SEROLOGICAL STUDIES IN RANIKHET (NEWCASTLE) DISEASE

THESIS

Submitted to the Indian Veterinary Research Institute, Izatnagar
in Partial Fulfilment of the requirements for the Award of

ASSOCIATESHIP

IN

VIROLOGY

By

PYATI MADHUSUDAN,

B. V. Sc.,

Division of Biological Products

INDIAN VETERINARY RESEARCH INSTITUTE,

IZATNAGAR (U. P.)

1967.

Dr.T.S.Gulrajani, G.B.V.C.,
Ph.D.(Cantab)
Head
Division of Biological Products

INDIAN VETERINARY RESEARCH INSTITUTE
IZATNAGAR, U.P.

December , 1967

This is to certify that the work embodied in this thesis entitled "Serological Studies in Ranikhet (Newcastle) Disease", has been authentically carried out by Shri Pyati Madhusudan under my supervision and guidance.

(T.S.GULRAJANI)

ACKNOWLEDGMENTS

The author wishes to place on record his indebtedness to Dr. T.S.Gulrajani, G.B.V.C., Ph.D. (Cantab), Head, Division of Biological Products, for his invaluable guidance.

The guidance given by Shri K.C. Sinha, Virologist, at the initial stages is also thankfully acknowledged.

He is extremely grateful to Dr. C.M. Singh, M.S.,Ph.D. (Michigan), Director of the Institute and to Shri M.R.Dhanda, L.V.P.(Hons.), M.S.(Mich.), Dip.Bact.(London), former Director of the Institute, for providing all the necessary facilities to carry out the present work.

The advice and encouragement given by Shri C.Seetharaman, B.Sc., B.V.Sc., M.Sc.(Vet.), F.A.O. Expert, and former Head, Division of Biological Products is acknowledged with pleasure.

The author is thankful to Shri Y.P. Nanda, B.V.Sc. & A.H., M.V.Sc., Assistant Virologist, Biological Products Division for his constructive criticism and help in the preparation of the manuscript.

Thanks are also due to my senior colleagues Shri D.R. Uppal, Inspector Control Laboratory, Shri S.S. Jawa, Poultry Pathologist, and Shri R.N. Seth, Assistant Serologist.

Lastly, the author extends his thanks to his junior colleagues and other laboratory staff for their whole-hearted cooperation and assistance.

CONTENTS

Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 5

III. MATERIALS AND METHODS 29

(a) Vaccine strain 29

(b) Seed Bank 29

(c) Standardisation 29

(d) The vaccine 29

(e) Sterility test 33

(f) Safety test 33

(g) Potency test 34

(i) Reconstitution of vaccine 34
(ii) Vaccination 34
(iii) Challenge virus 35

(h) Embryo infectivity of the vaccine .. 35

(i) Growth curve experiment 35

(j) Expression of titres 36

(k) Virus pool for tests 36

(l) Preparation of erythrocyte suspension for HA and HI tests 37

(m) Sera for HI and SN tests 37

(n) Sera for controls 38

(o) Cleaning of haemagglutination plates 38

CONTENTS

Page

(p) Spot test 38

(q) Diluent 38

(r) Room temperature 38

(s) Test animals 38

(t) Parallel titrations 39

(u) Biological test 40

(v) Haemagglutination 40

(w) Haemagglutination-inhibition test ... 41

(x) Serum neutralisation test 42

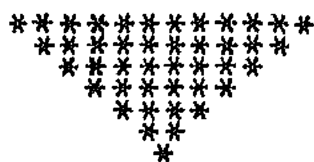
IV. RESULTS 43

V. DISCUSSION 94

VI. CONCLUSIONS 107

VII. SUMMARY 109

VIII. BIBLIOGRAPHY 110



INTRODUCTION

INTRODUCTION

Viruses are the smallest biological units which clearly manifest all the essential characteristics of life and many are known to be built up of nucleic acid and protein. With an uninterrupted succession of important discoveries leading to the development of new biochemical techniques useful in understanding the problems of virology, viruses have become the material "par excellence" for fundamental study (Burnet and Stanley, 1959). Research work on nucleic acid indicates that the nucleic acid structures contain the codes for the fabrication of every individual of every species. Virology has become an important and fascinating field of science in which the geneticist, cell physiologist and the biochemist have found, in the ground ploughed by the pathologist, a fertile soil for new approaches to fundamental problems of cell function and organisation.

With the invention of electron microscope and modern tissue culture techniques, rapid strides have been made in the understanding of the nature of the infective particles of animal viruses including their biological properties. The researches have been fruitful in understanding the 'virus-host cell' relationship as well.

One of the most extensively studied members of the 'myxo' group of viruses is the Ranikhet Disease virus (RDV) which is also known as Newcastle disease virus (NDV).

It exhibits a host of physico-chemical properties, some of which, it shares with influenza and mumps viruses. From the time the Newcastle disease virus was isolated and identified (Doyle, 1927) considerable amount of work has accumulated on various fundamental aspects of the virus including evolution of mesogenic and lentogenic strains of virus.

The demonstration by Burnet (1942) that NDV exhibits the so-called 'Hirst phenomenon' was followed by a series of interesting observations in technical virology resulting in its being recognised as a dependable tool not only in identifying the virus but also as a convenient means of studying the virus-host cell interaction. Secondly, the phenomenon of haemolysis (Kilham, 1949) which NDV shares with mumps virus has also provided a means of identification, in addition to its providing a clue to the possible relationship between the two viruses. The interesting observation that viruses of myxo virus group which get adsorbed to red cells elute with simultaneous destruction of receptors, has now been universally recognised and that the destruction of receptor is a preliminary step for entry into the cell.

A very convenient host for the experimental study of virus infection introduced in 1931 by Woodruff and Goodpasture opened a new era in the study of animal viruses for both theoretical and practical purposes. The adaptation of NDV to the developing chick embryo has made the study of

the behaviour and growth cycle of the virus easy in the tissues of the embryo. The role played by the living embryo and its fluids in bringing about attenuation of a virulent virus without impairing its antigenic properties cannot be explained with precision. However, it would appear that the virus adapts itself to survive and propagate under a changed environmental condition involving physio-chemical reactions resulting in a mutant. Wenner et al. (1950) have made extensive studies about the appearance of different antigens in embryonic and extra embryonic fluids.

It is probably true that no species of farm animals is afflicted with such a host of respiratory diseases as poultry. Among the maladies affecting poultry the Ranikhet disease (RD) stands foremost by virtue of its high virulence and communicability. A decade ago it was the largest single factor causing heavy losses in poultry and was a serious menace to the poultry industry. But in recent years, the disease has been brought well under control. Despite man's attempts to control if not eradicate the disease completely, RD still remains a crucial problem and stands foremost among the preventable diseases of poultry. In recent years, the disease has gained public health importance since it has been found to cause conjunctivitis among men handling infected birds or among laboratory workers (Burnet, 1943; Howitt et al. 1948; Keeney and Hunter, 1950; Pomeroy, 1951; Lippman, 1952 and Hanson and Brandly, 1958).

The enormous increase in poultry population throughout the world has focussed attention on it as a reservoir of disease both in man and animals.

In India, control of this disease has been mostly through vaccination and at present freeze dried egg adapted vaccine is manufactured by most biological products centres. This naturally demands that the vaccine produced for this purpose, should conform to high standards of safety and potency. At present the vaccine is standardised through active protection test in bird which is both expensive and time consuming. The present treatise is a compilation of the author's observations on the titration of the vaccine virus in the developing chicken embryo and its possible correlation with the protection afforded in fowls, as well as the serological response the vaccine virus evokes in the vaccinated birds. As an adjunct, growth curve of the virus in the developing chick embryo and the appearance of haemagglutinins has also been studied.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Ranikhet disease of fowls is a serious and a highly communicable virus disease affecting birds of all ages and attacking respiratory and nervous systems. Ever since the disease was recognised tremendous volume of work has been done on various aspects of the disease and its causal agent and therefore an exhaustive review in a treatise like this is not possible. The work has therefore been reviewed on aspects which have a bearing on the problem under study.

The myxo group of viruses to which the RDV also belongs, includes two classical influenza viruses types A and B, mumps and a growing list of minor respiratory pathogens of man as well as the other classical avian viruses. The unique contribution that has emerged out of the study of this group of viruses is the virus host cell interaction. All react with mucoproteins on the cell surface and in solution under appropriate conditions and carry an enzyme capable of splitting neuraminic acid or related components from the reactive mucoproteins. The nucleic acid content is low and wholly RNA. All have size of about 100 millimicrons.

PROPERTIES OF THE VIRUS

Shape and size

Ranikhet disease which is also known in the west as Newcastle disease was first described by Doyle in 1927 and

shown by him to be due to a filterable agent. The size of the parasite was estimated at 80-120 millimicrons by Burnet and Ferry (1934) using collodiol membranes.

Unstained and unshadowed preparations were examined in the electron microscope by Bang (1946, 1947, 1948), Cunha et al. (1947) and Elford et al. (1947) who described spherical, filamentous and tadpole-shaped structures. Later, Schafer and Rott (1959) showed a rod-like internal component. Further clarification of the structure using the negative staining technique was made by Horne and Waterson (1960).

Bang (1947) reported that in saline, the virus particles were filamentous, whereas they were round in water and chorio-allantoic fluid.

Elford et al. (1948) and Bang (1948) reported the average diameter value of RDV to be 150-190 millimicrons. Donald and Issacs (1954) estimated that 10 particles of virus were needed for one ID_{50} in chick embryos. The particles changed theor form in solutions of higher concentration, without loss in infectivity.

Granoff and Henle (1954) reported that the allantoic fluid and the allantoic membranes showed two components of the virus viz. 'L' and 'S'; the 'L' component being large complete, infective and possessed haemagglutinating and haemolytic activities while the 'S' was small, non-infective, haemagglutinating but did not exhibit haemolytic properties. Nadel and Eisenstark (1955) have reported that during growth

cycle of the virus in developing chick embryos it passed through a stage of "incomplete virus".

Waterson and Cruickshank (1963) reported that different strains of NDV showed minor variations in a standard basic structure.

Haemagglutination

The affinity of virus to erythrocytes was first detected by Doer and Gold (1932) who observed that most of the virus in fowls affected with fowl plague virus was attached to red cells and that repeated washings released the virus.

Hirst (1941) and McClelland and Hare in the same year independently reported that chick red cells were agglutinated by fluids from chick embryos infected with various strains of influenza virus.

Subsequently, Burnet (1942) showed that NDV possessed the same property of agglutination of fowl erythrocytes as the influenza virus. The phenomenon was extended to fowl plague (Lush, 1943), and mumps virus (Levens and Enders, 1945).

The common property of these viruses to agglutinate red cells by adsorbing to the mucin like material on the cell surface was used to define the group myxo virus to provide its name (Andrewes et al. 1955).

MNI group agglutinate the cells of chicken, man and guinea-pig. Many other species provide cells agglutinable by some strains of virus but the type of agglutination varies.

Comparative studies have been made in this regard by Clark and Nagler (1943), Chu (1948a), Sakkubai (1960). NDV has an unusual range of susceptible cells and the most susceptible species were cow, horse, sheep and fowls (Winslow et al. 1950). Sakkubai (loc. cit.) observed that different strains of NDV varied in their capacity to agglutinate cells of various species of animals and birds. A difference was also noted in the eluting capacity. This author further reported a zone phenomenon in case of cells of bull, cow, sheep and goat. Haemagglutinating viruses have been classified as under:-

1. MNI group in which the virus particle itself is the haemagglutinin and also carries an enzyme (neuraminidase) which allows elution of virus with destruction of red cell receptors.
2. Viruses in which the virus particle is believed to be the haemagglutinin but which possess no eluting enzyme and produce no destruction of cell receptors (Arbor, EMC, GD VII and pneumonia virus of mice).
3. Viruses which, during growth in suitable cells produce a haemagglutinin that is separate from the virus particle (pox group and some strains of Psittacosis and related viruses).

It is assumed that the haemagglutination is due to mechanical bridging of one or more red cells by virus particle which simultaneously adsorb to each red cell. Following

two hypotheses have been put forward to explain the virus adsorption to the red cells (Anderson, 1959).

Hypothesis of physical adsorption:- In this theory a clear distinction is made between adsorption of virus and subsequent enzymatic reaction. The red cell receptor is considered to be an area larger than the virus particles and to contain a large number of mucoid groupings. The active groups of the virus enzyme might form only a limited portion of the virus surface (Anderson, 1947).

It is assumed that after adsorption, the virus becomes orientated so that the enzyme reacts with mucoid substrate in the vicinity and each time one substrate grouping is enzymatically altered, the local attractive forces between virus and red cells are diminished. When a sufficient proportion of groupings in the vicinity has been altered, the virus particle is believed to roll or slide to an adjacent portion of the receptor area or to a nearby receptor area. This phenomenon has been termed "browsing". When sufficient red cell receptor areas have been browsed over this way, the virus leaves the red cell.

Hypothesis of enzyme substrate attraction:- In this hypothesis the union of virus and receptor is due primarily to the union between the functional group of virus enzyme and the substrate group in the receptor mucoid (Hirst, 1942b; Chu, 1948b; Burnet, 1948, 1951; Burnet and Lind, 1950).

Receptor gradient:- After complete elution of virus the red cells are no longer agglutinated by the eluted virus and are said to be stable. Cells stabilised by virus are not agglutinated by fresh supplies of the same strain (Hirst 1942b; Burnet et al. 1945). They may, however, be agglutinated by some other strains of MNI group. Any series of strains can be arranged in a linear order, called a receptor gradient (Burnet et al. 1946), so that when red cells are stabilised by one strain, they are not susceptible to agglutination by any virus earlier in the gradient but are agglutinated by every strain later in the gradient. In the receptor gradient order mumps is first and is followed by NDV and various strains of influenza viruses, MEL, WS, LEE, BEL, MIL, and swine. The order is same for both human and fowl cells.

Effect of Physical and Chemical Agents

A great variety of treatments and agents are known to inactivate viruses and during inactivation, the individual biological activity of the virus tends to disappear one after the other. Different environmental agents may inactivate virus particles in a variety of ways, by suppressing one or more of their activities, but the most important and generally, the most sensitive property of the virus particle involved is 'infectivity'.

NDV has according to the findings of Farinas (1930) and others cited by Beaudette (1943) indicated a substantial

survival capacity of the virus outside the living body (Doyle, 1933; Asplin, 1949).

Moses et al. (1947) found that the NDV was quite stable at comparatively wide pH range. After one hour's exposure at pH values of 2-12, the virus remained unaffected.

Farinas (1930) observed that the virus was inactivated in 30 minutes at 55°C and above, but remained infective at 50°C; at 37°C it was active after 24 but not 72 hours. Iyer (1943) reported that the NDV was killed by heating to 50°C or higher. Brandly et al. (1946b) observed that the infectivity was lost after 30 minutes at 50°C or within 45 minutes at 55°C.

Asplin (1949) found virus infected fluid active after 45 minutes but not after 60 minutes at 50°C. The thermal death point of the virus was observed to be between 58°C and 64°C after a 30 minutes exposure (Bushnell and Erwin, 1950).

Burnet and Ferry (1934) observed that photodynamic action of methylene blue was found to be rapid and so was destruction by infra red rays.

Brandly et al. (1946a) observed that exposure of the virus fluid to shorter ultraviolet rays (1600-1800A°) inactivated strain of NDV in 0.8 to 1.08 seconds. Sinha and Dutta (1950) reported that exposure to ultraviolet rays for 1-6 minutes did not inactivate the virus but by an exposure of 6-8 minutes, the virus lost its antigenicity and infectivity.

Beamer et al. (1950) reported that fumigation of incubators was found to be lethal for the virus.

Brandly et al. (1946b), Beaudette et al. (1948) and Thompson and Osteen (1948) reported that antibiotics like penicillin and streptomycin did not have any effect on virus.

The dehydrating and preserving effects of glycerine solution on the virus, especially in pieces of larger tissues were demonstrated by several workers (Hudson, 1937; Brandly et al. 1946b).

Chu (1948b) noted a progressive loss of infectivity of NDV along with parallel destruction of haemagglutinin activity when exposed to 54°-58°C for 15 minutes. He also found that 0.05% formalin rapidly inactivated NDV without impairing its haemagglutinating activity whereas higher concentration (0.5%) produced a slow destruction of the viral haemagglutinin.

Ultraviolet irradiation of NDV brought about a rapid decrease of infectivity but only a slow destruction of the property of haemagglutination (Atanasiu and Sout Patuleia 1952). Henle and Henle (1947) observed that ultraviolet light first destroyed the infectivity of PR 8 strain of influenza virus in dialyzed allantoic fluid and this was followed by destruction of other properties in the order; toxicity, ability to interfere, ability to immunize, ability to elute, haemagglutinin and complement fixing antigens.

SEROLOGICAL REACTIONS

Haemagglutination-inhibition

In Newcastle disease virus the inhibition of clumping of red cells by specific immune sera was first demomstrated by Burnet (1942) and Lush (1943). The specific H.I. antibody is stable at 62°C for 30 minutes and is not destroyed by incubation with Reactor Destroying Enzyme (RDE) or moderate concentrations of Periodate-ion. Union between virus and antibody is reversible during a short period after mixing (Burnet et al. 1945; Issacs, 1948).

