THE GROWTH OF DUCK HEPATITIS VIRUS IN DUCKLINGS

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SUMMARY

The growth patterns of the Houchin strain of duck hepatitis virus in different tissues of ducklings infected when 11 days old were similar but their magnitudes and the times at which virus growth occurred differed significantly. The virus multiplied best in the liver and less well in kidneys, spleen, small and large intestines, and pancreas. Virus present in bile was attributable to virus excreted from the liver. Virus in other tissues was attributable to the virus content of blood. Virus was recovered from cloacal swabs taken from infected ducklings from 24 hours after exposure and excretion continued for a further 11 days.

Age-related resistance to duck virus hepatitis was manifested in ducklings by a decline in the mortality rate as the birds grew older and was linked to a lengthening of the lag phase of virus growth, to a steady depression of virus growth, and to a shortening in the period of virus excretion. Multiplication rates of the virus, however, were not affected.

An age-related resistance was found also in chicken embryos and duck embryos infected with the Houchin strain of duck hepatitis virus. A strain, H_{56}, adapted to chicken embryos by serial passages overcame this age-related resistance in chicken embryos.

This thesis has been composed by me,
and describes my own work.

CONTENTS

INTRODUCTION

Duck virus hepatitis emerged as a new disease in 1949 and it is still recognised as one of the major problems of the duck industry. The rapid development of age-related resistance to duck hepatitis virus is striking and well documented but, surprisingly, there have been no adequate studies of the behaviour of the causal virus in ducks. An understanding of the interaction between a pathogen and its host is an essential pre-requisite for formulating rational control measures. Our objective was to study this interaction, particularly the age effects.

REVIEW OF THE LITERATURE

Duck virus hepatitis (DVH) is a highly infectious disease of ducklings characterised by a short course and high mortality. The livers of affected birds are enlarged and mottled with haemorrhages.

The disease and its viral aetiology were first recognised in the U.S.A. in 1949 (Levine & Fabricant, 1950). Since then it has been reported from many countries in all continents except Australia (Königshöfer, 1972).

THE VIRUS

Vindel (1963) reported that duck hepatitis virus (DHV) was an RNA-containing virus. Later Tauraso and his colleagues (1969) studied the physico-chemical properties of the virus and proposed that DHV was most aptly classified as a picornavirus. Their opinion was accepted by Andrewes and Pereira (1972). Crighton (1973), on the other hand, conceded that the virus was a small RNA virus with icosahedral symmetry but he refrained from classifying the virus. The virus was relatively resistant to heat, and chemical and environmental conditions (Asplin, 1961). Although indirect haemagglutination of sheep erythrocytes has been reported (Taylor & Hanson, 1967), DHV was not a haemagglutinating virus (Levine & Fabricant, 1950) nor was it a haemadsorbing virus (Tauraso et al., 1969).

Asplin (1965a,b) found that there were two distinct serotypes one of which, he suggested, was a variant of the

conventional type. Toth (1969b, 1972a) also reported the presence of a variant of DHV. DHV had an antigenic relationship with turkey hepatitis virus (Tzianabos & Snoeyenbos, 1965) but it was unrelated to other viruses known to cause hepatitis in birds or animals. Despite the antigenic link the viruses of duck and turkey hepatitis were considered to be distinct entities (Tzianabos & Snoeyenbos, 1965).

HOSTS

Natural Hosts

Rapid development of age-related resistance is the striking feature of infection with DHV; ducklings of less than 3 weeks of age were very susceptible and usually developed fatal disease after infection (Levine & Fabricant, 1950). Death was uncommon in 4 to 5 week-old ducklings and birds older than 6 weeks were usually refractory (Asplin, 1956).

Susceptibilities of ducklings of different breeds such as White Pekin, Penine, Aylesbury, Khaki Campbell, Rouen and domesticated mallard were similar (Hwang, 1974).

Using death as the criterion, Hwang (1974) concluded that guinea chicks, pheasant chicks and goslings were highly susceptible and quail chicks and poults were susceptible. No clinical signs or mortality were observed in Muscovy ducklings or chickens infected with DHV. The virus was, however, recovered from some of the infected birds 7 days after inoculation. These findings confirmed those of Asplin

and McLauchlan (1954) who reported earlier that chickens could contract inapparent infections. Infected chickens passed the virus through their faeces up to 3 days, and DHV was recovered from their livers up to 9 days after inoculation. Rhan cited by Levine (1972) found that day-old and week-old turkey poults infected with DHV developed clinical signs, lesions and neutralising antibodies. Lesions included mottled livers, enlarged gall bladders and spleens. DHV was isolated from livers of infected turkey poults up to 17 days.

Rabbits, guinea-pigs, and mice (Reuss, 1959; Sazawa, et al., 1963), dogs (Reuss, 1959) and pigeons (Sazawa et al., 1963) were unaffected after experimental exposure to DHV.

Experimental Hosts

Embryonated eggs: Duck and chicken embryos are very susceptible and support good growth of DHV. Infected chicken embryos developed characteristic lesions including stunting, generalised oedema, liver necrosis and greenish discolouration of the embryonic tissues and fluids (Levine & Fabricant, 1950). The lesions were found in infected chicken embryos either at death after 3 days (Asplin & McLauchlan, 1954) or when killed on the sixth day (Levine & Fabricant, 1950).

When DHV was passaged serially in chicken embryos it rapidly lost its pathogenicity for ducklings (Asplin, 1958; Hwang & Dougherty, 1962; Hwang, 1965b). Virus adapted to chicken embryos quickly regained its virulence for ducklings when passaged in ducklings (Asplin, 1958; Anon., 1973a).

Embryos of wild ducks (Ulbrich, 1971), geese (Akulov et al., 1972) and turkeys (Doroshko et al., 1966) were also susceptible.

Growth curves of a chicken embryo-adapted strain of DHV in whole embryos, allantoic fluids and chorioallantoic membranes (CAMs) were determined by Toth (1969a). The concentration of virus was always low 5 to 93 hours after inoculation in allantoic fluids. In CAMs and whole embryos, DHV apparently reached the plateau phase about 45 hours after inoculation and the plateau persisted up to the 93rd hour. According to Mason and his colleagues (1972) the lag phase of a vaccine strain of chicken embryo-passaged DHV was not less than 6 hours; there was some indication of virus growth at 12 hours after inoculation. Definite virus growth was found at 24 hours and the peak virus titre was reached by 48 hours. Hwang and Dougherty (1964) found that the titres of two virulent strains of DHV in chicken embryos harvested at 48 and 144 hours after inoculation were similar.

Cell-culture systems: DHV has been propagated in several cell-culture systems including chicken embryo explants (Pollard & Starr, 1959), chicken embryo cell cultures prepared with collagenase or trypsin (Pollard & Starr, 1960), and chicken embryo liver cell cultures (Kaeberle et al., 1961). After several attempts, Hwang (1966) concluded that duck embryo liver cell cultures were not suitable for the propagation of DHV.

Fitzgerald and his colleagues (1963) described cytopathi

effects (CPE) in duck embryo kidney cell cultures (DEK) infected with DHV. The CPE consistently occurred from the 16th to 26th passage but chicken embryo kidney cell cultures infected with virus from the 25th DEK passage did not develop CPE. The CPE was manifested as degeneration and necrosis of the cells of the monolayers within 24 to 36 hours of infection and most cells were affected by 72 hours. Nevertheless, when Fitzgerald and Hanson (1966) measured the growth curve of the 27th DEK passaged virus in DEK they used chicken embryos to assess the virus titres instead of DEK. Maiboroda and Kontrimavichus (1968), and Maiboroda (1972) respectively reported CPE in DHV-infected goose embryo kidney and duck kidney cell cultures. In contrast, Sazawa and his colleagues (1963) failed to observe CPE in duck kidney cell cultures but they did report plaque formations in duck embryo cell cultures. Hwang (1965a), however, found neither conspicious CPE nor plaque formation in DHV-infected duck embryo cell cultures. Sazawa and his colleagues (1963), Tauraso and his colleagues (1969) and Anon. (1973a) also failed to find CPE in chicken embryo cell cultures. To date, there have been no reports of titrations of DHV in cell cultures.

A possible explanation of these discrepant reports is that different isolates of the virus were used; Fitzgerald and his colleagues (1963) used a strain of DHV which had undergone several passages in chicken embryos and in several cell-culture systems. Sazawa and his colleagues (1963) used the Japanese Ciba strain. Maiboroda and Kontrimavichus (1968), and Maiboroda (1972) used three Russian strains of

DHV. Hwang (1965a) used a field isolated DHV. Tauraso and his colleagues (1969) and Anon. (1973a) studied different strains of chicken embryo-passaged DHV.

Kaeberle and his colleagues (1961) found that DHV titres assessed in chicken embryos decreased from the first to 6th passage in chicken embryo liver cell cultures and slightly increased in the 7th passage.

Hwang (1966) found that the titre of DHV in the first passage in duck embryo cell cultures was much lower than the dose used to infect the cultures. The titres remained low until the 10th passage when a slight increase occurred which was maintained through to the 20th passage.

In the study of the growth curve of a DEK passaged virus in DEK, Fitzgerald and Hanson (1966) found that the lag phase of the virus was 2 to 4 hours and was followed by an exponential increase phase which reached the highest titre at 24 hours. The plateau phase persisted from the 24th hour up to at least the 96th hour. Similar growth was also reported by Tauraso and his colleagues (1969) when they studied the behaviour of a vaccine strain of chicken embryo-passaged DHV in chicken embryo cell cultures at 0, 24, 48 and 72 hours; they also found a reduction in DHV titres in the cell cultures.

PATHOGENESIS

Virus has been reported excreted in faeces of infected ducklings (Reuss, 1959; Asplin, 1961; Rispens, 1969; Anon., 1974) and infected chickens (Asplin &

McLauchlan, 1954) and, under natural conditions, ducklings are likely to be infected with DHV by ingestion of contaminated feed and water (Asplin, 1961).

After oral inoculation with a 13 chicken embryo-passaged DHV day-old ducklings harboured the virus in the liver, spleen, brain, and bone marrow. Peak virus titres occurred in these organs at 48 hours (Barinsky & Tsypkin, 1966). Hwang and Dougherty (1964) reported that when newly hatched ducklings were infected by the intramuscular route with virulent strains of DHV, they died about 48 hours later. Virus was then found in the liver, kidney, spleen, heart, lung, brain, and leg muscles. When ducklings were inoculated with chicken embryo-attenuated strains, the virus was not detected in the brain and virus titres in the other organs were lower than those of virulent strains.

Decrease in the pathogenicity of DHV for ducklings through chicken embryo passages is well documented. Levine and Fabricant (1950) reported that the allantoic fluid of the first passage of DHV in chicken embryos did not kill day-old ducklings. Asplin (1958) found that his TN_5 and TN_6 viruses, the 5th and 6th chicken embryo-passaged DHV, failed to produce disease in 3- and 10 day-old ducklings by intramuscular inoculation, and the infected ducklings were resistant to challenge with a virulent virus 7 days later. Reuss (1959) showed that the pathogenicity of DHV for ducklings declined markedly when the virus was passaged in chicken embryos; the virulent virus killed 100 per cent of ducklings, whereas the 10th chicken embryo-passaged virus

killed only 32 per cent of ducklings. Hwang and Dougherty (1962) also found that the pathogenicity of DHV for ducklings declined when the virus was passaged; by the 20th chicken embryo-passage, the virus was completely non-pathogenic.

Hwang (1965a) reported that the pathogenicity of DHV for ducklings also decreased when the virus was passaged through duck embryo cell cultures. Although the virus became nonpathogenic for ducklings from the 6th passage it remained pathogenic for chicken embryos.

Target Organs

Kapp and Balázs (1970) examined liver cells of normal ducklings by electron microscopy and found that they were less electron dense and had fewer cytoplasmic organelles than the cells of birds more than 20 days old. They suggested that these pale, less electron dense cells favoured the growth of DHV.

Sublethal liver damage may also encourage growth of DHV. Friend and Trainer (1970) showed that ducklings fed with sublethal doses of polychlorinated biphenyl (PCB) suffered significantly higher mortality than birds which were not exposed to PCB.

Severe hepatic damage was observed 25 days after day-old ducklings were thyroidectomised by treatment with I^{131} (Kapp & Pethes, 1971). The liver cells were less electron dense than the cells from normal birds because there were fewer cell organelles and there was disorganisation of ribosomes and endoplasmic reticulum. When the thyroidec-

tomised birds were infected with DHV the mortality rate was higher than in control ducklings.

TRANSMISSION

Susceptible ducklings were experimentally infected by many routes including the oral, intranasal, intramuscular, intraperitoneal (Reuss, 1959; Friend & Trainer, 1972), and intravenous (Sazawa et al., 1963). Airborne infection has occurred (Reuss, 1959). Oral infection was considered to be the most likely under natural conditions (Asplin, 1961).

DHV was excreted in the faeces within a few hours of infection (Asplin, 1961) and excretion continued for at least 10 days (Anon. 1974). Reuss (1959) claimed that the excretion still occurred 6 and 8 weeks after infection. Reuss' opinion is, however, based on findings from one duckling experimentally infected. Rispens (1969) reported the presence of latent infection especially under intensive duck rearing conditions but he cited no supportive data. Moreover, he confused latent infection with inapparent infection using the phrases synonymously in his thesis (Rispens, 1966). Asplin (1961), on the other hand, reported that the latent infection was uncommon and recovered ducklings did not appear to remain carriers (Asplin, 1958).

Since DHV was relatively resistant to environmental conditions and disinfectants (Asplin, 1961) and remained viable for a long time in infected premises (Asplin, 1958; Rispens, 1969), fomites were considered to be the most likely source of infections in the field (Asplin, 1961).

Asplin (1964) suspected that an unknown reservoir host such as a wild bird acted as a healthy carrier of DHV. However, no neutralising antibodies to DHV have been detected in wild birds (Asplin, 1970; Ulbrich, 1971). Crighton (1973) suspected that adult ducks were subclinical carriers; the hypothesis, however, has still to be tested.

Vindel (1962) reported natural egg-transmission of DHV in France but it has not been confirmed elsewhere. Other workers (Levine & Fabricant, 1950; Asplin, 1958; Levine, 1972) on the other hand, considered that DHV was not egg-borne.

PATHOGRAPHY

The incubation period was usually short, about 1 to 2 days (Levine & Fabricant, 1950; Asplin & McLauchlan, 1954; Fabricant et al., 1957). Asplin (1964), however, cited a range of 2 to 5 days. Morbidity approached 100 per cent in infected young ducklings but was less in older birds (Levine, 1972). Mortality might be up to 95 per cent in some broods (Levine, 1972). The death rate decreased rapidly when ducklings were 3 weeks old and death was uncommon when ducklings were 6 weeks of age (Asplin, 1956; Rispens, 1969). Mortality increased when ducklings were stressed by chilling or frequent disturbance (Asplin, 1956). Hwang and his colleagues (1963) also suggested that concurrent infections, poor management and nutritional deficiencies might enhance the susceptibility of older ducklings.

The course of the illness was very short; ducklings died in less than one hour after the onset of symptoms. Clinical signs included sudden onset, dullness, sleepiness, inco-ordination, convulsions and death in the opisthotonic position (Levine & Fabricant, 1950). Most deaths of infected ducklings occurred within 4 days (Asplin, 1964; Levine, 1972).

PATHOLOGY

The most obvious lesions were in livers which became enlarged and ecchymotic. Necroses were usually seen when the livers were cut. Swollen kidneys with congested renal vessels were often observed (Levine & Fabricant, 1950; Asplin & McLauchlan, 1954; Levine, 1972). The spleen sometimes was enlarged (Asplin & McLauchlan, 1954; Levine, 1972). Primary histopathological changes were evident in livers and included various degrees of destruction and necrosis of the liver cells and proliferation of the bile ducts (Fabricant et al., 1957; Hanson, 1958; Richter, 1964). Regeneration of liver cells was seen in ducklings that survived (Fabricant et al., 1957).

Richter and his colleagues (1964) observed by electron microscopy, three arrangements of the virus particles in the cytoplasm of DHV-infected liver cells. They were either randomly scattered in the cytoplasm, packed into crystals or contained in membrane-limited vesicles. All three arrangements were sometimes found in the one cell.

DIAGNOSIS

Age incidence, clinical signs, morbidity, mortality and the post-mortem lesions especially of the livers were considered adequate for diagnosis of DVH (Asplin & McLauchlan, 1954; Asplin, 1964; Levine, 1965; Anon., 1973b). Confirmation of the field diagnosis when carried out usually consisted of isolation and identification of the isolates.

DHV was isolated by inoculation of a suspension of most organs including brain, blood, liver (Levine & Fabricant, 1950) and spleen (Asplin & McLauchlan, 1954) into chicken embryos. Neutralisation tests in ducklings or chicken embryos using specific antiserum were used to identify isolates (Levine & Fabricant, 1950; Asplin, 1964; Levine, 1972).

Success with agar gel tests have been reported from several groups (Murty & Hanson, 1961; Kaeberle et al., 1961; Gabridge & Newman, 1971). In contrast, others (Wachendörfer, 1965; Toth, 1972b; Anon., 1973a, 1974)failed. Wachendörfer (1965) was unable to produce specific precipitating antibodies against DHV. Toth (1972b), likewise, found that ducklings inoculated weekly with DHV by different routes for up to 7 weeks did not produce precipitating antibodies in their sera. Rabbits and chickens given a virulent DHV by the oral route also failed to produce precipitating antibodies. Rabbits injected with DHV-infected chicken embryos treated with fluoro-carbon also did not produce specific precipitating antibodies (Toth, 1972b). Others also failed to produce precipitating antibodies against DHV from rabbits (Anon.,

1973a) or from adult ducks (Anon., 1974).

Success with fluorescing antibody techniques was reported in Russia (Vertinskii et al., 1968; Maiboroda, 1972). Others have still to assess the technique and work on this is in progress (Toth, 1972c; Anon., 1973a).

IMMUNITY

Recovered ducks possessed a solid immunity and their sera neutralised the virus (Levine & Fabricant, 1950). Passive immunisation was induced by injecting the serum from convalescent birds into susceptible ducklings (Levine & Fabricant, 1950) or by immunising breeders from whom immunity was passed through the yolk to the ducklings (Asplin, 1956, 1958). Both virulent and adapted DHV were used.

Hanson and Alberts (1960) reported that intramuscular and intravenous injection of chicken embryo-modified virus into ducklings when 6 to 12 weeks of age resulted in an appreciable antibody response whereas oral administration to adult ducks failed to stimulate a measurable antibody. Asplin (1961) suggested that many factors including the age at vaccination, dose, route of vaccination, and interval between initial and subsequent vaccinations influenced the antibody response of the duck and, consequently, the strength and duration of passive resistance in the progeny. He confirmed that adult ducks injected or dosed with DHV failed to produce passively resistant ducklings implying that adult birds were not infected.

