PESTICIDE - INFECTIOUS DISEASE INTERACTION STUDIES

IN THE MALLARD

Part I: Studies on the effects of selected chemical and infectious disease agents in the mallard (Anas platyrhynchos).

Part II: Studies of possible interactions between selected chemical and infectious disease agents in the mallard (Anas platyrhynchos).

BY

MILTON FRIEND

A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(Veterinary Science and Wildlife Ecology)

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PESTICIDE - INFECTIOUS DISEASE INTERACTION STUDIES IN THE MALLARD

MILTON FRIEND

Under the supervision of Daniel O. Trainer

THESIS ABSTRACT

In recent years a great deal of information has been accumulated regarding both the lethal effects of various organochlorine pesticides on a variety of vertebrate and invertebrate species and the distribution of these compounds within the environment. However, limited data are available regarding the sublethal effects of these compounds singly, or in combination with other chemical or biological agents. One possible sublethal effect, that of increased susceptiblity of wildlife to infectious disease, was tested in this laboratory in a model system using the mallard, Anas platyrhynchos, as an experimental host.

A total of six different agents, Newcastle disease virus (NDV), duck hepatitis virus (DHV), Pasteurella multocida, DDT, dieldrin, and a polychlorinated biphenyl (PCB) were used in these experiments. The basic experimental design was to feed mallards one of the chemical compounds for 10 days, 5 days after the completion of chemical exposure the birds were challenged with one of the infectious agents, and mortality was recorded during the following 10 day period. Three ages of mallards (5-day, 30-day, and adult), two levels of the chemical, and two levels of the infectious agent were used in these experiments. In addition to mortality, other end-points utilized to assay for

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interactions were chemical residues, body weight, serum transaminases (SGO-T and SGP-T), antibody response, and pathology.

The studies were divided into two parts. In Part I, host response to the various agents, with the exception of PCB, was studied individually in different age mallards. Results of these studies indicated that DHV, P. multocida, DDT, and dieldrin were all suitable agents for use in the interaction model, and that NDV was unsuitable since mortality in mallards from this agent resulted from "viral toxicity" rather than an infectious process.

In Part II, combinations of a single organochlorine compound and an infectious agent were used to study interactions between the agents in the mallard. In pesticide - DHV interaction studies with wild ducklings 20 days old at DHV challenge, mortality of birds pretreated with either DDT or dieldrin was generally reduced from that of the controls. For ducklings 29 days old at DHV challenge, mortality among birds pretreated with DDT or dieldrin was greatly increased over that for controls. A similar increase in mortality was observed in ducklings 25 days old at DHV challenge which were pretreated with a PCB. When the exposure protocol was reversed and DHV inoculation preceded exposure to DDT or dieldrin by 48 hours, a delayed and reduced mortality response occurred among interaction test groups. With several combinations of DDT, dieldrin, PCB, and DHV, in different age mallards, statistically significant differences were observed for both synergistic and antagonsitic mortality responses.

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Results in P. multocida - pesticide interaction studies were inconclusive although there was a general trend of increasing mortality among interaction test groups as compared with controls.

The general conclusion reached from these studies was that under the conditions of the experimental model, an altered host response to challenge with an infectious disease agent often resulted among mallards preexposed to sublethal levels of DDT, dieldrin, or a PCB. These experiments demonstrate the feasibility of using an in vivo system to study pesticide - disease interactions and illustrate a real difference between "sublethal" and "no-effect" aspects of pesticides on a vertebrate host.

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INTRODUCTION

Historically, autumn of 1939 marked two events of world wide importance that occurred on the European continent, one political and one scientific. As often happens the significance of the political event was immediate while a considerable time lapse occurred between discovery and awareness of the implications of the scientific event. Thus, when Hitler's army attacked Poland at dawn on September 1, 1939 the die was cast for a conflict that expanded into World War II within a year. That same autumn Dr. Paul Müller while working in the Basle laboratories of the Swiss Company of J. R. Geigy S. A. noted that a chemical preparation assigned the name Gesarol appeared to be a highly effective insecticide. Further testing of the "active ingredient" of this compound resulted in confirmation of these properties and following the cessation of hostilities in World War II, this active ingredient became commercially available as DDT in larger quantities. In the years that followed, DDT became a household word and its use promiscuous. Now, 25 years later the biological effects of DDT and other related organochlorine compounds, whose discovery was enhanced by the early success of DDT, are still being determined and the ever increasing controversy over the use and misuse of pesticides has reached international importance.

This thesis is not a study of either World War II or DDT but rather, an exploration of the possible relationships that may exist between some organochlorine compounds and infectious disease. A relationship between pesticides and altered host resistance to disease

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is often suggested or implied by those concerned with the quality of the environment and the welfare of wild species. These allegations are strongly denied by others whose primary concern may be the production of food and fiber or control of disease in man and animals. A factual basis for this aspect of the pesticide controversy is generally lacking in scientific experimentation.

Three chemical and three infectious agents were studied in a model system in an attempt to provide scientific evidence for or against the hypothesis that preexposure to persistant organochlorine pollutants does not alter the susceptibility of a host to infectious disease. These studies were divided into two sections and appear as Part I and Part II of the thesis. In Part I the respective agents were studied to determine their suitability for use as part of the model and to establish base line data and parameters for assay. In Part II combinations of one pesticide and one infectious disease agent were used to study possible interactions between the two agents. DDT, dieldrin, and a polychlorinated biphenyl (Aroclor 1254) were the chemical agents selected and Newcastle disease virus, duck hepatitis virus, and Pasteurella multocida the infectious disease agents. The mallard (Anas platyrhynchos) was selected as the experimental host.

The Model

No attempt was made to construct a model that simulated "natural conditions" although the selection of agents was influenced, but not dictated, by their occurrence in nature. The intent in these studies was to establish whether interactions could occur in a laboratory model. In the event the model system proved productive, modification

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resulting in a greater similarity with "natural conditions" could be imposed for future studies.

The basic design of the interaction studies was to pretreat mallards by feeding a diet containing a predetermined amount of the selected organochlorine compound for 10 days after which the birds were to be maintained on pesticide free diets (Appendix C). After a 4 to 5 day period the birds were challenged with an infectious agent and the response in these birds measured against that of birds exposed to only the chemical or infectious agents. This challenge response period was 10 additional days. Generally, two levels of both the chemical and the infectious agent were used in mallards at three different ages.

Based on the results obtained in Part I, the chemical levels used in the model were selected to produce approximately 10 and 25 percent mortality. The intent was to obtain survivors with relatively high body burdens of the compound being used. Both dosage levels for the infectious agent in any particular trial were calculated to be less than the LD_{50}; the low dose was an approximation of the theoretical lowest limit for producing overt disease in the host and the high dose was chosen at a point between the low dose and the approximate LD_{50}.

The mallard was selected as the host species for the model on the basis of several criteria including its abundance and wide distribution in nature, availability, ease of maintenance in captivity, and ease of handling. This aspect of the model is the closest approximation of "natural conditions". The wild mallard and its

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domesticated variety are nearly ubiquitous in their distribution. In addition to this wide geographic distribution the mallard encounters a broad experience with a number of different environmental conditions including exposure to pesticide pollution and a variety of infectious pathogens. Thus, the mallard provides the experimental model with a species that is undoubtedly confronted with multiple insults from both chemical and biological agents throughout its life span.

The Agents

No attempt was made to undertake comprehensive studies for each of the agents involved. Instead, emphasis in Part I was placed on establishing baseline data that could be used as a basis for predicting the response of the mallard to each respective agent. By establishing these baseline parameters it was hoped that meaningful interaction studies could then be designed and the results of these interactions interpreted more fully.

Since this thesis is not an in depth study of each agent but rather a study involving a concept (the effect of pesticides on the susceptibility of a host to infectious disease) literature reviews have not been prepared for each agent. Literature citations utilized are those which have direct bearing on the interpretation of the interactions and understanding the properties of the agents involved as they may influence these results. When available, appropriate reviews for each agent are cited which provide the reader with sources for more comprehensive coverage.

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DDT: DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) is not a new compound; it was first synthesized in 1874 and remained relatively unknown until after its insecticidal properties were discovered by the Swiss chemist, Paul Müller in 1939 (Metcalf, 1955). It is an interesting paradox, in view of the present controversy which has resulted in the ban or restricted use of DDT in Sweden and a number of the states in the United States (including Wisconsin), that Müller was awarded the 1948 Nobel Prize in medicine and physiology for this discovery. Fourteen years later Rachel Carson's (1962) book, Silent Spring, dramatized the actual and potential dangers associated with the reckless and largely unrestrained introduction of DDT and other pesticides into the environment; and the battle lines were formed.

More scientific and semi-technical reports, encompassing a wide range of disciplines, have probably been written about DDT than any other synthetic compound known to man. A complete review of the literature would take years to accomplish and require several volumes to assemble. This voluminous background of information available on DDT adds credence to its selection in a model to study possible effects of pesticides with similar properties. In addition, DDT and its metabolites are the most frequently occurring and widespread synthetic chemical pollutants known in our environment (Rudd, 1969) and on this basis alone it becomes exceedingly important to gain as wide an understanding of its total effects as possible. Despite the vast collection of knowledge and decades of research, O'Brien (1967)

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noted that we are still far from understanding the mechanism of action of DDT in members of the animal kingdom.

As a result of the relatively specific objectives of the present experiments many aspects of organochlorine pesticide pollution and the specific effects of DDT, as well as dieldrin, are not cited or discussed. Additional citations to fill these voids are listed in a selected bibliography (Appendix A) which contain a vast amount of the current knowledge on the organochlorine problem and the effects of specific compounds.

Dieldrin: As with DDT, a Nobel prize appears in the history of dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,-4,4a,5,6,7,8,8a-octahydro-endo-exo-1,4:5,8-dimethanonaphthalene) but in an indirect manner. The physical conformation of cyclodiene compounds (which include dieldrin, aldrin, endrin, et al.) was made possible by the work of two German chemists, Otto Diels and Kurt Alder who discovered a method for adding the two ends of a carbon chain to opposite ends of a double bond in another carbon chain to form a ring of atoms (cyclic). This 1928 discovery became widely known as the Diels-Alder reaction and earned them the 1950 Nobel Prize for chemistry.

The discovery and development of the cyclodiene compounds as insecticides was largely the result of the work of Julius Hyman and his associates dating from 1945 (Metcalf, 1955). Although all cyclodiene compounds are cyclic, only a few of them are true dienes (possess two double bonds). Dieldrin, the epoxide of aldrin, is formed by the loss of the second double bond in aldrin (Figure 1) through an oxidation process (O'Brien, 1967). This transformation

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Figure 1. Structural formulas for DDT, polychlorinated biphenyls (PCB), aldrin, and dieldrin. Possible chlorine positions on the PCB molecule are indicated by X's. The epoxidation reaction resulting in the conversion of aldrin to dieldrin is indicated below the formulas for these compounds.

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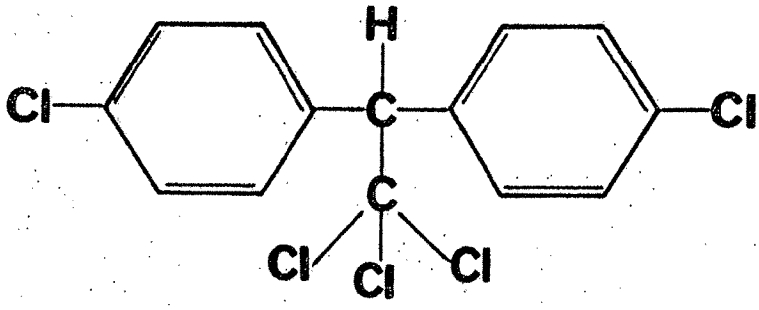
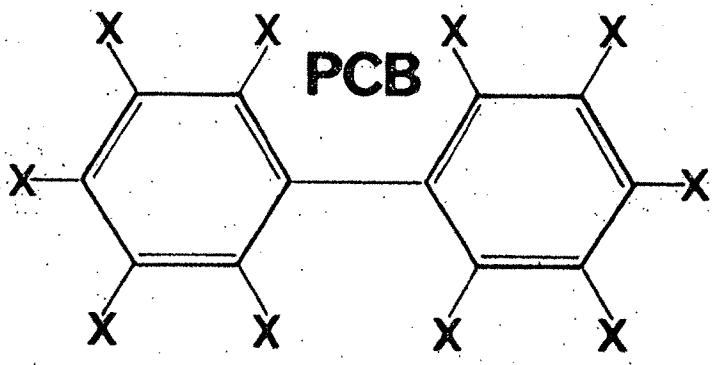
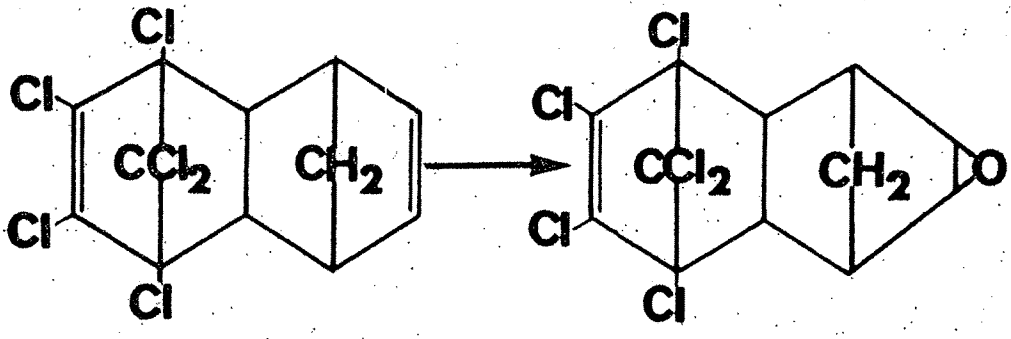
DDT

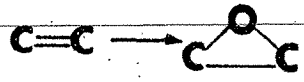


PCB





ALDRIN



DIELDRIN

not only results from manufacturing processes but readily occurs in nature (Bann et al., 1958; Gannon and Bigger, 1958; Gannon and Decker, 1958; Menzie, 1969). Thus, field applications of aldrin provide a source of dieldrin contamination.

Residues of dieldrin in nature have been reported to be second in frequency among persistant pesticides (Stickel et al., 1969). This wide distribution in nature, its high toxicity to many lower vertebrates, and relative lack of information regarding its effects on wildlife were all resaons for the selection of dieldrin in the model.

Polychlorinated biphenyl: Unlike the other chemical agents used in this model, preliminary studies were not carried out with polychlorinated biphenyls (PCB). When this research was initiated in 1967 the possible importance of PCB contamination had not been widely publicized or accepted and this group of compounds was essentially unknown among all but a small group of ecologically oriented chemists. As research progressed with the experimental model, PCB emerged into the ecological spotlight as another potential environmental hazard. This interest and the similarity in physical structure between DDT and PCB (Figure 1) prompted an interaction experiment to be undertaken despite the absence of preliminary studies. Because of the current interest in this class of compounds and the relatively recent ecological awareness of their existance it seems appropriate to deviate slightly from the previously stated policy of not presenting a literature review.

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PCB (also referred to as chlorinated diphenyls) are aromatic hydrocarbons (contain one or more benzine rings) of a diphenyl (biphenyl) or triphenyl structure to which one or more chlorines have been substituted during chlorination. Chlorinated diphenyls were described as early as 1881 in Justus Liebig's Annalin der Chemie., but did not come into wide use until after 1930 when the cost of diphenyl was reduced 100-fold by the development of a new commercial process. This catalyzed extensive research in the field of chemicals derivable from diphenyl (Penning, 1930).

PCB are produced under a variety of trade names, including the popular Aroclors manufactured by the Monsanto Chemical Company and are considered to be among the most versatile chemically produced materials available. They comprise a series of compounds that vary from mobile, oily liquids to white crystals to hard, transparent resins. PCB possess outstanding characteristics of inertness and heat stability resulting in their use in industry as lubricants, heat-transfer agents, and insulators. They are additives in many commercial products such as waxes, dental castings, printing inks, putty, and synthetic adhesives (Table 1). In addition, PCB are essential components of coating for flame-proofing cotton drill for outer garments and for rendering olive drab canvas fire retardent, water-repellent and rot-proof (Monsanto Chemical Company).

PCB are chemically inert (Penning, 1930) and although nothing is known about their biological decomposition, it is likely that they are more stable than DDT and its metabolites. Thus, their physical

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Table 1. Some uses of PCB.*

Used in industry as:
lubricants; heat transfer agents; insulators; plasticizers; protective coating; extenders; hydraulic fluids; grinding fluids; dielectric sealants; and dedusting agents.
Some common products in which they are additives:
wire and cable coating; impregnants for braided cotton-asbestos insulation; rotogravure and other printing inks; mimeograph ink; preparation of imitation gold leaf; pigment vehicle for decoration of glass and ceramics; essential components of coating for flameproofing cotton drill for outer garments and for rendering olive-drab cavas fire retardant, water-repellant, and rot-proof for tents, tarpaulins, etc; moisture-proof coatings for wood, paper, concrete, or brick; asphalt; roof coatings; high quality precision-casting wax; waxes used in making dental castings and costume jewelry; sealers for masonry, wood, fiberboard, and paper; window envelopes; polystrene; polyethylene; neoprene; polybutene; silicone rubber; crepe rubber; and plasticizers in paints.

*Monsanto Technical Bulletin O/PL-306; Reynolds (1969).

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and chemical characteristics are conducive for persistence in nature and accumulation within higher trophic levels of the food web (Peakall and Lincer, 1970). Risebrough (1969) and Risebrough et al. (1968) reported that regional PCB fallout patterns exist and that PCB peaks (generally of the 1200 series) detected on gas chromatograms indicate direct environmental contamination from industrial waste products, manufacturing processes, and combustion of materials containing PCB.

Although not appreciably insecticidal alone, PCB significantly increases the toxic effects of both DDT and dieldrin to house flies, Musca domestica, (Lichtenstein et al., 1969). In addition these compounds may be used as a means for prolonging the residual life and effectiveness of volatile insecticides. Aroclor 5460 was recommended 17 years ago as a means for improving both the appearance and persistance of lindane residues (Hornstein and Sullivan, 1953; Sullivan and Hornstein, 1953). In addition to lindane, the effective kill-life of expensive chlorinated insecticides containing chlordane, aldrin, dieldrin, and benzene hexachloride (BHC) has been extended as much as ten-fold by incorporation of equal parts of Aroclor 5460. Reynolds (1969) has stated that incorporation of Aroclor into pesticide formulations may be the most likely source of environmental PCB contamination.

PCB were first identified in the tissues of wildlife in Sweden (Anonymous, 1966) and shortly thereafter they were reported to occur in the environment in the same amounts as the chlorinated pesticides

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(Widmark, 1967). PCB have now been identified in the tissues of a variety of wildlife (Appendix B) from diverse areas of the world (Table 2), and along with DDT and its metabolites are reported to be the most abundant of the chlorinated hydrocarbon pollutants in the global ecosystem (Risebrough et al., 1968). The highest concentrations of these compounds are found among birds, expecially marine species at the top of complex oceanic food webs (Risebrough, 1969).