Besides the specific immune sera, there are certain non-specific thermolabile factors in the blood, tissues and body fluids which inhibit viral haemagglutinin and sometimes infectivity. Hirst (1942a, 1943) and McCrea (1946) described a factor in normal ferret serum and in ground ferret lung which inhibited haemagglutination by the influenza virus. Beveridge and Lind (1946) found in the allantoic fluid and yolk sac suspensions, a non-specific inhibitor for mumps virus. Polysaccharide of Klebsiella pneumonia type 2 had also been demonstrated to have similar property (Ginsberg et al. 1948). The non-specific inhibitors are known as "Chu inhibitors". Burnet et al. (1946) and McCrea (loc.cit.) showed that the inhibitors are thermolabile the range being 50°-62°C. Mucoids also inhibit viral haemagglutinin. McCrea (1948) demonstrated the presence of mucoid inhibitors

in the mammalian sera, Gottschalk and Lind (1949) in the egg white (ovomucin), Anderson (1948) in tears, Francis and Minuse (1948) in saliva and Tam and Horsfall (1952) in urine and other mucinous secretions. The mucoid inhibitor had little action against active virus but strongly inhibited haemagglutination by indicator virus. The inhibitory power is destroyed by the enzyme of the active virus, RDE (Anderson, 1948).

Haemagglutination inhibition (HI) test using the immune or infected serum has been considered to be more satisfactory for routine diagnosis than post-mortem examination in case of Newcastle disease (Puteanus, 1953; Nitzschke and Venske, 1956). Osten and Anderson (1948) considered HI and serum neutralisation tests to be of equal value for diagnostic purpose. HI test has been found to be satisfactory when organ or tissue extract or blood clots from the dead birds were used (Mitscherlich and Gurturk, 1952; Woernle and Siegmann, 1954; Nitzschke and Venske, 1956) or egg yolk (Schmittle and Millon, 1948; Bornstein et al. 1952).

Zargar and Pomeroy (1949) described a rapid HI plate test in which one loopful of whole blood from the wing vein of infected fowl was taken and mixed with ND antigen on a glass plate. Luginbuhl and Jungherr (1949) reported a similar plate HI test, but they took serum instead of whole blood.

Asplin et al. (1952) reported that a proportion of chickens exposed to Ranikhet and other infections simultaneously

remained negative to HI test. Hofstad (1951) showed that demonstable level of antibody was reached usually 5-10 days after infection with ND. Fabricant (1950) reported that the antibody could be detected 2 days after the first respiratory symptoms appeared.

There are two procedures that have been described for HI test:

Alpha procedure: in which serial two-fold dilution of the virus suspension is made and it is mixed with equal volume of serum to be tested (Anon. 1946; Fabricant, 1949).

Beta procedure: in which serial dilution of the serum is made and mixed with a constant amount of virus dilution containing a known number of HA units (Gentry, 1957; Chu, 1960).

A comparison of the two procedures has been made by Brandly *et al*. (1947).

Serum neutralisation test

It has been considered that the antibodies associated with HI and serum neutralisation tests do not appear to be the same and so also the mechanisms of the two reactions (Brandly *et al*. 1947; Beach, 1948; and Hanson *et al*. 1950). It was observed that the serum neutralisation titre (SN) persisted generally for longer periods than the HI titre (Hanson *et al*. *loc. cit*.). Rubin and Franklin (1957) noted that only one antibody molecule was required to inactivate one virus particle. Sakkubai (1960) noted that in SN test,

the sera gave higher neutralising titre against homologous strains than against the heterologous strains. Tissue culture technique was used by Crowther (1963) to study the neutralising activity of the ND serum and virus. Ginsberg and Horsfall (1949) reported that a labile component present in the serum of human beings, guinea-pig and rabbits neutralised the infectivity of mumps, ND and influenza A and B viruses and these labile components of the sera and also the sera of mouse inhibited HA of chick RBC by these viruses. Howitt et al. (1948) has also reported the presence of neutralising antibodies for ND virus in human sera.

CONTROL MEASURES

Right from ancient times man's chief interest in epizootiology of diseases has been economic and concerned with the development of satisfactory means for controlling disease outbreaks in valuable livestock. Man has one of two ends in view when he purposefully spreads viral agents; either he wants them to serve as mass immunising agents or as means for the mass slaughter of unwanted animal population. The practice of immunzing animal populations by planned infections was more common in the period before good methods for preparing effective vaccines were known. For instance, the practice of aphthisation in many European countries. Probably the most successful epizootic for the destruction of an

unwanted animal population was that in Australia for the purpose of ridding that Island of its billions of destructive rabbits. The agent used was the rabbit myxoma virus.

In combating Newcastle disease, considerations are given to the different situations that prevail in various countries or in areas within those countries. In areas where Newcastle disease is non-existent definite precaution in the form of quarantine is taken to prevent its introduction. Disease outbreaks that appear suspicious or resemble Newcastle disease should be properly checked for a definite diagnosis of the disease and the birds destroyed and properly disposed of. Recent episodes of Newcastle disease in various countries have revealed the importance of strict vigilance and effective regulatory measures for its eradication.

In areas where complete eradication appears impossible because of economic and other reasons an immunization programme has proved satisfactory in the prevention of the disease. Several types of vaccines have been recommended but more recent reports reveal that killed vaccines produce an immunity of a variable and low order and that chickens vaccinated in an enzootic area are likely to contract the disease. In such areas, the live modified virus vaccines are regarded as more effective biological tools.

The views of profounders of dead in preference to live vaccines may be summarised as:- A dead vaccine caused little or no stress to the birds, cannot initiate infection and

cause less interference with the laboratory diagnostic tests. The vaccine is also unable to stimulate a satisfactory response in chicks younger than 2-3 weeks of age. Against these features is the fact that the vaccine has to be administered individually which adds to the labour and cost of vaccination. The individual inoculation of dead vaccine, however, may not aggravate existing respiratory infections or precipitate an outbreak of chronic respiratory disease (CRD) which often may follow the use of even a modified vaccine given in drinking water or used as aerosol spray. Broiler growers particularly are apprehensive of infection during the first three weeks and may wish to vaccinate their chicks at birth, but it is unlikely that the immune response at this age would be sufficient to protect the chicks against their normal growing period of 10 weeks. Immune hens, however, transmit a passive immunity to their progeny which may last upto 21 days after hatching so that as more and more breeding birds are rendered immune by vaccination, more and more chicks will be protected during this early period and the necessity for vaccination before 3 weeks of age will cease.

The fears expressed by some workers that the live vaccine may exalt in virulence during the course of time due to the passage through susceptible hosts have been annulled with the increasing understanding of the biology of the viruses. In modern times it has always been the practice

to employ live vaccines for the control diseases of viral origin. A list of diseases for which live vaccines have been evolved and extensively employed in animals is presented below:

Live Vaccines used in prevention of virus infection of animals

Sl.No.	Disease	Source of vaccine
1	African horse sickness	Mouse brain
2	Distemper of dogs	Avianised
3	Fowl pox	Chick embryo propagated
4	Hog cholera	Lapinised virus
5	Newcastle disease	(i) Chick embryo attenuated (ii) Natural mutant
6	Rabies	Avianised (Flury)
7	Rinderpest	Goat adapted Lapinised Lapinised avianised
8	Swine fever	Lapinised
9	Foot and Mouth disease	Avianised Mouse adapted Cell culture

Burnet in his lectures to Sydenham University observed that an ideal living modified vaccine should be safe and reliable when administered in the proper manner and by the proper route; i.e. it should not induce fatal infections in susceptible hosts. It should be so attenuated as to be notably

innocuous but of low virulence. That transmission should not occur from one host to another so as to facilitate conceivable gain in virulence or pathogenicity, it should be adequately tested and at all times proved free from potentially pathogenic viruses, ricketsiae or bacterium even under remote conditions and should contain sufficient amount of antigen so as to cause immunizing infection as it is known that the live virus has to multiply in the tissues of the host to bring about this effect and it is, therefore, necessary that it should contain large number of infective particles. The optimal range of virus concentration should be quantitatively determined in each lot as minimal doses may sensitise and not immunise, thus causing harm to the host to subsequent exposure of the same virus. A qualitative and a quantitative assay should be conducted periodically to ensure a certain degree of invasiveness to cause sub-clinical infection and guarantee immunity; thus the virus should be so attenuated as to be innocuous but should not be allowed to get over attenuated so as to lose immunogenic potency. It should be stable i.e. its immunising property should remain unimpaired over long periods of time under adverse conditions of light, temperature, moisture and travel, it should be prepared in a practical way, not too expensive so that it can be had by those who need it. According to Burnet these specifications should be met with both by research scientists and production workers to make it a marketable vaccine of good reputation.

Both types of vaccines that have been employed for the control of Newcastle disease from time to time have been reviewed briefly in this treatise.

INACTIVATED VACCINES

Before actually the live vaccines against Ranikhet disease came into existence, the use of inactivated vaccines has been reported from all over the world with varying results. In some countries even now inactivated vaccines are being employed on a large scale for immunization, even though efficient modified strains or strains of low virulence are available.

Inactivation by Physical Agents:- Doyle (1927), Haddow and Idnani (1941) tried heat inactivated vaccines with varying results. Dutcher et al. (1960) reported inactivation of the ND virus at higher temperature for a shorter period without much loss in its antigenic factor.

Not much success was obtained with the use of ultra-violet irradiated vaccine (Brandly et al. 1946a)

Inactivation by Chemical Agents:- The use of formalin for inactivation of ND virus has been reported by the early workers (Doyle, 1927; Nakamura et al. 1937; Haddow and Idnani, 1941; Beach, 1948). Brandly et al. (1946a), Adler et al. (1951), Kaschula (1952), Dardiri and Yates (1962) suggested vaccination with formalised virus followed by live virus within two weeks to produce satisfactory results.

Haddow and Idnani (1941) could not get uniform results with formalised RD virus adsorbed on aluminium gel, whereas Coronel (1947) and Nakamura et al. (1956) considered it to be an effective immunising vaccine. Schoening et al. (1949) reported use of liquid paraffin and lanolin to be a better adjuvant in comparison to the aluminium gel.

Mitchell and Walker (1951, 1952) emulsified the ND virus in falba and mineral oil. Ninety per cent of the birds vaccinated with this vaccine were solidly immune even after 6 months.

Inactivation of ND virus with crystal violet was of not much use, since the results with this product were irregular and unsatisfactory (Iyer and Dobson 1941; Iyer, 1943; Thompson and Osteen, 1952; Van Wavern, 1955), but incorporation of ethanol glycol to crystal violet treated NDV could engender a durable immunity lasting at least 12 months (Doyle and Wright, 1950).

Use of Beta-propiolactone (BPL) for inactivation of viral agents was reported for the first time by Logrippo and Hartman (1955) and its successful application in concentrations varying from 0.05 to 0.03% for various virus inactivations. Logrippo (1959) reported that double inactivation with BPL and irradiation had better effect on the viruses of rabies and encephalomyelitis; and also tailing effect of BPL is diminished by the irradiation.

Mack and Chotisen (1955), Gill *et al*. (1959), Piercey *et al*. (1962) tried BPL inactivation of NDV, which alone or with adjuvants gave solid immunity to birds. Revaccination increased the degree of protection (Sullivan *et al*. 1958; Gill *et al*. 1959). The birds withstood challenge infection after 17 weeks (Simmins and Baldwin, 1963), 20 weeks (Gill *et al*. 1959), 52 weeks (Garside, 1962). Day-old chicks vaccinated with BPL inactivated vaccine adsorbed on aluminium gel were immune for 9-12 weeks. Keeble and Coid (1962) did not observe any physiological impairement in these. In older birds there was not any significant decrease in egg production nor any untoward symptoms (Cooper, 1963). Keeble and Wade (1963) studied the antibody response in chicks hatched out of the eggs of immune birds with BPL inactivated NDV. Keeble *et al*. (1963) advocated vaccination of such chicks at 3 weeks of age with BPL rather than at one day of age.

LIVE VACCINES

Mesogenic strains

Burnet and Ferry (1934) demonstrated the pathogenicity of Newcastle disease virus to the developing chicken embryos.

The first successful modification of Newcastle virus Hertfordshire strain, by serial passage in embryonating eggs

was reported by Iyer and Dobson (1940). After 33 egg passages in one series and 14 in another, the virus was virtually apathogenic for chickens, yet produced an immunity adequate to protect against 10^8 m.l.ds. of virus 14 to 17 days later. They further reported that inoculation of another bird with the spleen taken from a chicken sacrificed five days after injection with 41st egg passage virus was likewise nonpathogenic.

Chicken under 8 weeks of age were reported to be very susceptible (Iyer and Dobson, 1940; Schneider, 1954). The vaccine was reported to be safe for chickens over 12 weeks old (Schneider, loc.cit.).

The duration of immunity has been reported to be 3 to 5 months (Gualandi, 1951; Mazzarchio and Orfei, 1954) and a year (Teklinska, 1951).

Some cases of paralysis have been reported (Salyi and Hodosy, 1952 and Mazzarchio and Orfei (loc.cit.). Pagnini (1954) reported a severe drop in egg production following the use of this vaccine.

Mukteswar virus

The work of Iyer and Dobson (1940) opened the way of evolving an egg propagated and modified virus vaccine for the protection of poultry. Since then concerted efforts were made at the Indian Veterinary Research Institute, Mukteswar to adopt an Indian strain of Ranikhet (Newcastle)

disease virus. A particular Indian strain of virus which was observed by Iyer and Hashmi (1945) to produce 45 per cent mortality at the 92nd serial egg passage, was found to have become satisfactorily attenuated at the 115th passage (Haddow and Idnani, 1946). The latter workers used this strain of attenuated virus between 115th and 120th egg passage for immunising thousands of poultry during the late part of second world war with encouraging results. Continuous serial passage of the virus in eggs was not accompanied by loss in antigenicity (Dhanda, 1954). This strain has been designated as R_2B.

The vaccine was reported to induce a severe reaction in young chicks and some mortality (Haddow and Idnani, 1946; Gupta and Rao, 1959) which may reach 30% (Rao and Aggarwal, 1960) and also paralysis (Daubney and Mansi, 1948) in about 2% of the birds.

Dhanda et al. (1958) observed that the chickens of six weeks of age tolerated the vaccine well although mortality occurred which varied from 1 to 3 per cent (Generoso and Mendoza, 1950); to 6 per cent (Haddow and Idnani, 1946); to 16 per cent (Van Wavern and Zuidjam, 1953).

Like other mesogenic virus vaccine strains, the Mukteswar virus caused a marked reduction in egg production lasting 1 to 3 weeks (Haddow and Idnani, loc. cit.), to 6 weeks (Memo, 1955). During this period egg production may decrease by 10 to 16% (Agcanas and Rigor, 1951), even 60% (Dixit, 1950).

Nandi (1955) reported selective pathogenicity of the vaccine to White Leghorn chickens.

Immunity trials with this vaccine revealed solid protection against challenge virus for 9 to 15 months (Haddow and Idnani, 1946; Daubney and Mansi, loc.cit., Cakalowa et al. 1955). Seetharaman (1951) reported immunity period for 3 to 4 years. This was confirmed by Nilakantan et al. (1960b). On the other hand, Bornstein et al. (1949) were of the opinion that the immunity produced by the Mukteswar strain did not last for more than a year.

Nilakantan et al. (1960b) reported that birds vaccinated with this strain resisted challenge infection in 27 hours after vaccination, but detectable HI antibodies appeared 5 days post vaccination. Cakalowa et al. (1955) noted the highest HI titre in chicks vaccinated with Mukteswar strain one month after vaccination which gradually started falling, nevertheless the birds withstood challenge 13 months after vaccination.

Komarov and Haifa or Palestine strain

This strain was evolved as a result of intracerebral passage in ducklings (Komarov and Goldsmit, 1946). The same workers (1947) reported that the vaccine prepared with this strain did not result in a fall in egg production, general depression, loss of appetite, moulting and nervous symptoms.

Nilakantan et al. (1960a) concluded by various serological and immunological experiments that there was very little difference between the Mukteswar and Haifa strains.

In addition to the above, there are other mesogenic vaccine strains employed in U.S.A. as live vaccines. One strain 'ROAKIN' was identified during the screening of 105 strains (Beaudette et al. 1949). The other strain was MK 107 which was evolved as a result of serial passage in chick and duck embryos following its isolation (Clancy et al. 1949; Markham et al. 1949).

Both the strains caused severe morbidity and some mortality (Cole and Hutt, 1961; Van Roekel, 1956; Van Wavern, 1955). Reduced egg production has been noted with these strains too (Beach, 1949; Beaudette et al. loc.cit., Kaschula, 1950; Van Roekel, 1956).

The immunity engendered by Roakin strain was durable for 4 months (Van Wavern and Zuijdam, 1953), and that due to MK 107 strain was for at least 10 weeks (Markham et al. 1954) to 4 months (Clancy et al. loc.cit.).

Tissue culture vaccine

Bankowski and Boynton (1948) and Bankowski (1957) propagated a field strain of Newcastle disease virus designated as California 1914 in chick embryo cells. Bankowski (1958) observed that this strain got attenuated by serial

passage without loss in antigenicity. It could also be propagated in monolayer cultures of Hela cells as well as bovine or pig kidney cell cultures (Bankowski, 1958; Bankowski and Hyde, 1957). Bankowski et al. 1963) noted that in chickens, when vaccinated with B_1 strain at an age of 5 days followed by tissue culture vaccine, immunity lasted for 93 weeks.

Lentogenic strains

For immunisation of baby chicks a number of strains of low pathogenicity for chicks have been in use. These are B_1 (Hitchner and Johnson, 1948); F strain (Asplin, 1952) and Lasota (Winterfield et al. 1957). The duration of immunity and other characters of these strains have been reviewed by Lancaster (1964).

MATERIALS AND METHODS

MATERIALS AND METHODS

Vaccine virus strain

The Mukteswar vaccine virus strain viz. R_2B was used for preparing the vaccine in a freeze dried form.

Seed bank

The virus pool consisted of allanto-amniotic fluids from more than six developing chicken embryos infected with 0.1 ml. of 10^{-3} dilution of R_2B strain of Ranikhet disease virus and which was centrifuged at a speed of 1000 to 1500 r.p.m. for 10 minutes and the deposit discarded. The supernate was tested for sterility on aerobic and anaerobic media. The reading was made 72 hours later. The sterile fluid thus prepared was dispensed in suitable quantities in neutral glass ampoules, Edwards make, sealed and stored at -10°C in a deep freeze unit.

