Vaccinated group	12	13	11	10	12	9	10	11	11	10	10	14	12	10	11
Invaccinated control group	12	12	8	11	12	10	12	10	7	12	11	11	6	13	12

Vaccination 1.0 ml., i.m.

Days	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Vaccinated group	13	9	10	11	10	12	8	12	12	10	13	10	11	12	12
Invaccinated control group	14	12	9	14	13	9	9	14	13	9	12	12	8	12	8

Days	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Vaccinated group	12	10	9	13	8	11	10	7	10	10	10	11	11	12	9
Invaccinated control group	11	10	12	8	8	11	11	10	11	11	12	13	9	11	11

18 of White Leghorn hens were used per group

Considerations

It is generally believed that every virus particles, including that of the Newcastle disease virus, has a special architecture composed basically of protein and nucleic acid, and in addition, such other components as lipid and saccharide.

What is generally called 'inactivation' means a phenomenon of a certain deterrence caused in various biological activities of the virus particle by the action of some kind of physical or chemical agent on the fixed structure or components of the virus particle.

The viral biological activities are known to be manifold and exist in the form of in many properties such as infectivity, interference activity, HA activity and antigenic structure. It is also well known that some of these viral properties react to physical or chemical factors rather easily, while other do not.

An ideal inactivating chemical agent visualized by virologist in general is something which gives the least possible hindrance to the immunogenicity of the virus to be used in the production of a vaccine. Numerous inactivating agents have so far been used in attempting to produce virus vaccines of different types. But none seems to have proved so good as to be really worth calling idea.

Since it was first reported by LoGrippo & Hartman[60] in 1955 as effective in inactivating viruses, BPL has been widely recognized for its good inactivating capacity on many varieties of virus.

It has recently attracted more attention as a promising organic compound with such marked advantages as being very quick to inactivate viruses, losing all unnecessary inactivating action through hydrolysis, and being usable without regard to pH.

The author have also utilized BPL for inactivating his NDV viruses, and produced his own BPL vaccine with the addition of an aluminium phosphate gel as an adjuvant, scoring very satisfactory results by applying the vaccine to his trial to prevent the NDV infection.

While the inactivation of NDV with BPL will naturally change in attainable degree depending on the contents, that is, concentrations of BPL in the vaccine, it was confirmed that the concentrations of BPL in the vaccine also have varying effects on the hemagglutination (HA) titer in the serums of the subjects (Table 6). The author is confident this finding will pose an important problem concerning the immunogenicity of vaccines against NDV.

Speaking of the relationship between pH and inactivating agents, it is difficult to attain inactivation and produce a

vaccine retaining a certain adequate level of immunogenicity if the inactivating agent to be used is something susceptible to pH in its action, since it is impossible to obtain a fixed level of pH by the choice of virus materials.

As shown on Table 3, BPL seems to be highly stable against pH and to suggest the possibility of producing a vaccine with an extremely well stabilized level of immunogenicity. As for the inactivating temperatures, it also seems, as indicated by Table 2, that a vaccine of a very stabilized level of immunogenicity may be produced even under different temperatures from the normal level for such vaccine production if the BPL concentrations are propertly adjusted.

In inactivating NDV with BPL, It was also discovered that some difference occurred in inactivating action in the minimum BPL concentration depending on virus materials used.

As illusted by Table 5, there was such difference to be seen between the virus specimen suspended in a PBS (phosphate buffered saline) solution and that in the allantoic fluid, and also between the virus specimen suspended in a PBS solution plus the allantoic fluid and that in a simple allantoic fluid.

This results seems to suggest that there is some property in the allantoic fluid that deters the action of BPL.

A similar test had been held by Polly & Guerin[80] with

the influenza virus. They had reported that BPL shows some changes in its inactivating action between such different cases as when the allantoic fluid is dialysed, when the allantoic fluid is used as it is, and when the allantoic fluid is subjected to a freezing and thawing process. This should also testify to the author' belief that BPL's inactivating action varies according to virus materials.

Taking these into account, the author preferred as the virus material the supernatant fluid of a centrifuged mixture of a 30% PBS emulsion of infected chick embryo and the allantoic fluid of the same embryos.