Peakall and Lincer (1970) noted that despite recent studies the toxicology of PCB remains rather poorly known to the extent that LD_{50} values have not been published for the various formulations. Early reports of PCB indicated no toxic or systemic effects attributed to these compounds (Penning, 1930). However, with increased production their toxic hazard rapidly became evident as reports of industrial aciniform dermatosis (Brown, 1947; Jones and Alden, 1936; Mayers and Silverberg, 1938) and liver damage (Greenburg et al., 1939) associated with diphenyl manufacture began to appear. Laboratory studies with rats, guinea pigs, and rabbits reported similar liver damage and skin changes (Drinker et al., 1937; Miller, 1944). These findings led to the establishment of threshold limits (the maximum allowable concentration for an 8 hour working day) by the American Conference of Government Hygienists (Documentation of Threshold Limit Values) and PCB are now considered to have moderate to high toxicity hazard ratings on the scale of dangerous properties by which industrial materials are rated (Sax, 1963).

Results of recent studies have shown PCB to be one of the most

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Table 2. Reports of PCB distribution in the global ecosystem.

Geographical* location	Types of species	References
Sweden	Fish, birds, marine mammals, man, conifer needles	Anonymous, 1966; Jensen et al., 1969; Widmark, 1967
Great Britain	Marine mammals, birds, bird eggs	Holden and Marsden, 1967; Holmes et al., 1967; Presst and Jeffries, 1969; Presst et al., 1970
United States	Fish, birds, bird eggs	Anderson et al., 1969; Risebrough, 1969; Risebrough et al., 1968
Netherlands	Insects, fish, birds, shell fish	Koeman et al., 1969
Germany	Birds	Fiuczynski and Wendland, 1968; Koeman et al., 1967
Panama	Bird eggs	Risebrough et al., 1968
Canada	Marine mammals, birds and eggs	Anderson et al., 1969; Holden and Marsden, 1967; Reynolds, 1970
Hawii	Fish	Risebrough, 1969
Equador	Fish	Risebrough, 1969

*Including surrounding waters.

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toxic chemicals (among 100 tested) assayed by injections in hens eggs and to cause teratogenic effects (McLaughlin et al., 1963). In addition, PCB produce a disease syndrome similar to that of chick edema disease (Flick et al., 1965; McCune et al., 1962). Despite these findings the indication that PCB are a potential hazard in the natural environment has not yet been well supported by experimental data with wild species.

The minimum acute oral toxic dose for each of four different Aroclors tested in mallards appeared to be much greater than 2000 mg/kg, the highest level administered (Tucker and Crabtree, 1970), but all 20 Japanese quail (Coturnix c. japonica) fed a diet containing 2000 ppm of phenochlor DP6 died by 55 days after dosing started (Koeman et al., 1969). Presst et al. (1970) concluded that Aroclor 1254 is only 1/13 as toxic as DDT to Bengalese finches (Lonchura striata) and a review of the literature led Peakall and Lincer (1970) to the conclusion that lower vertebrates and invertebrates are much less susceptible than mammals to direct toxicity from PCB.

A significant finding associated with PCB is their ability to produce sublethal physiological effects in birds. They are powerful inducers of hepatic enzyme systems which increase the metabolism of progesterone, testosterone, and oestradiol. On a weight basis PCB preparations have an oestradiol degrading potential five times that of p,p'-DDE[1] or technical grade DDT (Risebrough et al., 1968). Induction of hepatic hydroxylating enzymes has been demonstrated in the pigeon, Columba livia, (Risebrough et al., 1968) and American

[1]DDE, 1, 1-dichloro-2,2-bis(p-chlorophenyl)ethylene.

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kestrel, Falco sparverius, (Lincer and Peakall, 1970). In addition, there is some evidence that the feeding of Aroclor in the diet caused thin eggshells in Japanese quail, mallards (Tucker, unpublished, cited by Peakall and Lincer, 1970), and ring-doves (Peakall and Lincer, 1970). There is also suggestive evidence that PCB affected eggshell thickness in double-crested cormorants, Phalacrocorax auritus, and white pelicans, Pelecanus erythrorhynchos, (Anderson et al., 1969).

Newcastle disease virus: NDV is generally considered a pathogen of domestic fowl with ducks being resistant to infection. Newcastle disease (ND) has been recognized as a clinical entity since 1926 when it was observed in Java and near Newcastle-on-Tyne, England, from where it derives its name. It has since been recognized in every poultry-producing area of the world and is an important economic disease in many areas (Brandly and Hanson, 1965).

NDV is a Myxovirus and is further classified as a member of the parainfluenza group (Wilner, 1969). Excellent reviews on ND are available (Lancaster, 1966; Palmer and Trainer, 1971); a good synopsis of the disease has also been prepared by Brandly and Hanson (1965); and in a relatively recent symposium the virus was considered in some detail (Hanson, 1964).

The velogenic Texas GB strain of NDV was selected for study in the model because of its high degree of pathogenicity and virulence for fowl. This virus attacks both the central nervous system (CNS) and respiratory systems, and generally produces a fatal disease syndrome. Involvement of the CNS was especially attractive in view of the disruptive action elicited within this system by many pesticides. Other considerations were the considerable amount of background data

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available on both the virus and the disease in domestic fowl (Brandly and Hanson, 1965; Hanson, 1964) and the relatively high prevalence of NDV antibody detected in wild waterfowl (Bradshaw and Trainer, 1966; Friend and Trainer, 1970; Palmer and Trainer, 1970; Wilson, 1968). These studies also provided an opportunity to add to our knowledge of diseases in the mallard of which currently little is known concerning the effect of the Texas GB strain of NDV in different age mallards.

Duck hepatitis virus: DHV was first recognized as a clinical entity in the spring of 1949 when the disease, duck virus hepatitis (DVH), occurred among white pekin ducklings on Long Island duck farms (Levine and Fabricant, 1950). An estimated 75,000 ducklings (15 percent of the total) died from DVH during these initial outbreaks (Levine, 1965) and since that time the disease has been diagnosed on all major continents except Australia (Piazza, 1969; Tauraso et al., 1969).

Classification of DHV is uncertain due to its incomplete characterization (Wilner, 1969) but Tauraso et al. (1969) concluded that this virus is best classified as a duck picornavirus. Physical and chemical properties supporting this classification have been described (Andrews and Pereira, 1967; Hanson et al., 1964; Piazza, 1969; Tauraso et al., 1969). DHV has at least two serotypes (Asplin, 1965 a, b) and it has no serologic relationship with infectious hepatitis of other animals or man (Koporowski, 1958).

DHV was selected for study because of the severity of liver damage which may result from infection. Pesticides and many other

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chemicals are metabolized primarily in the liver, and alterations in the ability of the liver to carry out these functions are ultimately reflected in host response to the insult presented. Another desirable factor is that gross liver lesions in ducklings under three weeks of age infected with DHV are considered to be pathognomonic (Richter et al., 1964) or nearly so (Levine, 1965); this provides an index to specificity of death. This characteristic is not absolute, however, since recent findings on Long Island (Toth, 1969 a) and elsewhere have disclosed agents which produce similar lesions, and that may or may not be related to DHV (Mansi et al., 1964; Wachnik and Nowacki, 1962).

DHV is usually an acute infectious disease of young domestic ducklings (Asplin, 1958). Age resistance starts to develop at the end of the third week, and by the end of the sixth week of age ducklings are refractory to clinical disease (Rispens, 1969).

Serologic procedures for the demonstration of DHV antibodies include virus-neutralization in chicken embryos (Hwang, 1969), an agar-gel-diffusion precipitin technique (Murty and Hanson, 1961), and indirect hemagglutination (Taylor and Hanson, 1967); although neither of the latter have been widely used.

DHV has been propogated in ducklings, duck and hens' eggs, and some tissue-culture systems. Adult ducks, chickens, turkeys, and standard laboratory animals are all refractory. Viral assay is not easily accomplished because of host specificity. In hens' eggs DHV end-points must be determined by inspections of embryos for specific lesions unless an egg-lethal strain of virus is employed. In the

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latter case the virus is not pathogenic for ducks. Serial passage in hens eggs attenuates the virus for ducklings and generally increases the pathogenicity for the chick embryo. Virulence for ducklings returns after passage through ducklings (Asplin, 1958).

DHV does not grow in mammalian tissue culture systems but has been grown with little or no cytopathic effect (CPE) in chick embryo (CE) explant cultures (Pollard and Starr, 1959), CE monolayer cultures (Pollard and Starr, 1960), and CE liver cell monolayer cultures (Kaeberle et al., 1961). CPE has been demonstrated with a tissue culture adapted strain of DHV in stained preparations of duck embryo kidney (DEK) cell cultures (Fitzgerald et al., 1963). A Japanese strain of DHV formed plaques on DE cell monolayer cultures under an agar overlay (Sazawa et al., 1963).

Pasteurella multocida: P. multocida is the causative agent of fowl cholera, a disease of domestic and wild birds that has been studied since the eighteenth century and occurs sporadically, often in the form of an epizootic, in most countries of the world (Harshfield, 1965). Recent reviews on fowl cholera adequately summarize the disease in domestic (Harshfield, 1965) and wild birds (Rosen, 1971) and need not be repeated here.

P. multocida is classified as a gram-negative, small, nonmotile coccobacillus, which when isolated in nature is encapsulated and exhibits characteristic bipolar morphology. Other bacteriological as well as biochemical and physical characteristics are listed in Bergey's Manual (1957). There are a number of strains of this

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organism and they have been variously classified on the basis of fermentation reactions (Rosenbusch and Merchant, 1939), immunological differences in cross-protection experiments with mice (Roberts, 1947), by hemagglutination tests utilizing the capsular antigen, and by a slide agglutination test with a somatic antigen (Namioka and Murata, 1961 a, b, c). At present no standard system of classification is universally accepted.

The selection of P. multocida for study in the model was based upon several criteria including the availability of the agent, use of a bacterial agent, and the importance of this disease for wild waterfowl (Jensen and Williams, 1964). Unlike the other two biological agents used in the model, P. multocida is an established pathogen of wild waterfowl and has been specifically studied in this respect (Rosen and Bischoff, 1950; Rosen and Morse, 1959).

Another reason for including this agent in the present studies was to test a hypothesis regarding the etiology of P. multocida in wild waterfowl. The epizootiology of fowl cholera in wild waterfowl has not been fully established but the popular hypothesis is that carrier birds among the population are stressed to a point where they "break" and initiate an epizootic (Rosen, 1971). While this hypothesis has not been proven or disproven, it is a tenable one based on reported carrier status in domestic birds (Hudson, 1959) and enzootics among chickens resulting from this type of reservoir (Webster et al., 1927; Pritchett et al., 1930 a, b; Pritchett and Hughes, 1932). Inclement weather and harassment from hunting are stresses suggested for causing carriers to "break" among wild waterfowl

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(Rosen, 1971), but it is also conceivable that organochlorine pollutants such as DDT could be the stress responsible by reducing both the general vigor of the birds and the body defense mechanisms. This theory has been considered in more detail previously (Friend and Trainer, 1970).

Losses of wild waterfowl from fowl cholera like other diseases of wildlife are poorly reported. The spectacular epizootics are detected but chronic outbreaks generally are not. Within the United States the vicinities of San Francisco Bay and the Muleshoe National Wildlife Refuge in the Texas Panhandle have continued as enzootic areas of the disease since the first reported outbreaks in early 1944 (Jensen and Williams, 1964). Annual mortality in these two areas have varied from small numbers in some years to tens of thousands in others. Among the more spectacular outbreaks in California are those that occurred in 1944 when 40,000 waterfowl and shore birds died (Rosen and Bischoff, 1949; 1950) and the winter of 1965-66 when more than 70,000 birds died (Rosen, 1971). In Texas, more than 60,000 birds died from this disease during the winter of 1956-57 (Jensen and Williams, 1964).

In recent years fowl cholera outbreaks have been reported in other prime waterfowl wintering and nesting areas such as the Squaw Creek National Wildlife Refuge in Missouri (Vaught et al., 1967) and along the East Coast (Gersham et al., 1964; Mendall, 1970; Reed and Cousineaus, 1967). The epizootic which occurred in Maryland during February and March of 1970 is estimated to have caused the deaths of 88,000 waterfowl, mostly sea ducks (Wildlife Management Institute,

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1970).

Pesticide-Infectious Disease Interactions

Finney (1952) stated, "any attempt to understand fully the toxic action of a group of insecticides or fungicides must ultimately involve a study of their behavior when two or more are applied in mixture...Precise meaning can only be given to these modes of action of mixtures after the establishment or definition of a normal mode of action, the results of any series of tests may then be compared with this standard in order to judge whether the toxicity is enhanced or reduced when the poisons are applied in mixture." The main objective of Part I of the present study was to establish the "normal mode of action" for each agent being tested for use in the interaction model. Part II of this study was designed to evaluate host response following exposure to two different agents, one a chemical compound and the other an infectious agent. Unlike the situation described by Finney, these agents were administered singly at different points in time rather than as a mixture at a single point in time, but the basic principles remain the same. Exposure to environmental pollutants in nature is continuous and insult from multiple agents (chemical, physical, and biological) during the life span of an individual is the rule rather than the exception. Therefore, evaluation of the biological effects of chemical agents must be considered from many aspects, including that of alterations in host response to subsequent exposures of infectious disease.

The importance of considering interactions between pesticides and

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other factors in the evaluation of the potential health hazard of pesticides was stressed by Durham (1967). His treatment of the subject like most interest in pesticide-disease relationships has been concerned with direct cause and effect aspects of the problem. Thus, considerable effort has been devoted to the carcinogenic potential of pesticides (Barnes, 1966; Falk et al., 1965; Oser, 1962) and to the role of pesticides in producing disease from direct exposure (Holmes, 1965; Sanchez-Mendal et al., 1963; West and Milby, 1965). Few studies have examined the indirect sublethal effects of pesticides that, when combined with other factors, may result in the establishment of overt disease. Initial research in this area was by Gablicks and Friedman (1969) who studied the response of cell cultures treated with organochlorine, organophosphorus, and dinitrophenol insecticides to polio and vaccinia viruses and diptheria toxin and by Wasserman et al. (1969) who studied the effects of organochlorine insecticides on body defense systems in the laboratory rat.

Evidence for and against health hazards of pesticides is largely of an epidemiological nature. In man for example, data includes autopsy findings and pesticide residues present in body fat and various other tissues, surveillance of health records of individuals in high risk operations (agriculture, pesticide manufacture, spray operators) and feeding experiments in volunteers. For lower animals, laboratory studies have been utilized (Durham, 1969; Hayes, 1959; Hoffman et al., 1967; Mastromattoo, 1964; Mengle et al., 1967; Radomski and Deichmann, 1969; Robinson et al., 1965; Yeary, 1966; Zapp, 1965). Results of these studies have led to the general conclusions

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by most investigators that pesticide residues stored in the fat of the general populace have no effect on human health and that these chemicals when properly used are not harmful to man.

There are, however, a number of instances of possible pesticide-disease relationships. Ganelin et al. (1964) found an unequivocal temporal relationship between exposure to parathion (a popular organophosphate) and asthmatic attacks. In another study, mean pesticide levels in autopsy fat of diseased humans were higher than those from fat of individuals dying from "natural causes". Significantly higher levels of p,p'-DDE and dieldrin occurred in 32 cases of portal cirrhosis of the liver (alcoholic, nutritional or Laennec's) and the greatest levels of DDT and dieldrin were measured from a single case of idiopathic amyloidosis. Other increased levels of DDT were found in cases of metastic malignancy of the liver, encephalomalacia due to cerebral thrombosis (here brain levels were also elevated), atherosclerosis, and hypertension. Dieldrin levels in body fat were elevated in cases of cerebral hemorrhage and both DDT and dieldrin levels were elevated 2 to 3 times normal in carcinoma cases (Radomski and Deichman, 1969). It is not known if any cause and effect relationship exist between the high residue levels and the disease state of these individuals.

Evidence of possible pesticide-disease relationships have also been observed among both warm and cold blooded lower animals. Gilbert (1969) concluded that DDE being used in studies with mink (Mustela vison) stressed these animals so that they became more vulnerable

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to other agents and resulted in several deaths from Chastek's paralysis. Among fish, the greatest numbers of cutthroat trout (Salmo clarkii lewisi) dying from infectious disease during DDT toxicity trials were those subjected to the highest levels of DDT exposure. The pattern of mortality that evolved, relative to dosage schedules and DDT levels, suggested that DDT increased mortality by reducing resistance to other, nonspecific, stresses including disease (Allison et al., 1964). A tabulation of deaths from specific diseases was not given by the authors but efforts to control furunculosis, Bacterium salmonicida, kidney disease, parasites, and fungal agents were mentioned.

Schoenthal (1963, cited by Cope, 1965) observed a higher incidence of furunculosis and fungus disease in DDT treated salmonids than in control fish, his interpretation being that treated fish were more susceptible to these diseases by the presence of DDT burdens in their bodies. In another study, Lowe (1967) suggested that brain parasitism noted in spot (Leiostomus xanthorus) exposed to Sevin, but not present in control fish, might have resulted from reduced resistance of the exposed fish to infestation by the parasite.

In the above reports the parasitic infections and infectious diseases observed were not planned introductions and these data are without quantitation regarding dose response. In addition, a large number of uncontrolled variables further complicate interpretation. The laboratory studies of Gabliks and Freedman (1969) and Wassermann et al. (1969) provide stronger evidence that pesticides do interact

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with disease agents in some systems. The following studies represent an attempt to test the hypothesis in an in vivo system that preexposure to pesticides does not alter susceptibility to infectious disease agents. The objectives of Part I of this study were to expose different age mallards to a number of individual agents (biological and chemical) and determine (1) baseline data regarding host response which could be utilized in the design and interpretation of interaction experiments and (2) to provide information regarding host response to the various agents that may be useful in interpretation of data collected under natural conditions. The objective of Part II was to expose different age mallards to pre-determined levels of chemical and biological agents to test the hypothesis that preexposure to pesticides does not alter susceptibility to infectious disease agents.

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PART I

STUDIES ON THE EFFECTS OF SELECTED CHEMCIAL AND INFECTIOUS DISEASE AGENTS IN THE MALLARD (*Anas platyrhynchos*)

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GENERAL EXPERIMENTAL PROTOCOL

There are a number of constants in the materials and methods used in these studies. These are identified at this time to avoid needless repetition each time a new agent is discussed. Deviations from these standards are appropriately identified when they occur and more detailed methodology is described when each agent is considered individually.

Experimental Birds

Mallards in these studies were generally obtained from the Max McGraw Wildlife Foundation, Dundee, Illinois on the day of hatch and raised to the desired age within tight or semi-isolation units at the Charmany Research Center of the University of Wisconsin, Madison. All McGraw mallards were fourth generation progeny from McGraw "wild stock".

Diets

Two specialized diets, one a duck starter and the other a duck grower, formulated by the University Poultry Department were fed ad libitum. These diets (Appendix C) were designed to provide adequate nutrition but minimize the formation of body fat. The switch from the starter to grower ration was dictated by the age of the birds at the start of each experiment. Birds under 30 days of age were fed the starter ration and those 30 days of age or older were fed the grower ration.