Standardization

The seed virus was standardized in relation to its infectivity to the developing chicken embryos system.

The vaccine

The vaccine consisted of a bacteriologically sterile suspension of embryo and allanto-amniotic fluids of developing chicken embryos infected with Mukteswar strain of Ranikhet disease virus, in a freeze dried form.

Nine to ten days old developing chicken embryos drawn from a known source free from _Salmonella pullorum_ infection were employed. The embryos were candled and a small mark was made over the well vascularised area in the shell and a puncture was made at the centre of the air sac with the help of a sharp needle. The mark over the vascularised area was sterilised with tincture of iodine and with the help of a dental drill, a small hole about 2-3 mm. was made without piercing the shell membrane.

The fertile eggs that were prepared as above were transferred to an inoculation hood which had been sterilised with ultraviolet light. The site of inoculation was sterilised again. The inoculum which consisted of 0.1 ml. of 10^{-3} dilution of the seed virus (10,800 m.i.ds.) that was prepared the previous day and tested for sterility was introduced into the allantois through the shell membrane and chorio-allantoic membrane with the help of a tuberculin syringe fitted with 26 g needle, 13 mm. long.

During drilling, care was taken to avoid damage to the chorio-allantoic membrane.

The openings at the air sac and over the allantois were sealed with a mixture of molten vaseline and hard paraffin and the eggs were returned to the incubator at 37°C.

The eggs were candled on the first day and the dead ones were discarded. On the second day, the eggs were candled and the dead ones were chilled in the refrigerator for 1 to 2 hours.

The chilled eggs were placed vertically in trays and the air sacs were painted with tincture of iodine. The air sac was opened and the allanto-amniotic fluids and the embryos were collected aseptically in a sterile stainless container, kept on ice. A drop of allantoic fluid was tested for spot haemagglutination test. At the Division of Biological Products, however, it was not practicable to test the 'aa' fluid from each egg for spot test, due to the large number of embryos employed for the mass production and so about 15% of the embryos were tested for spot haemagglutination test. The embryos were also examined for lesions which consisted of petechial haemorrhages on the dorsal and craneal surfaces of the embryo. Adequate sterility tests were also put up.

The tissues and fluids which were satisfactory in respect of virus content, were transferred to a sterile cool waring blender and blended for 3 minutes. Penicillin and streptomycin sulphate at the rate of 1000 i.u. and 2 mg./ml. respectively, were included in the homogenate before blending.

The suspension was dispensed in 0.5 ml. quantities in neutral glass ampoules with the help of an automatic 2 cc. syringe. Freeze-drying was carried out as under :-

The refrigerator of the centrifugal freeze dryer was switched on and when the temperature of the cooling coil

had reached -40° to -50°C, ampoules containing the liquid virus suspension were loaded in the centrifuge carrier plate fixed in the primary chamber and the cover of the primary chamber replaced and the centrifuge was run for 45 minutes. The centrifugation was done with a view to suppress the foaming and accelerate the drying by increasing the surface area of the material to be dried by causing it to take the form of a wedge against the wall of the ampoules.

Combined drain cock and air release valve of the unit were closed and primary drying pump was then switched on. As the pressure was reduced and the material in the chamber froze, the refrigerator temperature rapidly rose to about -10°C and after a few minutes, came back to -35°C to -40°C. The pressure in the primary chamber was indicated by the Pirani gauge of the unit.

The primary drying was carried out for 18-20 hours and during the course of this period the water vapour that escaped from the material was condensed on to the cooling coils of the refrigerator and only dry air and gases passed through the vacuum pump. When the material was sufficiently dry the vacuum was released and the ampoules were removed from the chamber.

The ampoules were necked using an oxy-acetylene flame to produce a constriction to facilitate quick sealing after drying on the secondary system.

The necked ampoules were loaded on to the secondary drying headers mounted on a manifold containing a desicant-phosphorus pentoxide. The manifold was evacuated to a very low pressure by means of a speedivac pump. The drying over the secondary system continued for 15 to 20 hours and the moisture content of the material was thus brought to the minimum. At the completion of the secondary drying and while still under vacuum, the ampoules were sealed using oxy-acetylene flame. The pressure under which the ampoules were sealed varied between 0.03 to 0.01 mm. of mercury. The sealed ampoules were transferred to a low temperature cabinet.

Sterility tests

The ampoules of vaccine were tested for bacteriological sterility on suitable media.

Safety test

Twelve healthy chickens aged 8-10 weeks from the same source, which were previously immunised against Ranikhet disease were taken and treated as under :-

Three of the test birds were injected subcutaneously with 0.1 ml. containing ten times the field dose of the vaccine. This group served to indicate if the product was free from viruses and organisms of septicaemia.

Three of the test birds were injected intratracheally with the same dose. This group served to indicate whether the product was free from viruses like infectious laryngotracheitis.

Three of the test birds were given the same dose intranasally and this group served to indicate freedom from other viral contaminants like coryza, infectious bronchitis etc.

The three remaining birds served as controls.

All the treated birds and the controls were observed daily for 14 days. All the test birds that succumbed during the period of observation were subjected to careful autopsy.

Potency Test

Reconstitution of vaccine

Two randomly selected ampoules of the vaccine were taken and to each 0.5 ml. of cold normal saline was added to bring it back to the original volume. This was taken as undiluted vaccine. Further progressive tenfold dilutions were made in the same diluent upto 10^{-6} dilution and also 10^{-7} wherever necessary.

Vaccination

Four susceptible chicks, 6-8 weeks old, were vaccinated by injecting subcutaneously in the wing web, with 1 ml. of

10^{-6} dilution of the vaccine. These were observed for a period of 14 days for any adverse post vaccinal reaction.

At the end of the 14 days period, these along with four susceptible control chicks of the same age group, were injected subcutaneously with 1 ml. of 1:100 dilution of virulent Ranikhet disease virus. The vaccinated and the control group were observed for 14 days. The controls invariably died on the 4th or 5th day, showing typical lesions of the disease while most of the vaccinated group survived the challenge.

<u>Challenge virus</u>

The virulent virus consisted of allanto-amniotic fluid, collected from twelve days old embryonated eggs infected with virulent strain of Ranikhet disease virus.

The virulent virus killed the embryos in 48 hours. The virus was stored in a refrigerator in suitable quantities.

<u>Embryo infectivity of the vaccine</u>

Serial ten fold dilutions of the vaccine were made in N.S.S. and dilutions from 10^{-6} to 10^{-8} were tested. Three embryos were used for each dilution. The embryos inoculated were incubated at 37°C for 48 hours. The presence of the virus was detected by spot haemagglutination test.

<u>Growth curve experiment</u>

The seed virus (aa fluid) was treated with penicillin (1000 I.U./ml.) and streptomycin (1 mg./ml.). The inoculum

was prepared one day prior to inoculation of embryos and kept in the refrigerator. In one of the batches of routine vaccine production, 150 developping chicken embryos were inoculated with 0.1 ml. of 10^{-3} dilution of the seed virus, in normal saline.

Batches of three eggs were removed from the incubator at durations of 0, 6, 12, 18, 24, 30, 36, 42 and 48 hours, candled and were chilled in a refrigerator for 1 to 2 hours prior to collection of allanto-amniotic fluid.

The 'aa' fluid collected above was centrifuged at 1000 r.p.m. for 10 minutes. Serial tenfold dilutions of the supernate were made in normal saline and suitable dilutions were used to inoculate the 9-10 days old embryos for each dilution. The normal 'aa' fluid served as the control. After incubation as usual, the presence of virus in each egg was ascertained by spot haemagglutination test and by the lesions in the embryos.

Expression of titres

Throughout the study, titres of virus have been expressed as $CELD_{50}$ and calculations were done according to the method of Reed and Muench (1938).

Virus pools for tests

Clear allanto-amniotic fluid from the infected embryos during the course of production of routine vaccine at the

Biological Products Division, was collected and kept in a deep freeze cabinet in quantities adequate for a test.

Preparation of erythrocyte suspension for haemagglutination (HA) and haemagglutination-inhibition (HI) tests

Blood was drawn from the wing veins of fowls in 2.5% of sodium citrate solution (1 ml. of the solution plus 4 ml. of blood). Two birds were employed at a time for collection. The two samples of blood were pooled, centrifuged at 1500 r.p.m. in a clinical centrifuge for five minutes. The buffy coat and plasma were removed and the cells were washed thrice in normal saline. The final centrifugation was done at 1500 r.p.m. for 10 minutes. A 30% suspension was made and kept in the refrigerator. The cells were used within two to three days of collection.

Sera for HI and SN tests

Blood was collected from the vaccinated chicks on the day of challenge from the wing vein in test tubes of 100 mm x 10 mm. and allowed to clot. The clot was released by means of a wooden applicator previously sterilised and kept in the incubator for 20 to 30 minutes and thereafter transferred to a refrigerator, serum was separated the next morning and was lightly centrifuged. The sera thus separated was employed for HA tests. For SNT, pooled sera from the birds of a batch of vaccine for testing, was employed.

Sera for controls

Normal healthy serum was prepared in the same way from chicks before they were vaccinated.

Cleaning of haemagglutination plates

The plates were washed in 2% washing soda and then washed off in the running water and stored in N/5 HCl until required for further use.

Spot test

A quick test for the presence of virus in the allanto-amniotic fluid harvested during the course of vaccine production consisted of taking a drop of the allantoic fluid on glass slide and mixing it with a drop of 10% suspension of erythrocytes. Agglutination occurred within 1/2 to 1 minute and could be clearly seen when the slide was carefully rotated.

Diluent

Throughout the study, only normal saline of 0.85% in distilled water and sterilised at 20 lb. pressure for 30 minutes was used.

Room temperature

The laboratory operations were carried out at 70-80°F.

Test animals

The developing chick embryos 9-10 days old, for vaccine production and the chicks for testing were drawn from the

same source, viz. poultry farm attached with the Division of Poultry Research of the Indian Veterinary Research Institute, Izatnagar.

Parallel titrations

Experiment I

The freeze dried Ranikhet disease vaccine was titrated in both the systems viz. chicks and developing chick embryos in dilutions of 10^{-5}, 10^{-6} and 10^{-7}. Batches 23, 24, 35 and 36 were included in the study.

Experiment II

The above experiment was repeated in respect of batches 50, 51 and 52 at two dilutions viz. 10^{-6} and 10^{-7}. The dilution of 10^{-5} was excluded because from the previous experiment it was known that the vaccine virus invariably killed the embryos at this dilution. Also from batch 47, 10^{-5} dilution was excluded for assay in chicks.

Experiment III

Twenty-two batches (103 to 121 and 168 to 170) of freeze dried Ranikhet disease vaccine were assayed in dilutions of 10^{-6}, 10^{-7} and 10^{-8} in the chick embryos, and have been dealt under embryo infectivity.

The titres were calculated on the basis of 50% end-point. For doing this, wherever there was no endpoint, the higher dilution was considered with negative scores for it.

This procedure was adopted with a view to facilitate presenting an uniform picture of the results in embryos.

Such of the batches of vaccine which were simultaneously titrated in chicks, the same method of determining 50% protective endpoint was introduced.

Biological test

This was conducted wherever the vaccinated birds died 3 to 5 days after challenge.

It consisted of inoculating 1% suspension of liver and spleen of the dead birds, into two susceptible chicks of 6-8 weeks and observed for one week. In all, four trials were made.

Haemagglutination

The haemagglutination (HA) test was carried out in persplex plates. The allanto-amniotic fluid was used as the source of the virus. Serial two-fold dilutions of the virus from 1:5 to 1:5120 were made in Normal Saline Solution (N.S.S.). To each cavity of 0.5 ml. of virus dilution, 0.5 ml. of 0.5 per cent suspension of fowl red blood cells (R.B.C.) was added by means of a tuberculin syringe fitted with 18 gauge needle with end truncated in such a way as to ensure thorough mixing. Controls having only saline and RBC suspensions were always kept. The test was performed at room temperature which varied from 70°-80°F.

Readings were taken after one hour. The pattern of sedimentation of the red cells in the control cavity was

taken into consideration while recording the results. In those cavities where there was complete agglutination, the cells sedimented in a uniform pinkish layer giving ground glass appearance. Partial agglutination was characterised by the formation of a central ring of unagglutinated cells with an irregular margin of a layer of agglutinated cells. Where there was no agglutination, the cells settled down in the form of a compact mass with a red button like appearance.

The highest dilution of the virus showing complete agglutination was taken as the end titre and was expressed as one haemagglutination (HA) unit.

Haemagglutination inhibition

Beta procedure was employed to carry out this test. Serial double fold dilutions of the serum were made in 0.25 ml. of saline in the persplex haemagglutination plates and 0.25 ml. of the virus suspension containing 2 HA units were added to each of the serum dilutions. The plate was kept at room temperature for 30 minutes and 0.5 ml. of a 0.5 per cent suspension of RBC was added to each cavity. The test was read one hour after the addition of cells. The highest dilution of the serum which caused complete inhibition of haemagglutination was taken as the HI titre. Adequate controls were included. The serum control consisted of 0.25 ml. of lowest dilution of serum plus0.25 ml.

normal saline solution, while antigen control consisted of 0.25 ml. of the virus plus 0.25 ml. of N.S.S. To each of the control 0.5 ml. of the 0.5% suspension of RBC was added.

Serum neutralisation test

Embryonated eggs 9-10 days old were used as the indicator system for this test. Serial ten fold dilution of the virus were made in cold 10% peptone broth saline. Sera were collected from the 6 to 8 weeks old birds before immunization and pooled and served as healthy serum control. None of the sera showed an HI titre of more than 1:10.

Immune sera from vaccinated birds were collected 2 weeks after vaccination, i.e. on the day of challenge and pooled. Sera-virus mixtures were then incubated at room temperature for 2 hours before inoculation into the indicator system. Embryonated eggs after inoculation with 0.2 ml.of serum virus mixtures were incubated at 37°C for 48 hours. The multiplication of the virus in the eggs was determined by spot haemagglutination test with the allantoic fluid of each embryo. Calculation of neutralising index in respect of each sample was done according to the method of Reed and Muench (1938).

RESULTS

RESULTS

VIRAL MULTIPLICATION

To assess the quantitative multiplication of Ranikhet disease vaccine virus in the developing chick embryos, a batch of embryonated eggs was inoculated with the seed virus and incubated. A group of eggs was removed at various intervals and chilled. The pooled amnio-allantoic fluids of the embryos in each group was collected and titrated in various decimal dilutions in the same host system with a view to assess the virus content of each pool as per procedure described earlier. The titres of virus obtained are presented in table 1 and graphically represented in Fig. 1.

It would be observed that during the first 6 hours, there was actually no multiplication of the virus and what was observed was only residual virus after adsorption from the quantity of inoculum used. There was a progressive rise in the virus content upto 36 hours and reaching to a maximum titre of $10^{-7.2}$. The embryo infective titre was nearly constant from 36 to 48 hours.

The 'aa' fluid collected at the various intervals was assessed for its haemagglutinin content. The results are presented in table 2, and graphically pictured in Fig. 1.

Table 1

Multiplication of Ranikhet Disease Vaccine Strain R_2B in chick embryos as adjudged by embryo infectivity

Sl. No.	Period of incubation (hours)	Titre (Log units)
1	0	1.50
2	6	∠1.0
3	12	3.50
4	18	5.20
5	24	5.70
6	30	6.20
7	36	7.20
8	42	7.20
9	48	7.00

Table 2

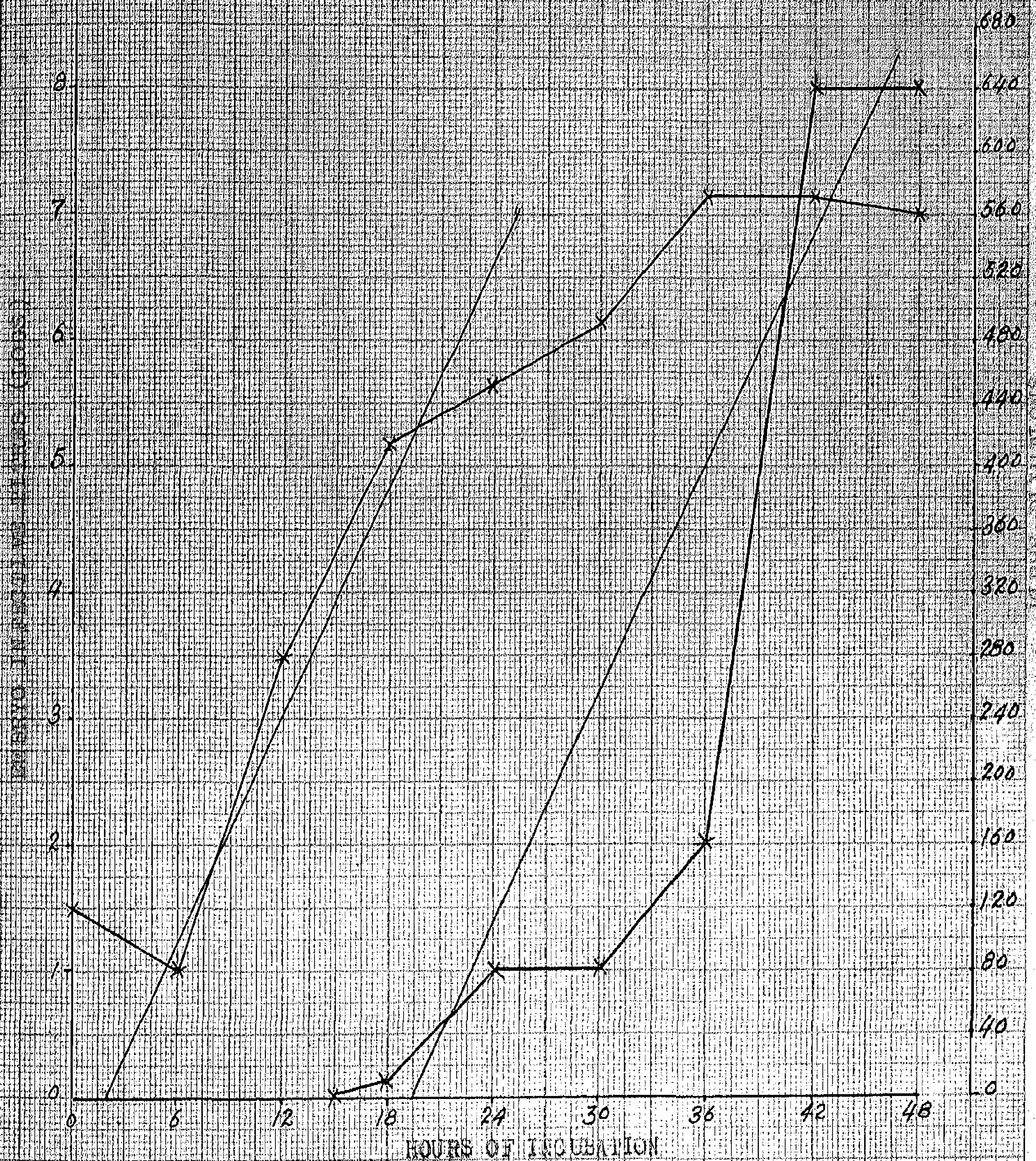
Development of Haemagglutinins

Sl. No.	Period of inocu-lation (hours)	Virus dilutions								*HA Titre
		10	20	40	80	160	320	640	1280	
1	0	-	-	-	-	-	-	-	-	Nil
2	6	-	-	-	-	-	-	-	-	Nil
3	12	-	-	-	-	-	-	-	-	Nil
4	18	+	±	-	-	-	-	-	-	10
5	24	+	+	+	+	-	-	-	-	80
6	30	+	+	+	+	±	-	-	-	80
7	36	+	+	+	+	+	-	-	-	160
8	42	+	+	+	+	+	+	+	-	640
9	48	+	+	+	+	+	+	+	-	640

* Titres expressed as the reciprocal of dilution of virus giving complete agglutination.