This virus material of mine seems to facilitate inactivation better than the allantoic fluid alone.

As indicated by Fig. 1, inactivation was difficult to attain with BPL at 0.01% in concentration, but was quite possible to attain with BPL at 0.025% in concentration.

Furthermore, judging from the results shown by Table 6, a degree of BPL concentration which had no effect on the HA action of the serum was considered the safest level for inactivation.

Thus, 0.05% was preferred as apparently the best and most practicable degree of BPL concentration for production of NDV vaccines.

Gill & Stone(30) had employed whole eggs minus their albumin

as their virus materials and produced and applied a BPL vaccine of 0.1 % in concentration. Mack & Chotisen[62, 63] used the allantoic fluid as their virus material and chose 0.025% as their best degree of BPL concentration in their vaccine.

The author's experiment suggested that such concentrations of BPL were inadequate for inactivation, but such variance in preference of BPL concentrations could not be explained by the testings as to whether it is attributable to difference in inactivating temperature, in the quality of BPL samples or other factors.

The immunogenicity of inactivated virus vaccines against Newcastle disease has been studied by many researchers with many different results, but it is generally recognized that the addition of some adjuvant boosts the immunizing actions of such vaccines.

Haddow & Idnani[32] have reported that by adding an aluminium hydroxide gel to their vaccine, they could attain a long period of immunization. Nakamura et al.[74] have also reported that the addition of an aluminium hydroxide gel to a formalin-inactivated virus vaccine produced a very strong immunizing effect. Ishida et al. [44] have reported that alum added to their vaccine as an adjuvant proved quite effective.

Now, an ideal method to prepare such a vaccine is to con-

centrate the virus specimen first and then add some adjuvant. But such a concentration seems impracticable in view of difficulties in obtaining a proper virus material as well as the cost of the process.

Under the circumstances, It was preferred an aluminium phosphate gel for the adjuvant. Now the adjuvant action of the this particular gel is said to rise in direct proportion to the rate of gel's absorption of the HA elements in the serum. This should suggest at the more the amount of gel to be added, the better will be the result.

However, as indicated by Table 6, attainment of the 100% absorption of the HA elements would require the addition of gel up to no less than 70% of the total volume of the vaccine. Such a density of gel in the vaccine will not only make the vaccine too sticky for use, but will pose a possible danger of a local reaction.

It follows that a better alternative in obtaining a vaccine with a high immunological capacity most free from reactions possibly lies in purifying the virus material to lessen all absorptive matters other than the antigenic component and thereby enhance the absorptivity of the antigenic component.

The author derived his virus material from the supernatant fluid of his virus preparation in order to eliminate all in-

herent matters in the chick embryos which have nothing to do with the antibody production for the purpose of attaining the highest possible absorptivity of the antigenic component in his preparation. Besides, his gel was purified by centrifugation to boost the absorptivity of the antigenic component of the preparation to produce the final vaccine.

Meanwhile, the author chose 4°C as his inactivating temperature in order to minimize the loss of the antigenicity in his vaccine. BPL's half-life in its solution at 4°C is believed to be somewhere between 16 and 20 hours (58).

BPL is known to lose its virucidal action when it turns into beta-oxy-propionic acid through hydrolysis. It was thus assumed that the temperature of 4°C was better for keeping all surplus volume of BPL at a certain level of potency without the loss of its antigenicity.

As given on Table 18, it was discovered that when the vaccine sample to which an aluminium phosphate gel was added and another to which no aluminium phosphate gel was added were preserved at 37°C, the former retained its immunological action, but the latter lost it. It was also found that some of the vaccine samples preserved at 5°C retained their immunological capacity as long as 17 months. This seems to indicate that such a gel works well not only as an adjuvant, but as a pro-

tector of the antigenicity of vaccines.

As to the time the immunization of the NDV vaccine starts, Nakamura et al.[74] have reported that it varies according to the age of chickens. They said that grown-up chickens usually acquire immunity about one week after vaccination, while some chicks acquire it more slowly depending on individual cases, but normally, the younger their age, the slower their acquirement of immunity.