DDT and dieldrin diets were formulated by adding the desired amounts of each compound to separate lots of standard duck rations.

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Formulation of premixes and mixing of these pesticide diets were done by the Wisconsin Alumni Research Foundation (WARF), Madison. The entire lot of feed for an experiment was mixed at one time and stored in covered galvanized barrels until used.

Laboratory Technique

Egg inoculations for virus propagation or assay were via the allantoic route in 10-day-old embryonating hens eggs. Ten-fold serial dilutions were generally employed whenever inoculums were prepared for assay. Tryptone broth (Difco) was used as a standard diluent and when 2,000 units of Potassium penicillin G/ml were added this diluent was referred to an antibiotic broth.

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NEWCASTLE DISEASE VIRUS

INTRODUCTION

Ducks and geese are considered to be more resistant than gallinaceous birds to Newcastle disease (ND), but natural outbreaks among domestic waterfowl have been reported and these species may play an important role in the dissemination of NDV (Lancaster, 1966). Little is known regarding the role of wild waterfowl in the epizootiology of ND although relatively large numbers of reactors have been detected in serologic surveys (Bradshaw and Trainer, 1966; Friend and Trainer, 1970a; Palmer and Trainer, 1970). In addition, other migratory water birds (shag, gannet, and cormorant) have been considered important agents in the spread of ND to poultry on the coast of Britain (Blaxland, 1951; MacPhearson, 1956; Wilson, 1950). The present NDV studies were designed to (1) determine the suitability of this agent for use in the interaction model, (2) provide base line data regarding host response, and (3) learn more about this virus in the mallard.

MATERIALS AND METHODS

Inoculum

The Texas GB strain of NDV used as inoculum in these studies was obtained from the World Newcastle Repository maintained in the Department of Veterinary Sciences, University of Wisconsin. Virus stock (10^9 $CELD_{50}$/0.1 ml) was prepared by inoculating 10-day-old embryonating chicken eggs via the allantoic route and harvesting allantoic fluid 48 hours after inoculation; the virus was stored in 3 ml aliquots at -20°C.

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Experimental Birds

Mallards used in these studies were from the Max McGraw Wildlife Foundation with the exception of those in the LD_{50} studies which were purchased from a commercial game breeder in northeastern Illinois. These birds were obtained from the breeder 2 days prior to NDV exposure with the exception of day-old ducklings which were secured on the day of use. High protein turkey rations obtained from the University's feed mill were fed rather than the standard duck rations since the duck diets had not been formulated at the time NDV studies were conducted. Group identity of all experimental birds was maintained by separate caging and colored leg bands. Chickens were obtained from the University poultry flock.

Route of Exposure

In the first trial individual day-old ducklings in groups of 10 birds each were exposed either intramuscularly (IM) or intranasally (IN) with 1 of 4 ten-fold dilutions of NDV (10^5 to 10^8 $CELD_{50}$). Ten controls for each route of exposure received sterile diluent. The same protocol was followed with 10-day-old ducklings. An additional group of ten 11-day-old ducklings was inoculated via the intracardiac route (intravascular or IV) with 10^8 $CELD_{50}$ of NDV.

Blood, brain, spleen, and lung were collected from birds that died during the 10 day experimental period and from a sample of survivors at the end of the experiment. Each tissue sample tested was inoculated as a 10 percent suspension in antibiotic broth into 5 eggs via the allantoic route, examined for 5 days, and allantoic fluids from dead

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eggs and a sample of survivors tested for hemagglutination (HA) properties as described in Methods for the Examination of Poultry Biologics (1963). Birds and eggs that died during the first 24 hours post-inoculation (PI) were discarded and not considered in the results.

LD_{50}

The protocol in these trials was essentially the same for each age class. Ducklings 1, 3, 6, 11, 16, 21, and 30 days old were inoculated intracardiacly while adults were inoculated intravenously via the brachial vein (no further distinction will be made between these two routes, hereafter, both will be considered intravascular). In each age class, groups of 10 birds were inoculated with 1 of 6 ten-fold dilutions of the stock virus (10^3 to 10^8 $CELD_{50}$) and a control group received only diluent (Table 1).

Each age class was assigned to individual holding units and the various treatment groups were identified by colored leg tapes and color marking of various body areas with paint. All treatment groups, including controls, within an age class were allowed to intermingle throughout the 10 day experimental period. Each holding unit was inspected a minimum of three times daily when clinical signs of disease were noted and dead birds removed and recorded. Survivors were killed 11 days PI.

Virus isolation attempts were made from 10 percent tissue suspensions prepared in antibiotic broth from brain, lung, and liver-spleen pools of birds that died, as well as from tissues of representative samples of survivors. Four ten-fold dilutions of these tissue

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Table 1. Percent mortality among different age mallards inoculated intravasuclarly with the Texas GB strain of NDV.

Age class (days)	Mortality (%)*							
	Inoculum ($CELD_{50}$)**							Total***
	C	10^3	10^4	10^5	10^6	10^7	10^8	
1	0	20	40	70	80	60	70	56.7
3	20	10	30	30	80	80	100	55.0
6	0	20	30	90	90	100	100	71.7
11	0	0	10	20	70	100	90	48.3
16	0	0	0	30	10	50	80	28.3
21	10	0	0	10	60	70	70	35.0
30	0	0	10	40	30	60	80	36.7
Adult	0	10	10	20	10	0	50	16.7
Total mortality	3.8	7.5	16.2	38.8	53.8	65.0	80.0	43.5

*Ten birds of each age class were inoculated at each level of inoculum.

**Chick embryo lethal doses; for 21 and 30-day-old birds add 0.3 and for adults 0.7 log units; C = diluent control.

***Excluding diluent controls.

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suspensions were assayed in hens eggs as described previously. Only tissues inoculated into eggs found to be HA positive were considered as NDV deaths.

Blood samples were taken from all birds at the end of the experimental period (11 days PI) and adults were bled prior to and after the trial period. Pre-inoculation blood samples were not taken from younger birds because of the mortality risk from cardiac puncture. Blood samples were allowed to clot overnight, serum samples were removed, heat inactivated (56° for 30 minutes), and stored at -20°C until tested for specific NDV antibody with the beta procedure of the hemagglutination-inhibition (HI) test (Methods for the Examination of Poultry Biologics, 1963). Erythrocytes for the HI test were obtained from adult mallard donors whose serums were negative for NDV antibody.

Pathogenesis Studies

Three trials involving virus distribution, viremia, and antibody response were done to obtain information on the pathogenesis of the Texas GB strain of NDV in the mallard.

Experiment 1 - virus distribution: Two groups of five male and five female adult mallards each were inoculated IV with either $10^{5.7}$ or $10^{8.7}$ $CELD_{50}$ of NDV. One bird from each group was killed every 24 hours PI at which time 10 ml of blood, the brain, spleen, and portions of liver and lung were removed, and 10 percent suspensions examined for the presence or absence of virus. Blood not used in virus assay was allowed to clot and serum collected.

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Experiment 2 - viremia: Four adult male mallards and a white leghorn rooster were inoculated IV, two of the mallards each received $10^{5.7}$ $CELD_{50}$ of NDV and the remaining birds $10^{8.7}$ $CELD_{50}$ each. Blood samples were collected from all birds hourly for the first 6 hours; every 2 hours for the remainder of the first 24 hours; and then at hours 30, 36, and 48 PI. Blood samples were tested for NDV virus as in Experiment 1. Surviving birds were killed 11 days PI and tissues assayed in eggs for NDV.

Experiment 3 - antibody response: Five adult female mallards were inoculated IV with $10^{5.7}$ $CELD_{50}$ of NDV, four with $10^{8.7}$ $CELD_{50}$, and two with antibiotic broth. Blood samples were taken from birds daily for the first 10 days and thereafter on days 15, 20, 25, 32, and 40 PI. Serum was collected from these samples and tested by the HI test as previously described.

In addition, two adult female mallards were sealed in a room in which 10^{10} $CELD_{50}$ of NDV had been added to four gallons of drinking water. The room was opened on day 10 PI at which time the birds were bled and the food and water supplies replenished. These birds were bled again on day 40 PI when the experiment was terminated.

RESULTS

Route of Exposure

In the IM and IN groups six ducklings died from other than accidental causes (crushed by waterer). Of these, NDV was only detected in the tissues of one bird in the IM group. Nine of the ten birds inoculated IV died and NDV was isolated from tissue pools of four

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birds each of spleen, lung, and brain but not from the blood (one bird died during the first 24 hours and was discarded). These findings resulted in selection of the IV route in subsequent NDV studies.

LD_{50}

The protocol and results of the LD_{50} trials are summarized in Table 1. Ducklings 1 and 3-days-old appeared to be more resistant to infection with NDV than those 6 days of age. Thereafter, mortality had a tendency to decrease with increasing age. Mortality among adults was less than half of that for any other age class with the exception of 16-day-old birds. Calculated LD_{50} values for each age class as approximated by the techniques of Reed and Muench (1938) and probit analysis (Finney, 1952) were similar (Table 2).

Regression analyses of these mortality data indicated similar response patterns for day-old ducklings and adults; a different magnitude and similar response patterns for 16, 21, and 30-day-old ducklings; and different response patterns for 6 and 11-day-old ducklings (Figure 1). The 3-day-old ducklings appear to represent a transitional relationship between decaying maternal antibody and increasing age resistance to NDV, the latter condition being reflected in the parallel but widely separated regression lines of 6 and 11-day-old ducklings and similar divergence between 11 day-old and older ducklings.

The degree of mortality was correlated with the quantity of virus in the challenge dose as might be expected, irrespective of age class (Table 1 and Figure 2). The greatest mortality occurred

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Table 2. LD_{50} values for mallards of different ages inoculated intravascularly with the Texas GB strain of NDV.

Age class (days)	Total mortality (%)	LD_{50}*		95%** Confidence intervals	
		R & M	Probit	Lower limit	Upper limit
1	57	5.0	4.8	3.3	6.3
3	55	5.3	5.2	4.5	5.8
6	72	4.3	4.2	3.6	4.7
11	48	5.4	5.6	5.1	6.1
16	28	6.9	6.9	6.2	7.6
21	35	6.8	6.8	6.2	7.4
30	37	6.9	6.7	6.0	7.5
Adult	17	>8.7	11.7	4.7	18.7

*Values expressed as $\log_{10}$ exponents; R & M = Reed and Muench; Probit = probit analysis.

**Calculated by probit analysis.

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Figure 1. Mortality as a function of dose in mallards inoculated intravascularly with the Texas GB strain of NDV. Mortality is expressed in probit values (probit number of 5 = 50 percent mortality) and dose in logs of $CELD_{50}$.

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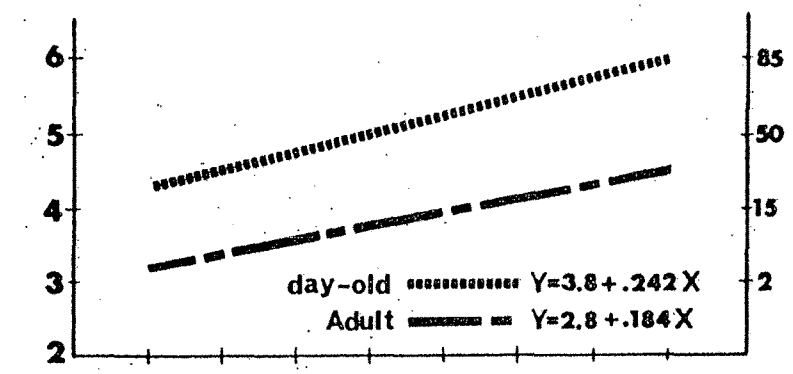

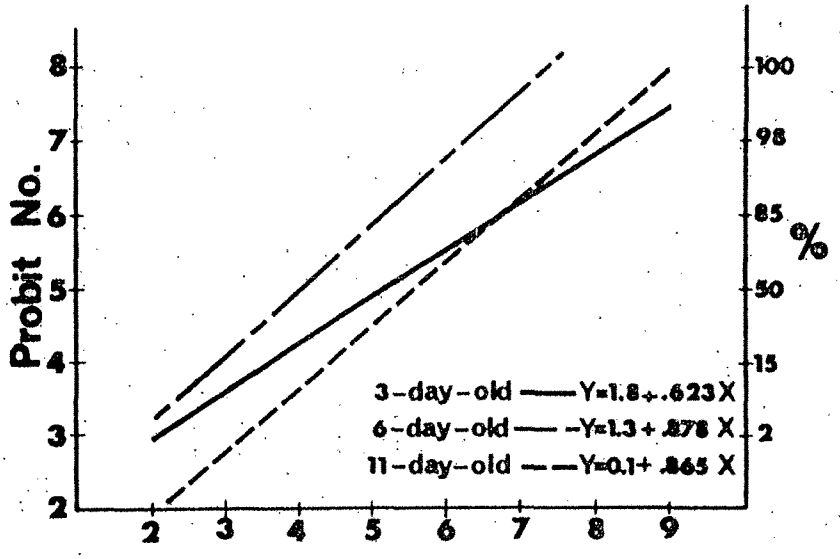

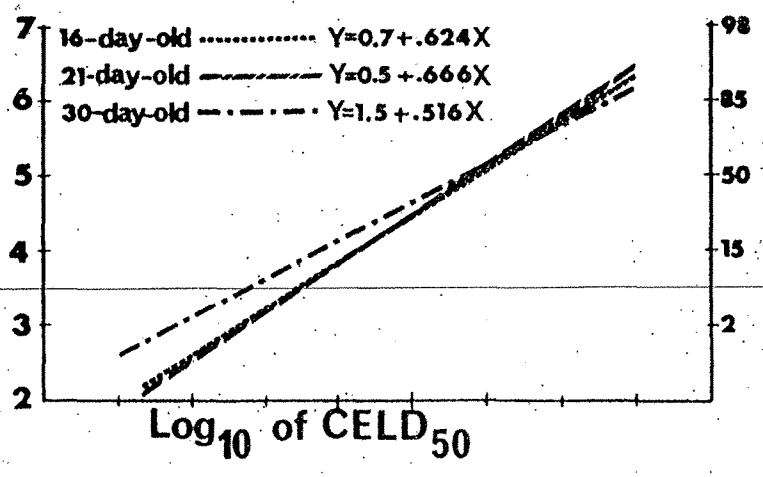

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Figure 2. Relationship between mortality and dose in mallards inoculated intravascularly with the Texas GB strain of NDV. Each point represents pooled data from all age classes.

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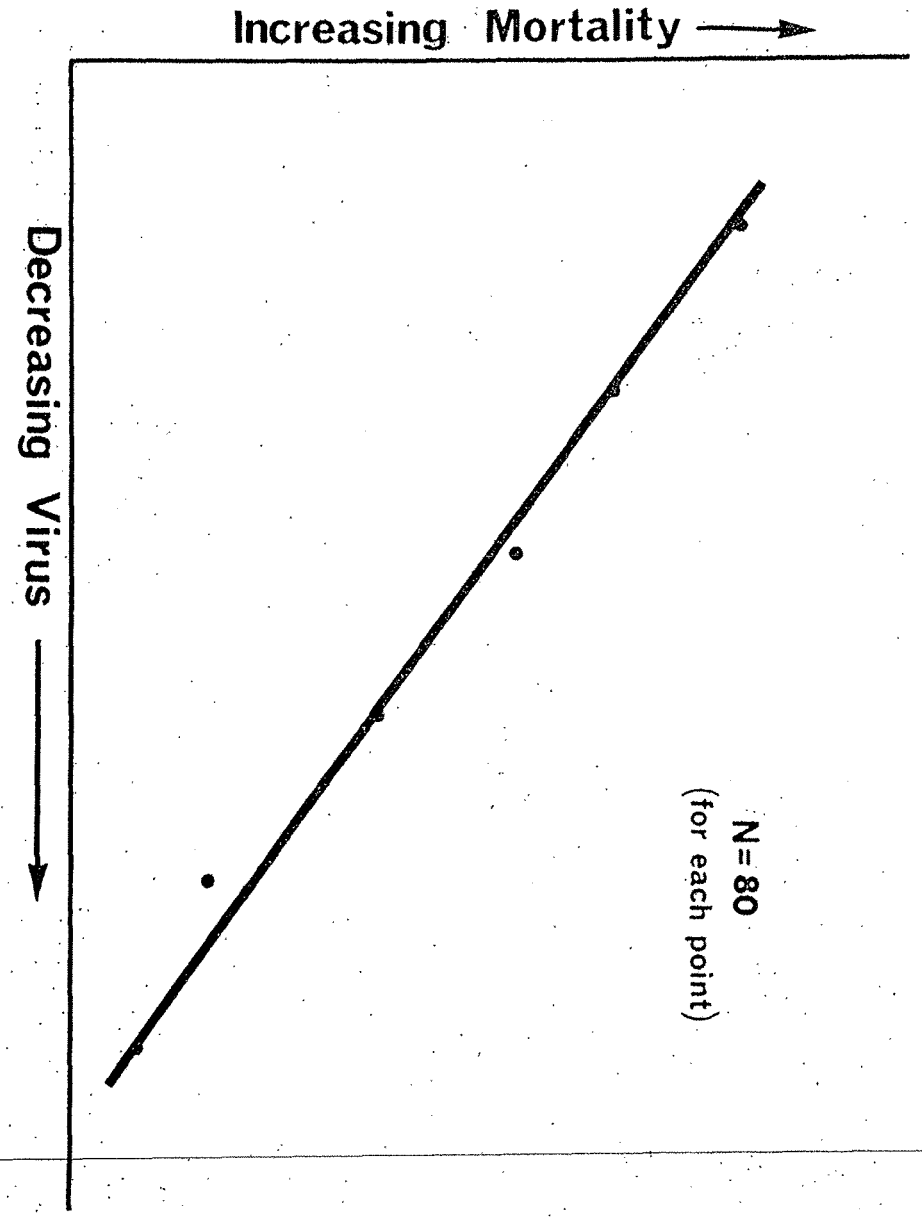

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at day 3 PI, after which the mortality-time-curve became markedly skewed to the right (Figure 3) primarily as a result of increased time to death with decreasing amounts of virus (Figure 4). This gradual increase in time to death did not appear to be influenced by age of the bird (Table 3), but by the amount of virus in the inoculum (Table 4).

Brain, lung, and liver-spleen suspensions of nearly all 212 birds that died during these experiments were assayed for the presence of NDV and virus was detected in 1/3 of the tissues of the birds tested. Virus was not detected in the tissues of any of the controls that died. There did not appear to be any correlation between success of detecting virus and the age of the birds, but a general increasing success ratio was suggested with increasing levels of virus inoculated (Table 5). Virus was not detected in the tissues of control mallards or treatment birds surviving NDV challenge, even though some of the latter had exhibited clinical signs of disease. A lone exception was the isolation of NDV from a liver-spleen suspension of a surviving adult.