FIGURE 1

GROWTH CURVE OF RANIKHET (NEWCASTLE) DISEASE VIRUS R_2B STRAIN AND DEVELOPMENT OF HAEMAGGLUTININS IN CHICK EMBRYO



Embryo infective titres

Haemagglutinin titres

The data reveals that the haemagglutinins started appearing from the 18th hour in low concentrations and increased gradually to a level of 640 at 42 hours and remained stationary till 48 hours.

A comparison of the appearance of infective particles and the haemagglutinins associated with it, has been shown in table 3.

Table 3

Correlation of embryo infectivity and haemagglutination titres of amnio-allantoic fluid at varying periods of incubation

Sl. No.	Period of incubation (hours)	Embryo infectivity titre (Log units)	HA Titre
1	0	1.5	Nil
2	6	1.0	Nil
3	12	3.50	Nil
4	18	5.20	10
5	24	5.70	80
6	30	6.20	80
7	36	7.20	160
8	42	7.20	640
9	48	7.00	640

EMBRYO INFECTIVITY

The freeze dried vaccine was reconstituted in the manner described under Materials and Methods and progressive ten-fold dilutions were made in normal saline. Three embryos for each dilution were inoculated with vaccine dilutions of 10^{-5} to 10^{-8} and incubated as usual.

The deaths at 24 hours were excluded and the 'aa' fluid from embryos which died next day, was put to spot haemagglutination test. Positive scores were included. The results are presented in table 4. A perusal of the data for 22 batches would reveal that the minimum embryo infective titre obtained was $10^{-5.6}$ (batch No.170) and the maximum titre obtained was $10^{-7.75}$ in respect of batch Nos. 103, 105 and 110. The remaining batches varied in between.

HAEMAGGLUTINATION INHIBITION

In addition to the studies described above, it was decided to find out if the birds vaccinated for potency test produced measurable quantities of HI antibodies before the challenge. For this purpose presence of HI antibody in each of the birds was looked for, 14 days after vaccination, i.e. on the day these birds were being challenged for potency. The sera before vaccination showed HI titre of 1 in 5 to 1 in 10.

The results of HI antibodies are presented in table 5. In all HI titres of 161 birds comprising 33 batches of vaccine are shown.

Table 4

Results of embryo titrations of Freeze-Dried Ranikhet Disease Vaccine

Batch No.	Dilution			Embryo infectivity titre ($CELD_{50}$)
	10^{-6}	10^{-7}	10^{-8}	
	Death/inoculated			
103	3/3	3/3	1/3	7.75
104	3/3	2/3	1/3	7.50
105	3/3	3/3	1/3	7.75
106	3/3	1/3	1/3	7.00
107	2/3	1/3	1/3	7.18
108	3/3	2/3	1/3	7.50
109	3/3	2/3	0/3	7.25
110	3/3	3/3	1/3	7.75
111	3/3	2/3	1/3	7.50
112	3/3	1/3	1/3	7.00
113	2/3	2/3	0/3	7.00
114	3/3	1/3	1/3	7.00
115	3/3	2/3	0/3	7.25
116	3/3	2/3	0/3	7.25
117	2/3	2/3	1/3	7.25
118	3/3	1/3	0/3	6.75
119	3/3	2/3	0/3	7.25
120	3/3	1/3	0/3	6.75
121	2/3	1/3	1/3	6.75
* 168	4/4	2/4	0/2	6.00
* 169	4/4	4/4	2/4	7.00
* 170	4/4	1/4	0/4	5.60

* Dilutions were 10^{-5}, 10^{-6} and 10^{-7}

Table 4

Results of embryo titrations of Freeze-Dried Ranikhet Disease Vaccine

Batch No.	Dilution 10^{-6}	Dilution 10^{-7}	Dilution 10^{-8}	Embryo infectivity titre ($CELD_{50}$)
	Death/inoculated			
103	3/3	3/3	1/3	7.75
104	3/3	2/3	1/3	7.50
105	3/3	3/3	1/3	7.75
106	3/3	1/3	1/3	7.00
107	2/3	1/3	1/3	7.18
108	3/3	2/3	1/3	7.50
109	3/3	2/3	0/3	7.25
110	3/3	3/3	1/3	7.75
111	3/3	2/3	1/3	7.50
112	3/3	1/3	1/3	7.00
113	2/3	2/3	0/3	7.00
114	3/3	1/3	1/3	7.00
115	3/3	2/3	0/3	7.25
116	3/3	2/3	0/3	7.25
117	2/3	2/3	1/3	7.25
118	3/3	1/3	0/3	6.75
119	3/3	2/3	0/3	7.25
120	3/3	1/3	0/3	6.75
121	2/3	1/3	1/3	6.75
* 168	4/4	2/4	0/2	6.00
* 169	4/4	4/4	2/4	7.00
* 170	4/4	1/4	0/4	5.60

* Dilutions were 10^{-5}, 10^{-6} and 10^{-7}

Table 5

Development of haemagglutination inhibiting antibodies during post vaccination period in birds

Batch No.	Sample No.	Serum Dilution									HI titre	Group average HI titre
		40	80	160	320	640	1280	2560	5120	10240		
103	1	+	+	+	+	+	+	+	-	-	2560	1280
	2	+	+	+	+	+	+	-	-	-	1280	
	3	+	+	+	+	+	-	-	-	-	640	
	4	+	+	+	+	+	+	-	-	-	1280	
	5	+	+	+	+	+	-	-	-	-	640	
104	6	+	+	+	+	+	+	-	-	-	1280	1013
	7	+	+	+	+	-	-	-	-	-	320	
	8	+	+	+	+	+	+	-	-	-	1280	
	9	+	+	+	+	+	+	-	-	-	1280	
	10	+	+	+	+	+	-	-	-	-	640	
	11	+	+	+	+	+	+	-	-	-	1280	
107	12	+	+	+	+	+	-	-	-	-	640	1152
	13	+	+	+	+	+	+	-	-	-	1280	
	14	+	+	+	+	+	-	-	-	-	640	
	15	+	+	+	+	+	+	+	-	-	2560	
	16	+	+	+	+	+	-	-	-	-	640	
108	17	+	+	+	+	+	-	-	-	-	640	746
	18	+	+	+	+	+	+	-	-	-	1280	
	19	+	+	+	+	-	-	-	-	-	320	
109	20	+	+	+	+	+	+	-	-	-	1280	586
	21	+	+	+	-	-	-	-	-	-	160	
	22	+	+	+	+	-	-	-	-	-	320	
116	23	+	+	+	+	+	-	-	-	-	640	640
	24	+	+	+	+	+	-	-	-	-	640	
	25	+	+	+	+	+	-	-	-	-	640	
	26	+	+	+	+	+	-	-	-	-	640	
117	27	+	+	+	+	+	+	-	-	-	1280	840

(continued)

Batch No.	Sample No.	Serum Dilution									HI titre	Group average HI titre
		40	80	160	320	640	1280	2560	5120	10240		
	28	+	+	+	+	+	-	-	-	-	640	
	29	+	+	+	+	+	+	-	-	-	1280	
	30	+	+	+	-	-	-	-	-	-	160	
118	31	+	+	+	+	+	+	-	-	-	1280	1253
	32	+	+	+	+	-	-	-	-	-	320	
	33	+	+	+	-	-	-	-	-	-	160	
	34	+	+	+	+	+	+	+	-	-	2560	
	35	+	+	+	+	+	+	+	-	-	2560	
	36	+	+	+	+	+	-	-	-	-	640	
119	37	+	+	+	+	+	+	+	-	-	2560	1600
	38	+	+	+	+	+	+	-	-	-	1280	
	39	+	+	+	+	+	+	-	-	-	1280	
	40	+	+	+	+	+	-	-	-	-	640	
	41	+	+	+	+	+	+	+	-	-	2560	
	42	+	+	+	+	+	+	-	-	-	1280	
120	43	+	+	+	+	+	+	-	-	-	1280	1280
	44	+	+	+	+	+	+	-	-	-	1280	
	45	+	+	+	+	+	+	+	-	-	2560	
	46	+	+	+	+	+	+	+	-	-	1280	
	47	+	+	+	+	+	-	-	-	-	640	
	48	+	+	+	+	+	-	-	-	-	640	
121	49	+	+	+	+	+	+	-	-	-	1280	1389
	50	+	+	+	+	+	+	+	-	-	2560	
	51	+	+	+	+	+	+	-	-	-	1280	
	52	+	+	+	+	+	-	-	-	-	640	
	53	+	+	+	+	+	+	-	-	-	1280	
	54	+	+	+	+	+	+	+	-	-	1280	
122	55	+	+	+	+	+	+	+	-	-	2560	1664
	56	+	+	+	+	+	+	+	-	-	2560	
	57	+	+	+	+	+	+	-	-	-	1280	

Batch No.	Sample No.	Serum Dilution									HI titre	Group average HI titre
		40	80	160	320	640	1280	2560	5120	10240		
	58	+	+	+	+	+	-	-	-	-	640	
	59	+	+	+	+	+	+	-	-	-	1280	
124	60	+	+	+	+	+	+	-	-	-	1280	1386
	61	+	+	+	+	+	+	-	-	-	1280	
	62	+	+	+	+	+	-	-	-	-	640	
	63	+	+	+	+	+	+	+	-	-	2560	
	64	+	+	+	+	+	+	-	-	-	1280	
	65	+	+	+	+	+	+	-	-	-	1280	
125	66	+	-	-	-	-	-	-	-	-	40	1726
	67	+	+	+	+	+	+	-	-	-	1280	
	68	+	+	+	+	+	+	+	-	-	2560	
	69	+	+	+	+	+	+	+	-	-	2560	
	70	+	+	-	-	-	-	-	-	-	80	
	71	+	+	+	+	+	+	+	-	-	2560	
126	72	+	+	+	+	+	-	-	-	-	640	1320
	73	+	+	+	+	+	+	-	-	-	1280	
	74	+	+	+	+	+	+	-	-	-	640	
	75	+	+	+	+	+	+	+	-	-	2560	
	76	+	+	+	+	+	+	+	-	-	2560	
127	77	+	+	+	+	+	+	-	-	-	1280	2026
	78	+	+	+	+	+	+	+	-	-	2560	
	79	+	+	+	+	+	-	-	-	-	640	
	80	+	+	+	+	+	+	+	-	-	2560	
	81	+	+	+	+	+	-	-	-	-	1280	
	82	+	+	+	+	+	+	+	-	-	2560	
128	83	+	+	+	+	+	+	+	-	-	2560	2240
	84	+	+	+	+	+	+	-	-	-	1280	
	85	+	+	+	+	+	+	+	-	-	2560	
	86	+	+	+	+	+	+	+	-	-	2560	

(continued)

Batch No.	Sample No.	Serum Dilution									HI titre	Group average HI titre
		40	80	160	320	640	1280	2560	5120	10240		
129	87	+	+	+	+	+	+	-	-	-	1280	1600
	88	+	+	+	+	+	+	+	-	-	2560	
	89	+	+	+	+	+	-	-	-	-	640	
	90	+	+	+	+	+	+	+	-	-	2560	
	91	+	+	+	+	+	+	-	-	-	1280	
	92	+	+	+	+	+	+	-	-	-	1280	
130	93	+	+	+	+	+	+	+	-	-	2560	1680
	94	+	+	+	+	+	+	-	-	-	1280	
	95	+	+	+	+	-	-	-	-	-	320	
	96	+	+	+	+	+	+	+	-	-	2560	
131	97	+	+	+	+	+	+	+	-	-	2560	1760
	98	+	+	+	+	+	+	-	-	-	1280	
	99	+	+	+	+	+	-	-	-	-	640	
	100	+	+	+	+	+	+	+	-	-	2560	
132	101	+	+	+	+	+	+	+	-	-	2560	2266
	102	+	+	+	+	+	+	+	-	-	2560	
	103	+	+	+	-	-	-	-	-	-	160	
	104	+	+	+	+	+	+	+	-	-	2560	
	105	+	+	+	+	+	-	-	-	-	640	
	106	+	+	+	+	+	+	+	+	-	5120	
133	107	+	+	+	+	+	+	-	-	-	1280	1493
	108	+	+	+	+	+	+	+	-	-	2560	
	109	+	+	+	+	+	+	-	-	-	1280	
	110	+	+	+	+	+	+	+	-	-	2560	
	111	+	+	+	+	+	-	-	-	-	640	
	112	+	+	+	+	+	-	-	-	-	640	
134	113	+	+	+	+	+	+	+	-	-	2560	
	114	+	+	+	+	+	+	-	-	-	1280	
	115	+	+	+	+	+	+	-	-	-	1280	
	116	+	+	+	+	+	+	-	-	-	1280	

(continued)

Batch No.	Sample No.	Serum dilution									HI titre	Group average HI titre
		40	80	160	320	640	1280	2560	5120	10240		
1	117	+	+	+	+	+	-	-	-	-	640	
	118	+	+	+	+	+	+	+	-	-	2560	
135	119	+	+	+	+	+	+	-	-	-	1280	1216
	120	+	+	+	+	+	+	-	-	-	1280	
	121	+	+	+	+	+	-	-	-	-	640	
	122	+	+	+	+	+	+	+	-	-	2560	
	123	+	+	+	+	-	-	-	-	-	320	
136	124	+	+	+	+	+	+	+	-	-	2560	1162
	125	+	+	+	+	+	+	-	-	-	1280	
	126	+	+	+	+	+	+	-	-	-	1280	
	127	+	+	+	+	+	-	-	-	-	640	
	128	+	+	-	-	-	-	-	-	-	80	
137	129	+	+	+	+	+	+	-	-	-	1280	1408
	130	+	+	+	+	+	+	-	-	-	1280	
	131	+	+	+	+	+	-	-	-	-	640	
	132	+	+	+	+	+	+	+	-	-	2560	
	133	+	+	+	+	+	+	-	-	-	1280	
138	134	+	+	+	+	+	+	-	-	-	1280	1493
	135	+	+	+	+	+	+	+	-	-	2560	
	136	+	+	+	+	+	-	-	-	-	640	
139	137	+	+	+	+	+	+	+	-	-	2560	1920
	138	+	+	+	+	+	+	+	-	-	2560	
	139	+	+	+	+	+	-	-	-	-	640	
140	140	+	+	+	+	+	+	+	-	-	2560	1386
	141	+	+	+	+	+	+	-	-	-	1280	
	142	+	+	+	+	-	-	-	-	-	320	

(continued)

Batch No.	Sample No.	Serum dilution									HI titre	Group average HI titre
		40	80	160	320	640	1280	2560	5120	10240		
141	143	+	+	+	+	+	+	-	-	-	1280	1333
	144	+	+	+	+	+	+	+	-	-	2560	
	145	+	+	+	-	-	-	-	-	-	160	
	146	+	+	+	+	+	+	+	-	-	2560	
142	147	+	+	+	+	-	-	-	-	-	320	1360
	148	+	+	+	+	+	+	-	-	-	1280	
	149	+	+	+	+	+	+	-	-	-	1280	
143	150	+	+	+	+	+	-	-	-	-	640	2133
	151	+	+	+	+	+	+	+	-	-	2560	
	152	+	+	+	+	+	-	-	-	-	640	
	153	+	+	+	+	+	+	+	+	-	5120	
	154	+	+	+	+	+	+	+	-	-	2560	
	155	+	+	+	+	+	+	-	-	-	1280	
144	156	+	+	+	+	+	+	+	-	-	2560	1200
	157	+	+	+	+	+	+	+	-	-	1280	
	158	+	+	+	+	+	+	-	-	-	1280	
	159	+	+	+	+	+	-	-	-	-	640	
	160	+	+	+	+	+	+	-	-	-	1280	
	161	+	+	+	-	-	-	-	-	-	160	

Titres expressed as reciprocal of serum dilution.