Susceptible ducklings were actively immunised by injecting them after hatching either with chicken embryo-propagated virus (Asplin, 1958, 1964; Hwang, 1972) or with cell culture-propagated virus (Hwang, 1971). Susceptible ducklings vaccinated with chicken embryo-propagated DHV developed resistance rapidly (Asplin, 1961), sometimes within 3 days (Asplin, 1958; Anon., 1971a; Hwang, 1972; Crighton, 1973). The rapid resistance was attributed to the production of interferon (Asplin, 1958, 1961; Crighton, 1973). Production of interferon in cell cultures in response to DHV has been shown by Sueltenfuss and Pollard (1963).

MATERIALS AND METHODS

THE VIRUS

Origin: Two strains of DHV, the Houchin and H_{53} strains, were received as freeze-dried suspensions from the Central Veterinary Laboratory, Weybridge through the courtesy of Mr. F.D. Asplin.

The Houchin strain was isolated from a field outbreak in England in 1954 by Asplin (1958) and maintained by passages in ducks. It was virulent for ducklings and in chicken embryos, it produced characteristic lesions but "was not rapidly lethal" (Asplin, personal communication).

The H_{53} strain was originally isolated from infected ducklings in Holland. It was adapted to and was lethal for chicken embryos. It was, however, attenuated for ducklings. The Houchin and H_{53} strains were antigenically similar but there was no known connection between them (Asplin, personal communication).

Viability: The freeze-dried suspensions were reconstituted with phosphate-buffered-saline (PBS) containing antibiotics and thereafter serial tenfold dilutions in PBS were prepared. The allantoic sacs of five 8-day old chicken embryos were inoculated with each dilution of the reconstituted H_{53} strain of DHV. Infectivity was indicated by death of the embryos with typical lesions within 6 days. Three serial passages using infected embryonic tissues and fluids were made. Stock from the third passage was prepared and stored as a bank. It was

designated as stock DHV (H_{56}).

The DHV (Houchin) was passaged five times in ducklings by the oral route. Stock virus was then prepared from the livers of infected ducklings and stored as a bank being designated as stock DHV (Houchin).

EXPERIMENTAL HOSTS

Ducks: Aylesbury ducklings were supplied by the University of Edinburgh Centre for Laboratory Animals on the day they hatched or when one day old. When necessary, they were reared until used. The ducklings came from a flock free from duck virus hepatitis.

Chicken embryos: Fertile eggs from White Leghorn hens were obtained twice weekly from the Centre for Laboratory Animals. They were incubated at 38-39C until required.

Duck embryos: Eight-to 18-day preincubated fertile Aylesbury duck eggs from the Poultry Research Centre, Roslin were used.

Other animals: Sheep, specific pathogen free (SPF) adult New Zealand White rabbits, SPF adult guinea pigs and White Leghorn chickens aged 2- to 42-weeks were used in attempts to produce specific DHV precipitating antibodies.

Cell cultures: Various cell cultures were inoculated in attempts to grow DHV for use as antigen to produce specific DHV precipitating antibodies; they included primary

chicken embryo cell cultures prepared by conventional methods, and cell lines such as the LLC-MK2 and Vero lines of monkey kidney cells and the BHK-21 line of baby hamster kidney cells. All cell cultures were grown in Falcon tissue culture flasks.[1]

Culture media, usually Medium 199[2] or when necessary Eagle's Medium-BHK,[2] were supplemented with foetal calf serum using 10 per cent for growth and 2 per cent for maintenance. Each ml of the medium contained 100 units penicillin and 100 µg streptomycin. The BHK-medium also contained 10 per cent tryptose-phosphate-buffer.

The cell culture monolayers were inoculated with 0.5 ml stock DHV(H_{56}) and incubated at 37C for 1 hour to allow virus absorption. The monolayers were then washed twice with PBS without antibiotics and maintenance medium was added. Inoculated cultures were incubated at 37C. The contents of one or two flasks were collected and stored at -20C until they were required.

Primary cell cultures were also prepared from DHV-infecte chicken embryos (Mason et al., 1972). Eight-day old chicken embryos were inoculated with 0.2 ml of neat stock DHV(H_{56}) or DHV(Houchin) and were incubated at 37C for 30- to 48-hours. The embryonic tissues were then harvested and used to prepare primary cell cultures by conventional methods. The presence of DHV in the fluid medium was checked by inoculation into chicken embryos.

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1. Becton, Dickinson (UK) Ltd., Middlesex.
2. Wellcome Reagents Ltd., Kent.

Primary cell cultures were also prepared from kidneys of 3-day old ducklings which had just died after infection with 0.1 ml of 10 per cent stock DHV(Houchin). Monolayers usually developed within 48 hours. The fluid medium was then collected and centrifuged lightly. The presence of DHV was checked by inoculating the harvest into day-old ducklings.

EXPERIMENTAL DESIGNS

The working hypothesis examined in these studies was that the age of the host at the time of infection had a predictable effect on the interaction between host and virus such that the severity of the interaction decreased as the age increased.

Virus Growth in Ducklings

Sighting experiment: Seventy-five ducklings were infected when 11 days old by the oral route with 0.1 ml doses of a 10^{-1} dilution of the stock DHV (Houchin) estimated to contain 10^{4} LD_{50}s. At frequent intervals after exposure 2 ducklings were selected at random, bled into bottles containing heparin, and then killed. Oral and cloacal swabs were made and tissues were collected. The swabs and infected tissues were stored individually at -20 C until required. The tissues harvested were the bile from the gall bladder, brain, bursa of Fabricius, heart muscle, kidney, large and small intestines, liver, lung, pancreas, spleen and thymus. After removing the contents, the large and small intestines were washed twice in PBS before storing.

All tissues were assayed for virus content.

Definitive experiment: The experimental design was similar to that of the sighting experiment but we limited our observations to five tissues. Four groups of 30 to 50 ducklings one, 21, 31 and 41 days old were infected by the oral route with 0.1 ml of 10^{-1} dilution of the same stock of DHV (Houchin). At frequent intervals after exposure 2 ducklings were selected at random and then killed. Oral and cloacal swabs were made and tissues were collected and stored at -20 C until required. The selected tissues were the liver, bile, kidney, spleen and pancreas.

Mortality Rate

Groups of 19 to 49 ducklings one to 41 days old were infected by the oral route using a dose of 0.1 ml of a 10^{-1} dilution of the stock Houchin strain of DHV estimated in ducklings infected when one day old to contain at least 10^4 LD_{50}s. The infected birds were kept under observation for 2 weeks. Deaths were confirmed pathologically as being due to DHV and recorded. The mortality rates were compared by the chi-square test (Snedecor & Cochran, 1967).

Livers of ducklings infected when one to 21 days old were collected and stored at -20 C until required. DHV titres in the livers were assessed in 8-day old chicken embryos.

Embryonic Susceptibility

The experimental design was to study whether age-related

resistance occurred in chicken or duck embryos and to determine the most suitable age of chicken embryos for DHV titration.

Stock DHV (H_{56}) and DHV (Houchin) were titrated in chicken embryos incubated for 7, 8, 9, 10, 11 and 12 days respectively. The allantoic sacs of 5 embryos were inoculated with each dilution of the stock viruses. Likewise, stock DHV (Houchin) was titrated in duck embryos incubated for 8, 9, 10, 11, 12, 14, 16 and 18 days respectively. The infectivity of the chicken or duck embryos was determined as described by Levine and Fabricant (1950).

Assay of Infected Tissues

Virus assays were based on titrations carried out in chicken embryos inoculated by the allantoic route when 8 days old. Organs were weighed and 10 per cent (w/v) tissue suspensions were made with PBS using mortars and pestles. Bile and blood were diluted with PBS to give 10 per cent (v/v) dilutions. Thereafter series of ten- or hundred-fold dilutions were made in PBS. Oral or cloacal swabs were immersed in 2.0 ml PBS and left overnight at 4 C.

Five to 6 chicken embryos were inoculated with each dilution. On the day of inoculation, each embryonated egg was candled, marked, swabbed with methyl alcohol, drilled and inoculated via the allantoic sac by the single hole method described by Blaškovič and Styk (1967) with 0.1 ml of the appropriate dilution of the tissue suspension or 0.2 ml of the appropriate dilution of extracts from oral and cloacal swabs. The hole was then re-swabbed and sealed with polythene adhesive tape[1] or nail varnish. The infected

1. Xlon Products Ltd., London.

chicken embryos were incubated in a laboratory incubator modified by the installation of a small electric fan. The humidity in the incubator was provided by two 500 ml beakers of water. The embryos were candled daily and those that died within 24 hours of inoculation were discarded as being due to non-specific causes.

Infection of chicken embryos with DHV was determined according to the criteria described by Levine and Fabricant (1950); death of the chicken embryos with specific lesions or the presence of the specific lesions alone when the embryos were opened and examined on the 6th day after inoculation. The 50 per cent embryonic infective dose (EID_{50}) was calculated by the Spearman-Kärber Method (Dougherty, 1964).

Precipitating Antibody Production

Chickens, ducks, guinea pigs, rabbits and sheep were used in attempts to produce specific precipitating antibodies to DHV using antigens prepared from many sources and treated with different techniques. The antigens used were obtained from livers, kidneys and plasma of ducklings infected with DHV (Houchin), from the fluid medium of cell cultures prepared from kidneys of ducklings which died after infection with DHV (Houchin), from the allantoic fluids of chicken embryos and the fluid media of cell cultures prepared from chicken embryos infected with DHV (Houchin) or DHV (H_{56}).

Techniques employed in treating the antigens included low speed centrifugation[1] at 1,000 to 1,400 g for 15 to 20

1. In an MSE centrifuge.

minutes, and high speed centrifugation[1] at 8,700 to 18,000 g for one to 24 hours. Treatments with chloroform, methyl alcohol, ammonium sulphate (Cramer, 1964), polyethylene glycol (Wagner et al., 1970) and acetone (Anon., 1971b) were tried. The treated materials were also purified by centrifugation[1] into caesium chloride at densities ranging from 1.35 to 1.45 g/ml at 11,000 to 18,000 g for 3 to 24 hours.

DHV in the treated antigens was checked by inoculation into ducklings or chicken embryos. Virus-containing antigens were injected into groups of 2 to 5 rabbits, guinea pigs or sheep. Some antigens were mixed with incomplete Freund's adjuvant. Doses were repeated 2 to 4 times after the first inoculation at weekly intervals. Serum was prepared from blood collected from the inoculated animals 2 to 4 weeks after the first inoculation.

Groups of 2 to 8 ducks aged 2 to 6 weeks or chickens aged 2 to 42 weeks were also injected with the antigens and were bled for serum 10 to 14 days after the first inoculation. They were also bled 10 days after the last inoculation if they were inoculated more than once.

Agar gel diffusion tests were carried out in petri dishes, 50 mm in diameter, and filled with 4.0 ml of one per cent Ionagar No. 2[2] in PBS. When duck or chicken serum was used, 8.0 per cent sodium chloride was added (Murty & Hanson, 1961).

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1. In a Beckman L2-65B ultracentrifuge using a Type-65-fixed angle rotor.
2. Oxoid Ltd., London.

STATISTICAL ANALYSES

Virus titres and their standard errors were calculated from titration data by the Spearman-Kärber method (Dougherty, 1964), and were plotted to depict virus growth against time.

The exponential increase phase of virus growth was subjected to a Z-transformation calculated according to Pearl (1940) from:

$$Z = \frac{K-(y-d)}{y-d}$$

where Z is the transformation of the observed virus titre, y. K is the maximum virus titre in a particular tissue subtracted by d which is the minimum titre attained. The maximum virus titre was obtained by adding one standard error to the observed highest virus titre. Log Z - transformations were then used to calculate linear regressions and were tested for significant linearity and compared by the method proposed by Dawkins (1968).

The lines of best fit derived from the Z-transformations data were logistic curves and they were calculated according to Pearl (1940) from:

$$Y = \frac{K}{1+C.e^{rt}} + d$$

where Y is the titre at time t recorded in hours such that t_o is the time at which the virus began to rise. The constant, C, is equal to Z_o at t_o and r is the rate of virus growth in a particular tissue being 2.30259 m where m is the slope of the linear regression of log Z on hours.

A practical difficulty associated with this type of analysis was recognition of the first and the last points

of the curve. Arbitrary rules were applied such that the first point was taken as being 2 places before the point clearly indicating multiplication of virus and the last point as being one place after the highest virus titre. Another snag of this type of analysis is that it embraces only the exponential increase phase.

The complete growth curves were skewed. When the hours were transformed into logarithms, the curves approached normality. The lines of best fit were second degree polynomials and were calculated by the method described by Snedecor and Cochran (1967). They were tested for significance by the analysis of variance (Snedecor & Cochran, 1967). Curvilinear regressions were compared by a modification of Dawkins' method for linear regressions (Dawkins, 1968).

Chi-square analyses were used to compare the mortality rates of ducklings infected with DHV(Houchin) at different ages.

RESULTS

GROWTH OF THE HOUCHIN STRAIN OF DUCK HEPATITIS VIRUS IN TISSUES OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

The growth of the Houchin strain of DHV in the tissues of ducklings infected when 11 days old conformed to the accepted pattern characterised by a sequence of eclipse, exponential increase, plateau, and exponential decline phases. Thereafter no virus was recovered from the tissues (Tables 1A, 1B & 1C).

The Lag Phase

After oral inoculation DHV (Houchin) apparently disappeared. Twelve hours elapsed before virus was found in the livers. Six hours later it appeared in the kidneys, spleens, small and large intestines, pancreases and bloods. Other tissues at this time contained no virus. Virus was found, however, in the other tissues at 24 hours after inoculation.

The Exponential Increase Phase

The growth of the virus in all tissues was exponential. The data gave a logistic plot (Fig. 1) and when they were transformed the regressions of the log Z-transformations were all found to be significantly linear (Table 2). Moreover, the slopes of the regressions were similar but the positions differed (Table 3; Fig. 2). Further analyses showed that the regressions fell into two groups similar in slopes but significantly different in positions. Regressions within

each group were statistically similar. The regressions of the livers and biles were in one group and those of the other tissues were in another group (Tables 4, 5, 6; Fig. 3).

The Plateau Phase

The limits of the plateau phases were difficult to estimate because the high titres fluctuated. Arbitrary limits were taken as the period between the first statistically significant rise between adjacent titres and the first significant decline between adjacent titres.

The plateau phases of DHV occurred 24 to 144 hours after inoculation in the livers of infected ducklings whereas they occurred later and were shorter in the other tissues (Tables 1A, 1B, 1C). The peak virus titres ranged from $10^{9.0}EID_{50}/g$ in the liver to $10^{4.2}EID_{50}/g$ in the brain (Tables 1A, 1B, 1C).

The Decline Phase

Virus titres fell exponentially after the plateau phase. The exponential decline phase was longer than the exponential increase phase. At the end of the exponential decline phase, virus was not recovered from the tissues (Table 1A, 1B, 1C).

The Complete Curve

The growth curves of DHV (Houchin) in all the tissues of the infected ducklings were skewed, the exponential increase phases being shorter than the exponential decline phases. When the time intervals were plotted on a logarithmi

scale the curves became symmetrical and all the lines of best fit were found to be significantly curvilinear (Table 7).

The shape of the growth curves were similar but the positions differed (Table 8). In other words, growth rates of DHV in all tissues of infected birds were the same but the times at which the virus growth occurred were different. Peaks of DHV titres in tissues were also different. The curve fell into five groups which differed significantly in position they were labelled in descending order of magnitude I, II, III, IV and V. Within each group the curves were similar in shapes and positions. The first, second, and the fourth groups contained only the curves of virus in one tissue, namely, liver, bile, and pancreas. Group III contained the curves of virus growth in the kidney, spleen, small and large intestines. Group V contained the curves of the remaining tissues (Tables 9, 10, 11; Fig. 4).

Lag phases of all growth curves lasted for 8-12 hours after inoculation (Table 12). The times of peak virus titres in tissues were 45-47 hours after inoculation in Groups I, III and V, 57 hours in Group II (bile) and 70 hours in Group IV (pancreas). The times at which virus was no longer recovered from tissues ranged from 141 hours in Group V to 366 hours in Group IV.

The growth curve of virus in liver (Group I) was earlier and reached a higher peak than in other tissues. The virus content in bile (Group II) reached a greater peak than other tissues except liver. The peak titre of Group III (kidney, spleen, small and large intestines) was the same as Group IV (pancreas) but occurred earlier.

TABLE 1 A

TITRES* OF THE HOUCHIN STRAIN OF DHV IN THE TISSUES OF 11-DAY OLD DUCKLINGS KILLED 8 TO 264 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Liver	Bile	Pancreas	Brain
8	<1.0	.	.	.
12	≤1.40 ± 0.40	<1.0	<1.0	<1.0
18	≤2.90 ± 0.49	<1.0	3.00 ± 0.00	<1.0
24	6.50 ± 0.80	4.60 ± 0.40	{≤2.17 ± 0.47 3.60 ± 0.20	<1.0
26	7.30 ± 0.53	.	3.00 ± 0.00	≤1.40 ± 0.40
28	9.00 ± 0.00	.	.	{≤1.80 ± 0.49 3.40 ± 0.40
30	8.25 ± 0.25	7.00 ± 0.57	5.00 ± 0.00	5.00 ± 0.00
32	7.70 ± 0.69	.	.	.
36	7.80 ± 0.49	7.00 ± 0.00	5.00 ± 0.00	4.20 ± 0.63
40	8.20 ± 0.49	.	.	.
42	.	6.60 ± 0.40	3.80 ± 0.49	3.00 ± 0.00
48	6.95 ± 0.32	7.00 ± 0.00	5.40 ± 0.40	4.20 ± 0.63
54	.	.	4.20 ± 0.49	3.00 ± 0.00
72	7.80 ± 0.49	5.00 ± 0.00	5.00 ± 0.00	3.20 ± 0.63
96	5.00 ± 0.00	5.00 ± 0.00	5.40 ± 0.40	≤1.80 ± 0.49
120	5.40 ± 0.40	.	6.60 ± 0.40	<1.0
144	5.00 ± 0.00	4.20 ± 0.49	5.40 ± 0.40	.
168	≤2.60 ± 0.62	3.00 ± 0.00	3.40 ± 0.40	.
216	≤2.20 ± 0.63	3.00 ± 0.00	4.20 ± 0.49	.
264	<1.0	<1.0	<1.0	.

* EID_{50} ± standard error, expressed as log 10/g. or ml.