All tissues were not tested from each bird which died (Table 6), but of those tested the greatest percentage of isolates were made from brain suspensions, the next from lung, and the least from liver-spleen suspensions. In all three instances if NDV was isolated from lung or liver-spleen suspensions it was also isolated from the brain of that bird. The success ratio of virus isolations appeared to be a function of daily mortality (Figure 3), that is, the ratio of percent

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Figure 3. Percent daily distribution of total mortality and virus isolations from tissues of dead mallards following intravascular inoculation with the Texas GB strain of NDV.

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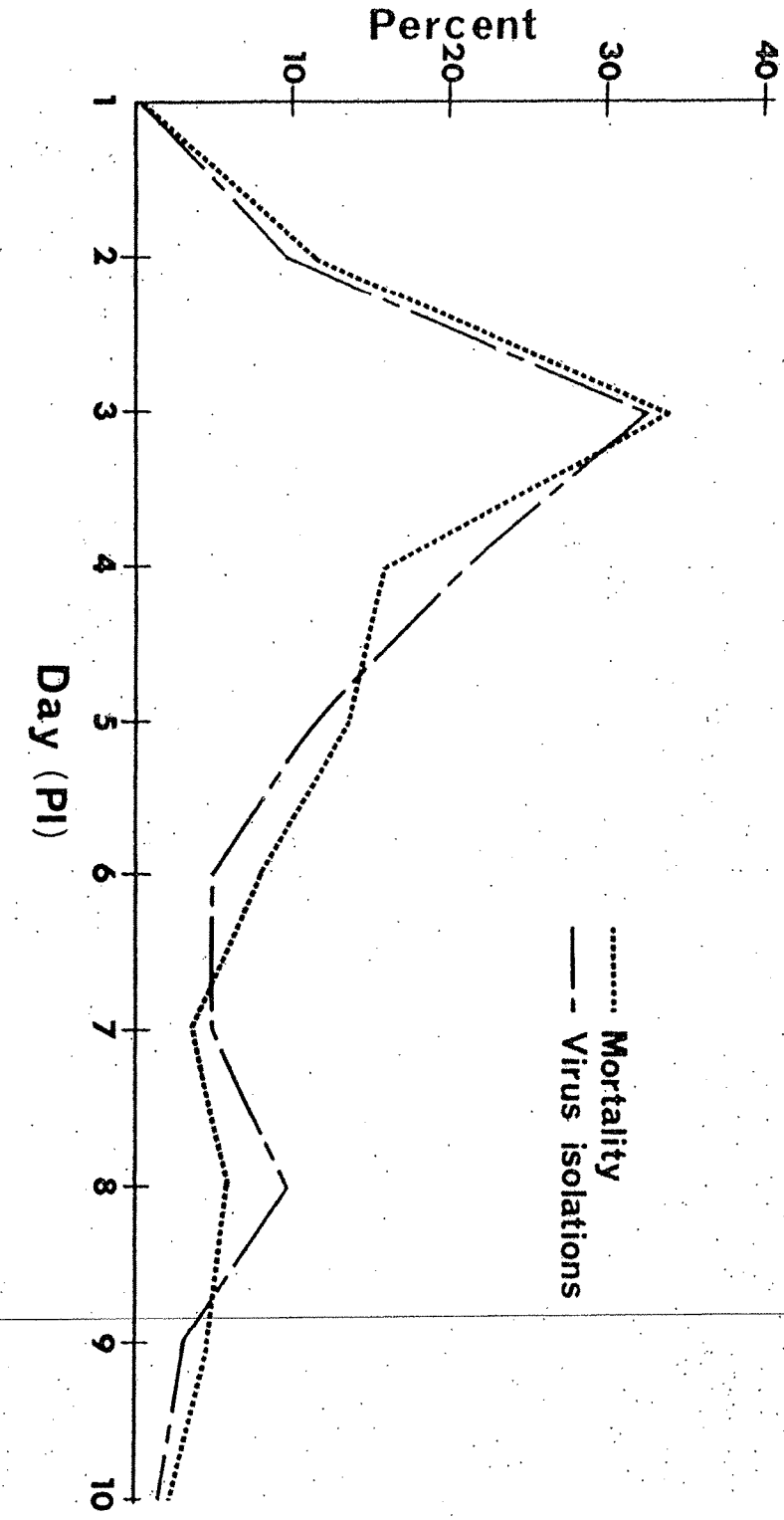

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Figure 4. Percent daily mortality, by dose, of mallards inoculated intravascularly with the Texas GB strain of NDV.

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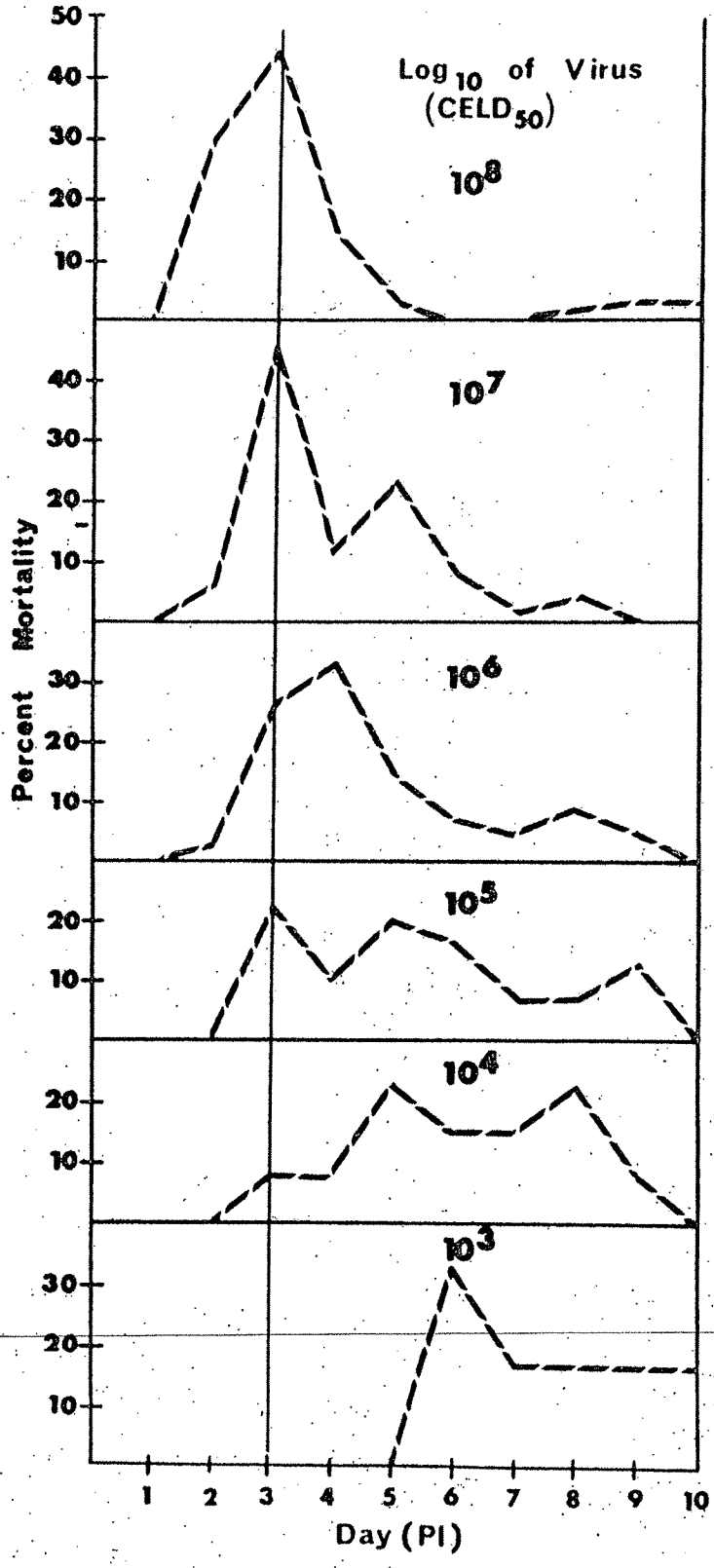

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Table 3. Percent daily mortality among mallards of different ages inoculated intravascularly with the Texas GB strain of NDV.

Age class* (days)	Percent mortality**										Total number of deaths
	Day post inoculation										
	1	2	3	4	5	6	7	8	9	10	
1	0.0	5.9	11.8	26.5	17.6	23.5	2.9	5.9	2.9	2.9	34
3	0.0	2.9	31.4	22.9	11.4	8.6	2.9	8.6	8.6	2.9	35
6	0.0	9.3	34.9	18.6	18.6	7.0	7.0	4.6	0.0	0.0	43
11	0.0	37.9	41.4	0.0	13.8	3.4	3.4	0.0	0.0	0.0	29
16	0.0	17.6	35.3	5.9	23.5	5.9	0.0	11.8	0.0	0.0	17
21	0.0	13.6	45.4	22.7	9.1	4.6	0.0	4.6	0.0	0.0	22
30	0.0	0.0	59.1	9.1	4.6	0.0	0.0	9.1	13.6	4.6	22
Adult	0.0	0.0	10.0	10.0	0.0	0.0	20.0	10.0	30.0	20.0	10

*Each age class contained 70 birds, 60 inoculated with NDV and 10 diluent controls.

**Total percent mortality for all levels of inoculum (see table 4).

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Table 4. Daily distribution of mortality among mallards inoculated intravascularly with the Texas GB strain of NDV.

Inoculum** ($CELD_{50}$)	Mortality (%)*											
	Day (PI)										Total	
	1	2	3	4	5	6	7	8	9	10	No.	%
C	0.0	0.0	33.3	0.0	0.0	33.3	0.0	0.0	0.0	33.3	3	3.8
10^3	0.0	0.0	0.0	0.0	0.0	33.3	16.7	16.7	16.7	16.7	6	7.5
10^4	0.0	0.0	7.7	7.7	23.1	15.4	15.4	23.1	7.7	0.0	13	16.2
10^5	0.0	0.0	22.6	12.9	19.4	16.1	6.4	6.4	12.9	3.2	31	38.8
10^6	0.0	2.3	25.6	32.6	14.0	7.0	4.6	9.3	4.6	0.0	43	53.8
10^7	0.0	5.8	46.2	11.5	23.1	7.7	1.9	3.8	0.0	0.0	52	65.0
10^8	0.0	31.2	43.8	14.1	3.1	0.0	0.0	1.6	3.1	3.1	64	80.0

*Total percent mortality for all eight age classes (see table 3).

**Ten birds of each age class were inoculated at each level of inoculum (sample size of 80); C = diluent control.

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Table 5. NDV isolations from tissues of different age mallards dying during LD_{50} experiments.*

Age class (days)	Inoculum ($CELD_{50}$)**							Total	
	C	10^3	10^4	10^5	10^6	10^7	10^8	No.	%
1	...	1/2***	1/4	3/7	4/8	2/6	2/7	13/34	38.2
3	0/2	1/1	1/3	2/3	1/8	6/8	6/10	17/35	48.6
6	...	0/1	0/1	0/3	2/7	3/7	3/4	8/23	34.8
11	...	...	0/1	...	0/5	0/10	2/9	2/25	8.0
16	...	...	...	0/1	...	4/5	4/7	8/13	61.5
21	0/1	...	...	0/1	1/6	3/7	3/7	7/22	31.8
30	...	...	0/1	0/4	0/2	0/6	2/8	2/21	10.0
Adult	...	1/1	0/1	0/2	0/1	0	3/5	4/10	40.0

*Total virus isolations from all tissues tested.

**For 21 and 30-day-old birds add 0.3 and for adults 0.7 log units; C = diluent control

***Number isolations/number tissues tested.

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Table 6. Virus isolations from tissues of different age mallards dying during LD_{50} trials following intravascular inoculation with the Texas GB strain of NDV.

Age class (days)	Inoculation					
	Brain		Lung		Liver-spleen pool	
	No.	%	No.	%	No.	%
1	13/34*	38.2	...	...	0/11	0.0
3	16/35	45.7	3/30	10.0	0/30	0.0
6	8/23	34.8	1/21	4.8	0/20	0.0
11	1/24	4.2	1/25	4.0	0/25	0.0
16	5/12	41.7	4/11	36.4	1/11	9.1
21	7/22	31.8	1/18	5.6	0/20	0.0
30	2/19	10.5	...	...	0/2	0.0
Adult	2/9	22.2	2/10	20.0	0/10	0.0
Total	54/178	30.3	12/115	10.4	1/129	0.8

*Number isolations/number tested.

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mortality to percent virus isolations was relatively constant with time. This relationship (when examined for brains only) was one of decreasing mean day of isolation with increasing virus dose and appeared to be unrelated to the age of the bird (Table 7). The greatest percentage of isolations were made on days 2 and 3 PI during which time 33 and 21 percent of all isolations were detected.

In these experiments antibody response appeared to be correlated with age. The number of ducks with HI titers of 1:20 or greater generally increased with increasing age as did the magnitude of these titers (Table 8). The high percentage (83 percent) of HI reactors among the day-old age class probably represents maternal antibody, which is also suspected in the reactors found in 3, 6, and perhaps 11-day-old age classes. Based on the magnitude of HI response, the first evidence of active antibody was detected in 16-day-old birds when 4 of 15 positive titers were 1:80 or greater. No significant titers were detected in any of the 50 controls tested.

All but four of the surviving adult mallards had NDV HI titers of 1:20 or greater (Table 9). An analysis of variance of the results obtained at different levels of inoculum indicated significant differences among mean titers ($F = 2.69$, $df = 5, 44$). When tested by the least significant differences (Steel and Torrie, 1960) significant ($p = <.05$) or highly significant ($p = <.01$) differences occurred between the low virus dose treatment mean ($10^{3.7}$ $CELD_{50}$) and all other treatment means except the high virus dose ($10^{8.7}$ $CELD_{50}$). No other treatment means were statistically different from one another ($p = >.05$).

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Table 7. Mean day of NDV isolations from brains of mallards dying during LD_{50} trials.

Age class (days)	Inoculum ($CELD_{50}$)*						
	10^3	10^4	10^5	10^6	10^7	10^8	Total**
1	10.0	8.0	6.3	4.2	6.0	2.5	5.5 ± 3.49(13)
3	8.0	8.0	7.0	3.0	3.7	3.2	4.2 ± 1.84(16)
6	...	...	...	5.0	4.3	4.7	4.6 ± 1.77(8)
11	...	...	...	...	...	7.0	7.0(1)
16	...	...	...	...	5.0	2.5	3.0 ± 1.22(5)
21	...	...	...	4.0	3.3	4.0	3.8 ± 1.83(8)
30	...	...	...	...	...	3.0	3.0(1)
Adult	8.0	...	...	...	...	9.0	8.5(2)
Total**	8.7 ± 1.16 (3)	8.0 0.0 (2)	6.5 ± 2.08 (4)	4.2 ± 1.04 (8)	4.1 ± 1.19 (15)	3.8 ± 2.14 (22)	

*For 21 and 30-day-old birds add 0.3 and for adults 0.7 log units.

**Mean ± standard deviation (Sample size).

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Table 8. Haemagglutination-inhibition antibody responses of different age mallards 11 days PI with the Texas GB strain of NDV.

Age class (days)	Reactors*		Serum HI titers of reactors**			
			Standard error			Range
	No.	%	Geometric mean	Lower limit	Upper limit	
1	5/6	83.3	24	21	27	20-40
3	2/13	15.4	58	...		40-80
6	2/12	16.7	29	...		20-40
11	4/12	33.3	29	...		20-40
16	15/18	83.3	41	34	50	20-160
21	12/19	63.1	44	36	53	20-160
30	18/21	85.7	60	51	70	20-160
Adult	46/50	92.0	214	192	239	20-640

*Number reactors (serum HI titer of 1:20 or greater)/number tested.

**HI titer expressed as reciprocal of endpoint; standard error calculated from log transformation of data; doubling dilutions of serum tested.

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Table 9. Haemagglutination-inhibition antibody response of adult mallards 11 days PI with the Texas GB strain of NDV.

Inoculum ($CELD_{50}$)	Reactors*		Serum HI titers**			
				Standard error		Range
	No.	%	Geometric mean	Lower limit	Upper limit	
$10^{3.7}$	6/9	67	30	12	71	0-640
$10^{4.7}$	9/9	100	219	131	291	40-640
$10^{5.7}$	8/8	100	270	241	302	160-320
$10^{6.7}$	9/9	100	219	158	303	20-640
$10^{7.7}$	10/10	100	185	144	298	40-640
$10^{8.7}$	4/5	80	88	28	272	0-320

*Number reactors (serum HI titer of 1:20 or greater)/number tested.

**HI titer expressed as reciprocal of end point; standard error calculated from log transformation of data; doubling dilutions of serum tested.

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Pathogenesis Studies

Experiment 1: Attempts to determine the distribution of NDV in selected tissues of mallards with time resulted in virus first being detected at 24 hours PI in the blood, liver, and lungs but not in the brain in the high dosage level group ($10^{8.7}$ $CELD_{50}$). Virus was present in the liver-spleen suspension of the low dosage level group ($10^{5.7}$ $CELD_{50}$) at 24 hours PI but not in other tissues. NDV was not detected in the tissues of birds from either group later than day 4 PI; virus persisted longest in the liver and spleen (4 days), next in lung (2 days), and was only detected on day 3 PI in brain (Figure 5).

Experiment 2: Results of the viremia study indicated that a viremia occurred in the rooster control and two of the four mallards inoculated IV with NDV. The rooster and one of these two mallards received $10^{8.7}$ $CELD_{50}$ of virus and the other mallard $10^{5.7}$ $CELD_{50}$ (Figure 6). Virus was detected in the blood of the rooster intermittently from 5 to 14 hours PI and uniformly thereafter for the remainder of the 48 hour experimental period, with the exception of 36 hours PI. In the mallard receiving the high level of inoculum, virus was detected at hours 4, 5, 6, 8, 16, and 30 PI. Virus isolations from the blood of the mallard receiving the low dose of inoculum were made at 18 and 30 hours PI. Virus was not detected in the blood of the contact control mallard or after 2 hours PI from either of the other two inoculated mallards.

The amount of virus detected in the mallard blood samples was quite low. In many instances only a single egg was killed from

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Figure 5. Virus isolations from the tissues of adult mallards killed daily following intravenous inoculation with the Texas GB strain of NDV.