+ = denotes complete inhibition against two HA units.

A summary of responses of individual birds and results of challenge are given in table 6. The results reveal that the antibody response to the experimental infection with freeze dried Ranikhet disease vaccine was very encouraging. The lowest titre obtained was 40 and the highest was 5120. The group average was between 640 to 2266. The protection afforded was also very encouraging.

A summary showing the number of birds in each HI titre group is given in table 7. On the basis of number of birds in each HI titre group percentages have been worked out which are also given in the same table.

SERUM NEUTRALISATION TEST

Pooled samples of sera of birds used in potency tests were screened for the presence of neutralising antibodies and the results are presented in table 8. A perusal of the data would reveal that the neutralising index of sera of vaccinated birds varied from 4.24 to 6.30.

The same table would also reveal that out of 12 samples of sera representing 12 batches, 11 batches had 100% protection in birds by potency test, while one batch No. 125 gave a protection of 66.6 per cent.

Table 6

Individual responses of 6-8 weeks old birds to Freeze Dried Ranikhet Disease Vaccine

Batch No.	Sample No.	Bird No.	H.I. antibody level	Results of challenge	Remarks
103	1	3767	2560	Immune	
	2	476	1280	"	
	3	3879	640	"	
	4	4021	1280	"	
	5	4029	640	"	
104	6	4385	1280	"	
	7	3878	320	"	
	8	7657	1280	"	
	9	7359	1280	"	
	10	7724	640	"	
	11	3778	1280	"	
107	12	1728	640	"	
	13	8594	1280	"	
	14	1645	640	"	
	15	8577	2560	"	
	16	1768	640	"	
108	17	1615	640	"	
	18	8599	1280	"	
	19	2120	320	"	
109	20	1193	1280	"	
	21	1197	160	"	
	22	7733	320	"	
116	23	6873	640	"	
	24	6824	640	"	
	25	5825	640	"	
	26	6604	640	"	
117	27	5714	1280	"	
	28	6640	640	"	
	29	5644	1280	"	
	30	5919	160	"	

(continued)

Batch No.	Sample No.	Bird No.	H.I. antibody level	Results of challenge	Remarks
118	31	5584	1280	Immune	
	32	6794	320	"	
	33	6593	160	Died on 5th day	Worms
	34	5947	2560	Immune	
	35	6750	2560	"	
	36	9808	640	"	
119	37	5327	2560	"	
	38	5930	1280	"	
	39	5370	1280	"	
	40	5739	640	"	
	41	5627	2560	"	
	42	5873	1280	"	
120	43	8243	1280	"	
	44	8324	1280	"	
	45	8132	2560	"	
	46	6919	1280	"	
	47	5923	640	"	
	48	5963	640	"	
121	49	6290	1280	"	
	50	6291	2560	"	
	51	6292	1280	"	
	52	6293	640	"	
	53	644	1280	"	
	54	440	1280	"	
122	55	9773	2560	"	
	56	9814	2560	"	
	57	8792	1280	"	
	58	7885	640	"	
	59	8632	1280	"	

(continued)

Batch No.	Sample No.	Bird No.	H.I. antibody level	Results of challenge	Remarks
124	60	7989	1280	Immune	
	61	9696	1280	"	
	62	8759	640	"	
	63	9662	2560	"	
	64	9751	1280	"	
	65	6982	1280	"	
125	66	7648	40	Died on 4th day	Debility
	67	7991	1280	Immune	
	68	7741	2560	"	
	69	7745	2560	"	
	70	9882	80	Died on 5th day	Worms and debility
	71	9102	2560	Immune	
126	72	1134	640	"	
	73	1135	1280	"	
	74	1136	640	"	
	75	1137	2560	"	
	76	1138	2560	"	
127	77	1140	1280	"	
	78	1141	2560	"	
	79	1142	640	"	
	80	1143	2560	"	
	81	1144	1280	"	
	82	1145	2560	"	
128	83	8793	2560	"	
	84	8748	1280	"	
	85	7591	2560	"	
	86	9810	2560	"	

(continued)

Batch No.	Sample No.	Bird No.	H.I. antibody level	Results of Challenge	Remarks
129	87	3451	1280	Immune	
	88	13852	2560	"	
	89	383	640	"	
	90	312	2560	"	
	91	4073	1280	"	
	92	346	1280	"	
130	93	8402	2560	"	
	94	7026	1280	"	
	95	8429	320	"	
	96	6324	2560	"	
131	97	6912	2560	"	
	98	8856	1280	"	
	99	8981	640	"	
	100	7038	2560	"	
132	101	6313	2560	"	
	102	5957	2560	"	
	103	9816	160	"	
	104	8988	2560	"	
	105	9961	640	"	
	106	4746	5120	"	
133	107	8853	1280	"	
	108	4394	2560	"	
	109	8074	1280	"	
	110	7902	2560	"	
	111	8898	640	"	
	112	4715	640	"	
134	113	9476	2560	"	
	114	14444	1280	"	
	115	4386	1280	"	

(continued)

Batch No.	Sample No.	Bird No.	H.I. antibody level	Result of challenge	Remarks
	116	6963	1280	Immune	
	117	7531	640	"	
	118	8850	2560	"	
135	119	4817	1280	"	
	120	4819	1280	"	
	121	4820	640	"	
	122	4821	2560	"	
	123	4822	320	"	
136	124	1133	2560	"	
	125	3618	1280	"	
	126	1132	1280	"	
	127	1130	640	"	
	128	3620	80	Died on 4th day	Worms and debility
137	129	4560	1280	Immune	
	130	4550	1280	"	
	131	4559	640	"	
	132	1524	2560	"	
	133	4556	1280	"	
138	134	4768	1280	"	
	135	4638	2560	"	
	136	744	640	"	
139	137	4756	2560	"	
	138	4786	2560	"	
	139	3216	640	"	
140	140	3632	2560	"	
	141	3296	1280	"	
	142	976	320	"	
141	143	1922	1280	"	

(continued)

Batch No.	Sample No.	Bird No.	H.I.antibody level	Result of challenge	Remarks
	144	4760	2560	Immune	
	145	3208	160	"	
	146	4754	2560	"	
142	147	592	320	"	
	148	573	1280	"	
	149	574	1280	"	
143	150	4577	640	"	
	151	4678	2560	"	
	152	6492	640	"	
	153	4861	5120	"	
	154	14669	2560	"	
	155	3250	1280	"	
144	156	589	2560	"	
	157	4673	1280	"	
	158	798	1280	"	
	159	4767	640	Died on 10th day	Debility
	160	4674	1280	Immune	
	161	3252	160	Died on 12th day	Debility

The H.I. titres are expressed as the reciprocal of serum dilutions against two haemagglutinating units. The sera were from birds which had received 1 ml. of 10^{-6} dilutions of freeze dried vaccine.

Table 7

Summary of H.I. responses

No. of birds tested	H.I. antibody level titres	Percentage of birds showing various titres
2	5120	1.2
47	2560	22.3
58	1280	46.5
36	640	21.1
8	320	4.9
6	160	3.7
2	80	1.2
1	40	0.6

Table 8

Neutralising indices of serum samples

Batch No.	Neutralising index	Protection index
124	6.30	100
125	4.90	66.6
126	4.90	100
127	5.0	100
128	5.50	100
129	6.00	100
130	4.24	100
131	4.50	100
132	5.00	100
133	5.25	100
134	5.25	100
135	5.50	100

SAFETY TEST

Initially when the manufacture of the vaccine was started at the Indian Veterinary Research Institute, the vaccine was tested for safety and freedom from abnormal toxicity in chicks of 6-8 weeksold, by injecting 0.5 ml. of liquid vaccine suspension before it was freeze dried.

Subsequently on the recommendation of the Committee for Standardisation of Veterinary Biological Products,** the freeze dried product was used for safety test. The outline for this has been dealt with under 'Materials and Methods'. Accordingly results of safety test from batch No.78 were carried out.

Ninety-three batches of vaccine were tested in this manner and the results are presented in table 9. It would be seen that the vaccine was safe. The birds which died during the observation period were autopsied and the caused ascertained. The deaths were found to be due to other causes.

** Report of the Expert Committee for Standardisation of Veterinary Biological Products, Ministry of Food and Agriculture, Government of India, 1954.

Table 9

Results of Safety Test

Batch No.	Subcutaneous group	Intranasal group	Intratracheal group	Incontacts	Survival	
					Ratio	Percentage
78	3/3	2/3	3/3	3/3	11/12	91.6
79	3/3	3/3	3/3	3/3	12/12	100
80	3/3	3/3	2/3	3/3	11/12	91.6
81	1/3	3/3	2/3	3/3	9/12	75
82	3/3	3/3	3/3	2/3	11/12	91.6
83	1/3	3/3	2/3	2/3	8/12	66.6
84	3/3	3/3	3/3	2/3	12/12	100
85	3/3	3/3	3/3	3/3	12/12	100
86	3/3	2/3	3/3	3/3	11/12	91.6
87	3/3	3/3	3/3	3/3	12/12	100
88	2/3	3/3	3/3	3/3	11/12	91.6
89	3/3	3/3	3/3	3/3	12/12	100
90	2/3	2/3	2/3	2/3	8/12	66.6
91	3/3	3/3	3/3	3/3	12/12	100
92	2/3	3/3	2/3	3/3	10/12	83.3
93	2/3	3/3	2/3	3/3	10/12	83.3
94	3/3	3/3	3/3	3/3	12/12	100
95	2/3	2/3	3/3	3/3	10/12	83.3
96	2/3	3/3	2/3	2/3	9/12	75
97	3/3	3/3	3/3	3/3	12/12	100
98	3/3	3/3	3/3	3/3	12/12	100
99	3/3	3/3	3/3	3/3	12/12	100
100	3/3	3/3	3/3	3/3	12/12	100
101	2/3	3/3	2/3	3/3	10/12	83.3
102	3/3	3/3	2/3	3/3	11/12	91.6
103	3/3	2/3	2/3	3/3	10/12	83.3
104	2/3	3/3	3/3	3/3	11/12	91.6
105	3/3	3/3	3/3	3/3	12/12	100

(continued)

Batch No.	Subcutaneous group	Intranasal group	Intratracheal group	Incontacts	Survival	
					Ratio	Percentage
106	3/3	3/3	3/3	3/3	12/12	100
107	3/3	3/3	3/3	3/3	12/12	100
108	2/3	2/3	3/3	3/3	10/12	83.3
109	3/3	3/3	3/3	2/3	11/12	91.6
110	2/3	3/3	3/3	2/3	10/12	83.3
111	3/3	3/3	3/3	2/3	11/12	91.6
112	3/3	3/3	3/3	3/3	12/12	100
113	3/3	3/3	3/3	3/3	12/12	100
114	3/3	3/3	3/3	3/3	12/12	100
115	3/3	3/3	3/3	3/3	12/12	100
116	2/3	3/3	3/3	3/3	11/12	91.6
117	3/3	3/3	3/3	3/3	12/12	100
118	3/3	2/3	3/3	3/3	11/12	91.6
119	2/3	3/3	2/3	2/3	9/12	75
120	2/3	2/3	2/3	3/3	9/12	75
121	2/3	3/3	3/3	3/3	11/12	91.6
122	3/3	3/3	2/3	3/3	11/12	91.6
123	2/3	3/3	3/3	3/3	11/12	91.6
124	3/3	3/3	3/3	3/3	12/12	100
125	3/3	3/3	3/3	3/3	12/12	100
126	2/3	3/3	3/3	3/3	11/12	91.6
127	3/3	3/3	3/3	2/3	11/12	91.6
128	3/3	3/3	3/3	3/3	12/12	100
129	3/3	3/3	3/3	3/3	12/12	100
130	3/3	3/3	3/3	3/3	12/12	100
131	3/3	2/3	3/3	3/3	11/12	91.6
132	3/3	3/3	3/3	3/3	12/12	100
133	3/3	3/3	3/3	2/3	11/12	91.6

(continued)

Batch No.	Subcutaneous group	Intranasal group	Intratracheal group	Incontacts	Survival	
					Ratio	Percentage
134	3/3	2/3	3/3	3/3	11/12	91.6
135	3/3	3/3	3/3	3/3	12/12	100
136	2/3	2/3	3/3	3/3	10/12	83.3
137	3/3	2/3	3/3	3/3	11/12	91.6
138	2/3	3/3	3/3	3/3	11/12	91.6
139	2/3	3/3	3/3	3/3	11/12	91.6
140	3/3	3/3	2/3	3/3	11/12	91.6
141	2/3	3/3	3/3	3/3	11/12	91.6
142	3/3	3/3	2/3	3/3	11/12	91.6
143	2/3	3/3	3/3	2/3	10/12	83.3
144	3/3	3/3	3/3	3/3	12/12	100
145	3/3	3/3	3/3	3/3	12/12	100
146	3/3	2/3	3/3	3/3	11/12	91.6
147	3/3	2/3	3/3	3/3	11/12	91.6
148	3/3	2/3	3/3	3/3	11/12	91.6
149	2/3	3/3	3/3	3/3	11/12	91.6
150	3/3	2/3	2/3	3/3	10/12	83.3
151	Tested at Mukteswar, 100% safe					
152	3/3	3/3	3/3	3/3	12/12	100
153	3/3	3/3	2/3	3/3	11/12	91.6
154	3/3	3/3	2/3	3/3	11/12	91.6
155	3/3	2/3	2/3	3/3	10/12	83.3
156	2/3	3/3	3/3	3/3	11/12	91.6
157	2/3	2/3	3/3	2/3	9/12	75
158	3/3	3/3	3/3	3/3	12/12	100
159	2/3	3/3	2/3	3/3	10/12	83.3
160	3/3	2/3	2/3	3/3	10/12	83.3

(continued)

Batch No.	Subcutaneous group	Intra-nasal group	Intra-tracheal group	Incontacts	Survival	
					Ratio	Percentage
161	3/3	2/3	2/3	1/3	8/12	66.6
162	2/3	3/3	3/3	3/3	11/12	91.6
163	3/3	3/3	2/3	2/3	10/12	83.3
164	2/3	3/3	3/3	2/3	10/12	83.3
165	3/3	2/3	3/3	3/3	11/12	91.6
166	3/3	2/3	3/3	2/3	10/12	83.3
167	2/3	2/3	2/3	3/3	9/12	75
168	3/3	3/3	3/3	3/3	12/12	100
169	3/3	3/3	2/3	2/3	10/12	83.3
170	2/3	2/3	3/3	2/3	9/12	75

Numerator : Number of birds survived

Denominator : Number of birds inoculated

POTENCY TEST

The freeze dried Ranikhet disease vaccine manufactured at the Indian Veterinary Research Institute, for the immunisation of birds of 6 weeks and above, was initially tested at the dilutions of 1:400, 10^{-5}, 10^{-6}, 10^{-7}. Each bird was injected with 1 ml. In this way, 46 batches were assayed and the results are presented in table 10. After gaining experience with these 46 batches of vaccine which were used to vaccinate birds in large number of poultry farms all over India, testing at the 10^{-5} dilution was excluded. Further 31 batches (Nos. 47 to 77) were therefore tested in the remaining dilutions, viz. 1:400, 10^{-6} and 10^{-7}. Results are presented in table 11.

The percentages of protection at the dilutions of 1:400, 10^{-5}, 10^{-6} and 10^{-7}, are presented in tables 12 and those for the dilutions of 1:400, 10^{-6} and 10^{-7} in table 13. The results reveal that at 1:400 dilution, 72 out of 77 batches; at 10^{-5} dilution 44 out of 46 batches; and at 10^{-7} dilution, 70 out of 77 batches had a protection index of 50% and above.

Subsequently, in the light of the recommendations of the Committee for Standardisation of Biological Products, the procedure for potency test was further modified to

limit the testing of the vaccine to a single dilution of 10^{-6}. So, from batches 78 to 170 the vaccine was assayed at this dilution and the results are presented in table 14 and the protection percentages in table 15.

It may be mentioned here that the dilution of 10^{-6} has been common throughout, that is from batch 1 to 170 (tables 10, 11, 12 and 13). Out of 170 batches of vaccine assayed, 163 batches had a protection index of 50% and above.

The percentages of protection indices at different dilutions of the vaccine tested have been worked out and presented in table 16.

Table 10

Results of challenge tests in birds

Sl. No.	Batch No.	1:400	Dilution 10^{-5}	10^{-6}	10^{-7}	Virus controls
1	1	2/4	4/4	2/3	2/4	0/2
2	2	3/4	3/3	1/3	1/4	0/2
3	3	4/4	4/4	4/4	2/4	0/2
4	4	4/4	4/4	1/2	3/3	0/2
5	5	4/4	2/2	2/2	2/3	0/3
6	6	2/3	3/3	3/3	2/3	0/2
7	7	3/4	1/2	4/4	3/3	0/4
8	8	2/4	3/4	2/2	2/3	0/4
9	9	3/4	3/3	3/4	3/4	0/2
10	10	1/4	2/2	2/3	2/3	0/2
11	11	3/4	3/4	2/3	3/3	0/2
12	12	3/4	4/4	1/4	3/4	0/2
13	13	2/2	2/2	2/2		
14	14	2/2	2/2	2/2		0/2
15	15	3/4	4/4	4/4	3/3	0/2
16	16	4/4	4/4	1/4	3/4	0/2
17	17	4/4	2/3	3/4	3/4	0/2
18	18	4/4	4/4	4/4	4/4	0/2
19	19	3/4	2/4	3/3	4/4	0/2
20	20	3/4	1/2	4/4	2/4	0/2
21	21	3/3	3/3	1/3	0/4	0/4
22	22	3/4	4/4	4/4	3/4	0/2
23	23	3/4	Not done	2/3	4/4	0/2
24	24	3/3	1/1	3/4	2/2	0/2
25	25	3/4	4/4	4/4	2/3	0/2

(continued)

Sl. No.	Batch No.	1:400	Dilution 10^{-5}	10^{-6}	10^{-7}	Virus controls
26	26	3/4	3/3	4/4	2/3	0/2
27	27	2/4	2/4	3/4	2/4	0/2
28	28	3/4	2/3	4/4	4/4	0/2
29	29	4/4	4/4	3/4	4/4	0/2
30	30	4/4	4/4	3/4	4/4	0/2
31	31	4/4	3/3	1/3	2/2	0/2
32	32	2/3	2/3	2/3	3/4	0/2
33	33	2/3	3/3	3/4	3/4	0/2
34	34	2/3	3/4	3/4	3/4	0/2
35	35	4/4	3/3	3/4	3/3	0/3
36	36	4/4	2/2	1/1	4/4	0/3
37	37	2/2	2/2	2/3	1/2	0/2
38	38	2/2	0/0	3/3	3/4	0/2
39	39	2/3	2/3	1/2	4/4	0/4
40	40	2/4	2/3	3/3	1/2	0/4
41	41	4/4	4/4	4/4	3/4	0/2
42	42	4/4	4/4	4/4	3/4	0/2
43	43	4/4	4/4	3/4	1/4	0/2
44	44	4/4	4/4	4/4	3/4	0/2
45	45	2/2	2/2	4/4	2/3	0/2
46	46	4/4	3/4	4/4	4/4	0/2

Numerator = Birds survived

Denominator = Birds challenged/used for controls

Batches 13 and 14 were tested at the Division of Bacteriology and Virology, Mukteswar in the dilutions of 1:200, 10^{-5} and 10^{-6} only. A cent per cent protection was found in all the dilutions.