The author's experiment has indicated that grown-up chickens will acquire immunity about two weeks after vaccination and chicks of two weeks of age in about 10 days. However, the time required for chicks' acquirement of immunity seems to need more studies.

The normal period of duration of immunity of the NDV vaccines is generally believed to be several months. But longer periods should be naturally desirable. In the author's studies, he has gone as far as seven months in checking the duration of his vaccine's immunity and confirmed that all his chickens, except those dying during processing, survived a challenge infection even at the end of that period, as shown on Table 12.

In respect to neutralizing antibodies, it was confirmed that the chickens retained them as long as nine months after vaccination.

Effects of vaccines on the oviposition of the chicken should be a question of particular importance in dealing with the accessory reactions of every attempt at vaccination against chicken epidemics.

Vaccination a carefully timed before the regular period of oviposition may be sometimes the answer to the problem. But when a Newcastle disease epidemic hits, it will be often necessary to vaccinate hens even during their period of oviposition. Chicken farmers will be commercially placed at a big disadvantage if a vaccination on egg-laying chickens affects their birds' egg production.

Bankowsky et al.[17] have reported that a decline in oviposition is unavoidable with the use of inactivated -virus vaccines, but it is possible if a tissue culture vaccine is used. But, if such a live-virus vaccine is to be used, it must be taken into consideration that depending on the degree of attenuation of the virus, there will be more or less ill effects on the health of chickens.

The author's experiment has convinced him that, as indicated by Table 19, that inactivated-virus vaccines, as far as his own version is concerned, are an excellent means of combating Newcastle diseass without any appreciable ill effect on oviposition.

All these considerations have led him to the conclusion that a Newcastle disease vaccine experimentally prepared is a very good kind with a high degree of stability and antigenicity in view of such special processes of production as: 1) The action of the allantoic fluid to deter inactivation was alleviated by diluting the fluid with a chick embryo emulsion; and 2) The adjuvant action of an aluminium phosphate gel added to the vaccine was increased by improving its absorption of antigenic materials through the previous elimination of surplus alubumin and order unnecessary components of the chick embryo emulsion by mean of centrifugation.

Conclusion

The following conclusion was obtained after the experimental production and application of a new variety of Newcastle disease vaccine.

The vaccine was produced from the virus material consisting of chick embryo infected with the Sato strain of the Newcastle disease virus (NDV) and the supernatant fluid of a centrifuged emulsion of their allantoic fluid and by inactivation of the viruses with beta-propiolactone and addition of an aluminium phosphate gel as an adjuvant.

1) The minimum concentration of BPL necessary for inactivating NDV was 0.025%, while the concentration of BPL adequate enough for inactivation of NDV without affecting the erythrocytic hemagglutination action of the virus was 0.1%. BPL's inactivating action was free from the effects of pH, but was effected by some virus materials depending on their characters.

2) Absorption of viruses by an aluminium phosphate gel increased according to the densities of the aluminium phosphate gel, and the increase in the rate of that absorption enhanced the immunizing action of the vaccine. An aluminium phosphate gel also is effective in the preservation of the vaccine.

3) The time required for the BPL-inactivated virus, an

aluminium phosphate gel-added Newcastle disease vaccine to attain immunity in chickens was approximately 10 days in case of chicks and about two week in case of grown-up chickens as shown by signs of their acquirement of immunity after vaccination. The vaccine applied once to chickens gives them at least seven months of immunity.

4) The vaccine may be effectively preserved for a period of at least 17 months.

5) Application of the vaccine to chickens had no ill effect either on chicks or grown-up chickens, neither did it have any adverse effect on the chickens' oviposition.

Summed up, the Newcastle disease vaccine produced by inactivating a 30% emulsion of NDV-infected chick embryos and the supernatant fluid of a centrifuged solution of their allantoic fluid with a 0.05% solution of Beta-propiolactone (BPL) and adding a 50% aluminium phosphate gel, is a good vaccine perfectly safe to use, preservable for a long period of time, and highly effective in imparting immunity to the chickens of all ages.