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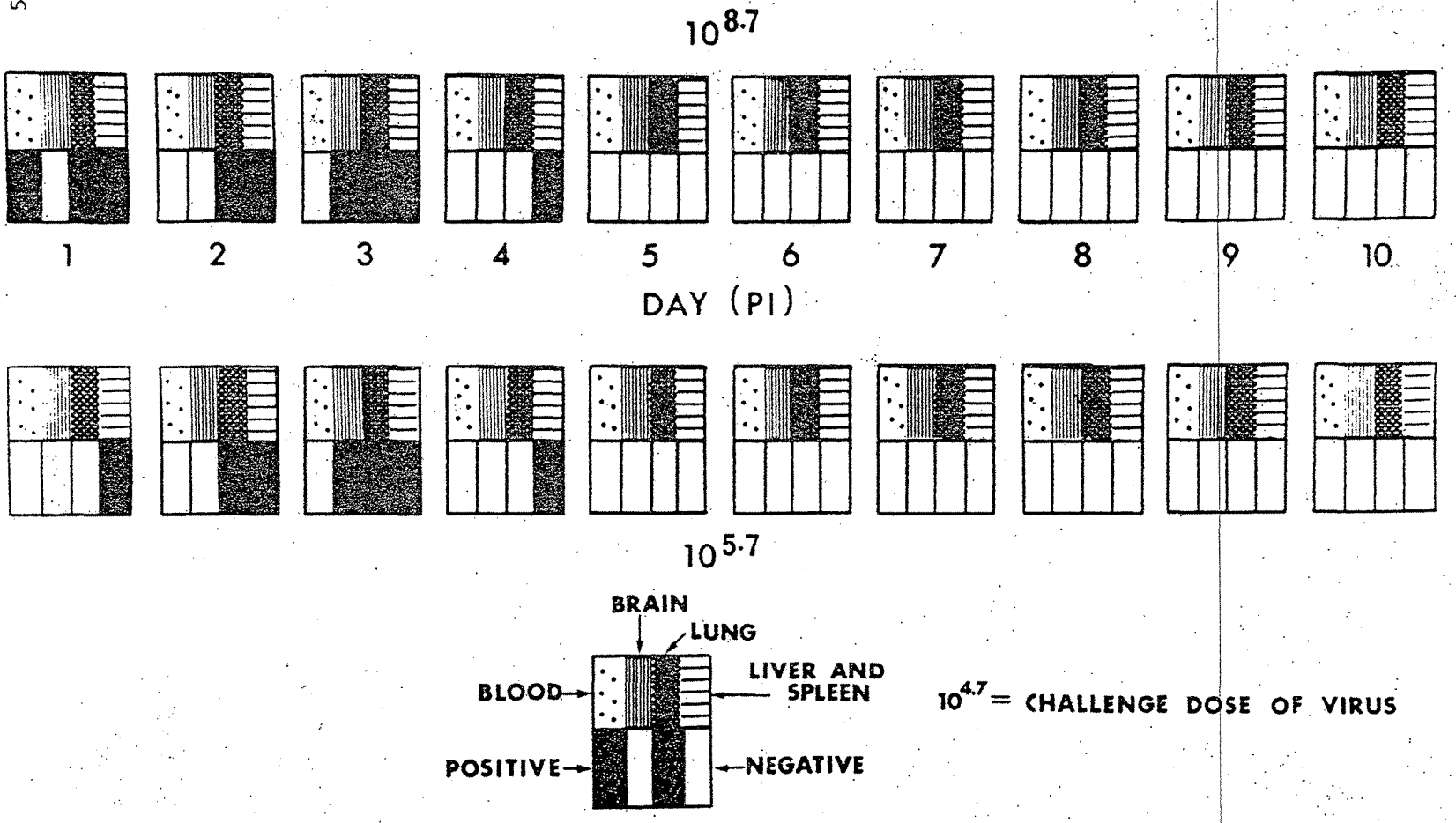

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Figure 6. Virus isolations from the blood of adult mallards and a White Leghorn rooster following intravenous inoculation with the Texas GB strain of NDV.

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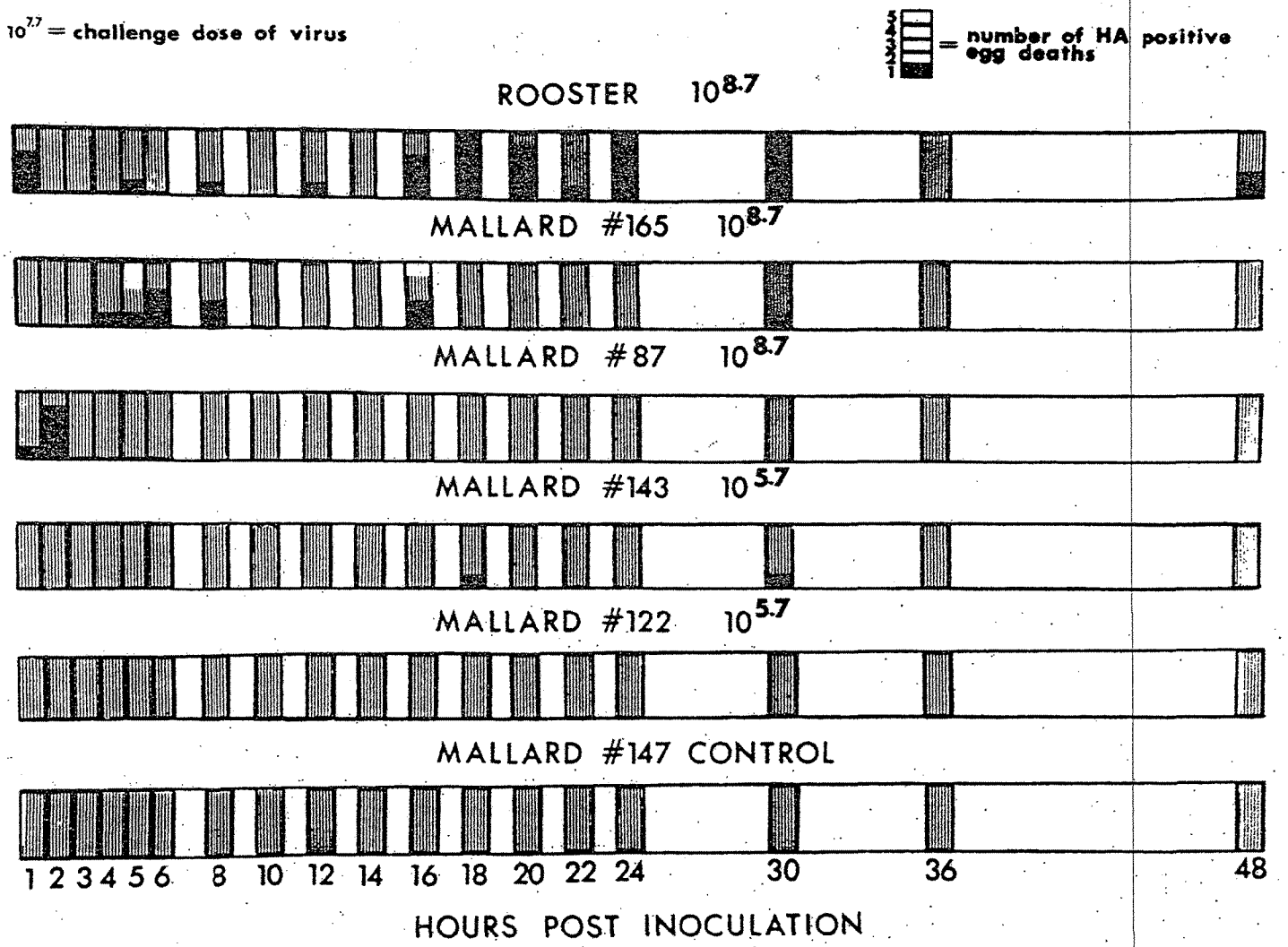

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inoculation of the 10 percent blood suspensions. A larger number of eggs were killed by the blood suspensions from the rooster control (Figure 6). Virus was detected in the tissues of the rooster control and one mallard following their deaths at days 4 and 5 PI, respectively, but not from any of the tissues of the mallards surviving the 11 day experimental period (Table 10).

Experiment 3: Studies of NDV antibody response in adult mallards indicated an early rise in HA antibody. Among the five mallards inoculated IV with either $10^{5.7}$ or $10^{8.7}$ $CELD_{50}$ of NDV, diagnostic HI titers of 1:20 or greater were observed on day 3 PI for the high dose of inoculum and on day 4 PI for the lower dosage level (Table 11). These titers peaked at 1:320 to 1:640 between days 7 and 8 PI and remained relatively constant through day 25 PI, after which they started to decline. The last measuremnt of antibody response was made 40 days PI at which time all serums had HI titers of 1:20 or greater. Contact controls which received only the diluent did not have detectable NDV HI titers.

The antibody response of two adult mallards exposed to NDV contaminated water was similar to that resulting from IV inoculation. One of these birds had a HI titer of 1:1280 at day 10 PI (the highest titer recorded during these studies) and the other mallard 1:80. At day 40 PI their respective titers were 1:160 and 1:20.

Clinical Signs

The signs of ND in the experimentally exposed mallards were primarily of CNS origin and began on day 2 PI. Initially birds would lie

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Table 10. Specific viral mortality in embryonating hens eggs resulting from inoculation of tissue suspensions from adult mallards previously inoculated intravenously with the Texas GB strain of NDV.

Species	Bird no.	Inoculum ($CELD_{50}$)	Tissue suspension*			Remarks**
			Brain	Lung	Liver-spleen pool	
Mallard	147	Control	0/5	0/5	0/5	
Mallard	143	$10^{5.7}$	0/5	0/5	0/5	Virus detected in blood at 1 and 2 hours PI
Mallard	122	$10^{5.7}$	0/5	0/5	0/5	Clinical signs of ND
Mallard	165	$10^{8.7}$	0/5	5/5	1/5	Viremia detected; died on day 5 PI
Mallard	85	$10^{8.7}$	0/5	0/5	0/5	Clinical signs; viremia detected
White Leghorn rooster	100	$10^{8.7}$	5/5	5/5	5/5	Viremia detected; died on day 4 PI

*Number HA positive egg deaths/number tested; virus isolation attempts on day 11 PI from tissues of surviving birds, day of death for other birds.

**See figure 6 for viremia study.

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Table 11. Haemagglutination-inhibition antibody responses of eleven adult mallards following intravenous inoculation with the Texas GB strain of NDV.

Inoculum ($CELD_{50}$)	Serum HI titer*														
	Day (PI)														
	0	1	2	3	4	5	7	8	9	10	15	20	25	32	40
$10^{5.7}$	0	0	0	0	10	160	320	...	...	160	320	320	320	160	40
$10^{5.7}$	5	0	0	0	40	320	320	...	...	320	...	320	320	160	80
$10^{5.7}$	0	0	0	0	10	80	640	...	...	320	640	320	160	160	40
$10^{5.7}$	0	0	0	0	...	40	80	...	...	80	160	80	80	80	20
$10^{5.7}$	0	...	0	0	40	160	80	640	640	...	...	...	...	...	...
Arithmetic mean	0	0	0	0	25	152	228	640	640	220	373	260	220	140	45
Geometric mean	0	0	0	0	20	121	211	640	640	190	320	226	190	135	40
$10^{8.7}$	0	0	5	...	Died	...	...	...	...	...	...	...	...	...	...
$10^{8.7}$	0	0	0	80	160	320	320	...	...	320	320	320	160	160	Died
$10^{8.7}$	0	0	...	40	Died	...	...	...	...	...	...	...	...	...	...
$10^{8.7}$	0	...	0	80	160	...	640	640	320	...	...	...	...	...	...

*HI titer expressed as reciprocal of endpoint; doubling dilutions of serum tested; ... indicates not tested.

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Table 11. Haemagglutination-inhibition antibody responses of eleven adult mallards following intravenous inoculation with the Texas GB strain of NDV (continued).

Inoculum ($CELD_{50}$)	Serum HI titer*														
	Day (PI)														
	0	1	2	3	4	5	7	8	9	10	15	20	25	32	40
Arithmetic mean	0	0	0	67	160	320	480	640	320	320	320	320	160	160	-
Geometric mean	0	0	0	63	160	320	452	640	320	320	320	320	160	160	-
Control	0	0	0	0	0	0	0	...	...	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	...	...	0	0	0	0	0	0

*HI titer expressed as reciprocal of endpoint; doubling dilutions of serum tested; ... indicates not tested.

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on their sternum with legs slightly extended to the sides. These birds were able to get up and move away when approached. More severely affected birds remained prostrate and were characteristically found lying on their sides exhibiting a swimming motion with both legs in vain attempts to escape. Breathing was both rapid and deep in these birds. Other affected birds with similar signs were unable to hold their heads up (Figure 7). By day 4 PI torticollis (Figure 8) and lateral deviation of the normal head position were evident in a number of birds, and wing drop (Figure 9) also began to appear. From this time on leg paralysis, involving one or both legs, and muscular tremors became increasingly noticeable among infected birds. Late in the experimental period a number of birds were observed sneezing but this was not a consistant finding.

DISCUSSION

Susceptibility

Based on the results of these experimental exposures, the mallard duck appears to be relativley resistant to the Texas GB strain of NDV. With the exception of a single duckling killed via the IM route, which could conceivably have been an intravascular inoculation, mallards could not be killed with this virus unless large doses were inoculated directly into the blood stream. Also, it did not appear that infective virus was shed by birds inoculated during these experiments since no virus isolations were made from, or antibody response detected in, contact controls (ducks or chickens).

Differences in susceptibility of mallards to NDV appeared to be

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Figure 7. Clinical signs of Newcastle disease in adult mallards following intravenous inoculation with the Texas GB strain of NDV. Note leg paralysis and inability of two of the birds to hold their heads erect.

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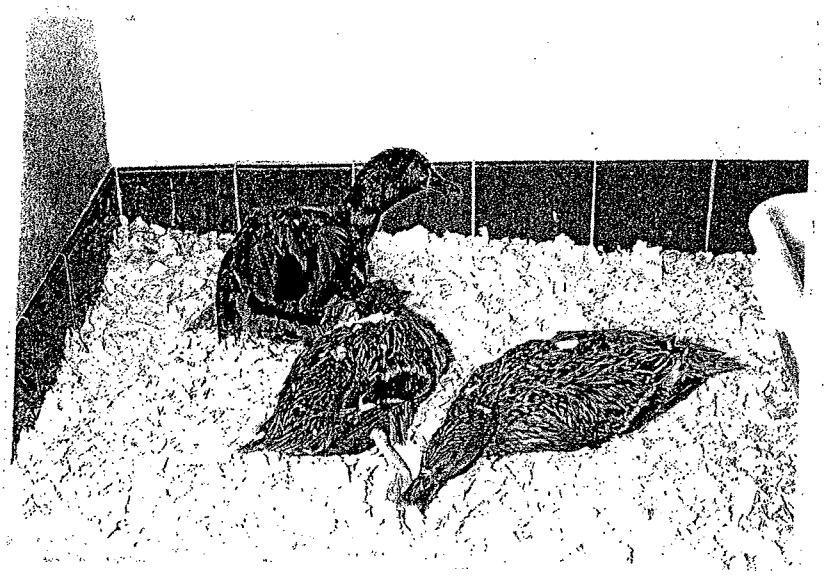

Figure 8. Torticollis in an adult female mallard following intravenous inoculation with the Texas GB strain of NDV.

Figure 9. Wing drop in an adult female mallard following intravenous inoculation with the Texas GB strain of NDV.

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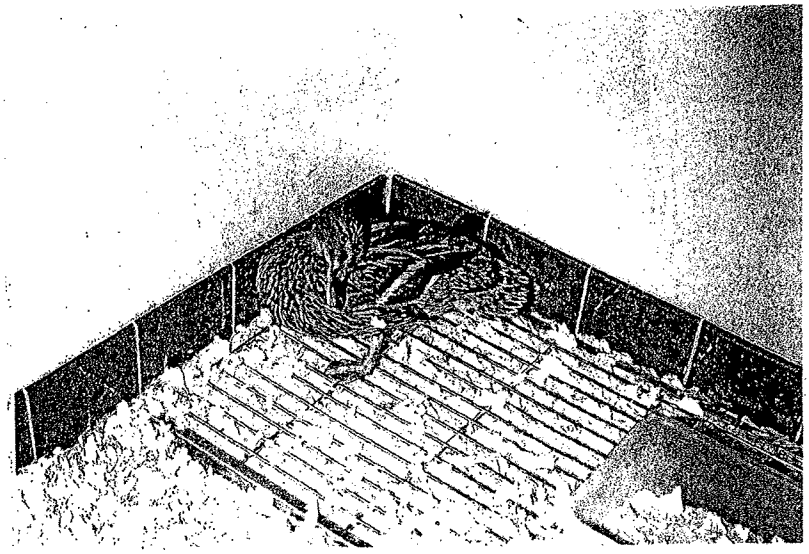

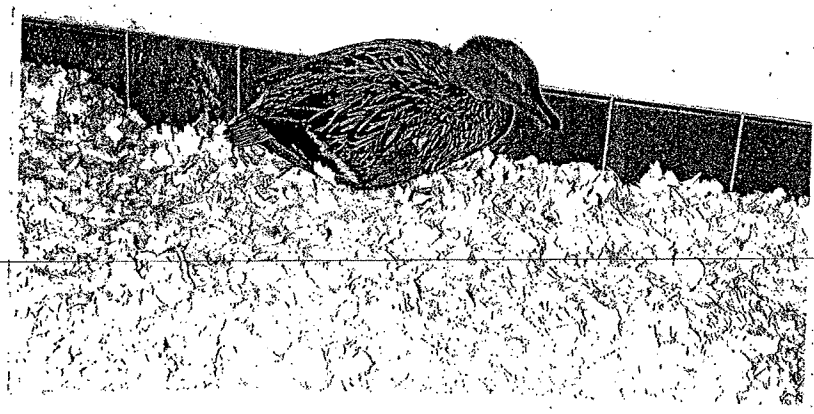

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related to both age and maternal antibody and fell into three broad age groups, ducklings 1 to 10 days of age, 11 to 30 days of age, and adults. The most susceptible ducklings were 6-day-old age birds, probably reflecting a greater decay of maternal antibody in this age class than in younger birds and less age resistance than older birds. Evidence for the presence of maternal antibody is suggested by the detection of low HI titers (1:20 to 1:40) from 5 of 6 three bird serum pools collected 10 days PI from ducklings inoculated on their day of hatch, at which time they are considered immunologically incompetent (Table 8). Several uninoculated controls also had similar levels of NDV antibody, as did half the day-old ducklings and 3/4 of the 3-day-old ducklings not used in the experiments (N = 4). Further support for this theory was the finding of 62 percent NDV reactors among breeders from the supplying flock (Friend and Trainer, 1970a).

Describing NDV in domestic fowl, Brandly and Hanson (1965) reported that, "the response of offspring of immune hens may be altered favorably by passive protection afforded by antibodies derived from the yolk." It has also been reported that fowl of all ages and breeds are susceptible to ND but that this susceptibility is reduced substantially with increasing age (Brandly, 1953; Brandly et al., 1946 a). Similar observations were seen in these mallards, although antibody decay appeared to be quite rapid when compared to domestic fowl (Boney and Stone, 1970).

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Viral Toxicity

Mortality data in the present study suggests that the response observed in mallards might be of a toxic nature rather than the result of a classical infectious process. "Viral toxicity" was coined by Burnet (1942; 1955) to describe the capability of NDV to provoke a pathologic response in a host system in the absence of viral multiplication. Since that time a wide variety of toxic effects have been described for other virus and rickettsial infections (Cooke, 1961; Cox, 1953; Platt, 1955).

The absence of viral multiplication in viral toxicity is no longer believed to be valid, and present evidence suggests that toxic action is due to a partial cycle of replication which leads to the death of the infected cell, but not to the production of infective progeny. Two salient features of viral toxicity are that the toxic effect can only be produced by inoculation of a massive dose of the virus and that the toxic activity is inseparable from the virus particle itself. Because of this latter condition toxicity is neutralized specifically by immune serum which neutralizes infectivity (Westwood, 1966).