Table 11

Results of challenge tests in birds

Sl. No.	Batch No.	1:400	Dilution 10^{-6}	Dilution 10^{-7}	Virus controls
1	47	2/2	2/3	3/4	1/2
2	48	4/4	4/4	2/3	1/2
3	49	3/3	2/2	4/4	0/2
4	50	4/4	3/3	3/3	0/2
5	51	4/4	3/4	1/4	1/2
6	52	4/4	1/4	3/4	0/2
7	53	4/4	4/4	3/3	0/2
8	54	4/4	4/4	4/4	2/3
9	55	3/4	4/4	4/4	0/2
10	56	3/4	4/4	4/4	0/2
11	57	3/3	2/3	2/2	0/2
12	58	4/4	3/4	3/4	0/2
13	59	2/4	4/4	4/4	1/2
14	60	3/3	3/3	1/2	0/2
15	61	2/2	3/3	2/2	0/2
16	62	0/4	3/3	2/2	0/2
17	63	0/4	3/3	2/3	0/2
18	64	0/4	2/2	3/3	0/2
19	65	1/1	2/2	3/3	0/2
20	66	2/3	3/4	2/3	0/2
21	67	3/4	4/4	4/4	0/4
22	68	4/4	3/3	2/3	0/2
23	69	4/4	3/3	3/3	0/2
24	70	4/4	3/3	2/3	0/2
25	71	0/2	2/3	1/1	0/2
26	72	3/3	0/0 (Not done)	3/4	0/2
27	73	4/4	4/4	3/3	0/4
28	74	4/4	4/4	4/4	0/2
29	75	3/4	4/4	3/4	0/2
31	76	4/4	3/4	1/4	0/2
31	77	4/4	4/4	4/4	0/2

Table 12

Immunity pattern in birds

Sl. No.	Batch No.	Protection - percentage at				Mortality % in the controls
		1:400	10^{-5}	10^{-6}	10^{-7}	
1	1	50	100	66.6	50	100
2	2	75	100	33.3	25	100
3	3	100	100	100	50	100
4	4	100	100	50	100	100
5	5	100	100	100	66.6	100
6	6	66.6	100	100	66.6	100
7	7	75	50	100	100	100
8	8	50	75	100	66.6	100
9	9	75	100	75	75	100
10	10	25	100	66.6	66.6	100
11	11	75	75	66.6	100	100
12	12	75	100	25	75	100
13	13	100	100	100	-	100
14	14	100	100	100	-	100
15	15	75	100	100	100	100
16	16	100	100	25	75	100
17	17	100	66.6	75	75	100
18	18	100	100	100	100	100
19	19	75	50	100	100	100
20	20	75	50	100	50	100
21	21	100	100	33.3	0	100
22	22	75	100	100	75	100
23	23	75	Not done	66.6	100	100
24	24	100	100	75	100	100
25	25	75	100	100	66.6	100
26	26	75	100	100	66.6	100

(continued)

Sl. No.	Batch No.	Protection - percentage at				Mortality % in the controls
		1:400	10^{-5}	10^{-6}	10^{-7}	
27	27	50	50	75	50	100
28	28	75	66.6	100	100	100
29	29	100	100	75	100	100
30	30	100	100	75	100	100
31	31	100	100	33.3	100	100
32	32	66.6	66.6	66.6	75	100
33	33	66.6	100	75	75	100
34	34	66.6	75	75	75	100
35	35	100	100	75	100	100
36	36	100	100	100	100	100
37	37	100	100	66.6	50	100
38	38	100	-	100	75	100
39	39	66.6	66.6	50	100	100
40	40	50	66.6	100	50	100
41	41	100	100	100	75	100
42	42	100	100	100	75	100
43	43	100	100	75	25	100
44	44	100	100	100	75	100
45	45	100	100	100	66.6	100
46	46	100	75	100	100	100

Table 13

Immunity pattern in birds

Sl. No.	Batch No.	1:400	Protection percentage 10^{-6}	Protection percentage 10^{-7}	Mortality percentage in the controls
1	47	100	66.6	75	50
2	48	100	100	66.6	50
3	49	100	100	100	100
4	50	100	100	100	100
5	51	100	75	25	50
6	52	100	25	75	100
7	53	100	100	100	100
8	54	100	100	100	33.3
9	55	75	100	100	100
10	56	100	100	100	100
11	57	100	66.6	100	100
12	58	100	75	75	100
13	59	50	100	100	50
14	60	100	100	50	100
15	61	100	100	100	100
16	62	0	100	100	100
17	63	0	100	66.6	100
18	64	0	100	100	100
19	65	100	100	100	100
20	66	66.6	75	66.6	100
21	67	75	100	100	100
22	68	100	100	66.6	100
23	69	100	100	100	100
24	70	100	100	66.6	100
25	71	0	66.6	100	100
26	72	100	Not done	75	100
27	73	100	100	100	100
28	74	100	100	100	100
29	75	75	100	75	100
30	76	100	75	25	100
31	77	100	100	100	100

Table 14

Results of challenge tests in birds

Sl. No.	Batch No.	Dilution 10^{-6}	Virus controls
1	78	3/3	0/2
2	69	3/3	0/2
3	80	3/3	0/2
4	81	3/4	0/4
5	82	4/4	0/4
6	83	2/2	0/4
7	84	4/4	0/4
8	85	2/2	0/4
9	86	4/4	2/4
10	87	4/4	1/4
11	88	2/3	0/2
12	89	2/3	0/3
13	90	2/3	0/4
14	91	1/2	0/4
15	92	3/4	0/3
16	93	2/4	0/4
17	94	2/4	0/4
18	95	3/4	2/4
19	96	2/2	2/4
20	97	3/4	3/4
21	98	5/5	1/4
22	99	4/4	0/4
23	100	4/5	0/4

Sl.No.	Batch No.	Dilution 10^{-6}	Virus controls
24	101	3/5	2/4
25	102	6/6	0/4
26	103	6/6	0/4
27	104	6/6	0/4
28	105	4/5	0/4
29	106	5/5	0/4
30	107	5/5	0/4
31	108	3/3	0/4
32	109	3/3	0/4
33	110	6/6	0/4
34	111	6/6	0/4
35	112	6/6	0/4
36	113	5/5	0/4
37	114	3/5	0/4
38	115	3/5	0/4
39	116	4/4	0/4
40	117	4/4	0/4
41	118	5/6	0/4
42	119	6/6	0/4
43	120	6/6	0/4
44	121	6/6	0/4
45	122	5/5	0/6
46	123	6/6	0/6
47	124	6/6	0/6
48	125	4/6	0/4

(continued)

Sl. No.	Batch No.	Dilution 10^{-6}	Virus control
49	126	5/5	0/4
50	127	6/6	0/4
51	128	4/4	0/4
52	129	6/6	0/4
53	130	4/4	0/4
54	131	4/4	0/4
55	132	6/6	0/4
56	133	6/6	0/4
57	134	6/6	0/4
58	135	5/5	0/4
59	136	4/5	0/4
60	137	5/5	0/4
61	138	3/3	0/4
62	139	3/3	0/4
63	140	3/3	0/4
64	141	4/4	0/4
65	142	3/3	0/4
66	143	6/6	0/4
67	144	4/6	0/4
68	145	5/6	0/4
69	146	6/6	1/4
70	147	6/6	2/4
71	148	6/6	0/4
72	149	3/5	0/4
73	150	5/5	0/4

(continued)

Sl. No.	Batch No.	Dilution 10^{-6}	Virus controls
74	151	6/6	0/4
75	152	5/6	0/4
76	153	3/3	0/4
77	154	4/4	0/4
78	155	3/4	0/4
79	156	3/4	0/4
80	157	4/5	0/4
81	158	3/4	0/4
82	159	3/4	0/4
83	160	4/5	0/4
84	161	5/5	0/4
85	162	3/4	0/4
86	163	4/5	0/4
87	164	6/6	0/4
88	165	3/4	0/4
89	166	4/5	0/4
90	167	3/3	0/4
91	168	5/5	0/1
92	169	4/5	0/2
93	170	3/4	0/4

Numerator = Number of birds survived

Denominator = Number of birds challenged

Table 15

Immunity pattern in birds

Sl. No.	Batch No.	Protection percentage at 10^{-6} dilution	Mortality % in controls
1	78	100	100
2	79	100	100
3	80	100	100
4	81	75	100
5	82	100	100
6	83	100	100
7	84	100	100
8	85	100	100
9	86	100	50
10	87	100	75
11	88	66.6	100
12	89	66.6	100
13	90	66.6	100
14	91	50	100
15	92	75	100
16	93	50	100
17	94	50	100
18	95	75	50
19	96	100	50
20	97	75	25
21	98	100	75
22	99	100	100
23	100	80	100

(continued)

Sl. No.	Batch No.	Protection percentage at 10^{-6} dilution	Mortality % in controls
24	101	60	50
25	102	100	100
26	103	100	100
27	104	100	100
29	105	80	100
30	106	100	100
31	107	100	100
32	108	100	100
33	109	100	100
34	110	100	100
35	111	100	100
36	113	100	100
37	114	100	100
38	115	60	100
39	116	100	100
40	117	100	100
41	118	83.3	100
42	119	100	100
43	120	100	100
44	121	100	100
45	122	100	100
46	123	100	100
47	124	100	100
48	125	66.6	100

(continued)

Sl. No.	Batch No.	Protection % at 10^{-6} dilution	Mortality% in controls
49	126	100	100
50	127	100	100
51	128	100	100
52	129	100	100
53	130	100	100
54	131	100	100
55	132	100	100
56	133	100	100
57	134	100	100
58	135	100	100
59	136	80	100
60	137	60	100
61	138	100	100
62	139	100	100
63	140	100	100
64	141	100	100
65	142	100	100
66	143	100	100
67	144	66.6	100
68	145	83.3	100
69	146	100	75
70	147	100	100
71	148	100	100
72	149	60	100

(continued)

Sl. No.	Batch No.	Protection % at 10^{-6} dilution	Mortality % in controls
73	150	100	100
74	151	100	100
75	152	83.3	100
76	153	100	100
77	154	100	100
78	155	75	100
79	156	75	100
80	157	80	100
81	158	75	100
82	159	75	100
83	160	80	100
84	161	100	100
85	162	75	100
86	163	80	100
87	164	100	100
88	165	75	100
89	166	80	100
90	167	100	100
91	168	100	100
92	169	80	100
93	170	75	100

Table 16

Percentage of protection at different levels

Dilution of vaccine	Percentage of protection					
	100%	75-83.3%	60-66.6%	50%	25-33%	0%
1:400	45/77	16/77	6/77	5/77	1/77	4/77
10^{-5}	31/46	4/46	5/46	4/46	0/46	2/46
10^{-6}	104/170	36/170	18/170	5/170	6/170	1/170
10^{-7}	34/77	17/77	12/77	7/77	4/77	3/77

Numerator = Number of batches showing protection.

Denominator = Number of batches tested

PARALLEL TITRATIONS

Experiment I

Parallel titrations of four batches of the freeze dried Ranikhet disease vaccine were done in the 9-10 days old embryos and in the chicks of 6 to 8 weeks of age. Results are presented in tables 17, and 18. In this experiment the dilutions used were 10^{-5}, 10^{-6} and 10^{-7}. The embryo infectivity titres were found to be $10^{-6.6}$, $10^{-6.0}$, $10^{-6.5}$ and $10^{-7.3}$ and the corresponding protection titres in chicks were $10^{-7.5}$, $10^{-7.2}$, $10^{-7.3}$ and $10^{-7.5}$.

Experiment II

In this experiment, only two dilutions viz. 10^{-6} and 10^{-7} of all the three batches were used in both the systems. The titres obtained were $10^{-7.3}$, $10^{-6.5}$ and $10^{-7.5}$ with corresponding titres of $10^{-7.5}$, $10^{-6.5}$ and $10^{-6.5}$ in chicks. Results are presented in table 19 and 20.

Experiment III

Consequent upon the adoption of the new outlines sugg ted by the Committee for Standardisation of Veterinary Biolo gical Products, a single dilution of the vaccine was tested in chicks. But decimal dilutions of 6, 7 and 8 were employ for titration in the chick embryos. Results are presented table 4, under embryo infectivity. 22 batches viz. 103 to 121, and 168 to 170 were titrated and results show that the minimum titre obtained was $10^{-5.6}$ and the maximum was $10^{7.5}$

Table 17

Chick embryo titration of Freeze Dried Ranikhet Disease Vaccine vis-a-vis immunity pattern in chicks

Batch No. —	23		24		35		36	
	MORTALITY							
Dilution	In embryos (48 hrs)	In birds	In embryos (48 hrs)	In birds	In embryos (48hrs)	In birds	In Embryos (48hrs)	in birds
10^{-7}	1/4	0/4	0/4	0/2	1/4	0/3	3/4	0/4
10^{-6}	4/4	0/2	2/4	1/4	3/4	1/4	4/4	0/1
10^{-5}	4/4	Not done	4/4	0/1	-	0/3	-	0/2

Numerator = Mortality
Denominator = Number inoculated

Table 18

Comparison of titres in chick embryos and in chicks
(calculated on the basis of 50% endpoint)

Batch No.	Titre in chick embryo per 0.1 ml.	Titre in chicks per 1 ml.
23	6.6	7.5
24	6.0	7.2
35	6.5	7.3
36	7.3	7.5

Table 19

Chick embryo titration of Freeze Dried Ranikhet Disease Vaccine vis-a-vis immunity pattern in chicks

Batch No. —	50		51		52	
Dilution	MORTALITY					
	In embryos	In birds	In embryos	In birds	In embryos	In birds
	48 hrs.		48 hrs.		48 hrs.	
10^{-7}	3/3	0/3	2/4	3/4	4/4	1/4
10^{-6}	1/2	0/3	2/4	1/4	3/3	3/4

Numerator = Mortality

Denominator = Number inoculated

Table 20

Comparison of titres in chick embryos and chicks (Calculated as LD_{50})

Batch No.	Titre in chick embryo per 0.1 ml.	Titre in chicks per 1 ml.
50	7.3	7.5
51	6.5	6.5
52	7.5	6.5

LYOPHILISATION

As discussed under 'Matérials and Methods', the liquid vaccine was dried from the frozen state. The primary drying was carried out for 19 hours and the pressure of the vacuum used to be 0.2 mm. at 2 hours of loading in the machines which gradually used to come down to 0.14 mm. at the end of the operation. Results are tabulated in table 21.

Secondary drying cycle was done for 19 hours on secondary system. The headers were maintained at a pressure of 0.14-0.12 mm. Observations are presented in table 22.

BIOLOGICAL TESTS

A biological test was conducted of the birds which used to die within 3 to 5 days of challenge. The results revealed that the vaccinated birds after challenge died of causes other than Ranikhet disease since the birds used for biological test survived.

FREEZING AND THAWING

During certain parts of the year, the vaccine could not be dried for want of freeze drying ampoules and it was stored i the deep freeze cabinet for periods varying from 7 days to 107 days (table 23).

The frozen vaccine had to be thawed from -10°C to 24-25° before it could be prepared for freeze drying. It took nearly two hours.

Results of titrations of these batches, 130-142, 157 and 167 are presented in table 15. It would be observed that none of the batches of vaccine which had undergone freezing and thawing had deteriorated in their immunogenic efficiency. The protection at 10^{-6} dilution was found to be 100% in ten out of 11 batches and 80% in the 11th batch, (batch No.157).