TABLE 1 B

TITRES* OF THE HOUCHIN STRAIN OF DHV IN THE TISSUES OF 11-DAY OLD DUCKLINGS KILLED 8 TO 168 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Kidney	Spleen	Small intestine	Large intestine	Blood
8	<1.0	<1.0	<1.0	<1.0	<0.1
12	<1.0	<1.0	<1.0	<1.0	<0.1
18	≤1.75 ± 0.52	≤2.90 ± 0.49	≤1.40 ± 0.40	≤1.40 ± 0.40	≤0.40 ± 0.40
24	3.40 ± 0.40 3.70 ± 0.80	≤1.70 ± 0.80	≤1.80 ± 0.49	3.00 ± 0.00	≤1.90 ± 0.24 3.20 ± 0.49
26	5.15 ± 0.40	5.00 ± 0.00	4.20 ± 0.63	.	3.20 ± 0.49
30	6.30 ± 0.20	8.00 ± 0.00	.	6.20 ± 0.63	.
32	5.40 ± 0.40	.	4.90 ± 0.28	.	5.20 ± 0.49
34	5.20 ± 0.69	.	4.95 ± 0.40	.	5.20 ± 0.49
36	7.00 ± 0.00	8.50 ± 0.52	5.90 ± 0.20	5.00 ± 0.57	4.40 ± 0.40
38	.	.	.	.	6.00 ± 0.00
40	5.50 ± 0.52	.	6.00 ± 0.57	.	.
42	5.40 ± 0.49	7.60 ± 0.63	5.00 ± 0.00	5.00 ± 0.00	5.80 ± 0.49
44	.	.	.	.	5.40 ± 0.49
46	5.80 ± 0.49	.	5.60 ± 0.40	.	4.40 ± 0.40
48	6.70 ± 0.35	6.30 ± 0.20	5.70 ± 0.20	5.00 ± 0.00	4.80 ± 0.49
54	.	.	.		4.00 ± 0.57
72	6.90 ± 0.42	6.60 ± 0.42	5.20 ± 0.78	5.00 ± 0.00	4.40 ± 0.49
96	5.40 ± 0.40	5.00 ± 0.00	3.40 ± 0.40	3.00 ± 0.00	<0.1
120	5.40 ± 0.40	<2.35 ± 0.66	≤2.60 ± 0.40	<1.0	.
144	≤1.40 ± 0.40	≤1.80 ± 0.49	<1.0	.	.
168	<1.0	<1.0	.	.	.

* EID_{50} ± standard error, expressed as log 10/g or ml.

TABLE 1 C

TITRES* OF THE HOUCHIN STRAIN OF DHV IN THE TISSUES OF 11-DAY OLD DUCKLINGS KILLED 12 TO 144 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Heart	Lung	Thymus	Bursa of Fabricius	Leg muscle
8	.	.	.	.	.
12	<1.0	<1.0	<1.0	<1.0	<1.0
18	<1.0	<1.0	<1.0	<1.0	<1.0
24	≤1.70 ± 0.20 ≤2.60 ± 0.40	≤1.90 ± 0.24 ≤2.20 ± 0.49	≤1.80 ± 0.49	≤1.40 ± 0.40 ≤1.70 ± 0.20	<1.0 ≤1.40 ± 0.40
26	≤2.20 ± 0.63	3.00 ± 0.57	≤2.20 ± 0.63	≤2.60 ± 0.49	≤2.20 ± 0.62
30	5.40 ± 0.40	4.80 ± 0.40	4.50 ± 0.52	5.40 ± 0.40	5.00 ± 0.00
36	5.00 ± 0.00	5.40 ± 0.40	5.40 ± 0.40	5.00 ± 0.00	5.00 ± 0.00
42	.	4.60 ± 0.40	.	3.80 ± 0.49	3.40 ± 0.40
48	4.60 ± 0.40	5.40 ± 0.40	5.40 ± 0.40	5.00 ± 0.00	3.80 ± 0.49
72	4.60 ± 0.40	3.00 ± 0.00	5.00 ± 0.00	4.60 ± 0.40	3.40 ± 0.40
96	≤2.60 ± 0.70	<1.0	≤2.00 ± 0.63	≤2.50 ± 0.57	3.00 ± 0.00
120	<1.0	.	≤1.80 ± 0.49	≤1.40 ± 0.40	≤1.40 ± 0.40
144	.	.	<1.0	<1.0	<1.0

* EID_{50} ± standard error, expressed as log 10/g.

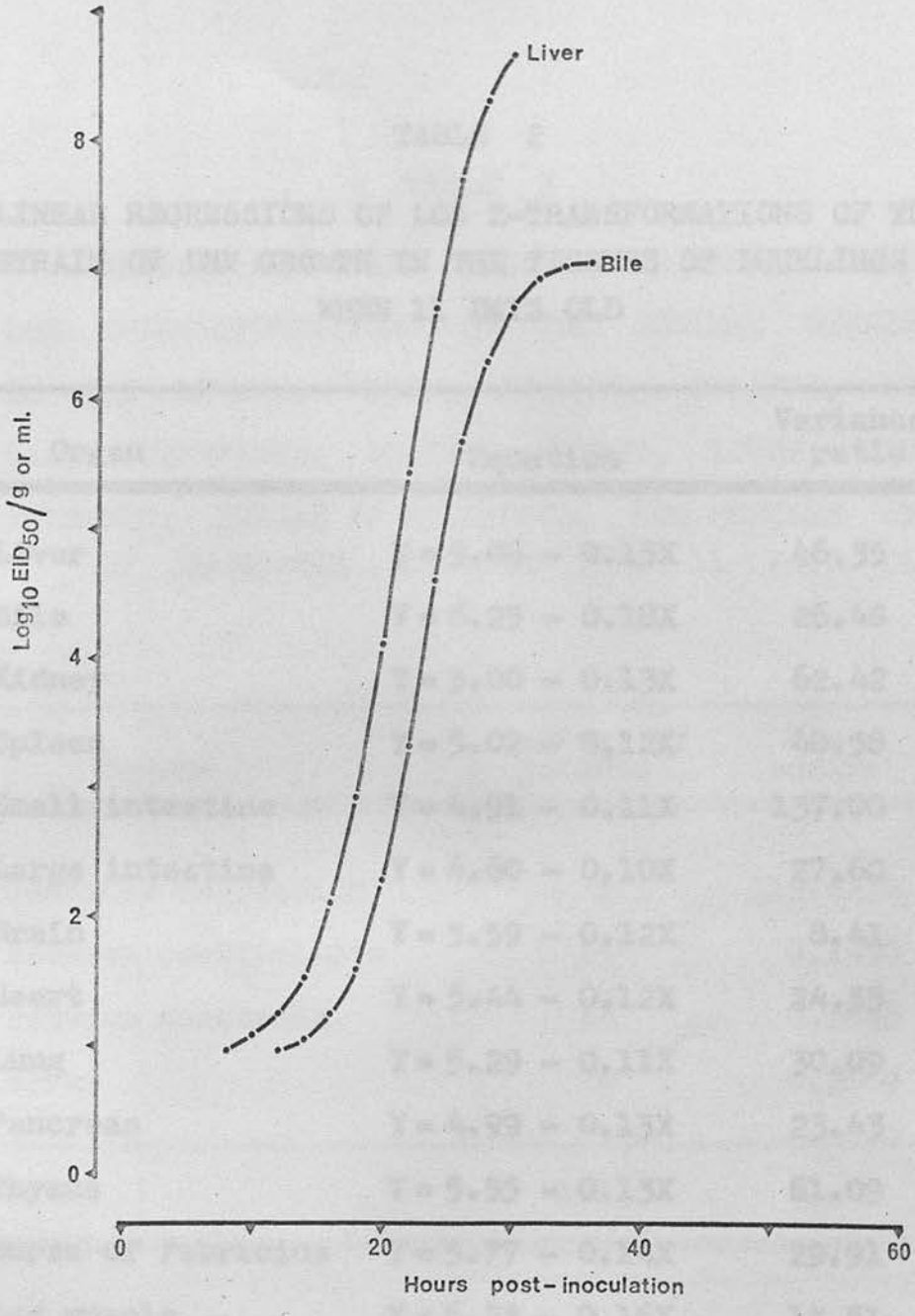


Figure 1. Examples of the logistic curves depicting the exponential increase phases of the growth of DHV (Houchin) in ducklings infected when 11 days old.

TABLE 2

LINEAR REGRESSIONS OF LOG Z-TRANSFORMATIONS OF THE HOUCHIN STRAIN OF DHV GROWTH IN THE TISSUES OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Organ	Equation	Variance ratio	P
Liver	Y = 5.06 - 0.15X	46.35	<0.01
Bile	Y = 6.25 - 0.18X	26.48	<0.05
Kidney	Y = 5.00 - 0.13X	62.42	<0.01
Spleen	Y = 5.02 - 0.12X	40.58	<0.01
Small intestine	Y = 4.91 - 0.11X	137.08	<0.01
Large intestine	Y = 4.60 - 0.10X	27.60	<0.01
Brain	Y = 5.59 - 0.12X	8.41	<0.05
Heart	Y = 5.44 - 0.12X	24.55	<0.01
Lung	Y = 5.29 - 0.11X	30.09	<0.01
Pancreas	Y = 4.99 - 0.13X	23.43	<0.01
Thymus	Y = 5.55 - 0.13X	61.09	<0.01
Bursa of Fabricius	Y = 5.77 - 0.14X	29.91	<0.01
Leg muscle	Y = 6.32 - 0.16X	13.51	<0.01
Blood	Y = 5.75 - 0.14X	100.22	<0.01

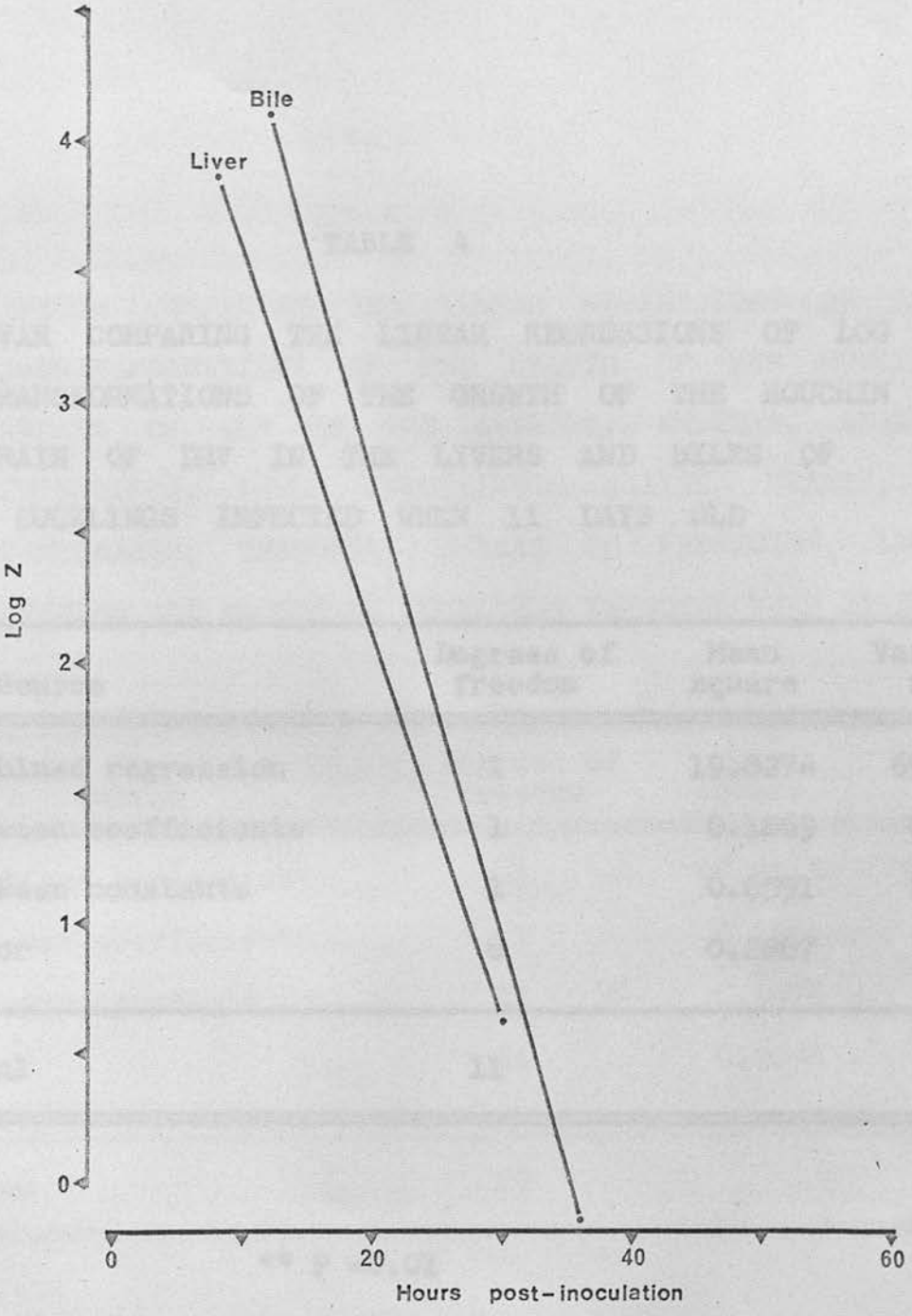


Figure 2. Regressions of the log Z-transformations on hours post-inoculation depicting exponential increase phases of the growth of DHV (Houchin) in ducklings infected when 11 days old.

TABLE 4

ANOVAR COMPARING THE LINEAR REGRESSIONS OF LOG Z-TRANSFORMATIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS AND BILES OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	1	19.8274	69.16**
Between coefficients	1	0.1869	0.65
Between constants	1	0.6591	2.30
Error	8	0.2867	
Total	11		

** P <0.01

TABLE 5

ANOVAR COMPARING THE LINEAR REGRESSIONS OF LOG Z-TRANSFORMATIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN THE KIDNEYS, SPLEENS, SMALL INTESTINES, LARGE INTESTINES, BRAINS, HEARTS, LUNGS, PANCREASES, THYMUSES, BURSAE OF FABRICIUS, LEG MUSCLES AND BLOODS OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	1	96.5016	364.98**
Between coefficients	11	0.1219	0.26
Between constants	11	0.4978	1.88
Error	66	0.2644	
Total	89		

** P <0.01

TABLE 6

SIGNIFICANT GROUPS OF SIMILAR LINEAR REGRESSIONS OF LOG Z-TRANSFORMATIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN THE TISSUES OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Group	Organ	Equation
I	Liver	Y = 5.44 - 0.16X
	Bile	
II	Kidney	Y = 5.30 - 0.12X
	Spleen	
	Small intestine	
	Large intestine	
	Brain	
	Heart	
	Lung	
	Pancreas	
	Thymus	
	Bursa of Fabricius	
	Leg muscle	
	Blood	

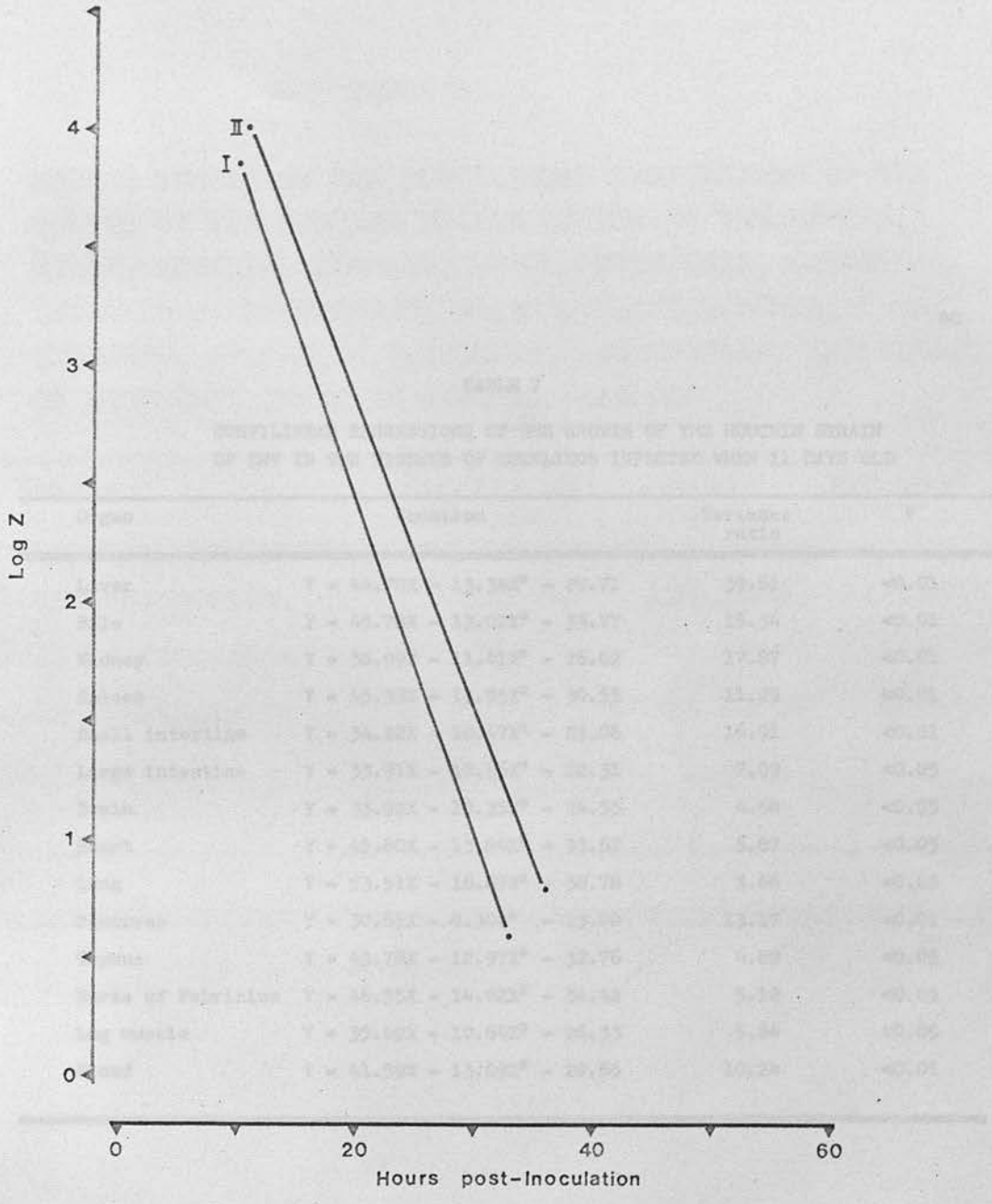


Figure 3. Significant groups of similar linear regressions of log Z-transformations of the growth of DHV (Houchin) in the tissues of ducklings infected when 11 days old.

I:- Liver, bile.

II:- Kidney, spleen, small and large intestines, brain, heart, lung, pancreas, thymus, bursa of Fabricius, leg muscle and blood.