Toxic response to NDV has been reported in mice and rabbits following IV inoculation. The toxic capability of the virus is also reported to be rapidly reduced by dilution (Brandly and Hanson, 1965). The relationship between the challenge dose of the virus and resulting mortality in the present studies was essentially linear over the span of dilutions used despite large amounts of virus being inoculated IV. These levels of virus are more than enough for establishing infection

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with most agents, especially considering the route of challenge, and appear to satisfy the criteria of massive dose at least at the higher levels. The sufficiency of the amount of virus used in the present study to produce a toxic response is further suggested by the finding that adsorption of a single viral particle was enough to lead to death of a cell as a result of toxic reaction, thereby negating the need for massive doses at the cellular level (Wilcox, 1959). The linearity of the mortality response is also suggestive of a toxic rather than an infectious response (Figure 2).

If the assumption of independent action can be extended to the present study (the infecting agents act independently of each other upon entry into the host with each having a certain probability of causing infection) the total probability of infection from a given dose is obtained by statistical combinations of the probabilities for the individual organisms. Data available for biological-response probabilities appear to be consistent with this hypothesis, with the possible exception of host-parasite systems requiring "overwhelming" doses to secure results (Shortley and Wilkins, 1965). Despite this possible exception the assumption of independent action is being accepted.

The consequence of independent action with host and treatment homogeneity is an exponential dose-response probability curve. That is, as d/do (expected dose/LD_{50}) is increased along the x axis on a linear scale the percentage response along the y axis will increase in a simple exponential manner. This relationship applies whether or not

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the infectious organisms are identical in strain or virulence, and whether or not the organisms land at equally receptive sites in the host. The critical factors in this relationship are host and treatment homogeneity (Shortley and Wilkins, 1965).

The uncomplicated quantal response curve to a toxic stimulus measured in dosage units is best described by a sigmoid distribution (Finney, 1952). With this type of distribution a linear relationship between d/do and percentage response is possible. That is, for each unit change in x, y will also change by some predictable and constant amount. Therefore, since the type of response obtained (Figure 2) is possible within the parameters of the sigmoid response curve but not within those of an exponential dose-response, these data are interpreted as being indicative of a toxic rather than an infectious response.

A complicating factor in the assumption of independent action is that of host heterogeneity. This can result in departures from the simple-exponential response-probability curve to more complex relationships. Such heterogeneity has been shown to arise from a number of sources including host and pathogen strain and host age (Shortley and Wilkins, 1965). However, these more complex relationships do not increase the possiblity of the dose-response curve of an infectious process being linear over an appreciable range of challenge doses.

The criteria of virus and toxic activity being inseparable and thus neutralizable by specific immune serum is accounted for in the present studies by protection afforded through maternal antibody.

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Viral toxicity cannot be differentiated from an infectious process on this basis since specific antibody will afford protection in both instances. The toxic effect is largely explained by incomplete cycles of viral replication within the cells during which infective virus is not produced. The essential difference in cell deaths produced by viral toxicity and that from a full cycle of normal replication is that the toxic effect cannot be transmitted from cell to cell (Westwood, 1966). Antibody to be effective then, must prevent adsorption of the virion to the cellular surface since entry is paramount to cellular death. Prevention of release by antibody of new infectious particles from infected cells is of little or no value under these circumstances.

Other data suggesting a toxic rather than infectious response are those regarding virus isolation attempts. Despite large amounts of virus being inoculated, recovery of virus from the tissues of dead birds was both quantitatively and qualitatively low. Of the 212 birds of all age classes that died during the LD_{50} trials, only 67 virus isolations were made from 61 of the 183 birds tested (33 percent). In some instances only a single HA positive egg death, at a ten-fold dilution of the 10 percent tissue suspension, was recorded and seldom were all five eggs positive. Only on rare occasions were one or more eggs at a 10^{-3} dilution killed. Retesting of several tissue samples producing single egg deaths gave identical or nearly identical results.

The majority of virus isolations were made from brain suspensions and the fewest from liver-spleen suspensions. Also, the distribution of these isolations was related to the amount of virus in the inoculum

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and not to the age of the birds. These observations, plus the finding that the probability of making a virus isolation from the brain on a given day PI was related to the number of birds dying rather than time PI, are also interpreted as evidence against NDV causing an infectious disease response in the mallards. In typical NDV infection of fowl, younger individuals are more likely to yield virus (Brandly et al., 1946 b), and the tissues most likely to have a high viral content are the spleen and lungs, with wide distribution and concentration of virus occurring in other tissues following infection with the more pathogenic and virulent strains (Hofstad, 1951; Karzon and Bang, 1951). In addition, although NDV may be isolated from the brain when circulating HI antibodies are present (Walker and McKercher, 1954), NDV generally "disappears" from the tissues following or coinciding with the development of demonstrable circulating antibodies (VN and HI) and detectable immunity (Brandly et al., 1946 b); Hofstad, 1951). Virus isolations were made from brains of mallards throughout the 10-day PI period despite diagnostic level HI titers by days 3 to 4 PI, however, the degree of protection afforded by this antibody is unknown.

Pathogenesis

Pathogenesis studies in mallards, while not complete, suggest that ND is not an important disease of wild mallards in nature and that the mallard is not important in the epizootiology of the disease. It appears from the present studies that additional factors are necessary if mallards are to respond to the Texas GB strain of NDV in

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a manner "typical" of ND in fowl.

NDV was found to be of low pathogenicity in the mallard, there was no evidence that virus was shed by these birds, and little evidence that any significant amount of viral multiplication occurred. Virus was only isolated from organs of birds that died following inoculation, and no susceptible baby chicks or uninoculated mallards housed with treatment birds as contact controls died or reacted serologically. The amounts of virus isolated from tissues of birds that died and from blood samples during viremia studies were small.

The presence of a 24 hour, low grade viremia was detected in two adult mallards following IV inoculation with NDV. Virus detected in the circulatory blood prior to 4 hours PI was interpreted as residual virus from the inoculum. The length of time necessary to obtain total clearance of NDV from the blood following IV inoculation is not known but probably does not exceed 4 to 6 hours. In fowl, the replication time for NDV is 4 hours after invasion of a suitable host cell (Rott, 1964). Primary multiplication of the virus is at the site of introduction and is followed by a liberation of virus into the bloodstream, followed by a second cycle of multiplication in visceral organs, and a second release into the blood stream, followed in some instances by viral passage into the CNS (Asdell and Hanson, 1960). The conclusion reached was that while some viral replication took place in two of the adult mallards inoculated with NDV, the total response was atypical of infection with a virulent strain of NDV in domestic fowl.

NDV was highly antigenic for mallards even when administered by

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the oral route. Studies in Canada geese (Branta canadensis) have disclosed similar antibody response as well as the lack of clinical reponse (Bradshaw, 1965; Page, 1958; Palmer, 1969). It appears that wild waterfowl react antigenically to NDV, but there is no evidence in the present studies to indicate that the mallard is involved in its epizootiology.

Statistical Comments

The geometric rather than the arithmetic means of serum titers were used for analyses since the principal use of the former is in averaging a sequence of ratios. The value obtained is the nth root of the product of all values in the series (Wallis and Roberts, 1965). It was desirable to place some estimate of variation around the mean for these data but a search through a number of standard statistical textbooks did not disclose any method for calculating a variance term associated with the geometric mean. The followed method was devised to calculate an estimation of variance and its use is proposed for other data of this type.

The value of one was added to all reciprocal of serum titers, since the geometric mean is only meaningful for sets of observations which are all positive, and these adjusted values transformed to logarithms ($base_{10}$). Since the log of one is zero no real change in values had been made and these transformed values were then used as real numbers to calculate variance associated with normal statistics. The standard error was then calculated and used as an estimate of

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variance around the mean. This value was added and subtracted from the calculated geometric mean to obtain the upper and lower limits of one standard error and then all three values (the mean and upper and lower limits) were retransformed from logarithms to real numbers by looking up their anitlogarithms in tables.

No alteration of the procedure for calculating the geometric mean was actually done since this value is obtained by taking the nth root of the sum of the product of all members in the series multiplied together, which is the same as the $\log_{10}$ for each observation summated and divided by the total number of observations. The adjustment of 1 to give all members of the series positive values can be removed if desired but is of insignificant consequence in the total result.

A second point of statistical results worthy of mention is the nearly identical results obtained from LD_{50} calculations with both the techniques of Reed and Muench (1938) and probit analysis (Finney, 1952). The technique of Reed and Muench while giving a reliable estimate of the LD_{50} for data of this type does not give any estimate of variance about that LD_{50}. Probit analysis provides the investigator with the full complement of statistical parameters associated with regression analysis, thereby allowing meaningful statistical comparisons to be made between different treatments in a quantal response assay.

Specificity

The HA properties of the virus were used as the basis of confirming specific egg deaths during virus isolation attempts and as an assay of antibody response. The pattern of egg deaths from 1 to 5 days PI,

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lack of specific gross lesions in dead embryos or mallard ducks, the clinical disease produced in the rooster control, and original source of the virus are all consistant with NDV being the agent involved. Constant sterility checks throughout these studies failed to disclose the presence of bacterial contaminants and sterile laboratory technique was utilized to minimize the possibility of contamination with other pathogens. Birds utilized in these studies had no known experience with other Myxoviruses, and all studies were carried out in isolation facilities.

In similar NDV studies, but in Canada geese, Palmer (1969) explored the question of HI antibody specificity and found no evidence of nonspecificity in goose sera and a high correlation between HI and VN titers of 90 serums tested. All of 35 serums with HI titers of 1:20 or 1:40 in his study also had VN titers considered diagnostic of ND in domestic fowl. In addition, nonspecific NDV HI has been reported in the serums of mammals but not birds, and, while mumps virus may induce antibody cross-reactions with NDV, no avian pathogens are known that will give similar cross-reactivity with NDV (Lancaster, 1966).

General Conclusions

Studies with NDV in the mallard were discontinued after concluding that this agent was not suitable for use in the experimental interaction model, since the type of infectious response desired could not be demonstrated in the mallard following exposure to the Texas GB strain of NDV. As a result, virus neutralization studies were not conducted to further confirm antibody or virus-isolation findings. The limitations of this study

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are appreciated but the results are considered to represent exposure with the Texas GB strain of NDV in the mallard.

SUMMARY

Different aged mallards were exposed by different routes to the Texas GB strain of NDV to determine clinical, virologic, and serologic responses. Only large intravascular doses of NDV produced clinical signs of ND or mortality indicating that the mallard is highly resistant to clinical disease from this strain of NDV. Mortality in mallards resulting from intravascular inoculation with large doses of NDV appeared to be induced by "viral toxicity" rather than an infectious process and mortality within age classes appeared to be altered by the combined effects of maternal antibody and age-resistance.

Clinical signs of disease, when present, were suggestive of CNS involvement. The greatest percentage of virus isolations were made from the brain and only a small number of isolates were made from lung or liver-spleen pools. NDV isolations were made from brain tissue of mallards that died throughout the 10 day PI period. A low grade 24 hour viremia was detected in 2 of 4 adult mallards inoculated intravascularly with NDV. There was no evidence that infective virus was shed by birds inoculated with NDV during these experiments.

NDV was found to be highly antigenic in the mallard when administered intravascularly or orally in large doses. The HA antibody response in adults appeared within 3 to 4 days PI, peaked between days 7 and 8 PI, and persisted at diagnostic levels during the 40 day experimental period.

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The Texas GB strain of NDV was considered unsuitable for further studies in the experimental model since the type of infectious response desired could not be demonstrated in the mallard following inoculation with this agent. Information gained relative to LD_{50} values and antibody response provided useful knowledge of NDV exposure in the mallard.

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DUCK HEPATITIS VIRUS

INTRODUCTION

Duck virus hepatitis is an important disease of the domestic duck industry in many areas of the world but its occurrence in wild waterfowl has not yet been reported. The causative agent of this malady, duck hepatitis virus (DHV), was selected for study primarily because of the severe liver damage that often results from infection with this agent. Since many chemicals, including a number of pesticides, are metabolized in the liver, biological agents which cause liver damage were considered desirable for interaction studies. Availability of DHV isolates, distinctive pathology, stability of the agent, and host specificity, providing a laboratory safety factor, were other reasons resulting in the selection of DHV. The objectives of this study were to determine the suitability of DHV for use in the interaction model and to obtain base line data regarding the response of different age mallards to this agent.

MATERIALS AND METHODS

Two different isolates of DHV were used as inoculum in these studies. The first (R85952), obtained from Dr. Lyle Hanson, University of Illinois, was isolated from a Pekin duckling in 1955 and has undergone several egg passages. This stock, hereafter referred to as DHVI (duck hepatitis virus Illinois), titered $10^{4.5}$ $CELD_{50}$/ml. Pathogenesis and interaction studies utilized a DHV isolate obtained from the Cornell University Duck Research Laboratory, Eastport. This virus was received as a 10 percent liver suspension with a titer of $10^{6.8}$

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duck lethal dose$_{50}$ (DLD_{50}) in day-old Pekin ducklings and will hereafter be referred to as DHVLI (duck hepatitis virus Long Island). If neither virus is specified then use of DHVLI should be assumed.

Virus Assay

Embryonating hens eggs were used, in addition to susceptible ducklings, for the propagation of virus stock and assay of virus activity. Specificity of death was based on a combination of factors including clinical signs, pathological findings (pathognomonic liver lesions), and the capacity of liver suspensions from dead birds to produce characteristic mortality patterns in embryonating hens eggs.

Route of Exposure

Day-old mallard ducklings were assigned to 10 bird experimental groups and inoculated by either the intranasal (IN), intraperitoneal (IP), intramuscular (IM), or oral route with $10^{2.5}$ $CELD_{50}$ of DHVI stock. These inoculated birds as well as 45 uninoculated contact ducklings were held in an isolation unit where free intermixing was permitted, group identity being maintained by colored leg bands.

Ten percent liver suspensions from ducklings that died during the study were pooled and inoculated into eggs. Undiluted allantoic fluid ($10^{3.9}$ $CELD_{50}$/ml) harvested from dead eggs was used as inoculum for IP challenge of surviving ducklings and 15 previously unexposed ducklings of the same age (11-days-old).

Viremia Experiment

Two groups of 10-day-old ducklings were used to study the presence and duration of viremia following IP inoculation with DHVLI. Each

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of 151 ducklings in group A received $10^{0.5}$ DLD_{50} and the 158 ducklings in group B received $10^{3.5}$ DLD_{50}/bird. Each group was housed in a separate isolation unit and two birds from each group were killed every two hours (hr) for the first 72 hr; then at hr 76, 84, 92, 110, and 135 PI. Clinically sick birds were not selected for viremia sampling, but allowed to develop typical DHV infection. Deaths were recorded at each sample hr. Ducklings selected at a sample hr were anesthetized with 0.5 to 1 ml of sodium pentabarbital (1 grain/cc) given IP. An incision was then made which exposed the liver for gross examination of lesions, following which an aseptic blood sample was taken from the heart, and the bird killed by an intracardiac injection of sodium pentabarbital. All blood samples within a given group and time interval were pooled by mixing with an equal volume of Alsever's solution and stored at $4^{o}C$ in sterile labeled screw cap vials. In addition, two pools of livers from four birds that died in each group on day 3 PI were also refrigerated until virus assay was conducted.

Within 20 hr of completion of the above phase of the experiment mallard ducklings were used to screen for the presence of virus. Only 14-day-old susceptible ducklings were available and groups of four birds each were inoculated IP with 0.5 ml of undiluted aliquots of the different PI blood samples. Group A blood samples were tested at 6 hr intervals, with the exception of 36 hr PI, and group B samples at 2 hr intervals. The last blood sample tested for both groups was 92 hr PI. Concurrent titrations were done with the 10, 20, 30, and 44 hr blood samples from group B, using four ducklings per dilution; with

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10 percent liver suspensions from both groups using 3 ducklings per dilution; and with the stock virus using 6 ducklings per dilution. Each titration was done in a separate room, birds representing individual dilutions being caged separately. Susceptible ducklings were used in preference to hens eggs because of their reported sensitivity and the irregular and low mortality of chick embryos inoculated with field strains of DHV (Hwang, 1965).

As a precaution against possible cage to cage transmission of DHV during these trials, approximately one-half (every other sample) of the birds inoculated with group B blood samples were housed in individual Horsfall isolation units and the remaining birds held in standard laboratory cages. Parallel results were obtained from both segments regardless of the housing. Ducklings inoculated with the viremia blood samples were observed twice daily at 20, 28; 44, 52; 68, 76; 92, 100; and 116 hr PI. Dead birds were removed and the time of death recorded as the time the bird was removed. All birds that died were examined for gross lesions. A similar procedure was carried out for ducklings in which titrations were being made, except that these birds were checked less frequently, observations being made at 21, 41, 48, 66, 94, 117, and 141 hr PI.

Pathology

No attempt was made to undertake detailed studies of the pathology of DHV in the mallard since this would be a full scale thesis problem in itself. Studies of this type have been done in domestic ducks (Hanson, 1958; Kapp and Toth, 1968) and in the developing chicken embryo (Fitzgerald et al., 1969) and were used as guides to the type

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of changes which might be expected in the mallard. Clinical response of mallards to DHV is essentially the same as that in domestic ducks (Hanson, 1968) suggesting that pathological changes might also be similar. The liver, spleen, and brain were selected for study on the basis of changes reported in the literature for domestic ducks. Nineteen-day-old mallard ducklings inoculated with DHV were killed by decapitation every 6 hr during the first 36 hr PI, at 40 hr PI, and every 8 hr thereafter through 76 hr PI, and then at 88, 100, 120, 144, and 168 hr PI. The closer time intervals early in the study were selected to establish the onset of various lesions, while the greater time intervals after 76 hr PI were selected to establish the relative persistance of these lesions. Although liver, spleen, and brain tissues were collected, processed, and stained with hematoxylin-eosin, only liver and brain sections were examined from birds that died and from those killed at daily intervals in other studies. Approximately 200 tissue sections of liver and 20 tissue sections of brain were examined microscopically for lesions.

Indirect Hemagglutination Test

Consultation with immunologists at the Medical Microbiology Department of the University of Wisconsin Medical School resulted in the substitution of bis-diazotized benzidene (BDB) for tannic acid in the indirect hemagglutination (IHA) test protocol described by Taylor and Hanson (1967). The antigen was prepared utilizing methodology of Taylor and Hanson (1967) except that DHV was propagated in hens eggs rather than tissue-culture. Partial purification and

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concentration of the antigen was accomplished by ammonium sulfate $(NH_4)_2SO_4$ precipitation (Hanson et al., 1964). BDB was prepared as recommended by Sehon (1958) and modified for classroom use by the Medical Microbiology Department, University of Wisconsin Medical School.