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ニューカッスル病ワクチンに関する研究
特にBeta-Propiolactone 不活化，ならびに燐酸アルミニウムゲル添加によるワクチンの効力について。

五十嵐　義　輝

ニューカッスル病ワクチンに関しては、本病が1つの独立した疾病として認められて以来、生ワクチン、不活化ワクチンともほぼ同時期に開発され、今日までに種々研究されている。両者の長短を比較するのにはまだ数多くの難点があるが、従来生ワクチンの長所と云われてきた点は、免疫力が強く持続性の長いこと、投与法に容易さのあること、および米国やその他の諸国で長年余に亘つて使用されてきているので有効であるらしい、などの点である。しかし生ワクチンの免疫力の強さや持続性の長いことは、病原性のmildなウイルス株（lentogenic strain）から作成された場合にのみあてはまり、逆にこのようなmildな株ではワクチン接種それ自身で発病するものがみられたり、他のニワトリへ病気を伝播することが知られている。また投与法についても、飲水法やエロゾール法のように水に溶かして投与する場合、ニワトリ個体による投与法の不確実性など、使用に際して最大の注意が払われなければならないし、このような方法で投与された場合、ワクチンウイルスの気道感染によつてCRD（Chronic respiratory disease）など、他の呼吸器疾患を誘発する可能性もある。さらに生ワクチンの場合、ワクチン材料中に迷入した他の病原体をワクチン自身が伝播する危険性もある。またニューカッスル病ウイルスは元来、変異性の顕著なものであるため、生ワクチンに用いられている弱毒ウイルスがその病原性を復帰しえないと云う保

証がない。以上の点を不活化ワクチンの場合にあてはめてみると、不活化ワクチンでも使用法が適切であれば、差がみられないし、何よりも安全であると云う点である。すなわちニワトリのageに関係なく使用でき、接種によつてワクチンそれ自身による感染を起したり、その感染を他のニワトリに伝播させる危険性もないし、ワクチン材料中に含まれる可能性のある他の病原体を伝播することもない。さらに不活化ワクチンは個体別に接種されるため、接種量が厳格に規定でき、また筋肉内注射によることが多いため、ＣＲＤその他の気道感染症を誘発することもない。

不活化ニューカッスル病ワクチンについては、本病が独立した疾病であることが認められて以来、種々研究され、いろいろなタイプのワクチンが作成されている。不活化剤としては、クリスタル・ビオレット、紫外線の照射、ホルマリンなどが主として用いられてきたが、１９５５年Hartman らによつてβ－Propiolactoneにウイルス不活化作用が報告されて以来、種々のウイルスの不活化に応用され、ニューカッスル病ワクチンでは、Mack およびChotisen（１９５５）によつて始めて応用され、以来多くの研究者によつてＢＰＬによるニューカッスル病ウイルスの不活化について検討されてきている。ＢＰＬは短時間で完全な不活化ができること、不活化は広いｐＨ域内で行うことができ、さらに残余ＢＰＬはすみやかに加水分解してβ－oxy－Propionic acidとなり不活化作用が失われるため、残余ＢＰＬによる抗原性の損失を防ぐことができる。などの利点があり、これらの点はすべて不活化ウイルスワクチン作成のために好適である。またＢＰＬは、ホルマリンや石炭酸のごとき蛋白変性剤とは異り、ウイルス粒子の外被蛋白に対する作用が弱いため、抗原性蛋白はほとんど変化のないままに保持され、高度の抗原性を有するワクチンの作成が可能であると云われている。日本に於ては不活化ワクチンの作成に不活化剤としてＢＰＬを使用した報告は少くわずかに狂犬病ワクチンの試作および日本脳炎ワクチンの不活化実験にホルマリンなどとの比較実験に使用されているにすぎず、いずれもまだ実用の域に達していない。

著者は不活化剤としてＢＰＬを使用し、アジュバントとして、燐酸アルミニウムゲルを使用したワクチンを作成して、以下のごとき種々の実験を試みた。

すなわちニューカッスル病ウイルス（ＮＤＶ）佐藤株に感染した鶏胎児の３０％ＰＢＳ乳剤にその感染漿尿液を加えて遠心した上清をウイルス材料とした。これに対するＢＰＬの不活化作用をしらべるとともに、アジュバントとしての燐酸アルミニウムゲルの作用を検討し、さらにこのようにして作成されたワクチンの抗原性、ニワトリに接種した場合の免疫原性、免疫の持続期間、安全性、およびこのワクチンの保存性について検討した。