TABLE 7

CURVILINEAR REGRESSIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN THE TISSUES OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Organ	Equation	Variance ratio	P
Liver	$Y = 44.70X - 13.34X^2 - 29.71$	39.61	<0.01
Bile	$Y = 45.78X - 13.02X^2 - 33.77$	15.34	<0.01
Kidney	$Y = 38.09X - 11.41X^2 - 26.02$	17.87	<0.01
Spleen	$Y = 45.33X - 13.95X^2 - 30.55$	11.29	<0.01
Small intestine	$Y = 34.22X - 10.47X^2 - 23.06$	16.91	<0.01
Large intestine	$Y = 33.91X - 10.66X^2 - 22.31$	7.09	<0.05
Brain	$Y = 33.92X - 10.35X^2 - 24.55$	4.68	<0.05
Heart	$Y = 45.80X - 13.84X^2 - 33.67$	5.87	<0.05
Lung	$Y = 53.51X - 16.67X^2 - 38.78$	5.66	<0.05
Pancreas	$Y = 30.65X - 8.30X^2 - 23.00$	13.17	<0.01
Thymus	$Y = 43.78X - 12.97X^2 - 32.76$	4.89	<0.05
Bursa of Fabricius	$Y = 46.55X - 14.02X^2 - 34.42$	5.12	<0.05
Leg muscle	$Y = 35.69X - 10.64X^2 - 26.33$	5.64	<0.05
Blood	$Y = 41.59X - 13.09X^2 - 28.66$	10.24	<0.01

TABLE 8

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS, BILES, KIDNEYS, SPLEENS, SMALL INTESTINES, LARGE INTESTINES, PANCREASES, BRAINS, HEARTS, LUNGS, THYMUSES, BURSAE OF FABRICIUS, LEG MUSCLES, AND BLOODS OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	198.1060	122.18**
Between coefficients	26	2.4257	1.49
Between constants	13	15.6949	9.68**
Error	155	1.6216	
Total	196		

** P <0.01

TABLE 9

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN KIDNEYS, SPLEENS, SMALL INTESTINES AND LARGE INTESTINES OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Source	Degrees of freedom	Mean square	F
Combined regression	2	94.0856	49.94**
Between coefficients	6	0.9717	0.52
Between constants	3	3.9515	2.10
Error	50	1.8838	
Total	61		

**P <0.01

TABLE 10

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH OF THE HOUCHIN STRAIN OF DHV IN HEARTS, LUNGS, BRAINS, THYMUSES, BURSAE OF FABRICIUS, LEG MUSCLES AND BLOODS OF DUCKLINGS INFECTED WHEN 11 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	68.3505	41.13**
Between coefficients	12	0.4923	0.30
Between constants	6	1.6644	1.00
Error	66	1.6618	
Total	86		

**P <0.01

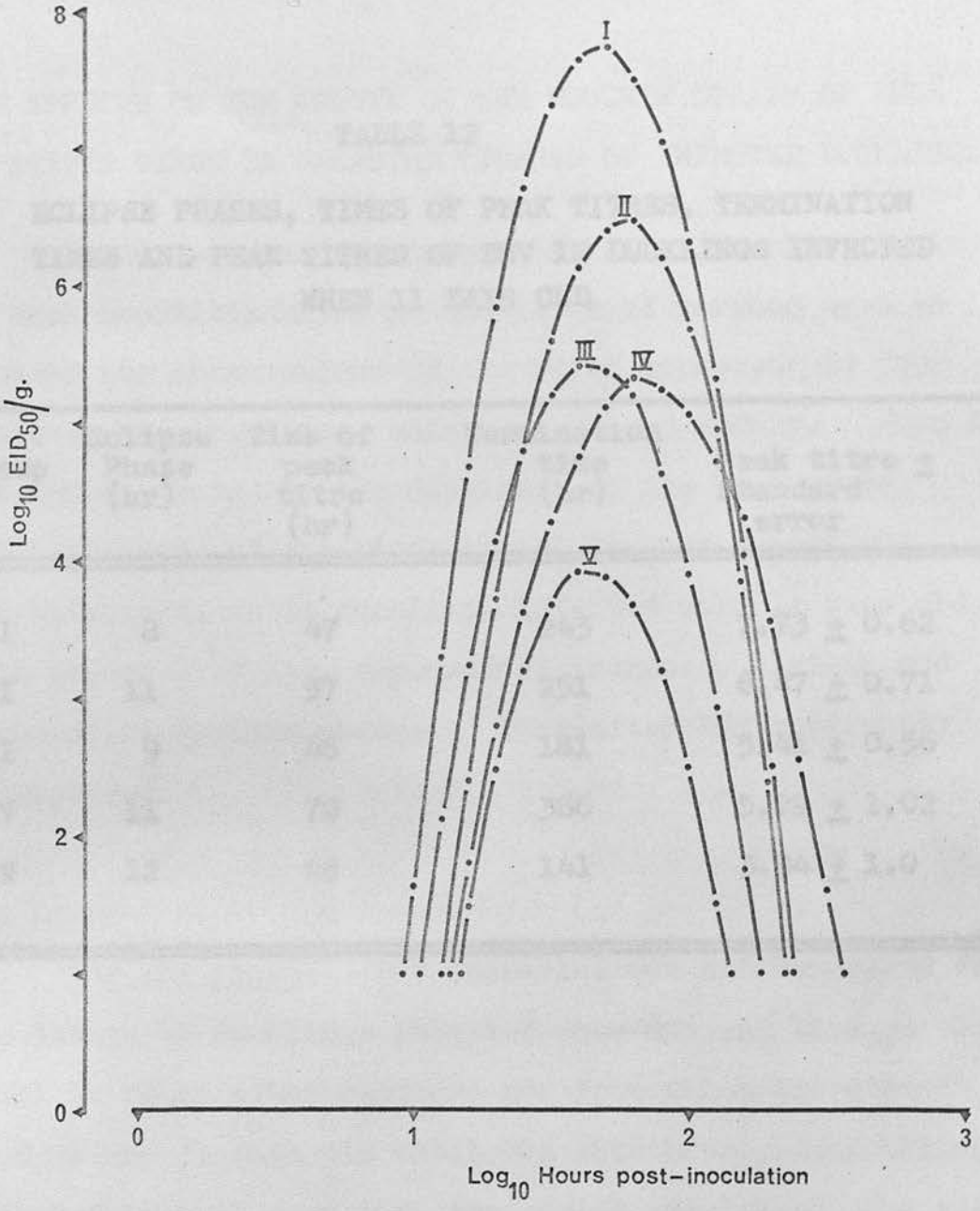


Figure 4. Significant groups of similar growth curves of DHV (Houchin) in the tissues of ducklings infected when 11 days old.

I:- Liver.

II:- Bile.

III:- Kidney, spleen, small and large intestines.

IV:- Pancreas.

V:- Brain, heart, lung, thymus, bursa of Fabricius, leg muscle and blood.

TABLE 12

ECLIPSE PHASES, TIMES OF PEAK TITRES, TERMINATION TIMES AND PEAK TITRES OF DHV IN DUCKLINGS INFECTED WHEN 11 DAYS OLD

Group	Eclipse Phase (hr)	Time of peak titre (hr)	Termination time (hr)	Peak titre ± Standard error
I	8	47	243	7.73 ± 0.62
II	11	57	251	6.47 ± 0.71
III	9	46	181	5.41 ± 0.56
IV	11	70	366	5.29 ± 1.02
V	12	45	141	3.94 ± 1.0

AGE EFFECTS ON THE GROWTH OF THE HOUCHIN STRAIN OF DUCK HEPATITIS VIRUS IN SELECTED TISSUES OF INFECTED DUCKLINGS

In our studies of the growth of the Houchin strain of duck hepatitis virus in ducklings of varying ages we limited our observations of the virus behaviour to four tissues, namely, liver, bile, spleen and kidney. When DHV was administrated to the ducklings by the oral route, multiplication of the virus in all tissues tested was characterised, as in ducklings infected when 11 days old, by a sequence of lag, exponential increase, plateau and exponential decline phases. Thereafter DHV apparently disappeared.

The Liver

The lag phase: DHV (Houchin) was not recovered from the livers of ducklings infected when one and 11 days old until 12 hours after exposure nor from those infected when 21 and 31 days old until the 18th hour. In ducklings infected when 41 days old, the period was longer, the virus being first recovered in the livers at 24 hours (Table 13).

The exponential increase phase: The growth of DHV in the livers of ducklings infected at all ages was exponential after the end of the lag phase (Table 13).

The plateau phase: After the exponential increase phases DHV titres fluctuated at high levels (Table 13). Because the limits of the plateau phases were difficult to

estimate the arbitrary limits used in ducklings infected when 11 days old were applied, namely, the period between the first statistically significant rise between adjacent titres and the first significant decline between adjacent titres. The plateau phases of DHV in the livers were thus estimated to be 144, 120, 96, 90 and 54 hours long in ducklings infected when one, 11, 21, 31 and 41 days old respectively. The estimated durations of the plateaus were inversely related to ages at which the ducklings were infected ($r = -0.983$, $P < 0.01$).

The peak titres declined with the ages at which ducklings were infected being $10^{7.8}$, $10^{9.0}$, $10^{7.4}$, $10^{7.2}$ and $10^{6.6}$ EID_{50}/g in ducklings infected when one, 11, 21, 31 and 41 days old respectively. The regression of the peak titres on age at inoculation was not significant ($r = -0.742$, $P > 0.05$). Nevertheless the difference between the peak titres of the five age groups was statistically significant ($F_{[4,20]} = 5.97$, $P < 0.01$); the difference between peak titres in ducklings infected when 11 days old and the rest was highly significant ($t_{[23]} = 4.581$, $P < 0.001$). The difference between peak titres in ducklings infected when one, 21, 31 and 41 days old was, however, not significant ($F_{[3,16]} = 1.49$, $P > 0.05$).

The exponential decline phase: The titres of DHV (Houchin) in the livers of infected ducklings declined after the plateau phases until no virus was recovered from the livers (Table 13). There was no clear relationship between the ages when ducklings were infected and the virus

extinction times which ranged from the 216th to 312nd hour after inoculation.

The complete curve: The growth curves of DHV in livers of all age groups were skewed, the exponential increase phases being shorter than the exponential decline phases. When the time intervals were plotted on a logarithmic scale the curves became symmetrical and all the lines of best fit were significantly curvilinear (Table 14).

The peak titres in the livers of younger infected ducklings were higher than in the livers of older infected birds, except in birds infected when day old (Table 37; Fig. 5).

The shapes of the growth curves of DHV in the livers were similar. The positions of the curves, however, differed significantly (Table 15). The growth curves fell into three groups which were similar in shapes but differed in positions. The curves within each group were similar both in shapes and positions (Tables 16, 17, 18; Fig. 6). The groups were labelled in descending order of peak titres: I, II, and III. Group I consisted of the growth curves in ducklings infected when one and 11 days old. Group II comprised the curves in ducklings infected when 21 and 31 days old. Group III contained only the curve in ducklings infected when 41 days old.

TABLE 13

VIRUS TITRES* IN THE LIVERS OF ONE- TO 41-DAY OLD DUCKLINGS KILLED 8 TO 312 HOURS AFTER ORAL INOCULATION WITH DHV (HOUCHIN)

Hours after inoculation	Age (day)				
	1	11	21	31	41
8	<1.0	<1.0	<1.0	<1.0	.
12	≤2.70±0.20	≤1.40±0.40	<1.0	<1.0	.
18	3.40±0.40	≤2.90±0.49	≤1.40±0.40	≤1.80±0.49	<1.0
24	5.80±0.80	6.50±0.80	5.37±0.48	4.20±0.49	{≤2.35±0.40 3.00±0.00
26	.	7.30±0.53	4.65±0.98	.	.
28	.	9.00±0.00	.	.	.
30	7.40±0.80	8.25±0.25	7.00±0.00	5.80±0.49	4.00±0.57
32	.	7.70±0.69	6.80±0.56	.	.
34	.	.	6.40±0.30	.	.
36	6.40±0.40	7.80±0.49	7.40±0.57	7.00±0.00	3.40±0.40
40	.	8.20±0.49	.	.	.
42	7.66±0.66	.	7.00±0.00	7.20±0.40	6.60±0.40
48	7.80±0.16	6.95±0.32	5.40±0.49	6.20±0.49	5.40±0.40
72	7.00±0.00	7.80±0.49	6.60±0.40	5.80±0.40	5.40±0.40
96	7.00±0.00	5.00±0.00	6.20±0.49	5.20±0.40	5.00±0.00
120	6.60±0.40	5.40±0.49	5.40±0.40	5.20±0.40	3.40±0.40
144	5.00±0.00	5.00±0.00	3.80±0.49	3.00±0.69	3.40±0.52
168	5.80±0.40	≤2.60±0.63	3.20±0.76	4.20±0.49	3.80±0.49
216	3.40±0.69	≤2.20±0.63	3.20±0.69	<1.0	≤1.80±0.49
264	≤1.40±0.40	<1.0	3.00±0.00	.	<1.0
312	.	.	<1.0	.	.

* EID_{50} ± standard error, expressed as log 10/g.

TABLE 14

CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD.

Age (day)	Equation	Variance ratio	P
1	$Y = 43.72X - 12.49X^2 - 30.69$	51.09	<0.01
11	$Y = 44.70X - 13.34X^2 - 29.71$	39.61	<0.01
21	$Y = 36.45X - 10.58X^2 - 24.90$	34.30	<0.01
31	$Y = 35.47X - 10.39X^2 - 24.08$	14.32	<0.01
41	$Y = 49.06X - 13.46X^2 - 39.28$	26.13	<0.01

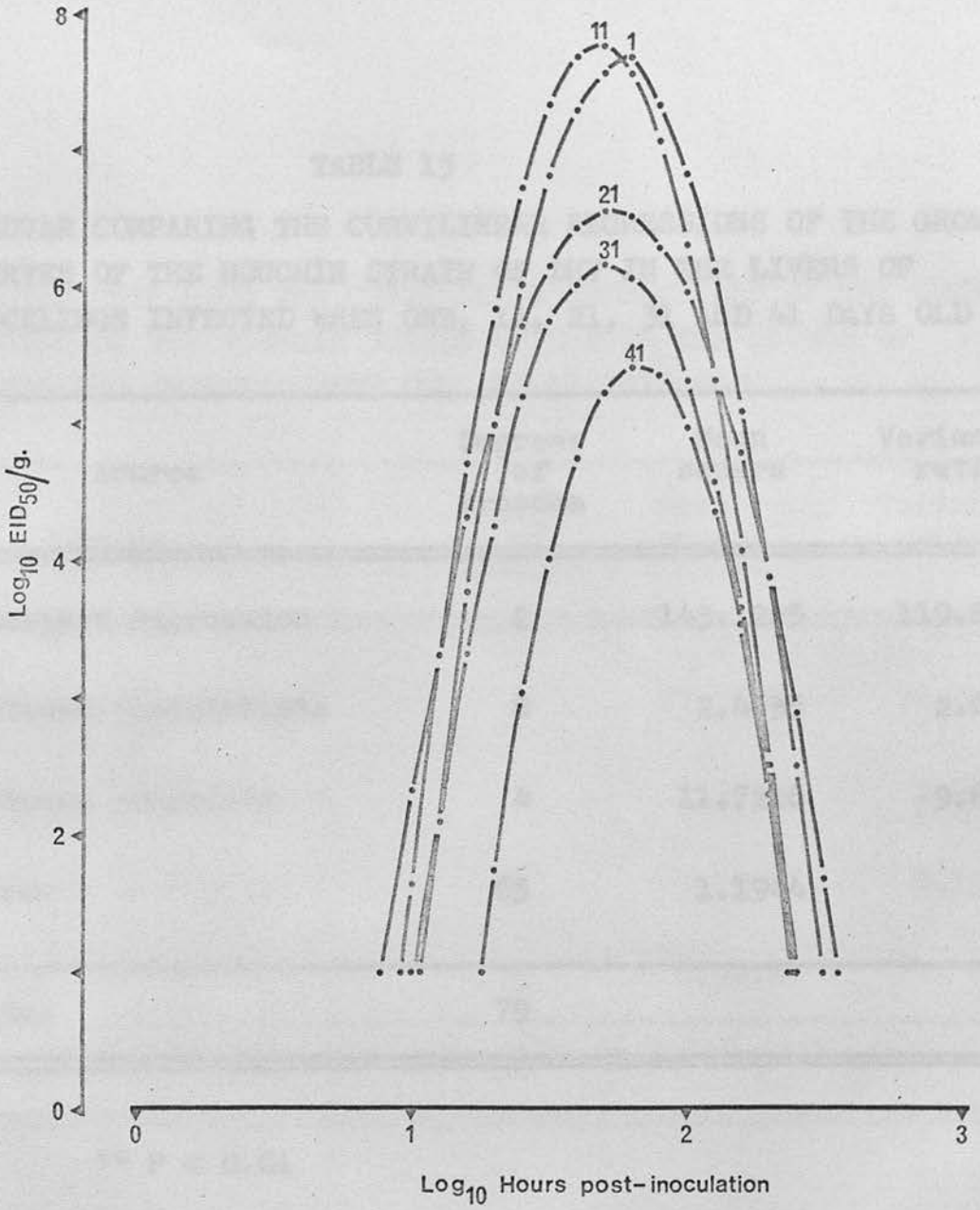


Figure 5. Growth curves of DHV (Houchin) in the livers of ducklings infected when 1 to 41 days old.

TABLE 15

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS OF DUCKLINGS INFECTED WHEN ONE, 11, 21, 31 AND 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	143.1225	119.83**
Between coefficients	8	2.4635	2.06
Between constants	4	11.7216	9.81**
Error	65	1.1944	
Total	79		

** $P < 0.01$

TABLE 16

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS OF DUCKLINGS INFECTED WHEN ONE AND 11 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	77.5636	78.54**
Between coefficients	2	2.7630	2.80
Between constants	1	0.1830	0.19
Error	26	0.9876	
Total	31		

** $P < 0.01$

TABLE 17

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS OF DUCKLINGS INFECTED WHEN 21 AND 31 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	63.2586	39.46**
Between coefficients	2	0.0869	0.05
Between constants	1	0.0978	0.10
Error	28	1.6031	
Total	33		

** $P < 0.01$

TABLE 18

SIGNIFICANT GROUPS OF SIMILAR GROWTH CURVES, ECLIPSE PHASES AND PEAK TITRES OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Group	Age (day)	Equation	Eclipse phase (hr)	Peak titre ± standard error
I	1 & 11	$Y = 43.23X - 12.67X^2 - 29.23$	9	7.65±0.42
II	21 & 31	$Y = 35.91X - 10.47X^2 - 24.45$	10	6.34±0.37
III	41	$Y = 49.06X - 13.46X^2 - 39.28$	18	5.41±0.25

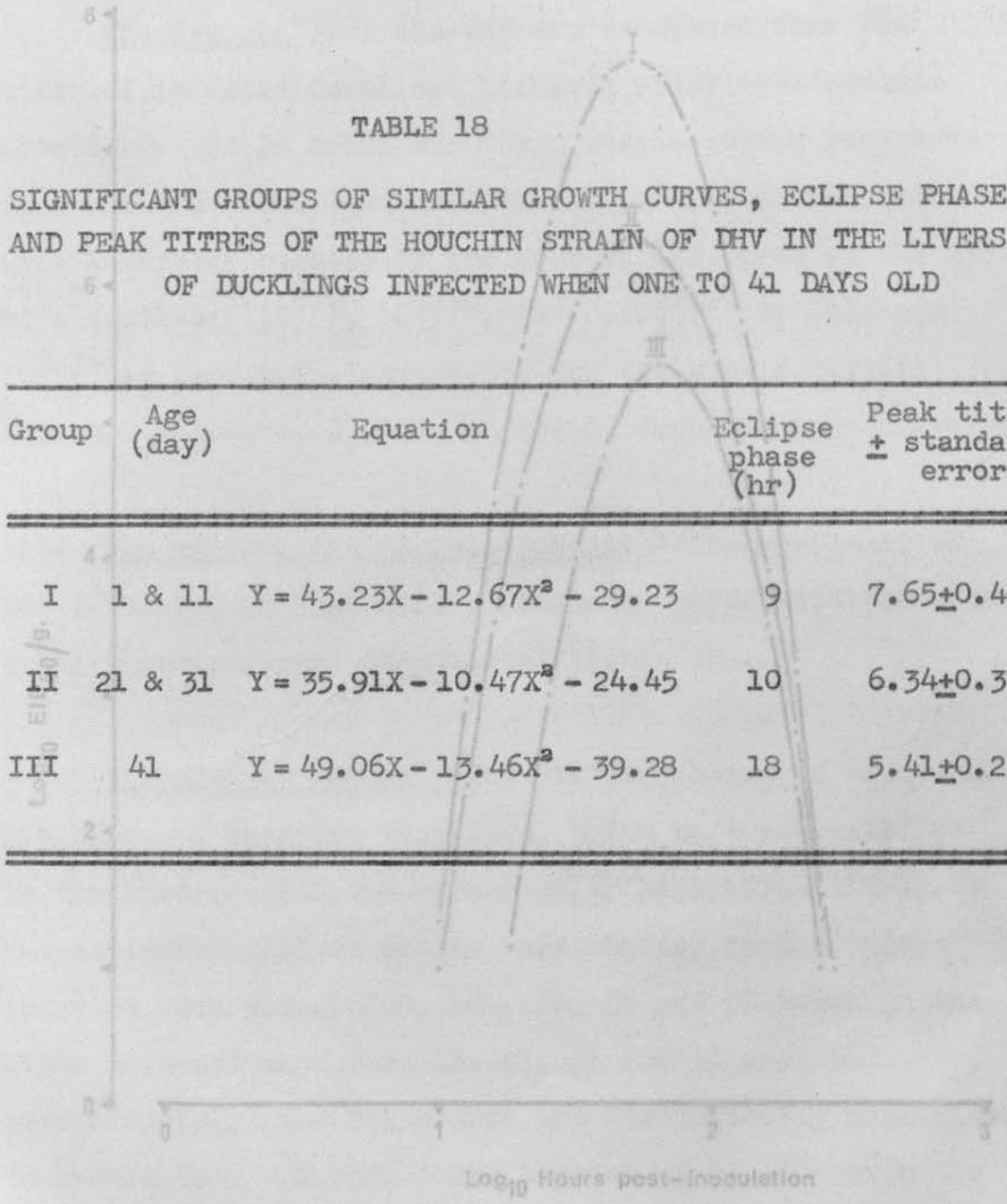


Figure 6. Significant groups of similar growth curves of DHV (Houchin) in the livers of ducklings infected when 1 to 41 days old.