RESULTS

Route of Exposure

Three age classes of mallards were used to determine the best route of inoculation in future studies. Among day-old ducklings inoculated with $10^{2.5}$ $CELD_{50}$ of DHVI, mortality of 60 percent occurred by either the IN, IM, or IP route while approximately half as many ducklings died from oral inoculation or from contact exposure (Table 1). Mortality was generally greatest on day 2 PI and declined steadily thereafter, however, this varied with the route of exposure. Deaths began on day 2 PI among ducklings inoculated IM, IN, or IP, on day 3 PI among contact controls, and on day 4 PI among those inoculated orally. The appearance of prominent liver lesions (Figure 1) coincided with the onset of mortality in each of these groups.

Four of 15 previously unexposed 11-day-old ducklings died from DHV following IP inoculation with $10^{3.2}$ $CELD_{50}$ of virus. No deaths occurred among ducklings (11-day-old) that had previously survived exposure to DHV at one day of age. Virus used as inoculum in this trial was harvested from the first egg passage of liver suspensions from ducklings that died during the day-old duckling trial.

Adult mallards were used in the final trial in this experiment,

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Table 1. Mortality among day-old mallard ducklings experimentally exposed to $10^{2.5}$ $CELD_{50}$ of DHV by different routes.

Route of exposure	No. exposed	No. dead	Mortality (%)
Intransal	10	6	60
Intraperitoneal	10	6	60
Intramuscular	10	6	60
Oral	10	3	30
Contact	45	15	33

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Figure 1. Typical gross liver lesions associated with DHV infection in mallard ducklings (A). Note the change in coloration and enlargement of infected livers as compared with the liver from an uninfected duckling of the same age (B).

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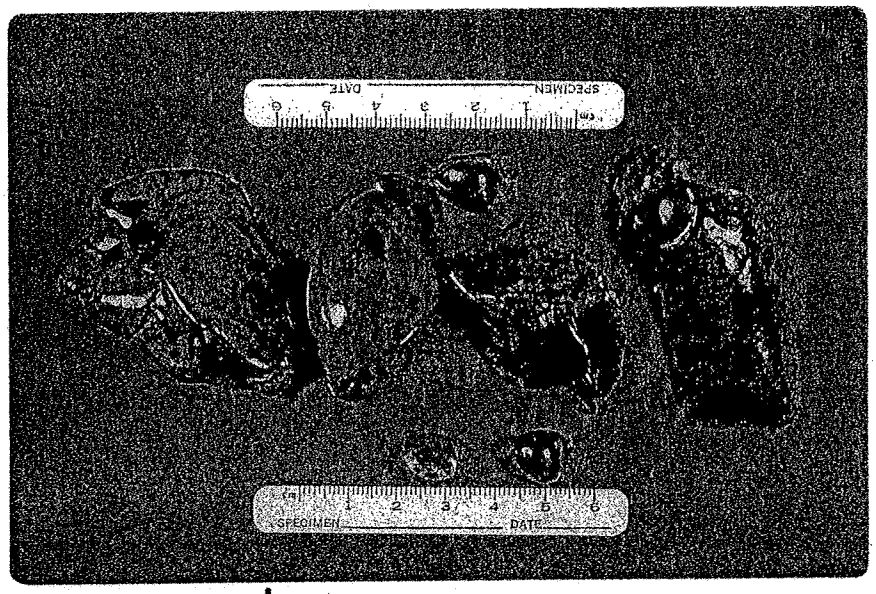



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None of 20 mallards inoculated IV with $10^{2.5}$ $CELD_{50}$ of DHVI stock died or became clinically ill with DVH.

Viremia Experiment

Ducklings receiving $10^{3.5}$ DLD_{50} of DHVLI (group B) suffered greater mortality and died earlier than ducklings inoculated with $10^{0.5}$ DLD_{50} (group A) of the same virus stock (Table 2). More than 90 percent of the deaths in group B occurred on day 2 PI while the combined totals for days 2, 3, and 4 PI for group A barely equaled this amount. Cumulative mortality curves for the two groups were nearly parallel (Figure 2).

During the experimental period a viremia was detected in both experimental groups. DHV was detected in all blood samples collected between 18 to 92 hr PI from group B. The duration of viremia was not established since virus was still detected at 92 hr PI, the last sample hr tested for both groups.

An attempt was made to quantitate the amount of virus present in each blood sample tested by utilizing a combination of percent mortality and mean death time resulting from each of the four bird test groups inoculated with a single blood sample. This assay system was identified as the relative index (RI) method. The RI value was obtained by dividing the number of deaths among each four bird test group by four to obtain the percent mortality; this value was then divided by the mean death time for those birds that died within that test group. The greatest RI value would be obtained if all four ducklings in a test group inoculated with a single blood sample died with the lowest

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Table 2. Mortality among 10-day-old mallard ducklings experimentally exposed to DHV via the intraperitoneal route.

Day (PI)	Mortality*			
	Inoculum			
	Group A (151 birds) 3 DLD_{50}		Group B (158 birds) 3140 DLD_{50}	
	No.	%	No.	%
1	0/67	0	1/81	1.2
2	17/67	25.4	74/81	91.4
3	36/67	53.7	6/81	7.4
4	10/67	14.9	...	
5	3/67	4.5	...	
6	1/67	1.5	...	
Total	67/67	100	81/81	100

*Number of deaths on designated day PI/total number of deaths during experimental period.

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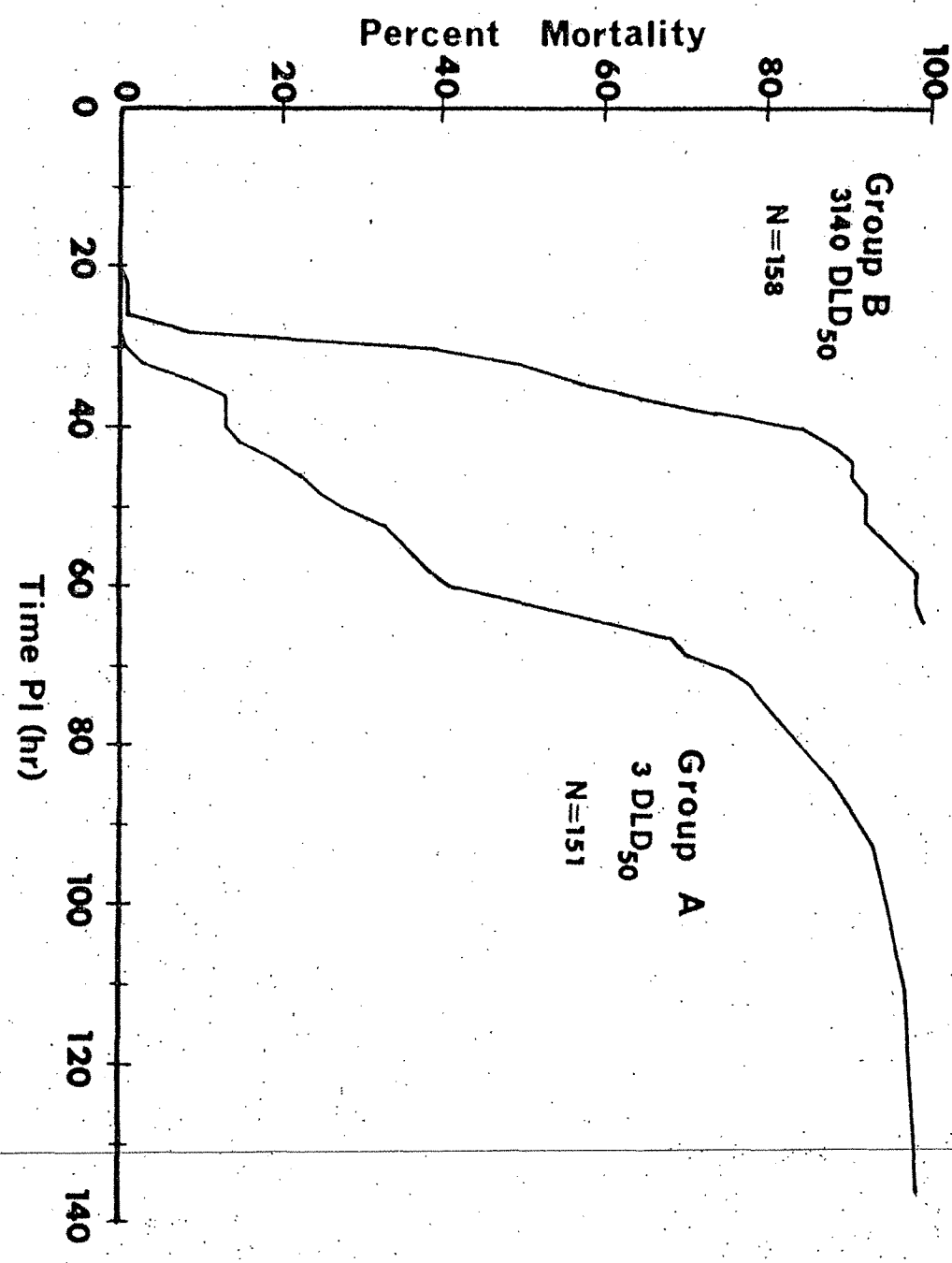

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mean death time, while the smallest RI values would be obtained from combinations of less than 100 percent mortality and greater mean death times. Within this system, greater RI values indicate greater amounts of virus present in the sample.

For, the group B samples, a plot of RI values against time PI suggested three peaks of virus activity (Figure 3). A peak of virus activity was defined by having lows on either side; a low by definition was identified by having at least three consecutive samples with RI values at least 1.0 less than the furthest right hand point of the adjacent peak. The group B peaks occurred between 10 and 14 hr, 26 and 40 hr, and from 54 to 85 hr PI. The greatest RI values occurred during the 2nd peak and the smallest during the first peak. Virus titrations of the 10, 20, 30, and 44 hr PI blood samples lend support to this peak theory. The DLD_{50} value for the 20 hr sample was the lowest obtained ($10^{2.6}$/ml) and was within a low (Figure 3). The RI value for this sample was 0.6. The 30 hr sample had the greatest DLD_{50} ($10^{6.6}$/ml) and also the greatest RI value (3.8). Both the 10 and 44 hr samples and similar DLD_{50} ($10^{4.1}$ and $10^{4.6}$/ml) and RI values (1.5 and 1.4). In addition, within test groups mean death time of ducklings used to assay blood samples generally decreased as mortality rates increased, suggesting greater amounts of virus with decreasing time to death (Table 3).

The virus titer of pooled liver samples (3 livers each) for ducklings from both groups A and B at day 3 PI was also determined and compared with the titer of the stock virus. Virus titrations, in

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Figure 3. Relative virus levels in the blood of 10-day-old mallard ducklings following intraperitoneal inoculation with DHV. Group A ducklings received 3 DLD_{50} of DHV each. Blood samples from these birds were assayed for virus at 6 hour intervals. Group B ducklings received 3140 DLD_{50} of DHV each and blood samples from these birds were assayed for virus at 2 hour intervals.

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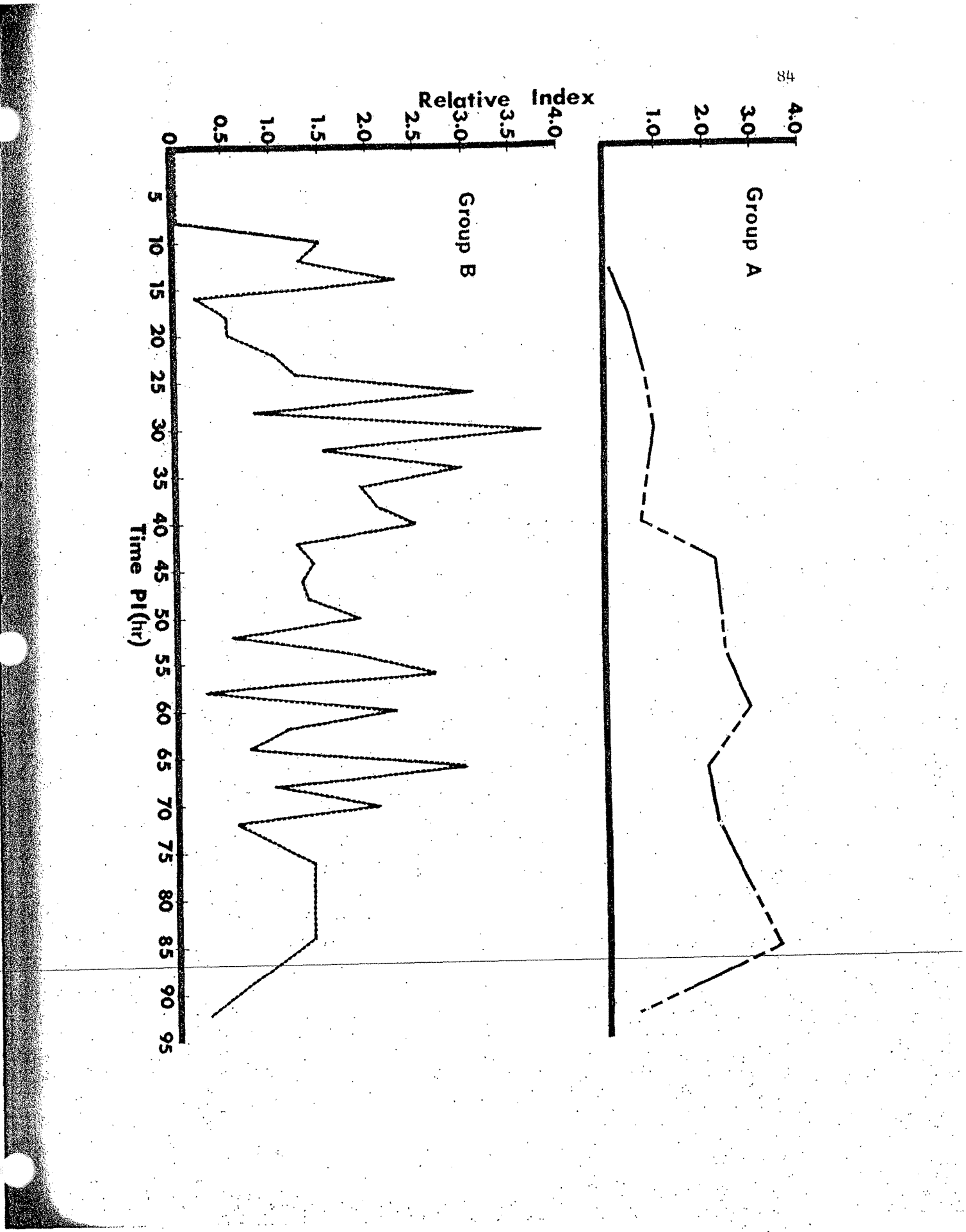

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Table 3. Mean relative index values from blood of ducklings inoculated with DHV.

Mortality in test ducklings*			Relative** index
No. samples	Mortality (%)	Mean death time (hr)	
5	25	63.2 ± 17.64	0.4
11	50	56.0 ± 7.55	0.9
14	75	43.2 ± 3.73	1.7
5	100	47.0 ± 10.45	2.1

*Each sample inoculated into four ducklings; only birds that died used to calculate mean death time.

**Percent mortality/mean death time.

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a duckling assay system, resulted in titers of $10^{6.1}$ DLD_{50}/ml for the group A sample, $10^{6.5}$ DLD_{50}/ml for the group B sample, and $10^{6.7}$ DLD_{50}/ml for the stock virus (in the form of a ten percent liver suspension).

An arbitrary classification system, grades I, II, and III, was established based on the severity of gross liver lesions. Grade I livers had less than 25 percent of their surface covered with discrete petechial or larger hemorrhages (Figure 4); grade II livers had generalized hemorrhages involving most of the surface area but with little or no coalescence (Figure 5); and grade III livers had generalized hemorrhages with considerable coalescence between hemorrhages and often were very friable (Figure 6).

In group A birds (low dose of virus) which died liver lesions were first detected at 30 hr PI. Among birds in this same group killed for blood samples, liver lesions were not detected until 46 hr PI; thereafter, approximately half of the birds killed through 84 hr PI had gross liver lesions. Among birds that died of DVH, the number of liver lesions increased with time PI. Liver lesions were detected in 56 percent of the birds that died between 30 and 36 hr PI, in 83 percent of those which died between 42 and 46 hr PI, and in 88 percent of those dying between 62 and 110 hr PI.

Gross liver lesions were detected earlier in group B birds (high dose of virus); 22 hr PI in the first bird that died of DVH and at 24 hr PI in birds killed for blood samples. All birds which died and were necropsied from group B had liver lesions, as did approximately half of the birds killed for blood samples between 24 and 70 hr PI.

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Figure 4. Gross DHV liver lesions (Grade I) in a 15-day-old mallard duckling.

Figure 5. Gross DHV liver lesions (Grade II) in a 15-day-old mallard duckling.

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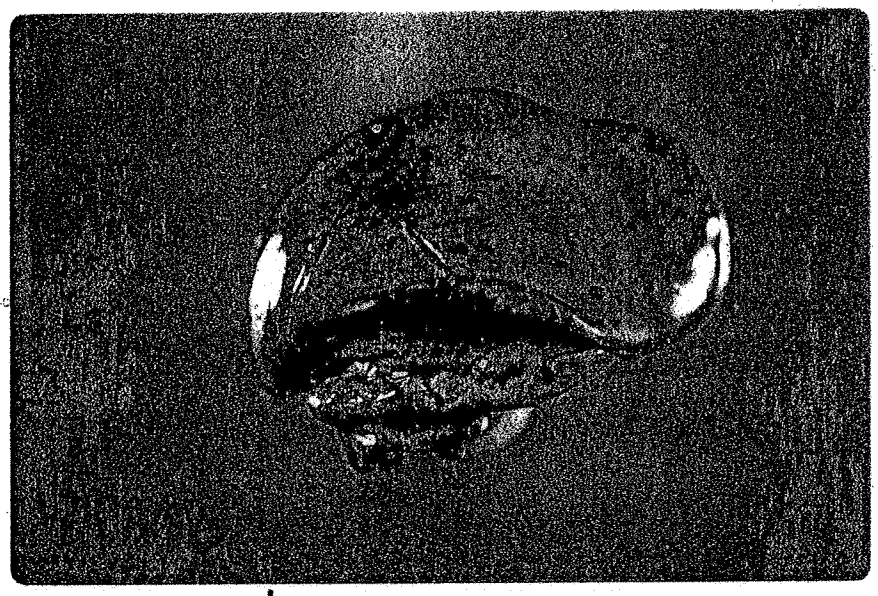

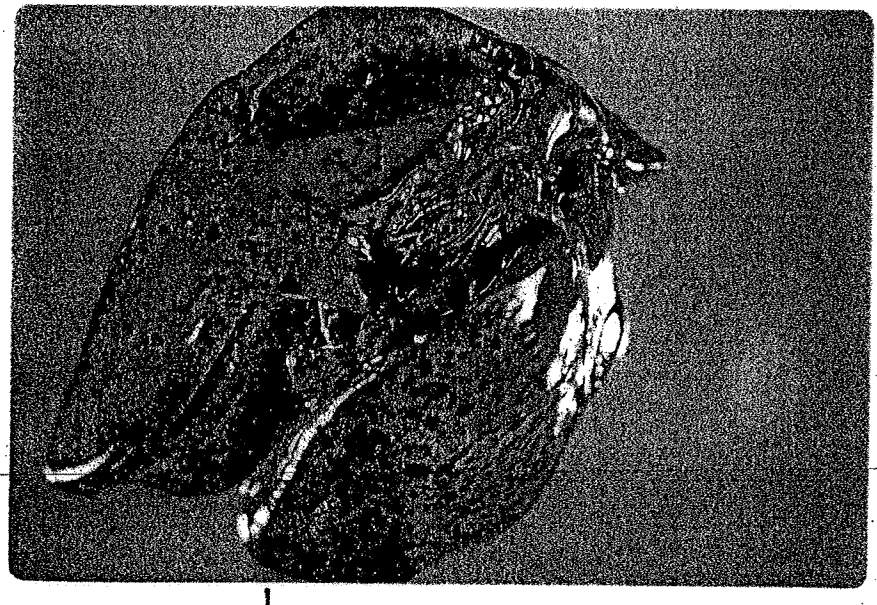

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Figure 6. Gross DHV liver lesions (Grade III) in a 15-day-old mallard duckling. Splits in the liver surface are a result of extreme friability.