ＮＤＶの不活化に必要なＢＰＬの最少有効濃度は３７℃および４℃２４時間の不活化で、いずれの場合にも0.０２５％であつた。４℃不活化の場合最少濃度が0.１％以下であれば赤血球凝集素に対する影響はみられなかつた。このような不活化はウイルス材料のｐＨが6.０～8.０の範囲内であれば、３７℃、４℃の場合とも、ほとんど差がみられなかつたが、用いるウイルス材料により差がみられた。すなわち３０％ニワトリ胎児乳剤、それに漿尿液を加えたもの、および精製ウイルスの場合には不活化が容易に行われるが、漿尿液のみの場合、0.１％以下のＢＰＬ濃度では不活化は不完全であつた。なお、0.２５％以上のＢＰＬ濃度では抗原性が損われるため、安全な濃度域は0.０５～0.１％と考えられる。

ウイルスの燐酸アルミニウムゲルの吸着は、ゲル濃度に比例して高まり、ゲル濃度が１０～２０％では５０％、３０％では75 ~~５０~~％、４０～６０％では８7.５％、および７０％以上では１００％のウイルスが吸着することが認められ、ワクチンの免疫能を増強することが解つた。さらにホルマリン不活化したウイルス材料とＢＰＬ不活化したウイルス材料とについて、燐酸アルミニウムゲルを添加したものと、添加しないものとをニワトリに接種して、その感染防禦能を比較したところ、ＢＰＬ不活化－燐酸アルミニウムゲル添加ワクチンを接種した場合には１００％、ホルマリン不活化－燐酸アルミニウムゲル添加ワクチンでは８０％の防禦率がみられた。それに対し、ゲルを添加しなかつた場合にはいずれも６０％の防禦率を示した。このことからＢＰＬ不活化の場合でも、ホルマリン不活化の場合でも、燐酸アルミニウムゲルのアジュバント効果が認められた。さらにこの結果からＢＰＬ不活化の方が、ホルマリン不活化したものよりも免疫原性の強いことが認められた。また燐酸アルミニウムゲルは、水酸化アルミニウムゲルよりもアジュバント効果が

強く、油性アジュバント（Freundの不完全アジュバント）とはほぼ同程度であつた。

上記のごとくＢＰＬで不活化し、アジュバントとして燐酸アルミニムウゲルを添加したニューカツスル病ワクチンの免疫発現時期はヒナでは１０日前後、成鶏では２週間前後であり、このワクチンの１回接種によつて、少くとも７ケ月間の感染防禦能が認められ、中和抗体は少くとも９ケ月間残存した。

１００mℓづつバイアルびんに分注して４℃および３７℃保存したワクチンをふ化３ケ月令のニワトリに接種したところ、４℃で１４ケ月保存したものでは１００％の防禦率が認められ、１７ケ月保存したものではわずかに抗原性が損われたが、防禦能は充分認められた。３７℃保存したものでは少くとも２１日間は感染防禦能が認められた。また燐酸アルミニウムゲルを添加しないワクチンでは３７℃で１週間の保存でも効力が認められなかつたところから、燐酸アルミニウムゲルはアジュバント効果のみならず、保存中の抗原性の保護作用のあることも認められた。

このワクチンをニワトルに接種し、臨床所見を観察したが、何らの異常を認めなかつた。また産卵中の雌鶏に接種して１ケ月産卵率を観察したが、ワクチン接種によると思われるような産卵に対する影響は認められなかつた。

以上の結果から、０.０５％ＢＰＬで、３０％感染鶏胎児乳剤およびその漿尿液の遠心上清を不活化し、５０％に燐酸アルミニウムゲルを添加したワクチンは、安全であり、保存有効期間も長く、良好な免疫力を賦与するワクチンであることが証明された。