I:- 1 & 11 days old.

II:- 21 & 31 days old.

III:- 41 days old.

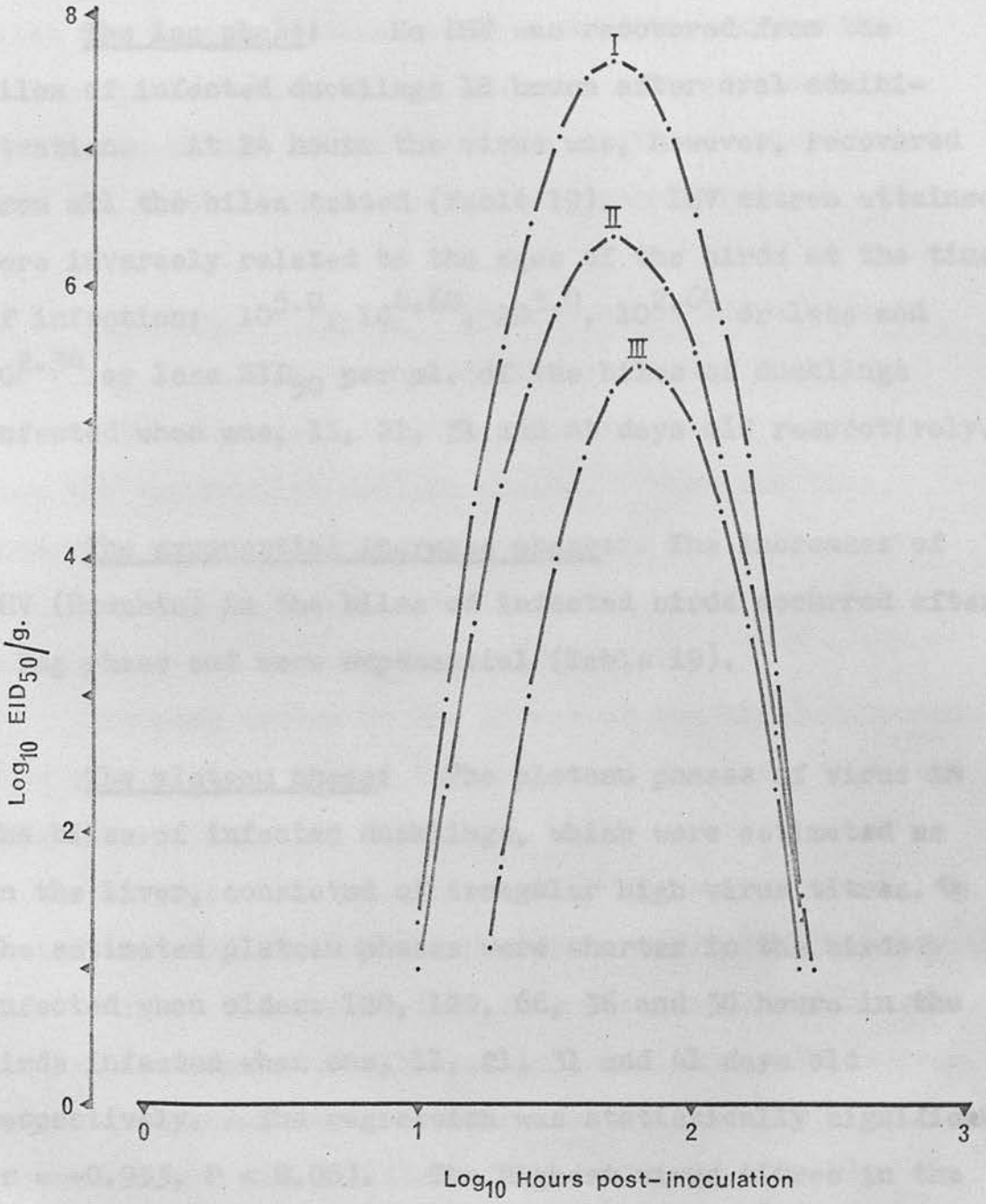


Figure 6. Significant groups of similar growth curves of DHV (Houchin) in the livers of ducklings infected when 1 to 41 days old.

I:- 1 & 11 days old.

II:- 21 & 31 days old.

III:- 41 days old.

The Bile

The lag phase: No DHV was recovered from the biles of infected ducklings 18 hours after oral administration. At 24 hours the virus was, however, recovered from all the biles tested (Table 19). DHV titres attained were inversely related to the ages of the birds at the time of infection; $10^{5.0}$, $10^{4.60}$, $10^{3.0}$, $10^{2.60}$ or less and $10^{2.34}$ or less EID_{50} per ml. of the biles of ducklings infected when one, 11, 21, 31 and 41 days old respectively.

The exponential increase phase: The increases of DHV (Houchin) in the biles of infected birds occurred after a lag phase and were exponential (Table 19).

The plateau phase: The plateau phases of virus in the biles of infected ducklings, which were estimated as in the liver, consisted of irregular high virus titres. The estimated plateau phases were shorter in the birds infected when older: 120, 120, 66, 36 and 30 hours in the birds infected when one, 11, 21, 31 and 41 days old respectively. The regression was statistically significant ($r = -0.953$, $P < 0.05$). The highest virus titres in the biles were $10^{7.0}$, $10^{7.0}$, $10^{7.0}$, $10^{6.2}$, and $10^{6.5}$ EID_{50}/ml in birds infected when one, 11, 21, 31 and 41 days old respectively (Table 19).

The exponential decline phase: The exponential decline phases were longer than the exponential increase

phases (Table 19). Failure to recover virus from the biles was used to determine the end of the exponential decline phases which varied and were not related to the ages of ducklings at the time of infection; they ranged from the 216th to 312nd hour after inoculation.

The complete curve: The curves of DHV titres in the biles of all age groups of infected ducklings were skewed, the exponential increase phases being shorter than the exponential decline phases. When the time intervals were plotted on a logarithmic scale the curves became symmetrical and all the lines of best fit were significantly curvilinear (Table 20).

The peak titres in the livers of the birds infected when younger were higher than those of the birds infected when older (Table 37; Fig. 7).

The shapes of the curves of DHV growth in all biles tested were similar but the positions differed significantly (Table 21). The curves fell into two groups which were similar in shapes but differed in positions. The curves within each group were, however, similar in both shapes and positions (Tables 22, 23, 24; Fig. 8). The curves of DHV in ducklings infected when one and 11 days old were similar and were designated Group I. Similarly, all the curves of DHV in ducklings infected when older were in the Group II.

TABLE 19

TITRES* OF THE HOUCHIN STRAIN OF DHV IN THE BILES OF ONE- TO 41-DAY OLD DUCKLINGS KILLED 12 TO 312 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Age (day)				
	1	11	21	31	41
8	.	.	.	.	.
12	<1.0	<1.0	.	.	.
18	<1.0	<1.0	<1.0	<1.0	<1.0
24	5.00±0.00	4.60±0.40	<1.0 3.00±0.00	≤2.60±0.63	≤2.34±0.70
30	7.00±0.00	7.00±0.57	7.00±0.00	3.80±0.49	≤1.40±0.40
36	6.20±0.49	7.00±0.00	7.00±0.00	5.00±0.00	3.00±0.00
42	7.00±0.00	6.60±0.40	7.00±0.00	5.40±0.40	6.50±0.52
48	6.60±0.40	7.00±0.00	5.00±0.57	6.20±0.40	{3.80±0.40 4.60±0.40
72	.	5.00±0.00	5.40±0.40	4.60±0.40	5.00±0.76
96	5.00±0.00	5.00±0.00	4.60±0.40	3.20±0.49	3.30±0.70
120	.	.	3.00±0.00	2.20±0.49	3.00±0.00
144	4.60±0.40	4.20±0.49	3.00±0.00	3.00±0.00	3.00±0.00
168	≤2.06±0.78	3.00±0.00	.	≤2.00±0.29	≤2.90±0.64
216	<1.0	3.00±0.00	≤1.40±0.40	<1.0	≤1.40±0.40
264	<1.0	<1.0	.	.	<1.0
312	.	.	<1.0	.	.

* EID_{50} ± standard error, expressed as log 10/ml.

TABLE 20

CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE BILES OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Age (day)	Equation	Variance ratio	P
1	$Y = 50.07X - 14.39X^2 - 36.97$	25.92	<0.01
11	$Y = 45.78X - 13.02X^2 - 33.77$	15.34	<0.01
21	$Y = 46.24X - 12.96X^2 - 35.58$	6.86	<0.05
31	$Y = 40.38X - 11.41X^2 - 30.98$	11.22	<0.01
41	$Y = 40.55X - 11.11X^2 - 32.58$	8.62	<0.01

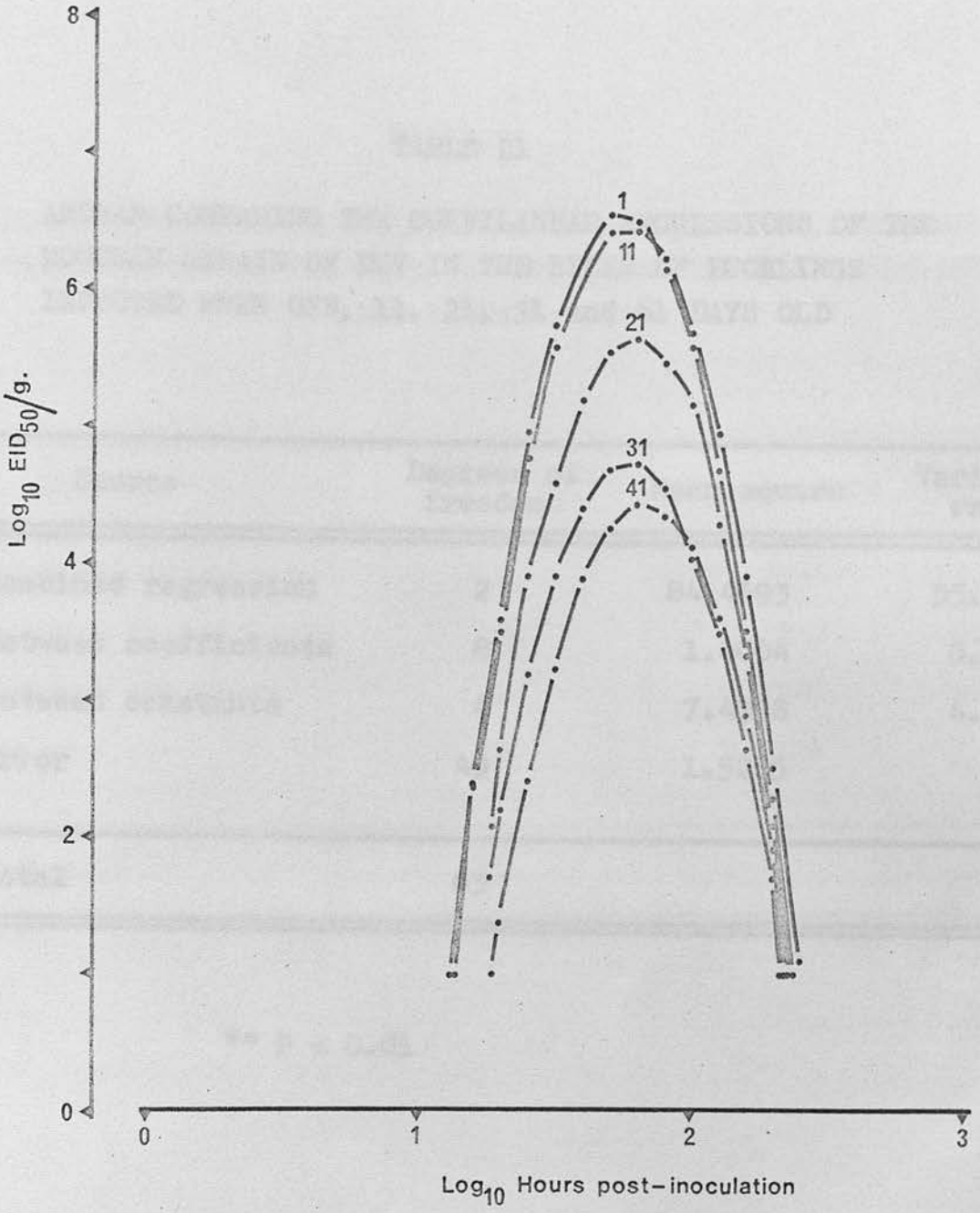


Figure 7. Growth curves of DHV (Houchin) in the biles of ducklings infected when 1 to 41 days old.

TABLE 21

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE BILES OF DUCKLINGS INFECTED WHEN ONE, 11, 21, 31 and 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	84.4493	55.32**
Between coefficients	8	1.0004	0.66
Between constants	4	7.4396	4.87**
Error	49	1.5265	
Total	63		

** $P < 0.01$

TABLE 22

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE BILES OF DUCKLINGS INFECTED WHEN ONE AND 11 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	55.7546	43.82**
Between coefficients	2	0.3237	0.25
Between constants	1	0.2247	0.18
Error	19	1.2724	
Total	24		

** $P < 0.01$

TABLE 23

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE BILES OF DUCKLINGS INFECTED WHEN 21, 31 AND 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	39.0404	23.14**
Between coefficients	4	0.4473	0.27
Between constants	2	3.0771	1.82
Error	30	1.6874	
Total	38		

** $P < 0.01$

TABLE 24

SIGNIFICANT GROUPS OF SIMILAR GROWTH CURVES, ECLIPSE PHASES AND PEAK TITRES OF THE HOUCHIN STRAIN OF DHV IN THE BILES OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Group	Age (day)	Equation	Eclipse phase	Peak titre ± standard error
I	1 & 11	$Y = 47.85X - 13.68X^2 - 35.30$	13	6.52±0.57
II	21, 31 & 41	$Y = 41.51X - 11.57X^2 - 32.30$	16	4.90±0.68

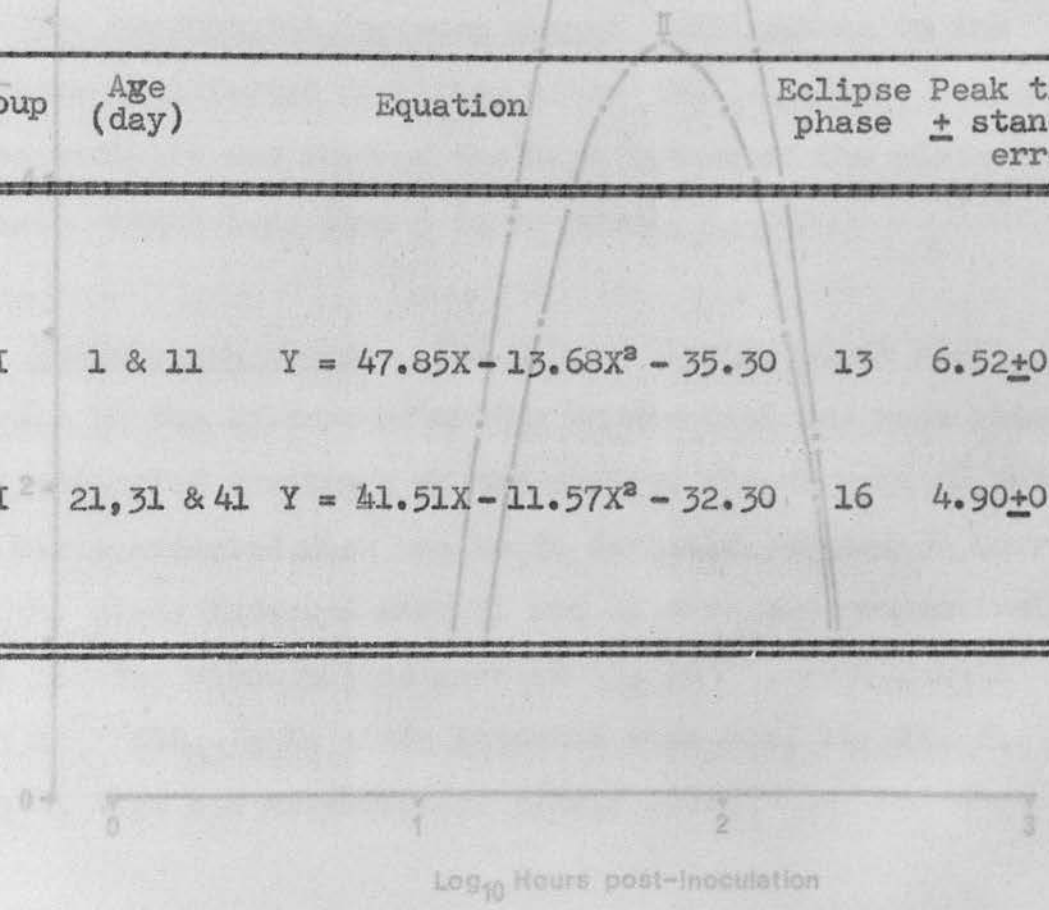


Figure 8. Significant groups of similar growth curves of DHV (Houchin) in the biles of ducklings infected when 1 to 41 days old.