Table 21

Observations - Primary drying

Hours	Ref. temperature °C	Pressure	Room temp. °C	Remarks
0	-42	-	22	Centrifuge on
0.45	-38	-	24	Centrifuge off
2	-38	0.2	24	
4	-40	0.18	24	
6	-40	0.18	24	
8	-42	0.18	24	
10	-43	0.18	24	
12	-43	0.16	24	
14	-43	0.16	24	
16	-45	0.16	22	
18	-45	0.14	22	
19	-45	0.14	24	

Table 22

Observations - Secondary drying

Hours	Pressure mm. of mercury	Room temp. °C
0	0.14	24
2	0.15	24
4	0.13	24
6	0.12	23
8	0.12	23
10	0.12	24
12	0.12	24
14	0.12	24
16	0.12	24
18	0.12	24
19	0.12	24

Table 23

Effect of freezing and thawing on the Ranikhet Disease Vaccine virus (Mukteswar)

Batch Np.	Date of harvesting	Date of drying	Period of storage at −10°C	Immunising titre in chicks	Protection percentage
130	23-8-1965	6- 9-65	14 days	10^{-6}	100
131	30-8-1965	7- 9-65	8 "	-do-	100
132	6-9-1965	15- 9-65	9 "	-do-	100
137	1-11-1965	15- 2-66	107 "	-do-	100
138	3- 1-1966	22- 2-66	50 "	-do-	100
139	17-1-1966	28- 2-66	42 "	-do-	100
140	31-1-1966	3- 3-66	31 "	-do-	100
141	7-2-1966	8- 3-66	29 "	-do-	100
142	21-2-1966	14- 3-66	21 "	-do-	100
157	19-9-1966	26- 9-66	7 "	-do-	80
167	20- 3- 67	28- 3-67	8 "	-do-	100

DISCUSSION

DISCUSSION

VIRUS MULTIPLICATION

Several workers have exploited the usefulness of developing chicken embryo not only as a medium for virus growth for vaccine production but also for assay. This is particularly true in respect of influenza and Newcastle disease viruses.

Many a worker have attempted to trace the development of the infective particle in the embryonic fluids and also the significance of relative concentration of the infective unit and the haemagglutinins at different intervals in the fluids and tissues of the embryo (Henle and Henle, 1949; Hoyle, 1950; Wenner et al. 1950; Liu and Henle, 1951; Sakkubai, 1960). These workers have noted that at first the infective virus particle appeared in the embryo fluids and thereafter the haemagglutinin particles could be detected in demonstrable quantities. Furthermore the quantity of haemagglutinins produced was intimately linked up with the active multiplication of the infective units.

In the present study, it was observed that the infective particle could be detected at the 6th hour of incubation and that there was a progressive rise in the infectivity titre of the allantoic fluid as is evident from table 1 and fig. 1. The appearance of haemagglutinins was detected at the 18th hour of incubation which also increased in concentration as the virus titre rose (tables 2 and 3). Hill (1957) observed that there was corresponding

increase in the HA titre of the ND virus with the increase of embryo infective titre. The observations made in this study concur with those of Hill as well as those of Sakkubai.

Another interesting observation that has been noticed is that the HA activity is demonstrable only when the embryo infective titre is about $10^{-5.2}$ $CELD_{50}$. Hanson *et al.* (1947) working with NDV pointed out that the virus suspensions having an embryo LD_{50} of 10^{-5} or less failed to produce HA and the present observations bear a similarity with the observations of these workers.

From table 3 it will be seen that while the infective titre reached its peak at 36th hour of incubation and remained almost stationary till 48 hours, the HA activity increased gradually upto 48th hour. This is explained by the fact that after the death of the embryo the increase in HA titre is due to the reaction of both viable and non-viable virus particles. Similar observations have been reported with influenza virus by Bernkopf (1950) and with NDV by Wenner *et al.* (1950) and Sakkubai (1960).

HAEMAGGLUTINATION-INHIBITION ANTIBODY LEVELS

In the experimental and field investigations, the haemagglutination-inhibition test has been employed by various workers as a convenient tool for measuring the

the degree of response to vaccination. This serological method provides a convenient technique for obtaining quantitative data on the degree of response since it measures a specific antibody induced by the virus.

Hofstad (1951) showed that a demonstrable level of HI antibody was reached usually 5-10 days after infection with NDV.

In the present study, the HI reaction has been employed as a means of evaluating the response of birds to the freeze-dried Ranikhet disease vaccine.

A perusal of the data in table 6 would reveal that out of sera of 161 birds screened for HI antibody, in 97% of them the HI antibody was correlated with the protection against challenge virus. It was only in 3% of cases that the birds which had comparatively low levels of HI antibody (40, 80 and 160) died on the 4 to 5th day after challenge. These were autopsied and Ranikhet disease was ruled out.

However, it may be mentioned that these birds could not possibly produce a high level of HI antibody due to intercurrent infections. Unfortunately the serum samples from such birds were not screened for the presence of neutralising antibody which, however, could have explained the significance of HI antibodies vis-a-vis protection at this level. The protection of HI antibody levels of

1:10 was also not noticed as the controls invariably succumbed to challenge virus.

Positive HI reactions are always correlated with ability to withstand challenge tests. HI negative reactions, unless accompanied by serum neutralisation tests or challenge data, are far leas meaningful because they may or may not be associated with resistance to challenge and the presence of neutralising antibody (Markham *et al*. 1954).

Because of the signifidance of the HI positive reaction, it might be assumed that a method of vaccination which elicits an increased HI titre is superior to a method which fails to do so.

To the extent that positive HI antibody levels are accompanied by protection, the findings in the present study are in agreement. The data also support that the vaccine virus viz. R_2B strain of R.D. virus is capable of evoking a high degree of immune response.

NEUTRALISING ANTIBODIES

Circulating antibody is the basis of immunity against reinfection. The most direct and biologically significant way of detecting and measuring viral antibodies is by the capacity of a serum to prevent or hinder the infectivity of a virus preparation.

In the present studies, 12 batches (124 to 135) of pooled sera were subjected to neutralisation test by using constant serum plus varying dilution of virus. Normal sera were used as controls. SNI was calculated according to Reed and Muench (1938). Data are presented in table 8. The lowest neutralising index obtained was 4.24 and the highest 6.30. Protection at both these levels was 100%. Thus it would be seen that serum neutralising antibodies are sufficient indicators of immune status of the birds.

The interrelationship of the HI and SN antibodies has also been studied in detail and the available reports indicate that neither these two antibodies nor the mechanism of their formation is the same. It has been established that the SN antibodies persist substantially longer than HI antibodies (Brandly et al. 1947 and Hanson et al. 1950). It has been discussed by several workers (Luginbuhl and Jungherr, 1949; Zargar and Pomeroy, 1949; Doll et al. 1950; Nilakantan et al. 1960b) that the HI response may differ according to the strain of virus used, the time of development, titre and its persistence, route of infection and other factors. However, this aspect has not been studied in this treatise. The stress was more on the response that the freeze dried Ranikhet disease vaccine was able to elicit in the vaccinated birds through the

well established procedures of HI and SN tests, and evolving a simpler and rapid method for potency assay of the vaccine.

POTENCY ASSAY

Various criteria have been suggested for the determination and evaluation of immunity including the intradermal test (Wasserman and Yates, 1953; Yates *et al*.1954).

Lancaster (1962) discussed the use of HI test as a quantitative measure of immune response. Doll *et al*. (1950a, 1950b, 1950c); Hitchner and Reising (1953); Hamann (1958); Markham *et al*. (1954), Raggi and Lee (1962) and Simmins and Baldwin (1963) emphasised that the HI response could not be compared directly with immune status as measured by challenge tests. On the other hand, Nilakantan *et al*. (1960b) observed that a HI response of 1:20 was a dependable guide for probability of protection through challenge tests.

The observations of Keeble and Wade (1963) and Raggi and Lee (1960) that SN antibody and HI antibody did not always develop or decline at the same rate following vaccination, makes it impossible to regard as a single entity, the immune bodies associated with HI, virus neutralization and specific refractivity to infection (Brandly *et al*. 1947; Hanson *et al*. 1950 and Dardiri and Yates, 1962).

Nakamura et al. (1956) did not observe any definite relationship between HI and SN tests and respiratory infection. However, SN test has been considered to portray a truer picture of the immunity than the HI test (Hitchner and Reising, 1954).

It therefore follows from the work of the above authors that the criteria adopted for evaluating the immunity engendered by Newcastle disease vaccines have been variable and thus a close relationship of all the results made difficult.

Bankowski and Cortsvet (1960) were of the view that immunity in Newcastle disease was composed of many measurable and unmeasurable factors.

However, after weighing the pros and cons of the problem, a better procedure for determining the immunity appears to be to expose vaccinated and non-vaccinated chickens to virulent virus. This has been the view of Taylor (1953) and Bankowski and Cortsvet (1962). The criterion laid down for a positive result being the absence of mortality or sign of infection.

In the light of these, 170 batches of freeze dried Ranikhet disease vaccine manufactured at the Indian Veterinary Research Institute, were assayed, through direct challenge test. The data of survivals after challenge and

the percentages of protection indices have been presented in tables 10 to 15.

The summary of batches of vaccine which gave protection at different dilutions and at different percentages of protection in table 16 shows that 72 out of 77 batches (93.5%) at 1:400 dilution; 44 out of 46 batches (95.6%) at 10^{-5} dilution; 163 out of 170 (95.8%) at 10^{-6} dilution, and 70 out of 77 (nearly 91%) at 10^{-7} dilution, had a protection level of 50% and above.

Taking the dilutions of 10^{-6} and the protection of even 75% and above, the data works out to 82.3%. This dilution is being discussed in the light of recommendations of the Committee for Standardisation of Veterinary Biological Products and also the sera that were employed for assay of HI and SN titres, was from the birds that received 10^{-6} dilution of the vaccine. On the basis of 75% protection the titre works out to 10^{-6}/1 ml. which consisted 1000,000 infective units and in the field dose it works out to 5,000 minimum infective units for a vaccinating dose which has a wide margin of safety.

The immune response through HI was very encouraging (tables 5 and 6). The immune HI antibody response and the protection (table 6) have already been discussed above.

The serum neutralizing index (SNI) of immune sera (table 8) was also quite good. The lowest SNI was 4.24

and the highest was 6.30 which corresponded to neutralisation of 16,000 to 19,95000 LD_{50} units of the virus (4.2 logs to 6.2 logs). Sakkubai (1960) reported that the immune serum gave a neutralising index of 7.27.

Beach (1944), Osteen and Anderson (1948) who employed chickens and embryos for SNT, reported that 1000 infective doses of virus were neutralised but they employed virulent virus.

Absolute protection is generally not possible in biological systems of assays due to individual variations and genetic make up (Cole and Hutt, 1961). The vaccinated birds during the challenge period develop a temporary viraemia (Hofstad, 1956; Gill et al.1959) which may gain upper hand in case of birds having worms or some other stress factors. The low level of antibodies may not be sufficient to warrant protection against virulent virus, and the birds may succumb. In the present study the birds which died were subjected to careful autopsy and the cause ascertained as far as possible. In most of the cases heavy worm infestation and debility were the causes.

On such occasions where the birds died 3 to 5 days after challenge, biological test was performed. The results of the biological tests proved that the birds which died after challenge in these cases, did not suffer

from Ranikhet disease. This explains the discrepancies at certain dilutions of the vaccine in the potency test.

Burnet and Ferry (1934) demonstrated the pathogenicity of Newcastle disease virus to the chicken embryos. Chick embryo, besides providing a source for vaccine production, has been employed for assay purposes. This is all the more true of Newcastle disease since the systems in both cases (CE and chicken) is almost identical.

British Veterinary Codex lays down that the Newcastle disease vaccines (living) are standardised so that one dose for intramuscular or subcutaneous inoculation in the field contains 1000 to 100,000 infective doses for the chick embryo. With a view to evolve a simpler and rapid method of assay of the Ranikhet disease vaccine it was decided to titrate this vaccine in the developing chicken embryo.

Before this was initiated, to get a preliminary idea about the correlation between embryo infective titres and the protection through challenge tests and also to simulate identical conditions, the freeze dried vaccine was titrated in developing chick embryo and in the chicks which were used as a routine for biological assay.

The results of parallel titrations at dilutions of 10^{-5}, 10^{-6} and 10^{-7} are presented in tables 17, 18, 19 and 20.

The four batches, namely, 23, 24, 35 and 36 had embryo infective titres of $10^{-6.6}$, $10^{-6.0}$, $10^{-6.5}$ and $10^{-7.3}$ with corresponding chicken titres of $10^{-7.5}$, $10^{-7.2}$, $10^{-7.3}$ and $10^{-7.5}$ (tables 17 and 18). A reference to table 12 would show that these batches had 100% protection at the highest dilution of 10^{-7}. Protection was 75% and above at dilution of 10^{-6} (batches 24, 35 and 36) while batch 23 gave 66.6% protection at this dilution.

Reducing the figures to arithmetical units, the above batches of vaccine had 3981000, 1000000, 3162000 and 19950000 embryo infective units which works out to 40 (39.81), 10, 31 and 199 vaccinating doses based on 100,000 embryo infective units per vaccinating dose.

The results obtained in experiment I were repeated in experiment II. Batches 50, 51 and 52 were titrated at 10^{-6} and 10^{-7} dilution in both the systems. The titres obtained in chick embryos were $10^{-7.3}$, $10^{-6.5}$ and $10^{-7.5}$, while the titres in chicks worked out to $10^{-7.5}$, $10^{-6.5}$ and $10^{-6.5}$ (tables 19 and 20). The protection was absolute in the case of batch 50 while protection at 10^{-6} dilution of batch 51 was 75%. However in respect of batch 52, 75% protection was noted at higher dilutions i.e. 10^{-7} as compared to 25% at the next lower dilution i.e. 10^{-6}. This may be due to the skipping of dilution, commonly noted with viruses (table 13).

Encouraged by the results of the two experiments discussed in the preceding pages, 22 batches (103 to 121 and 168 to 170) of freeze dried Ranikhet Disease vaccine were assayed in the chick embryos and their results presented in table 4 would show that the titres varied from $10^{-5.60}$ to $10^{-7.75}$ which corresponds to 4 and 562 vaccinating doses. As per the standards laid down by the British Veterinary Codex, this is quite satisfactory and the results of chick embryo titration could be relied upon as a method for the assay of freeze dried Ranikhet disease vaccine. The protection afforded by these batches was also high (table 15).

In the light of the findings presented in this dissertation and the experience of previous workers it is clear that the embryo infectivity test which bears a close relationship with the immunizing efficiency of the vaccine can be depended upon as a suitable substitute for the direct challenge test.

From the results of freezing and thawing of Ranikhet disease virus presented in table 23, it would be observed that there was no deleterious effect in the immunising efficiency of the vaccine virus, even when the storage period was upto 107 days at -10°C. This would facilitate

storing of liquid vaccine in times of scarcity of freeze drying ampoules or any other mechanical breakdown.

From the observations on drying cycles presented in tables 21 and 22, it would be observed that the huge quantities of Ranikhet disease vaccine prepared at this Institute was dried in a thoroughly convincing manner. The ampoules were finally sealed under a pressure of 0.05 to 0.01 mm. of mercury.

CONCLUSIONS AND SUMMARY

CONCLUSIONS

In the present studies the sequence of development of the infective particle and the haemagglutinins in the embryonic tissues during the growth of the virus was followed. It has been found that infective units appear after 6 hours of incubation while the haemagglutinins appear at 18 hour (tables 1 and 2).

These observations support the findings of Bang (1948), Hill (1957) and Sakkubai (1960).

Within the limits of observations presented it appears that an embryo infectivity titre of $10^{-5.20}$ is necessary for the detection of haemagglutinins (table 3).

The observation recorded by Sakkubai (loc. cit.) and Negi (1963), that at certain stage of multiplication of virus the infectivity titre becomes stationary while a rise in HA titre is noted, has been corroborated in the present study. This may be due to the fact that at a certain stage the virus multiplication slows down and death of the already formed virus particles occurs, resulting in a stationary infectivity titre. The increase in HA titre on the other hand can be attributed to the sum total of reaction of both viable and non-viable virus particles.

The immune response of chicks to the freeze dried Ranikhet (Newcastle) disease vaccine was measured through haemagglutination inhibition and serum neutralisation tests. It has been found that the freeze dried vaccine induced a good degree of HI antibody 14 days after experimental infection. The group average titres varied between 640 and 2266. The correlation between HI antibody and the protection observed through challenge test has been quite satisfactory (table 6).

Serum neutralising indices of 12 batches of pooled sera varied from 4.24 to 6.30. Protection in all these batches has been cent per cent except in one case (tables 8 and 15).

The vaccine could be conveniently titrated in the developing chicken embryo and the results reveal a good correlation with the protection afforded through challenge tests (tables 4, 12, 13 and 15). In view of these findings the assay of the Ranikhet Disease vaccine can be safely depended upon by employing chick embryo titration which is more economical and quicker than the challenge test in birds.

SUMMARY

The literature on the properties of the aetiological agent of Ranikhet (Newcastle) disease of fowls with special reference to serological reactions and the various biologicals used for its control has been reviewed.

The detection of infective virus particles and the development of haemagglutinins during the growth cycle of the virus in the developing chicken embryo have been studied in this treatise.

The serological response of the birds to the freeze dried Ranikhet disease vaccine through haemagglutination-inhibition and serum neutralisation tests have been recorded in these studies.

The potency test results of 170 batches of freeze dried Ranikhet disease vaccine have been analysed.

The possibility of utilising the embryo infectivity as a substitute for expensive and time consuming potency test in birds has been studied. The results reveal a good correlation between the embryo infectivity titre of the vaccine and its immunising efficiency.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adler, H.E., Willers, E.H. and Campbell, J. (1952).
Am. J. Vet. Res., 12 : 44.

Agcanas, P.B. and Rigor, T.V. (1951).
Philipp. J. Anim. Ind., 12 : 19.

Anderson, S.G. (1947 and 1959).
[Cited by the author in "The viruses" (1959) edited by Burnet and Stanley, Publishers, Acad. Press, N.Y., vol. 3.].

____________ (1948).
Aust. J. exp. Biol. Med., 26 : 347.

Andrewes, C.H., Bang, F.B. and Burnet, F.M. (1955).
Virology, 1, 176.

Annon. (1946). HA and HI tests for diagnosis of Newcastle Disease. U.S. Dept. Agri. Bur. Anim. Ind., Path. Divn., Oct. 1946.

Asplin, F.D. (1949).
Vet. Rec., 59 : 621.

____________ (1952).
Ibid, 64 : 245.

Asplin, F.D., Gordon, R.F. and Reid, J. (1952).
Rep. 14th Int. vet. Congr., London, 1949, 2 : 366.