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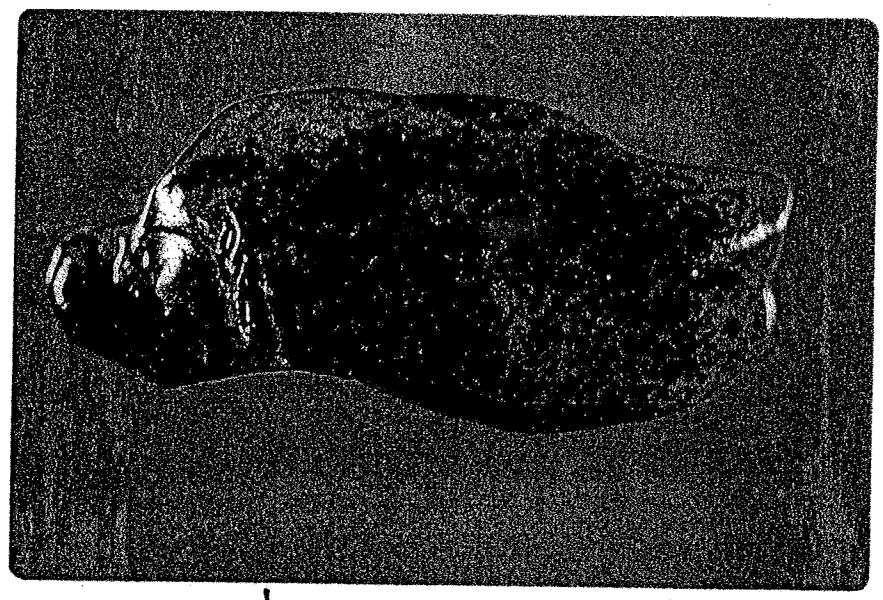

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No lesions were observed prior to or after this period.

Signs and Pathology

The first signs of DVH in mallards occurred 2 to 4 hr prior to the onset of mortality and consisted of depression and listlessness followed by incoordination which often resulted in the birds falling on their sides. Once in this position spasmodic kicking with both legs occurred and the birds died shortly thereafter with heads drawn back. Most dead birds were found in this typical opisthotonic posture (Figure 7).

The most characteristic lesion observed on necropsy was a greatly enlarged and swollen liver that contained numerous petechial and/or ecchymotic hemorrhages over varying amounts of the surface (Figures 1, 4, 5, and 6). In severe cases the liver was extremely friable and ruptured at the slightest pressure (Figure 6). Hemorrhages in the liver were most pronounced from day 2 through day 4 PI. Livers of infected birds were swollen to such an extent that they often filled much of the abdominal cavity and pressure on this organ from ribs and musculature resulted in hemorrhagic bands being formed on the liver surface (Figure 8). This type of lesion was most frequently observed in ducklings that had died of DVH but was also seen in a small number of birds killed for blood samples. The spleen of most infected birds was enlarged 2 to 4 times normal size (Figure 9) and its surface was often mottled or hemorrhagic.

The principal histological lesions in livers of mallards were infiltration of chronic inflammatory cells (Figure 10), bile duct proliferation (Figure 11), hepatic cell necrosis (Figure 12), and

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Figure 7. Typical opisthonic posture of a mallard duckling that died from DVH.

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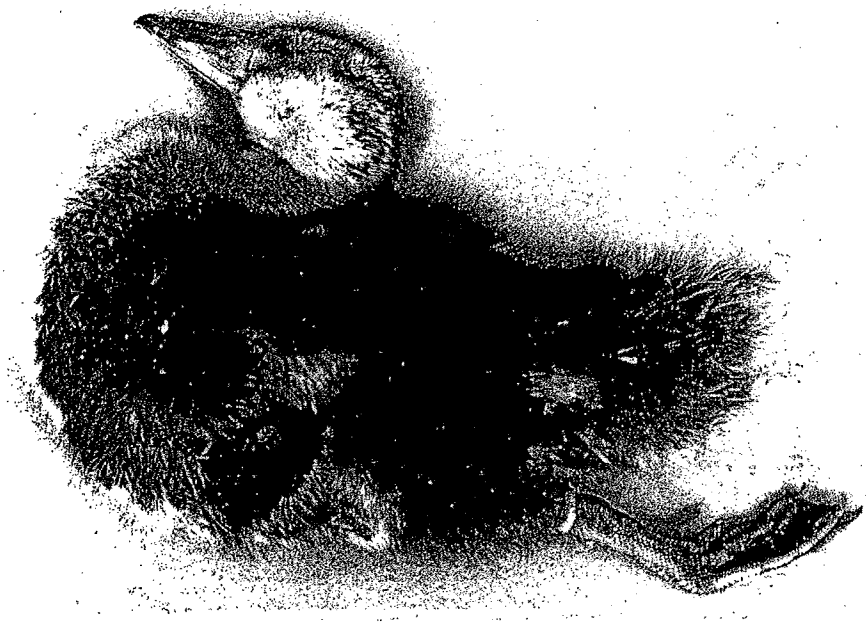

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Figure 8. Hemorrhagic bands on the liver of a mallard duckling killed approximately 48 hours after inoculation with DHV. These bands result from pressure on the liver from ribs and musculature due to the swollen condition of the liver.

Figure 9. Comparative sizes of spleens from 15-day-old mallard ducklings dying of DVH (A) and the spleen from a non-DHV exposed control duckling of the same age.

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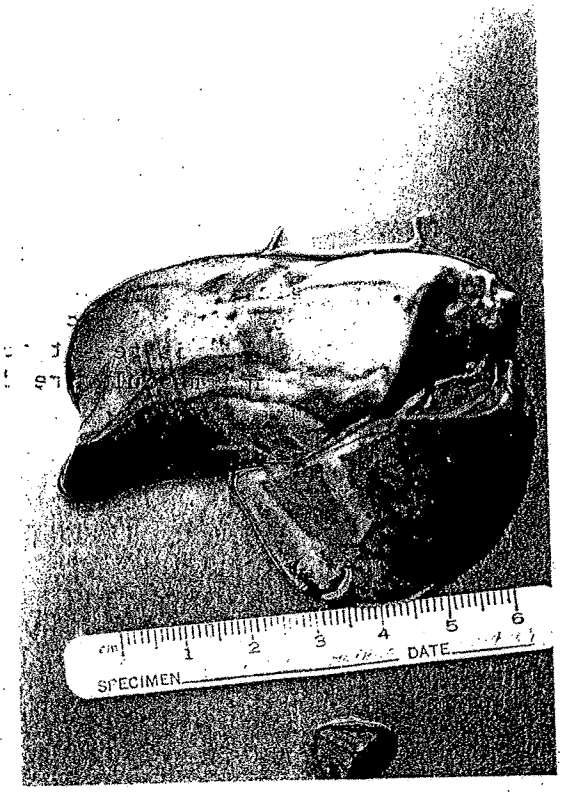

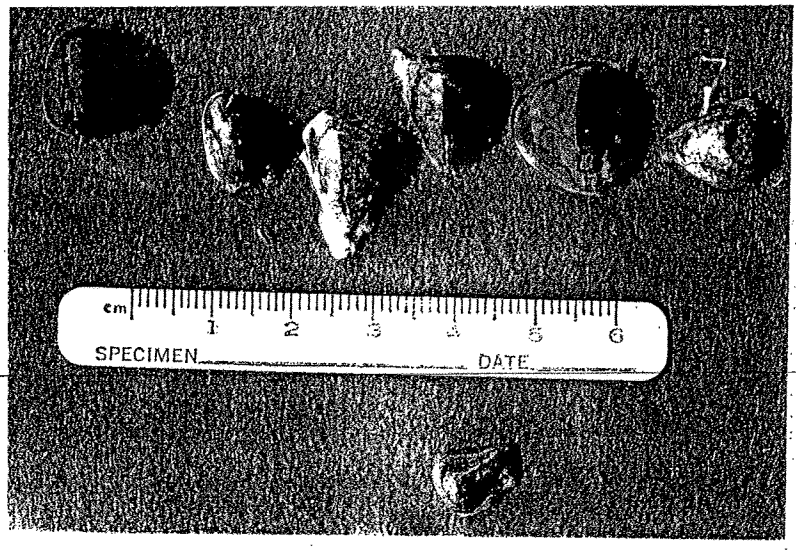

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Figure 10. Chronic inflammatory response in the liver of a 15-day-old mallard duckling approximately 24 hours after intraperitoneal inoculation with DHV. H & E stain; x 400.

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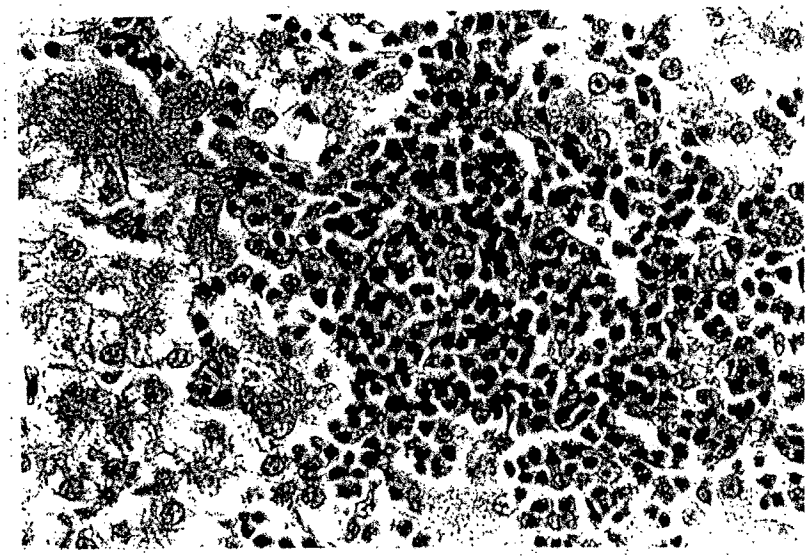

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Figure 11. Liver of a mallard duckling approximately 48 hours after intraperitoneal inoculation with DHV, showing (A) bile duct proliferation and (B) infiltration of chronic inflammatory cells. H & E stain; x 400.

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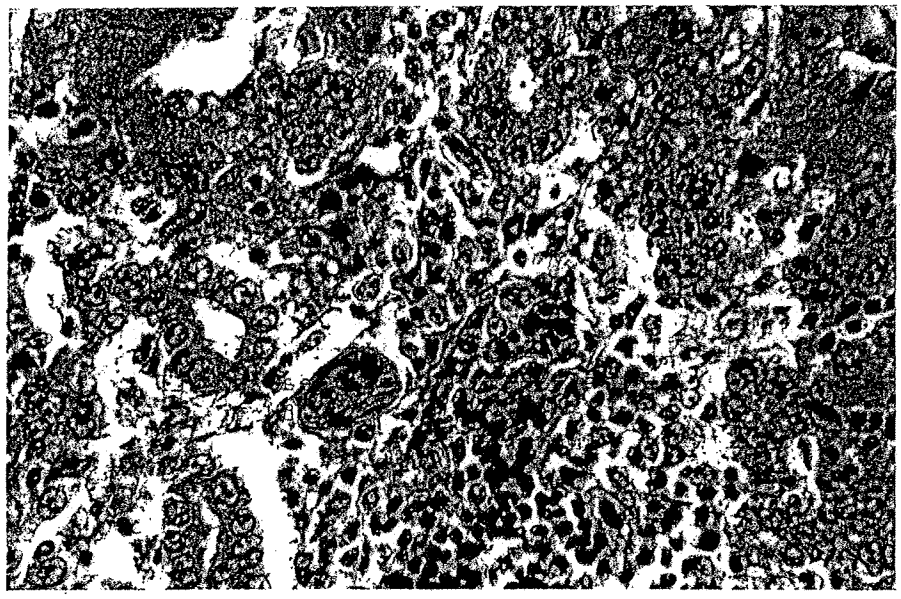

Figure 12. Livers of mallard ducklings 36 to 50 hours after intraperitoneal inoculation with DHV. (A) early severe degenerative changes. H & E stain; x 630. (B) Necrosis; note kayorrhexis and pyknosis of hepatic cell nucleoli (indicated by arrows). H & E stain; x 400.

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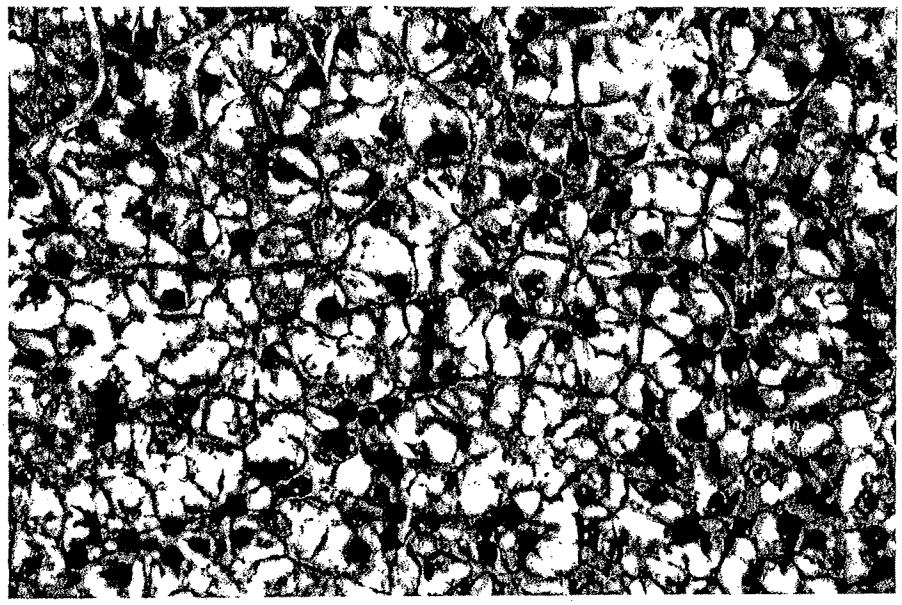

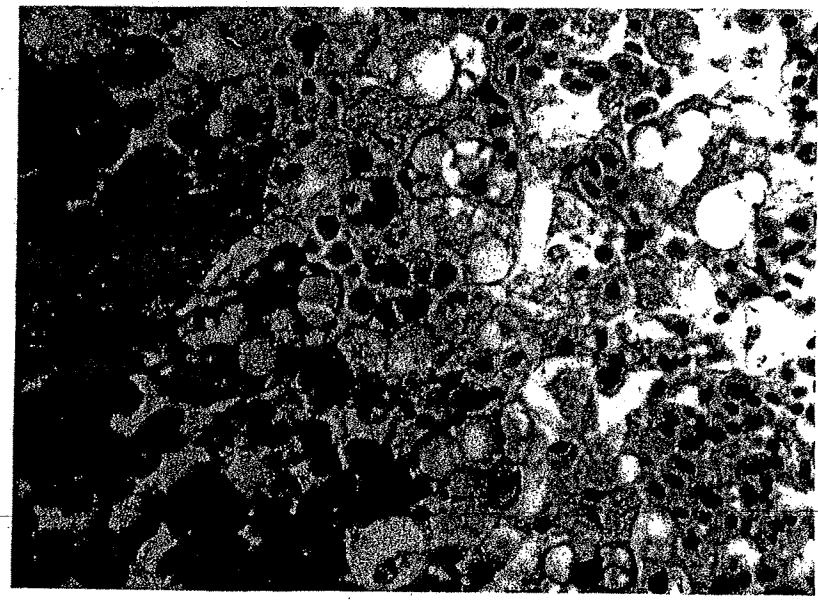

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hemorrhage. No diagnostic lesions were detected in brain sections examined. Some evidence of degeneration of purkinjie cells and necrosis of neurons in the cerebrum were noted, but these changes were not impressive or considered to be important.

Indirect Hemagglutination Test

Specific hemagglutination of DHV could not be achieved with the indirect hemagglutination system as described in these studies. The use of this test and pitfalls encountered are elaborated upon in the discussion.

DISCUSSION

Route of Exposure

It was apparent from the limited trials within this experiment that exposure to DHV by all routes tried resulted in clinical DVH in mallard ducklings. Direct inoculation rather than oral or contact exposure appeared more suitable for use in the model because of the shorter incubation period produced, greater ability for uniform exposure of quantitated doses, and the ease of inoculation. The failure of adult mallards to respond clinically to DHV was not unexpected since a similar clinical response in domestic ducks has been reported after the birds reach six weeks of age (Rispens, 1969).

Viremia Experiment

A viremia occurred in mallard ducklings inoculated IP with DHV. The onset of this viremia was apparently related to dose. Virus was first detected at 10 hr PI in group B ducklings receiving the high dose of DHV ($10^{3.5}$ DLD_{50}) and at 18 hr PI in group A ducklings

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receiving the low dose of DHV ($10^{0.5}$ DLD_{50}). Relative virus titers during the viremia were determined by use of a RI method which allowed a quantitative comparison to be made between blood samples tested at various time intervals. From the RI values obtained, the viremia in both groups of ducklings appeared to be diminishing by 92 hr PI, the last sample tested in each group, and by 100 to 112 hr PI the viremia had probably ceased.

The RI method provided information (quantitative titers) that could not be easily obtained by other methods, and the validity of this method was supported by titrations in a duckling assay system, of blood samples collected during the viremia. The blood sample tested with the highest RI value also had the highest virus titer; mid range RI values had lower virus titers; and the lowest RI value tested had the lowest virus titer. These observations indicate that the RI method is suitable for determining relative amounts of DHV activity, and in addition to blood would probably be useful for assaying DHV activity in other tissues.

Whether the peaks that appear from a plot of RI vs. hr PI are real or an artifact of the RI system is unknown. As previously stated, lows were delineated by having a minimum of three consecutive samples with an index value at least 1.0 less than the value of the furthest right hand point of the peak. This arbitrary establishment of peaks can result in similar index values being in both a low and a high peak, and this happened with the 10 and 44 hr samples of group B. The 10 hr sample occurred in the lowest peak while the 44 hr sample

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