I:- 1 & 11 days old.

II:- 21, 31 & 41 days old.

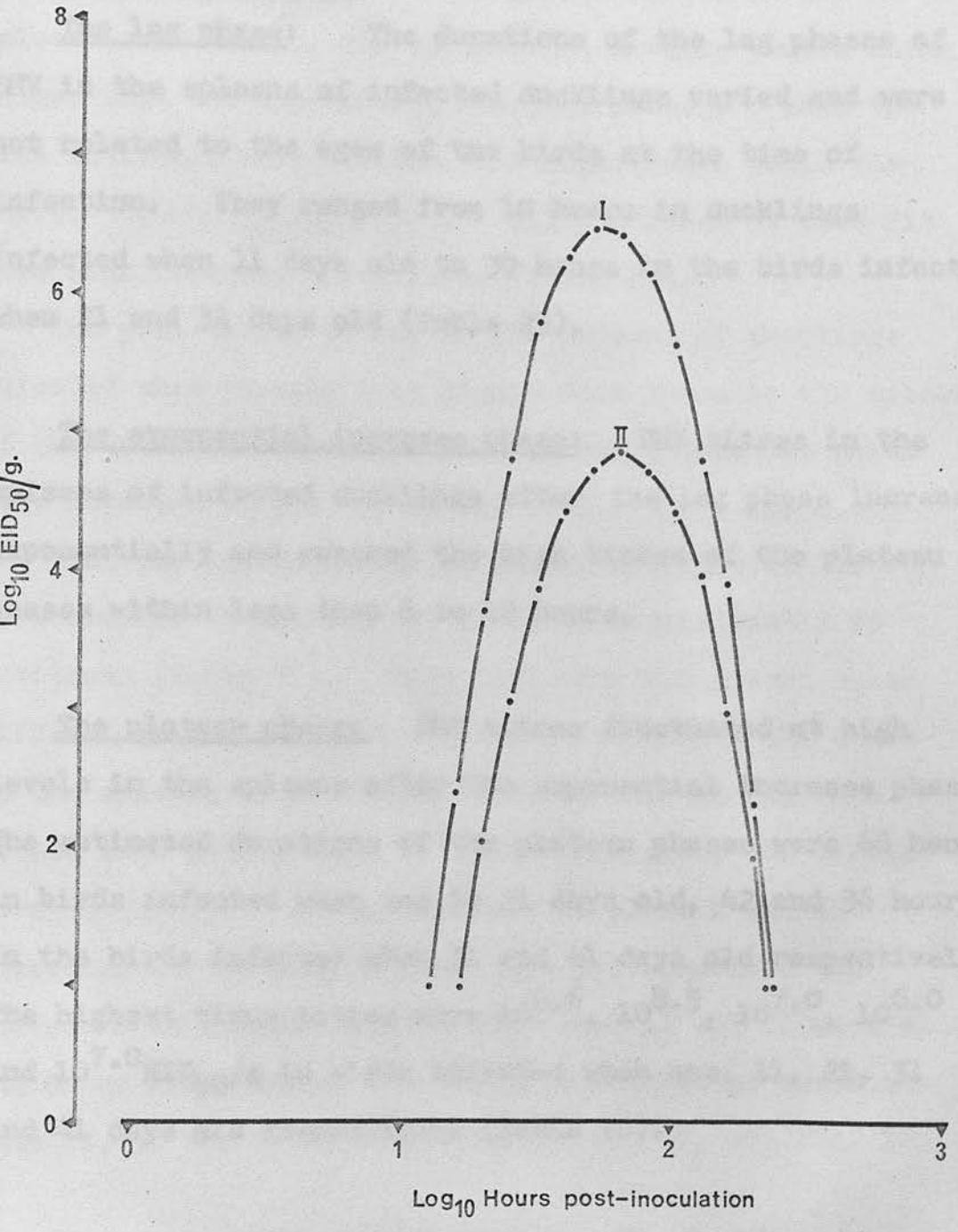


Figure 8. Significant groups of similar growth curves of DHV (Houchin) in the biles of ducklings infected when 1 to 41 days old.

I:- 1 & 11 days old.

II:- 21, 31 & 41 days old.

The Spleen

The lag phase: The durations of the lag phases of DHV in the spleens of infected ducklings varied and were not related to the ages of the birds at the time of infection. They ranged from 18 hours in ducklings infected when 11 days old to 30 hours in the birds infected when 21 and 31 days old (Table 25).

The exponential increase phase: DHV titres in the spleens of infected ducklings after the lag phase increased exponentially and reached the high titres of the plateau phases within less than 6 to 12 hours.

The plateau phase: DHV titres fluctuated at high levels in the spleens after the exponential increase phases. The estimated durations of the plateau phases were 66 hours in birds infected when one to 21 days old, 42 and 36 hours in the birds infected when 31 and 41 days old respectively. The highest virus titres were $10^{6.6}$, $10^{8.5}$, $10^{7.0}$, $10^{6.0}$ and $10^{7.0}$ EID_{50}/g in birds infected when one, 11, 21, 31 and 41 days old respectively (Table 25).

The exponential decline phase: The decline phases of DHV in the spleens of ducklings were not related to the ages of the birds at the time of infection and varied from less than 24 hours in ducklings infected when one and 31 days old to 72 hours in ducklings infected when 11 days old (Table 25).

The complete curve: The growth curves of DHV in the spleens were skewed, the exponential decline phases being longer than the exponential increase phases. When the time intervals were plotted in a logarithmic scale the curves became symmetrical. All the lines of best fit were significantly curvilinear (Table 26).

The peak titres of DHV in the spleens of ducklings infected when younger were higher than those in the spleens of the birds infected when older, except when the birds were infected at one day of age (Table 37; Fig. 9).

All the growth curves of DHV in the spleens tested were similar in shapes, but differed significantly in positions (Table 27). They fell into two groups which differed in position but overlapped such that the curve in the spleens of ducklings infected when 21 days old fitted into both groups. Within each group the curves were similar. The groups were labelled I and II according to the peak titres; Group I comprised the curves in ducklings infected when one, 11 and 21 days old, and Group II contained the curves in ducklings infected when 21, 31 and 41 days old (Tables 28, 29, 30; Fig. 10).

TABLE 25

TITRES* OF THE HOUCHIN STRAIN OF DHV IN THE SPLEENS OF ONE- TO 41-DAY OLD DUCKLINGS KILLED 8 TO 168 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Age (day)				
	1	11	21	31	41
8	.	<1.0	.	.	.
12	<1.0	<1.0	.	.	.
18	<1.0	≤2.90±0.49	<1.0	<1.0	<1.0
24	≤2.00±0.00	≤1.70±0.80	<1.0	<1.0	≤2.20±0.49
26	.	5.00±0.00	.	.	.
30	5.40±0.40	8.00±0.00	5.00±0.00	5.50±0.52	3.00±0.00
36	5.20±0.49	8.50±0.52	7.00±0.00	5.00±0.00	4.20±0.49
42	6.00±0.57	7.60±0.63	5.00±0.49	6.00±0.57	7.00±0.00
48	6.60±0.40	6.30±0.20	.	3.00±0.00	5.00±0.00
72	6.00±0.57	6.60±0.40	5.00±0.00	5.00±0.00	5.00±0.00
96	5.40±0.40	5.00±0.00	4.00±0.57	<1.0	≤1.40±0.40
120	<1.0	≤2.35±0.66	≤1.40±0.40	<1.0	<1.0
144	.	≤1.80±0.49	<1.0	.	.
168	.	<1.0	.		

* EID_{50} ± standard error, expressed as log 10/g.

TABLE 26

CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE SPLEENS OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Age (day)	Equation	Variance ratio	P
1	$Y = 56.17X - 16.89X^2 - 40.95$	7.46	<0.05
11	$Y = 45.33X - 13.95X^2 - 30.55$	11.29	<0.01
21	$Y = 56.40X - 16.94X^2 - 41.74$	6.25	<0.01
31	$Y = 89.91X - 26.99X^2 - 69.90$	5.65	<0.05
41	$Y = 95.51X - 28.49X^2 - 74.81$	10.78	<0.05

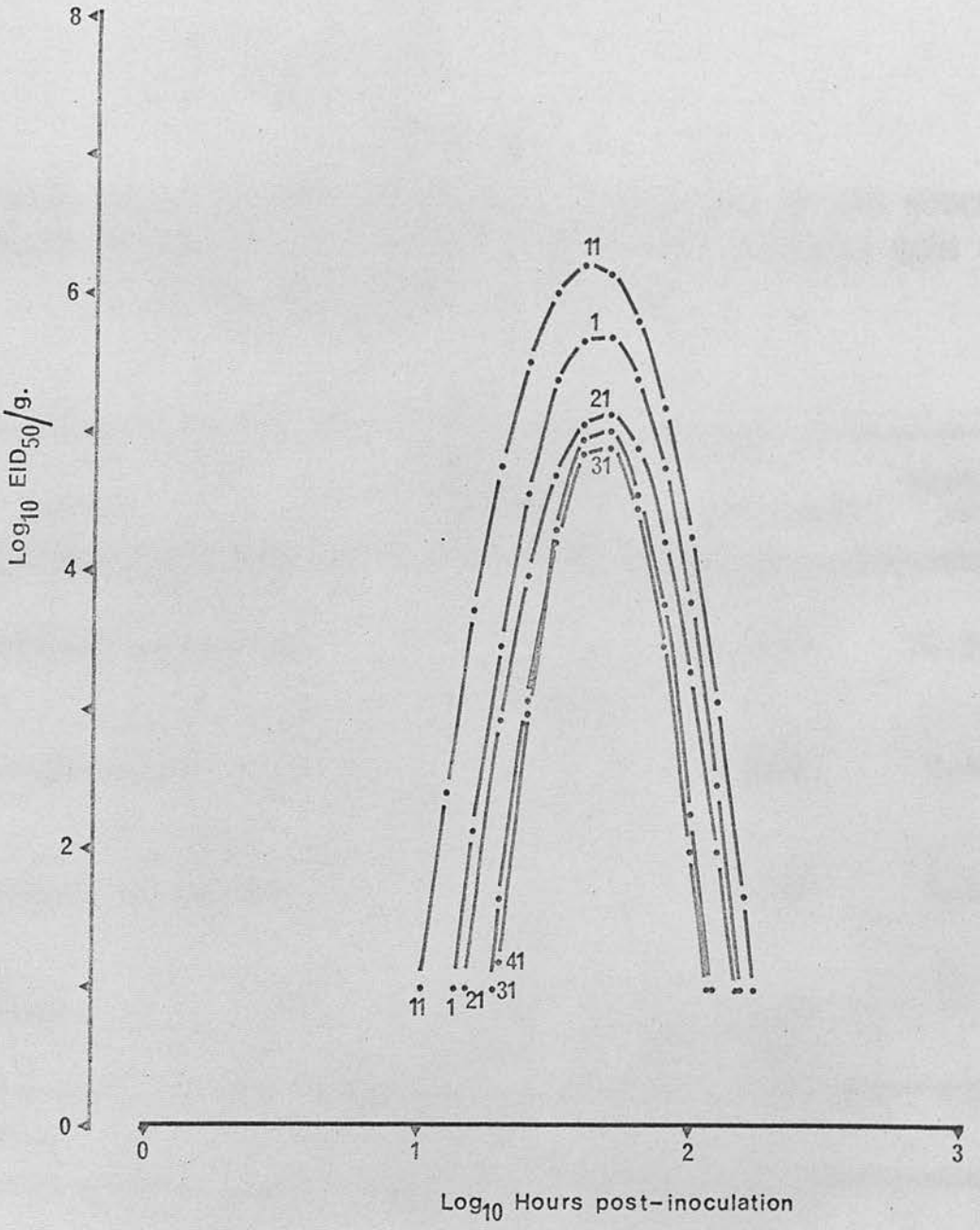


Figure 9. Growth curves of DHV (Houchin) in the spleens of ducklings infected when 1 to 41 days old.

TABLE 27

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE SPLEENS OF DUCKLINGS INFECTED WHEN ONE, 11, 21, 31 AND 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	76.8067	31.56**
Between coefficients	8	0.9822	0.40
Between constants	4	9.9708	4.10**
Error	40	2.4338	
Total	54		

** P < 0.01

TABLE 28

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE SPLEENS OF DUCKLINGS INFECTED ONE, 11 AND 21 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	59.9892	23.02**
Between coefficients	4	0.7550	0.29
Between constants	2	6.8431	2.63
Error	26	2.6056	
Total	34		

** $P < 0.01$

TABLE 29

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE SPLEEN OF DUCKLINGS INFECTED WHEN 21, 31 AND 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	38.6882	15.15**
Between coefficients	4	1.0422	0.41
Between constants	2	2.3149	0.91
Error	21	2.5540	
Total	29		

** $P < 0.01$

TABLE 30

SIGNIFICANT GROUPS OF SIMILAR GROWTH CURVES, ECLIPSE PHASES, AND PEAK TITRES OF THE HOUCHIN STRAIN OF DHV IN THE SPLEEN OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Group	Age (day)	Equation	Eclipse phase	Peak titre ± standard error
I	1,11 & 21	$Y = 47.27X - 14.37X^2 - 33.18$	12	5.69±0.56
II	21,31 & 41	$Y = 54.74X - 16.54X^2 - 40.58$	15	4.71±0.62

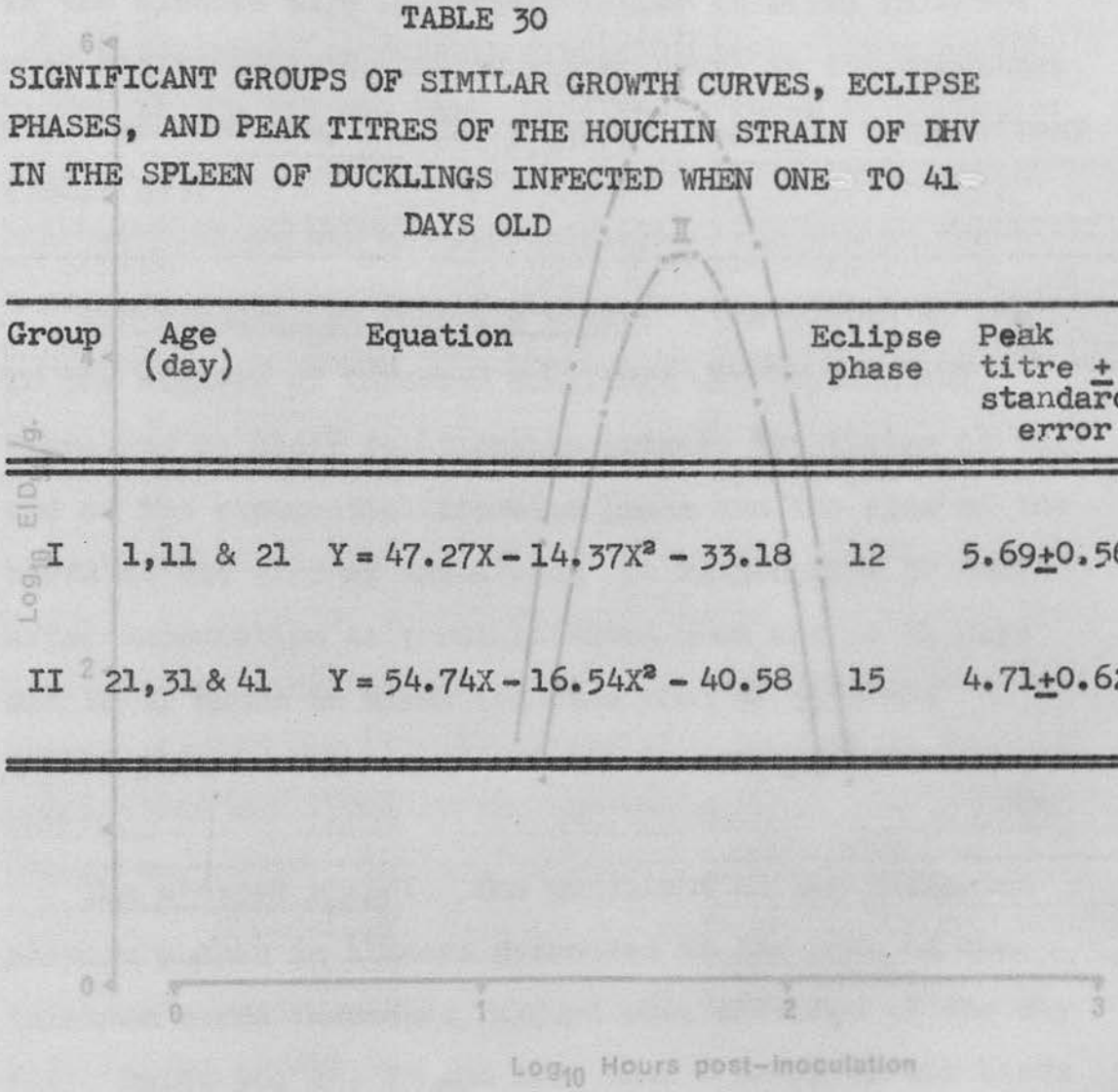


Figure 10. Significant groups of similar growth curves of DHV (Houchin) in the spleens of ducklings infected when 1 to 41 days old.

I:- 1, 11, & 21 days old.

II:- 21, 31, & 41 days old.