Atanasiu, P. and Soutopatuleia, M.C. (1952).
Ann. Inst. Past., 82 : 247.
(Vet. Bull., 23 : 202).

Bang, F.B. (1946).
Proc. Soc. exp. Biol. Med., 63 : 5.

__________ (1947).
Ibid, 64 : 135.

__________ (1948).
J. exp. Med., 88 : 251.

Bankowski, R.A. (1957).
Proc. Soc. exp. Biol., N.Y., 96 : 114.

__________ (1958).
Avian Dis., 2 : 197.

Bankowski, R.A. and Boynton, W.H. (1948).
Vet. Med., 43 : 305.

Bankowski, R.A. and Cortsvet, R.E. (1960).
Am. J. vet. Res., 21 : 60.

__________ and __________ (1962).
Avian Dis., 6 : 333.

Bankowski, R.A., Cortsvet, R.E. and Pannu, J. (1963).
Ibid, 7 : 135.

Bankowski, R.A. and Hyde, J. (1957).
Am. J. vet. Res., 18 : 743.

Beach, J.R. (1944).
Science, N.Y., 100 : 361.

Beach, J.R. (1948).
J. Am. vet. Med. Ass., 58 : 301.

__________ (1949).
Bull. Calif. Dep. Agric. Reprint P. 67.

Beamer, P.D. and Prier, J.E. (1950).
Cornell Vet., 40 : 56.

Beaudette, F.R. (1943).
Proc. 47th Ann. Meet. Livestock Sanit. Ass., p. 112.

Beaudette, F.R., Bivins, J.A. and Miller, B.R. (1948).
Am. J. vet. Res., 9 : 97.

__________ __________ and __________ (1949).
Cornell Vet., 39 : 302.

Bernkopf, H. (1950).
J. Immun., 65 : 571.

Beveridge, W.I.B. and Lind, P.E. (1946).
Aust. J. exp. Biol. Med., 24 : 127.

Bornstein, S., Rautenstein, A. and Moses, E. (1949)
Refuah vet., 6 : 127.

Bornstein, S., Rautenstein, A. and Samberg, Y. (1952).
Am. J. vet. Res., 13 : 379.

Brandly, C.A., Moses, H.E. and Jungherr, E.L. (1946a).
Ibid, 7 : 302.

______ ______ and ______ (1946b).
Ibid, 7 : 333.

Brandly, C.A., Lewis, S.H., Winslow, N.S., Pritchard, W.R.
Hout, H.H., Hanson, R.P. and Nerlinger, C.M.
(1947).
Cornell Vet., 37 : 324.

British Veterinary Codex, 1963.

Burnet, F.M. (1942).
Aust. J. exp. Biol. med. Sci., 20 : 81.

______ (1943).
Med. J. Aust., 2 : 313.

______ (1948).
Lancet, 1 : 7.

______ (1951).
Physiol. Revs., 31 : 131.

Burnet, F.M. and Ferry, J.D. (1934).
Br. J. exp. Path., 15 : 56.

Burnet, F.M., Beveridge, W.I.B., McKwin, J. and Boake, W.C. (1945).
Aust. J. exp. Biol. med. Sci., 23 : 177.

Burnet, F.M. and Lind, R.E. (1950).
Ibid, 28 : 129.

Burnet, F.M., McCrea, J.F. and Stone, J.D. (1946).
Br. J. Exp. Path., 27 : 228.

Burnet, F.M. and Stanley, W.M. (1959).
"The Viruses". Acad. Press, N.Y.

Bushnell, L.D. and Erwin, L.E. (1950).
Kansas Acad. Sci., Trans., 53 : 58.
(Biol. Abst., 24 : 1950).

Cakalowa, A.A., Teklinska, M. and Karezewski, W. (1955).
Med. Wet., 11, 393 (Vet. Bull., 26 : 74).

Chu, C.M. (1948a).
J. Hyg. Camb., 46 : 239.

__________(1948b).
Ibid, 46 : 247.

Chu, H.P. (1960).
A laboratory handbook on the diagnosis of poultry diseases, Rome, F.A.O. Animal Health Res. Monograph, No.2.

Clancy, C.F., Cox, H.R. and Bottorff, C.A. (1949).
Poul. Sci., 28 : 58.

Clark, E. and Nagler, F.P.O. (1943).
Aust. J. exp. Biol. Med., 21 : 103.

Cole, R.K. and Hutt, F.B. (1961).
Avian Dis., 5 : 205.

Cooper, D.M. (1963). Vet. Rec., 25 : 75.

Coronel, A.B. (1947).
Am. J. vet. Res., 8 : 120.

Crowther, R.W. (1963).
J. exp. Path., 74 : 151.

Cunha, R., Well, M.L., Beard, D., Taylor, A.R., Sharp, D.G. and Beard, J.W. (1947).
J. Immun., 55 : 69.

Dardiri, A.H. and Yates, V.J. (1962).
Am. J. vet. Res., 23 : 330.

Daubney, R. and Mansi, W. (1948).
J. Comp. Path., 58 : 189.

Dhanda, M.R. (1954).
Ann. Rep. Ind. Vet. Res. Inst.

Dhanda, M.R., Nilakantan, P.R. and Sakkubai, P.R. (1958).
Indian Vet. J., 35 : 5.

Dixit, S.G. (1950).
Indian J. Vet. Sci., 20 : 277.

Doer, R. and Gold, E. (1932).
Z. Hyg., 113 : 645.
(Cited by Burnet, F.M., 1960, Principles of Animal Virology).

Doll, E.R., McCollum, W.H. and Wallace, M.E. (1950a).
Vet. Med., 45 : 231.

________ ________ and ________ (1950b).
Ibid, 45 : 365.

________ ________ and ________ (1950c).
Am. J. vet. Res., 12 : 232.

Doll, E.R., McCollum, W.H. and Wallance, M.E. (1950).
Ibid, 11 : 4337.

Donald, H.B. and Issacs, A. (1954).
J. Gen. Microbiol., 10 : 457.

Doyle, T.M. (1927).
J. Comp. Path., 40 : 144.

__________ (1933).
Ibid, 46 : 90.

Doyle, T.M. and Wright, R.C. (1950).
Brit. vet. J., 106 : 139.

Dutcher, R.M., Read, R.G.jr. and Litsky, W. (1960).
Avian Dis., 4 : 205.

Elford, W.J., Chu, C.M., Dawson, I.M., Dudgeon, J.A., Fulton, F. and Smiles, J. (1948).
Brit. J. exp. Path., 29 : 590.

Elford, W.J., Smiles, J., Chu, C.M. and Dudgeon, J.A.(1947).
Biochem.J., 41 : 25.

Fabricant, J. (1949).
Cornell Vet., 39 : 202.

__________ (1950).
Ibid, 40 : 39.

Farinas, E.C. (1930).
Philip. J. Agri., 1 : 311.

Francis, T. jr. and Minuse, E. (1948).
Proc. Soc. exp. Biol. Med., 69 : 291.

Garside, J.S. (1962).
Vet. Rec., 76 : 1497.

Generoso, J.D. and Mendoza, I.L. (1950).
Philip. J. anim. Ind., 10 : 163.

Gentry, R.F. (1957).
Avian Dis., 1 : 75.

Gill, E., Sullivan, J.F., Stone, H.D. and Hundaman, A.S. (1959). Am. J. vet. Res., 20 : 357.

Ginsberg, H.S., Goebel, W.F. and Horsfall, F.L. jr. (1948). J. exp. Med., 87 : 385.

Ginsberg, H.S., Horsfall, F.L.jr. (1949). Ibid, 90 : 475.

Granoff, A. and Henle, W. (1954). J. Immun., 72 : 329.

Gottschalk, A. and Lind, P.S. (1949). Brit. J. exp. Path., 30 : 85.

Gualandi, G.L. (1951). Arch. vet. Ital., 2 : 283.

Gupta, B.R. and Rao, S.B.V. (1959). Indian vet. J., 36 : 365.

Haddow, J.R. and Idnani, J.A. (1941). Indian J. vet. Sci., 11 : 113.

________ and ________ (1946). Ibid, 16 : 45.

Hamann, I. (1958). Mh. Vet. Med., 13 : 99.

Hanson, R.P. and Brandly, C.A. (1958). Ann. N.Y. Acad. Sci., 70 : 585.

Hanson, R.P., Winslow, N.S. and Brandly, C.A. (1947). Am. J. vet. Res., 8 : 416.

Hanson, R.P., Winslow, N.S., Brandly, C.A. and Upton, E. (1950). J. Bact., 60 : 557.

Henle, W. and Henle, G. (1947). J. exp. Med., 85 : 347.

________ and ________ (1949). Ibid, 90 : 23.

Hill, W.K.W. (1957).
Vet. Bull., 27 : 411.

Hirst, G.K. (1941).
Science, 94 : 22.

__________ (1942a).
J. exp. Med., 75 : 49.

__________ (1942b).
Ibid, 76 :195.

__________ (1943) .
Ibid, 78 : 99.

Hitchner, S.B. and Johnson, E.P. (1948).
Vet. Med., 43 : 525.

Hitchner, S.B. and Reising, G. (1953).
Proc. 25th Ann. Meet. N. Conf. Laby. Workers, Pullorum Dis. Control, Amherst, Mass.

__________ and __________ (1954).
Proc. 90th Ann. Meet. Am. vet. med. Assoc., p.350.

Hofstad, M.S. (1951).
Am. J. vet. Res., 12 : 334.

__________ (1956).
Ibid, 17 : 738.

Horne, R.W. and Waterson, A.P. (1960).
J. molec. Biol., 2 : 75.

Howitt, B.F., Bishop, L.K., Gorrie, R.H., Kissling, R.E., House, G.H. and Trueting, W.L. (1948).
Proc. Soc. exp. Med., 68 : 70.

Hoyle, L. (1960). J. Hyg., 48 : 277.

Hudson, J.R. (1937).
E. Afr. Agric. J., 2 : 495.

Issacs, A. (1948).
Brit. J. exp. Path., 29 : 529.

Iyer, S.G. (1943).
Indian J. vet. Sci., 13 : 1.

Iyer, S.G. and Dobson, N. (1940).
Vet. Rec., 52 : 839.

________ and ________ (1941).
Ibid, 53 : 381.

Iyer, S.G. and Hashmi, Z.A. (1945).
Indian J. vet. Sci., 15 : 155.

Kaschula, V.R. (1950).
J.S. Afr. vet. med. Ass., 21 : 134.

____________ (1952).
Onderstepoort J. vet. Res., 25 : No.4, p.29.

Keeble, S.A. and Coid, C.R. (1962).
Vet. Rec., 74, 1112.

Keeble, S.A. and Wade, J.A. (1963).
J. comp. Path., 73 : 186.

Keeble, S.A., Box, P.G. and Christie, D.W. (1963).
Vet. Rec., 75 : 151.

Keeney, A.H. and Hunter, N.C. (1950).
J. Ophthal., 44 : 573.

Kilham, L. (1949).
Proc. Soc. exp. Biol. Med., 71 : 63.

Komarov, A. and Goldsmidt, L. (1946).
Refuah vet., 3 : 111.
(Vet. Bull., 17 : 540).

__________ and ___________ (1947).
Cornell Vet., 37 : 368.

Lancaster, J.E. (1962).
Canad. J. Comp. Med., 26 : 285.

________________ (1964).
Vet. Bull., 34, No.2

Levens, J.H. and Enders, J.F. (1945).
Science, 102 : 117.

Lippman, I. (1952).
J. Ophthal., 35 : 1021.

Liu, O.C. and Henle, W. (1951). J. exp. Med., 94 : 291.

Logrippo, G.A. (1959).
J. Immun., 80 : 198.

Logrippo, G.A. and Hartman, F.W. (1955).
Ibid, 175 : 123.

Luginbuhl, R.E. and Jungherr, E.L. (1949).
Poul. Sci., 28 : 622.

Lush, D. (1943). J. Comp. Path., 53 : 157.

Mazzaracchio, V. and Orfei, Z. (1956).
(Cited by Lancaster, J.E. Vet.Bull., 34 : 2).

Memorandum (1955). Government of India, Ministry of Food and Agriculture, 1955.

McClelland, L. and Hare, R. (1941).
Canad. J. publ. Hlth., 32 : 530.

McCrea, J.F. (1946).
Aust. J. exp. Biol. Med., 24 : 283.

________________ (1948).
Ibid, 26 : 335.

Mack, W.N. and Chotisen, A. (1955).
Poul. Sci., 34 : 1010.

Markham, F.S., Cox, H.R. and Bottorff , C.A. (1949).
Ibid, 28 : 52.

Markham, F.S., Cox, H.R. and Bottorff, C.A. (1954).
Cornell Vet., 44 : 324.

Mitchell, C.A. and Walker, R.V.L. (1951).
Canad. J. Comp. Med., 15 : 219.

______________ and ______________ (1952).
Ibid, 16 : 409.

Mitscherlich, E. and Gurturk, S. (1952).
(Vet. Bull., 24 : 1896)

Moses, H.E., Brandly, C.A., Jones, E.E. (1947).
Science, 105 : 477.

Nadel, M.K. and Eisenstark, A. (1955).
J. Bact., 69 (2), 173.

Nakamura, J., Dyama, S. and Wagetsuma, S. (1937).
J. Japan Soc. vet. Sci., 16 : 55.

Nakamura, J., Miyamoto, T. and Nagashima, H. (1956).
Nibs Bull. biol. Res., 1 : 69.

Nandi, S.N. (1955). Indian vet. J., 31 : 271.

Negi, S.K. (1963).
Studies on Newcastle disease- Antigenic and chemical changes in infected embryonated eggs- Thesis submitted to Agra University, 1963.

Nilakantan, P.R., Sakkubai, P.R. and Dhanda, M.R. (1960a).
Indian vet. J., 37 : 503.

______________ ______________ and ______________ (1960b).
Ibid, 37 : 548.

Nitzschke, E. and Venske, W. (1956). (Vet. Bull., 27 : 476).

Osteen, O.L. and Anderson, W.A. (1948).
J. Am. vet. med. Ass., 112 : 40.

Pagnini, U. (1954).
Alti. Soc. ital. Sci. vet., 8 : 644.

Piercy, S.E., Macleoid, A.J. and Blaxland, J.D. (1962).
Vet. Rec., 74 : 1478.

Pomeroy, B.S. (1951).
Proc. 88th Ann. Meet. vet. med. Ass., 1951, p.215.

Puteanus, I. (1953).
Vet. Bull., 24 : 1512.

Raggi, L.G. and Lee, G.G. (1960).
Avian Dis., 4 : 187.

________ and ________ (1962).
Ibid, 6 : 297.

Rao, S.B.V. and Aggarwal, K.K. (1960).
Indian vet. J., 37 : 6.

Reed, L.J. and Muench, H. (1938).
Am. J. Hyg., 27 : 493.

Rubin, H. and Frankling, R.M. (1957).
Virology, 3 : 587.

Sakkubai, P.R. (1960).
Studies on Newcastle Disease Virus - Thesis submitted to Madras University, (1960).

Salyi, J. and Hodosy, J. (1952).
Acta. vet. Hung., 2 : 169.

Schafer, W. and Rott, R. (1959).
Z. Naturforsch., 14b : 629.

Schmittle, S.G. and Millon, T.W. (1948).
Cornell Vet., 38 : 306.

Schneider, B. (1954).
Vet. Med. Nachr., 2 : 65.
(Cited by Lancaster, J.E.- Vet. Bull., 34 : 2).

Schoening, H.W., Osteen, O.L., Legeahusen, D.H., Anderson,W.A. and Hall, W.J. (1949).
Am. J. vet. Res., 10 : 176.

Seetharaman, C. (1951). Indian vet. J., 28 : 13.

Simmins, G.B. and Bladwin, B.A. (1963).
Res. vet. Sci., 4 : 286.

Sinha, K.C. and Dutta, S. (1950). Current Sci., 19 : 343.

Sullivan, J.F., Gill, E. and Somer, A.M. (1958).
Am. J. vet. Res., 19 : 483.

Tam, I. and Horsfall, F.L.jr. (1952).
J. exp. Med., 95 : 71.

Taylor, J.R.E. (1953). Canad. J. comp. Med., 17 : 321.

Teklinska, M. (1951).
Med. Wet., 7 : 13. ?

Thompson, Jr. C.H. and Osteen, O.L. (1948).
Am. J. vet. Res., 9 : 303.

__________ and __________ (1952).
Ibid, 13 : 407.

Van Roekel, H. (1956).
Proc. 92nd Ann. Meet. Am. vet. med. Ass., 1955, p. 324.

Van Wavern, G.M. (1955).
Bull Off. Int. Epiz., 44 : 107.

Van Wavern, G.M. and Zuidjam, D.H. (1953).
Vet. Bull., 56 : 126.

Wasserman, B. and Yates, V.J. (1953).
Mich. Coll. Vet., 13 : 91.

Waterson, A.P. and Cruickshank, J.G. (1963)
Z. Naturforsch, 18 : 114.

Wenner, H.A., Monley, A. and Todd, R.N. (1950).
J. Immun., 64 : 323.

Winslow, N.S., Hanson, R.P., Upton, E. and Brandly, C.A. (1950).
Proc. Soc. exp. Biol. Med., 74 : 174.

Winterfield, R.W., Goldman, C.L. and Sendale, E.H. (1957).
Poul. Sci., 36 : 1076.

Woernle, H. and Seigmann, O. (1954).
(Cited by Lancaster, J.E., Vet.Bull., 34 :2).

Woodruff, A.H. and Goodpasture, E.W. (1931).
Am. J. Path., 7 : 209.

Yates, V.J., Wassermann, B. and Fry, D.E. (1954).
(Cited by Lancaster, J.E., Vet. Bull., 34: 2).

Zargar, S.L. and Pomeroy, B.S. (1949).
J. Am. vet. med. Ass., 115 : 354.