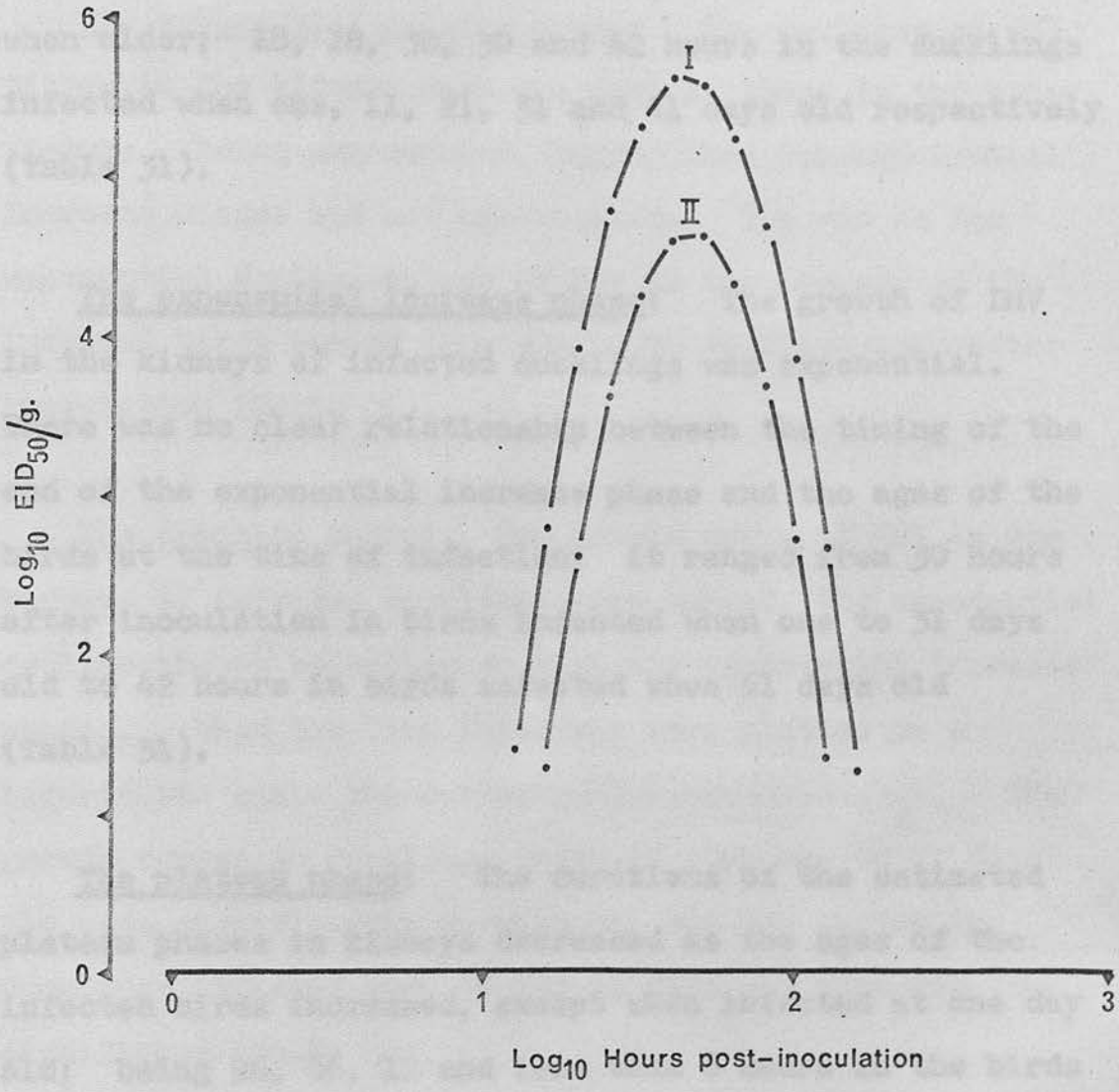


Figure 10. Significant groups of similar growth curves of DHV (Houchin) in the spleens of ducklings infected when 1 to 41 days old.

I:- 1, 11, & 21 days old.

II:- 21, 31, & 41 days old.

The Kidney

The lag phase: The durations of the lag phases of DHV in the kidneys were apparently longer in birds infected when older; 18, 18, 30, 30 and 42 hours in the ducklings infected when one, 11, 21, 31 and 41 days old respectively (Table 31).

The exponential increase phase: The growth of DHV in the kidneys of infected ducklings was exponential. There was no clear relationship between the timing of the end of the exponential increase phase and the ages of the birds at the time of infection; it ranged from 30 hours after inoculation in birds infected when one to 31 days old to 42 hours in birds infected when 41 days old (Table 31).

The plateau phase: The durations of the estimated plateau phases in kidneys decreased as the ages of the infected birds increased, except when infected at one day old; being 96, 66, 12 and less than 6 hours in the birds infected when 11, 21, 31 and 41 days old respectively (Table 31). There was no discernible plateau in ducklings infected when 41 days old. When ducklings were infected at one day of age, the estimated duration of the plateau was 66 hours. The highest titres of virus in the kidneys did not vary in ducklings infected when one, 11, 21 and 31 days old; being $10^{6.3}$, $10^{7.0}$, $10^{6.5}$ and $10^{7.0}$ EID_{50}/g respectively ($F_{[3,16]} = 3.13$, $P > 0.05$). The highest titre of the virus in ducklings infected when

41 days old was significantly lower at $10^{3.0}$ EID_{50}/g ($t = 14.718$, $P < 0.001$).

The exponential decline phase: The declines of DHV titres in the kidneys were the same as those in the other tissues being exponential, longer than the exponential increase phases and not age-related. The end of the exponential decline phases of DHV in the kidneys of the infected birds varied from the 120th to 216th hour after inoculation (Table 31).

The complete curve: The growth curves of DHV in the kidneys of infected ducklings were skewed, the exponential decline phases being longer than the exponential increase phases. When the time intervals were plotted on a logarithmic scale the curves became symmetrical. The growth curves in ducklings infected when one to 21 days old were significantly curvilinear but the curves in ducklings infected when 31 and 41 days old were not significant (Table 32).

The peak titres were inversely related to the ages of infected ducklings except in ducklings infected when one day old. The peak titre in the birds infected when 41 days old was much lower than the titres in the others (Fig. 11).

The curves in ducklings infected when one to 31 days old were similar both in shapes and positions (Table 33). When the curves in duckling infected when one to 41 days old were compared only the

shapes of the curves were the same; the positions differed significantly (Table 34). There were thus two groups of growth curves of DHV in the kidneys of birds infected when one to 41 days old. The growth curve of virus in birds infected when 41 days old was in one group and all the rest were in the second group (Table 35; Fig. 12).

TABLE 31

TITRES* OF THE HOUCHIN STRAIN OF DHV IN THE KIDNEYS OF ONE- TO 41-DAY OLD DUCKLINGS KILLED 8 TO 216 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Age (day)				
	1	11	21	31	41
8	.	<1.0	.	.	.
12	<1.0	<1.0	.	.	.
18	≤2.90±0.69	≤1.75±0.52	.	.	.
24	≤2.20±0.69 3.00±0.69	3.40±0.40 3.70±0.80	<1.0	<1.0	.
26	.	5.15±0.40	.	.	.
30	5.30±0.42	6.30±0.20	5.00±0.00	7.00±0.00	.
32	.	5.40±0.40	.	.	.
34	.	5.20±0.69	.	.	.
36	6.20±0.63	7.00±0.00	6.50±0.26	5.00±0.00	<1.0
40	.	5.50±0.52	.	.	.
42	6.20±0.49	5.40±0.49	4.20±0.49	6.00±0.59	3.00±0.00
46	.	5.80±0.49	.	.	.
48	6.30±0.32	6.70±0.35	4.20±0.85 5.00±0.00	3.00±0.00	≤2.20±0.49
72	3.80±0.49	6.90±0.42	5.20±0.49 5.00±0.00	3.00±0.00	≤2.20±0.49
96	5.00±0.00	5.40±0.40	4.00±0.57	.	≤1.40±0.40
120	3.00±0.00	5.40±0.40	≤1.80±0.49	≤1.40±0.40	<1.0
144	3.00±0.00	≤1.40±0.40	≤1.80±0.49	≤2.60±0.40	.
168	<1.0	<1.0	≤2.30±0.69	<1.0	.
216	<1.0	.	<1.0	.	.

* EID_{50} ± standard error, expressed as log 10/g.

TABLE 32

CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE KIDNEYS OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Age (day)	Equation	Variance ratio	P
1	$Y = 40.79X - 11.86X^2 - 29.68$	16.05	<0.01
11	$Y = 38.09X - 11.41X^2 - 26.02$	17.87	<0.01
21	$Y = 50.30X - 14.32X^2 - 39.25$	8.39	<0.01
31	$Y = 40.57X - 12.15X^2 - 29.33$	2.03	>0.05
41	$Y = 62.00X - 17.50X^2 - 52.47$	1.63	>0.05

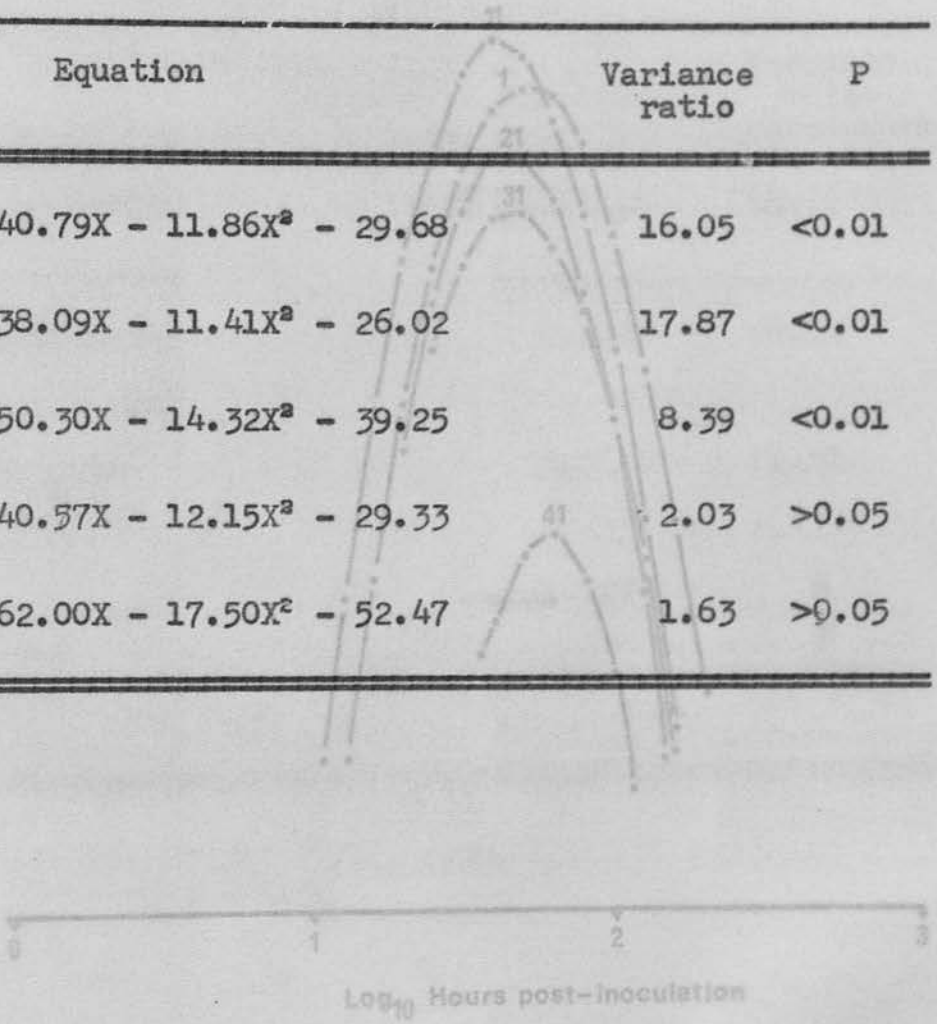


Figure 11. Growth curves of DHV (Houchin) in the kidneys of ducklings infected when 1 to 41 days old.

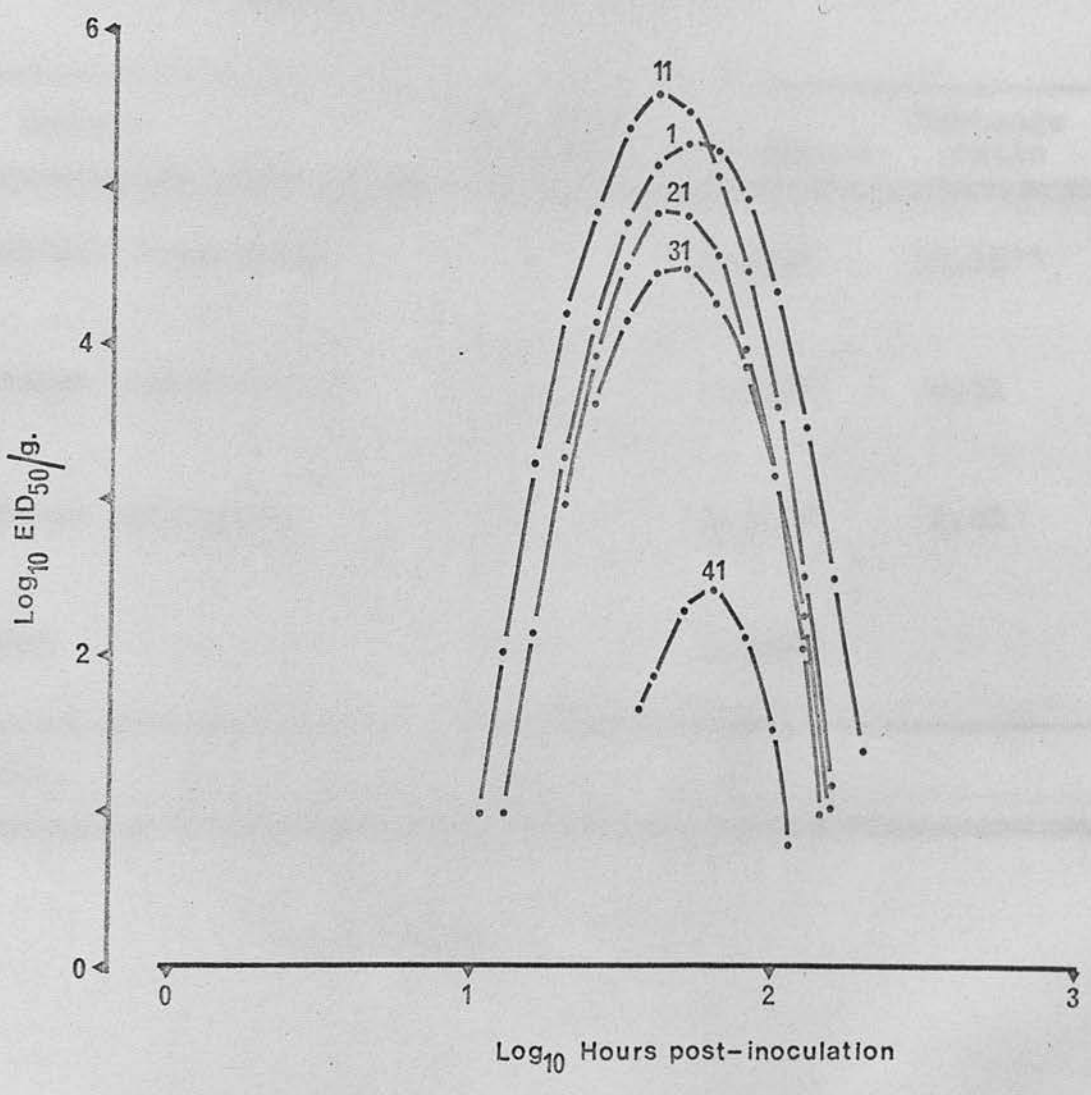


Figure 11. Growth curves of DHV (Houchin) in the kidneys of ducklings infected when 1 to 41 days old.

TABLE 33

ANOVAR COMPARING THE CURVILINEAR REGRESSION OF THE HOUCHIN STRAIN OF DHV IN THE KIDNEYS OF DUCKLINGS INFECTED WHEN ONE, 11, 21 AND 31 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	60.7358	35.16**
Between coefficients	6	0.5331	0.31
Between constants	3	4.5130	2.61
Error	43	1.7274	
Total	54		

** $P < 0.01$

TABLE 34

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE HOUCHIN STRAIN OF DHV IN THE KIDNEYS OF DUCKLINGS INFECTED WHEN ONE, 11, 31 AND 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	52.6793	31.68**
Between coefficients	8	0.4540	0.28
Between constants	4	11.6949	7.09**
Error	46	1.6481	
Total	60		

** $P < 0.01$

TABLE 35

SIGNIFICANT GROUPS OF SIMILAR GROWTH CURVES, ECLIPSE PHASES AND PEAK TITRES OF THE HOUCHIN STRAIN OF DHV IN THE KIDNEYS OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

Group	Age (day)	Equation	Eclipse phase	Peak titre ± standard error
I	1,11,21 & 31	$Y = 36.31X - 10.83X^2 - 25.27$	11	5.16 ± 0.63
II	41	$Y = 62.00X - 17.50X^2 - 52.47$	30	2.45 ± 1.33

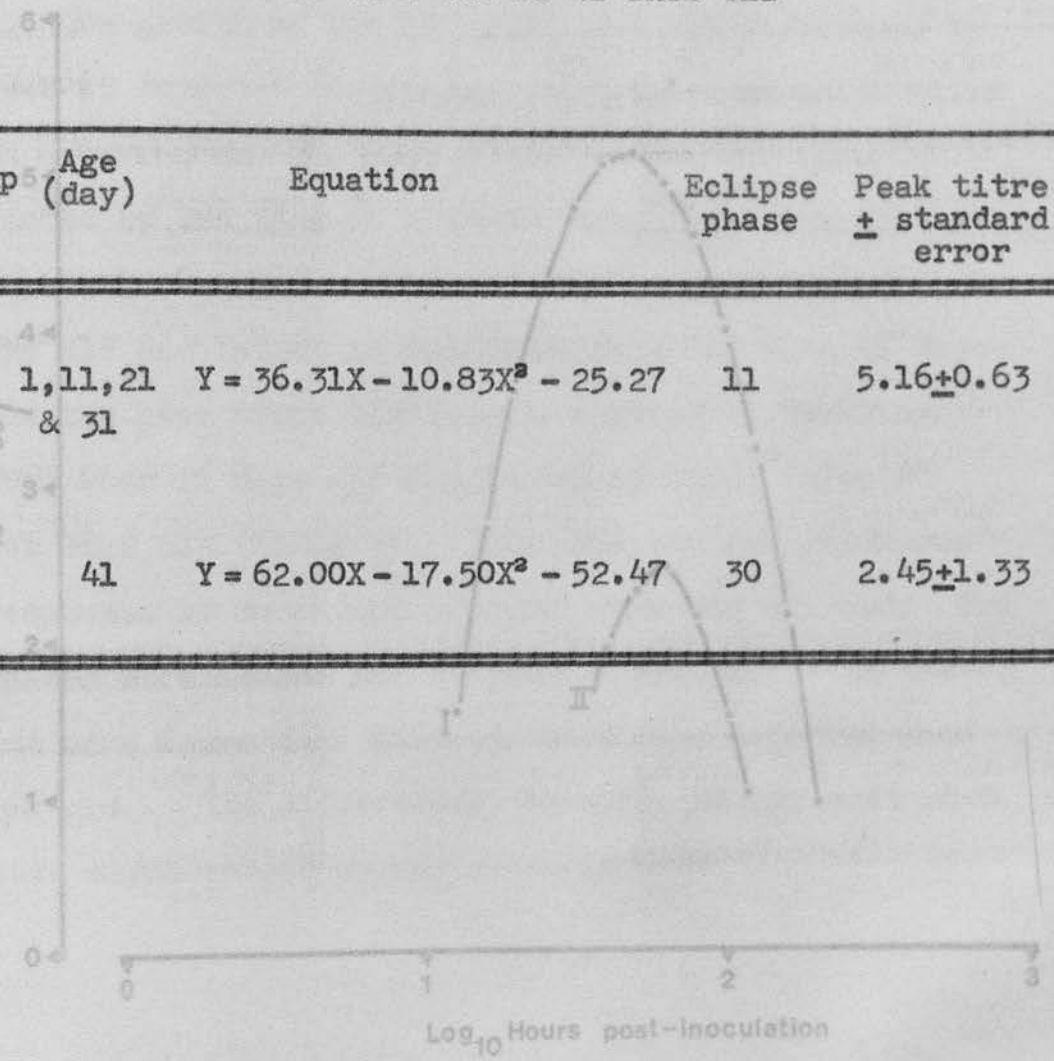


Figure 12. Significant groups of similar growth curves of DHV (Houchin) in the kidneys of ducklings infected when 1 to 41 days old.

I:- 1, 11, 21 & 31 days old.

II:- 41 days old.

The Comparison Between Tissues

The growth curves of DHV in the tissues of ducklings infected when one to 41 days old were compared. The shapes of the curves were similar, but the positions differed significantly (Table 36). In other words, the rate of the growth of DHV (Houchin) was not influenced by the ages of infected ducklings. The times at which virus growth occurred and the peak virus titres were apparently influenced by the ages of infected ducklings such that the virus growth occurred earliest in ducklings infected when 11 days old and latest in ducklings infected when 41 days old and the peak virus titres were highest in ducklings infected when 11 days old and lowest in birds infected when 41 days old (Table 37). An apparent exception was the responses of ducklings infected when one day old; the lag phases were longer and the peak virus titres in the tissues were lower than those in ducklings infected when 11 days old. The differences, however, disappeared when complete virus growth curves were compared.

TABLE 36

ANOVAR COMPARING THE CURVILINEAR REGRESSIONS OF THE GROWTH CURVES OF THE HOUCHIN STRAIN OF DHV IN THE LIVERS, BILES, KIDNEYS AND SPLEENS OF DUCKLINGS INFECTED WHEN ONE, 11, 21, 31, AND 41 DAYS OLD

Source	Degrees of freedom	Mean square	Variance ratio
Combined regression	2	296.9007	163.19**
Between coefficients	38	2.2285	1.22
Between constants	19	14.1738	7.79**
Error	200	1.8194	
Total	259		

** P < 0.01

TABLE 37

CALCULATED PARAMETERS OF THE HOUCHIN STRAIN OF DUCK HEPATITIS VIRUS IN TISSUES OF DUCKLINGS INFECTED WHEN ONE TO 41 DAYS OLD

(i) LAG PHASES (Hours)

Age when infected	Tissues			
	Liver	Bile	Spleen	Kidneys
1	11	13	13	13
11	9	13	10	11
21	10	15	15	17
31	10	16	19	14
41	18	19	20	30
$r_{[1-41]}$	0.650	0.953*	0.874	0.772
$r_{[11-41]}$	0.831	0.982*	0.965*	0.833

(ii) PEAK TITRES ($Log_{10}EID_{50}/g$)

Age when infected	Tissues			
	Liver	Bile	Spleen	Kidneys
1	7.59	6.59	5.74	5.38
11	7.73	6.47	6.28	5.77
21	6.49	5.65	6.04	4.91
31	6.18	4.75	4.97	4.51
41	5.41	4.44	4.52	2.45
$r_{[1-41]}$	-0.955*	-0.976**	-0.800	-0.866
$r_{[11-41]}$	-0.972*	-0.982*	-0.972*	-0.950*

* $P < 0.05$ ** $P < 0.01$

OTHER AGE EFFECTS

Virus in Pancreas

In ducklings infected when one to 21 days old, DHV (Houchin) appeared in the pancreases after it had occurred in the livers and it persisted as long or longer than in the livers (38A, 38B, 38C). In older birds, the persistence of virus in the pancreases was, however, shorter than in the livers (38D, 38E).

Virus Excretion

DHV (Houchin) was recovered from cloacal swabs serially taken from ducklings infected when one and 11 days old over a longer period than from birds infected when older (Table 39). The virus was recovered from only one cloacal swab out of 18 tested samples taken from ducklings infected when 21 days old.

DHV was isolated from cloacal swabs when virus was isolated from bile. It was not isolated from cloacal swabs when it was not present in bile. No virus was found 312 hours after inoculation.

Oral excretion, on the other hand, was limited. DHV was recovered from a small number of samples taken from ducklings infected when 11 days old but all the oral swabs taken from the other age groups were negative (Table 40).

Duckling Mortality

The mortality rates in ducklings infected when they were one, 11, 21, 31 and 41 days old by the oral route differed significantly ($\text{Chi-square}_{[4]} = 46.554, P < 0.001$)

such that the rates decreased as the ages of the ducklings at the time of infection increased, dropping from 70 per cent when 11-day old ducklings were infected to 5 per cent when 31-day old ducklings were infected (Table 41). None out of 19 ducklings infected when 41 days old died. The regression of mortality rate on age was significantly linear ($r = -0.910$, $P < 0.05$).

An exception was the mortality rate in ducklings, infected when they were one day old; the rate was lower than that of ducklings infected when 11 days old (Table 41) but the difference was not significant (Chi-square = 0.065, $P > 0.70$).

Virus Titres of Livers from Dead Ducklings

Livers were collected from ducklings that died within 48 hours of oral administration of DHV (Houchin) and suspensions of the livers were titrated in 8-day old chicken embryos. All the titres were similar (Table 42). There was no relationship between the virus titres in the livers of ducklings which died from DVH and the ages of the ducklings at the time of infection ($r = -0.202$, $P > 0.05$). Virus titres in the livers of ducklings that died or were killed after infection when one or 21 days were similar ($t_{[8]} = 1.883$, $P > 0.05$ and $t_{[8]} = 1.596$, $P > 0.05$ respectively).

Virus Growth in Duck Embryos

Death more than 24 hours after inoculation, was used as the parameter for assessing the virus titres of duck embryos. The titres in duck embryos infected when 8 to 14 days old were similar ranging from $10^{7.3}$ to $10^{7.0}$ LD_{50} per ml, but thereafter dropped to $10^{5.80}LD_{50}$ per ml in embryos infected when 16 and 18 days old (Table 43). The regression of DHV titres on age in duck embryos infected when 8 to 18 days old was linear and significant ($r = -0.883$, $P < 0.01$).

The virus titres in ducklings infected one day after hatching was $10^{4.30}LD_{50}$ per ml. The titre fitted the regression of titres in duck embryos on age and the new regression was linear and significant ($r = -0.965$, $P < 0.01$) (Fig. 13).

Virus Growth in Chicken Embryos

Titres of the duckling virulent Houchin strain of DHV fell as the ages of chicken embryos at the time of inoculation increased; from $10^{7.0}EID_{50}$ per ml of the inoculum in the embryos inoculated when 7 days old to less than $10^{1.0}EID_{50}$ per ml in the embryos inoculated when 12 days old (Table 44). The fall was linear and significant ($r = -0.974$, $P < 0.01$) (Fig. 14). In contrast, titres of the chicken embryo-adapted H_{56} strain of DHV, were similar in all ages of embryos tested ($F_{[5,24]} = 0.32$, $P > 0.05$).

TABLE 38A

THE PRESENCE OF THE HOUCHIN STRAIN OF DHV IN ORGANS OF ONE-DAY OLD DUCKLINGS KILLED 8 TO 312 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Liver	Kidney	Spleen	Pancreas
8	-	-	-	-
12	+	-	-	-
18	+	+	-	+
24	+	+	+	+
30	+	+	+	+
36	+	+	+	+
48	+	+	+	+
72	+	+	+	+
96	+	+	+	+
120	+	+	-	+
144	+	+	-	+
168	+	-	-	+
216	+	-	-	+
264	+	•	•	+
312	-	•	•	-

+ virus present

- no virus present

TABLE 38B

THE PRESENCE OF THE HOUCHIN STRAIN OF DHV IN ORGANS OF 11-DAY OLD DUCKLINGS KILLED 8 TO 264 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Liver	Kidney	Spleen	Pancreas
8	-	-	-	-
12	+	-	-	-
18	+	+	+	+
24	+	+	+	+
30	+	+	+	+
36	+	+	+	+
48	+	+	+	+
72	+	+	+	+
96	+	+	+	+
120	+	+	+	+
144	+	+	+	+
168	+	-	-	+
216	+	-	-	+
264	-			-

\+ virus present

\- no virus present

TABLE 38C

THE PRESENCE OF THE HOUCHIN STRAIN OF DHV IN ORGANS OF 21-DAY OLD DUCKLINGS KILLED 8 TO 264 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Liver	Kidney	Spleen	Pancreas
8	-	.	.	.
12	-	.	.	.
18	+	-	-	-
24	+	-	-	-
30	+	+	+	+
36	+	+	+	+
48	+	+	+	+
72	+	+	+	+
96	+	+	+	+
120	+	+	+	+
144	+	+	-	+
168	+	+	-	+
216	+	-	-	+
264	+	-	.	+
312	-	.	.	+
360	-	.	.	+

+ virus present

- no virus present

TABLE 38D

THE PRESENCE OF THE HOUCHIN STRAIN OF DHV IN ORGANS OF 31-DAY OLD DUCKLINGS KILLED 12 TO 216 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Liver	Kidney	Spleen	Pancreas
8	•	•	•	•
12	-	•	•	•
18	+	•	•	•
24	+	-	-	
30	+	+	+	-
36	+	+	+	+
48	+	+	+	+
72	+	+	+	+
96	+	+	-	+
120	+	+	•	+
144	+	+	•	+
168	+	-	•	-
216	-	•	•	

+ virus present

- no virus present

TABLE 38E

THE PRESENCE OF THE HOUCHIN STRAIN OF DHV IN ORGANS OF 41-DAY OLD DUCKLINGS KILLED 12 TO 264 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Liver	Kidney	Spleen	Pancreas
8	•	•	•	•
12	–	•	–	•
18	–	–	–	–
24	+	–	+	–
30	+	–	+	–
36	+	–	+	+
48	+	+	+	+
72	+	+	+	+
96	+	+	+	–
120	+	–	–	–
144	+	–	–	–
168	+	–	–	•
216	+	–	•	•
264	–	•	•	•

\+ virus present

– no virus present

TABLE 39

TITRES* OF THE HOUCHIN STRAIN OF DHV FROM CLOACAL SWABS OF ONE- TO 41-DAY OLD DUCKLINGS KILLED 2 TO 360 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Age (Day)				
	1	11	21	31	41
2	.	<0.40	<0.40	.	<0.40
4	.	.	<0.40	.	<0.40
8	.	.	<0.40	.	<0.40
12	<0.40	<0.40	<0.40	.	<0.40
18	<0.40	<0.40	<0.40	<0.40	<0.40
24	1.20±0.49	≤0.90±0.24	<0.40	<0.40	<0.40
30	≤0.80±0.40	1.10±0.24	<0.40	1.60±0.49	<0.40
36	≤0.80±0.40	1.50±0.24	<0.40	3.10±0.49	1.75±0.52
42	≤0.80±0.40	<0.70	<0.40	<0.40	2.90±0.24
48	1.20±0.49	2.25±0.26	<0.40	1.60±0.49	1.20±0.49
72	<0.40	1.00±0.29	1.20±0.49	2.00±0.00	≤0.80±0.40
96	≤0.96±0.56	≤0.85±0.20	<0.40	<0.40	<0.40
120	1.75±0.52	≥2.30±0.00	<0.40	<0.40	<0.40
144	<0.40	1.10±0.40	<0.40	≤0.80±0.40	<0.40
168	<0.40	≥1.50±0.00	<0.40	<0.40	<0.40
216	≤0.80±0.40	1.50±0.49	<0.40	.	.
264	<0.40	1.60±0.49	<0.40	<0.40	<0.40
312	<0.40	<0.40	<0.40	.	.
360	.	.	.	.	<0.40

* EID_{50} ± standard error, expressed as log 10/ml.

TABLE 40

TITRES* OF THE HOUCHIN STRAIN OF DHV FROM ORAL SWABS OF ONE- TO 41-DAY OLD DUCKLINGS KILLED 2 TO 384 HOURS AFTER ORAL INOCULATION

Hours after inoculation	Age (Day)				
	1	11	21	31	41
2	.	<0.40	<0.40	.	<0.40
4	.	<0.40	<0.40	.	<0.40
8	.	<0.40	.	.	<0.40
12	<0.40	<0.40	<0.40	.	<0.40
18	<0.40	<0.40	<0.40	<0.40	<0.40
24	<0.40	1.10±0.24	<0.40	.	<0.40
30	<0.40	1.00±0.29	<0.40	<0.40	<0.40
36	<0.40	1.10±0.20	<0.40	<0.40	<0.40
42	<0.40	1.50±0.24	<0.40	<0.40	<0.40
48	<0.40	≤0.90±0.24	<0.40	<0.40	<0.40
72	<0.40	1.00±0.29	<0.40	<0.40	<0.40
96	<0.40	<0.75	<0.40	.	<0.40
120	<0.40	.	<0.40	<0.40	<0.40
144	<0.40	<0.75	<0.40	.	<0.40
168	<0.40	.	<0.40	<0.40	<0.40
216	<0.40	<0.40	<0.40	.	<0.40
264	<0.40	.	<0.40	.	<0.40
312	<0.40	≤0.80±0.40	.	.	.
360	.	.	.	.	.
384	.	≤0.80±0.40	.	.	.

* EID_{50} ± standard error, expressed as log 10/ml.

TABLE 41

MORTALITY IN DUCKLINGS AFTER ORAL INOCULATION WITH THE HOUCHIN STRAIN OF DHV

Age (day)	Number inoculation	Number died	Case mortality (%)	Mean day of death ± standard error
1	49	31	63	2.42±0.14
11	20	14	70	2.29±0.61
21	19	3	16	3.00±0.58
31	20	1	5	3.00
41	19	0	0	.

TABLE 42

TITRES* OF THE HOUCHIN STRAIN OF DUCK HEPATITIS VIRUS IN LIVERS OF DUCKLINGS WHICH DIED WITHIN 48 HOURS OR KILLED AFTER ORAL INOCULATION

Age (day)	Titre at death	Peak titre when killed
1	6.17±0.66	7.80±0.56
7	7.30±0.63	ND
9	7.00±0.00	ND
21	6.20±0.49	7.40±0.57

* EID_{50} ± standard error, expressed as log 10/g.

ND - Not done.

TABLE 43

TITRES* OF THE HOUCHIN STRAIN OF DUCK HEPATITIS VIRUS IN DUCK EMBRYOS AFTER ALLANTOIC INOCULATION

Age (day)	Titres*
8	7.32±0.39
9	7.00±0.00
10	7.00±0.00
11	7.00±0.00
12	7.00±0.00
14	7.00±0.00
16	5.80±0.49
18	5.80±0.49
28**	4.30±0.45

* LD_{50} ± standard error, expressed as log 10/g.

** one day after hatching.

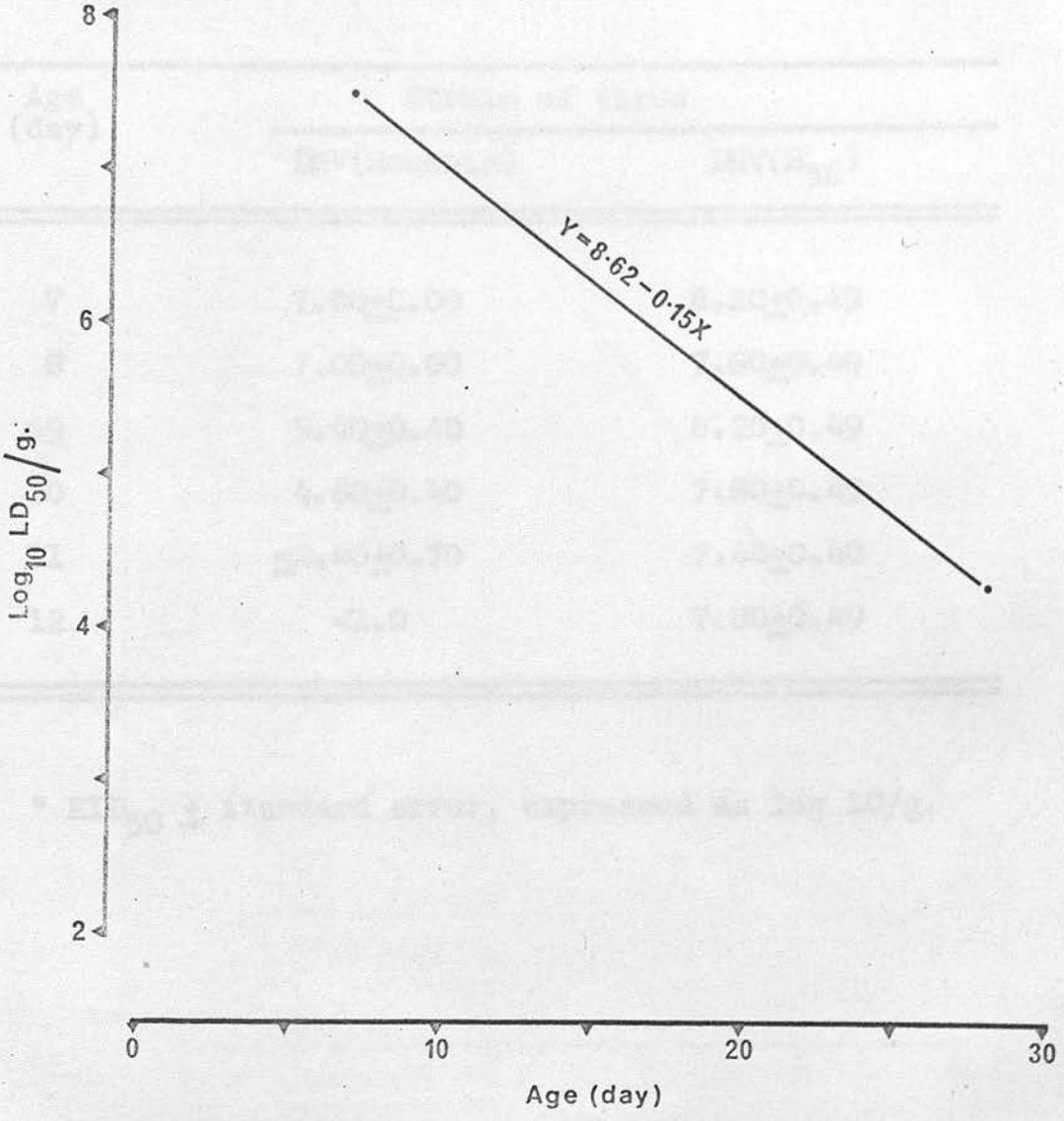


Figure 13. The linear regression of the titres of DHV (Houchin) in duck embryos infected when 8 to 28 days old.

TABLE 44

TITRES* OF THE HOUCHIN AND H_{56} STRAINS OF DHV IN CHICKEN EMBRYOS AFTER ALLANTOIC INOCULATION

Age (day)	Strain of virus	
	DHV(Houchin)	DHV(H_{56})
7	7.00±0.00	8.20±0.49
8	7.00±0.00	7.80±0.49
9	5.40±0.40	8.20±0.49
10	4.60±0.40	7.80±0.49
11	≤2.40±0.70	7.40±0.40
12	<1.0	7.80±0.49

* EID_{50} ± standard error, expressed as log 10/g.

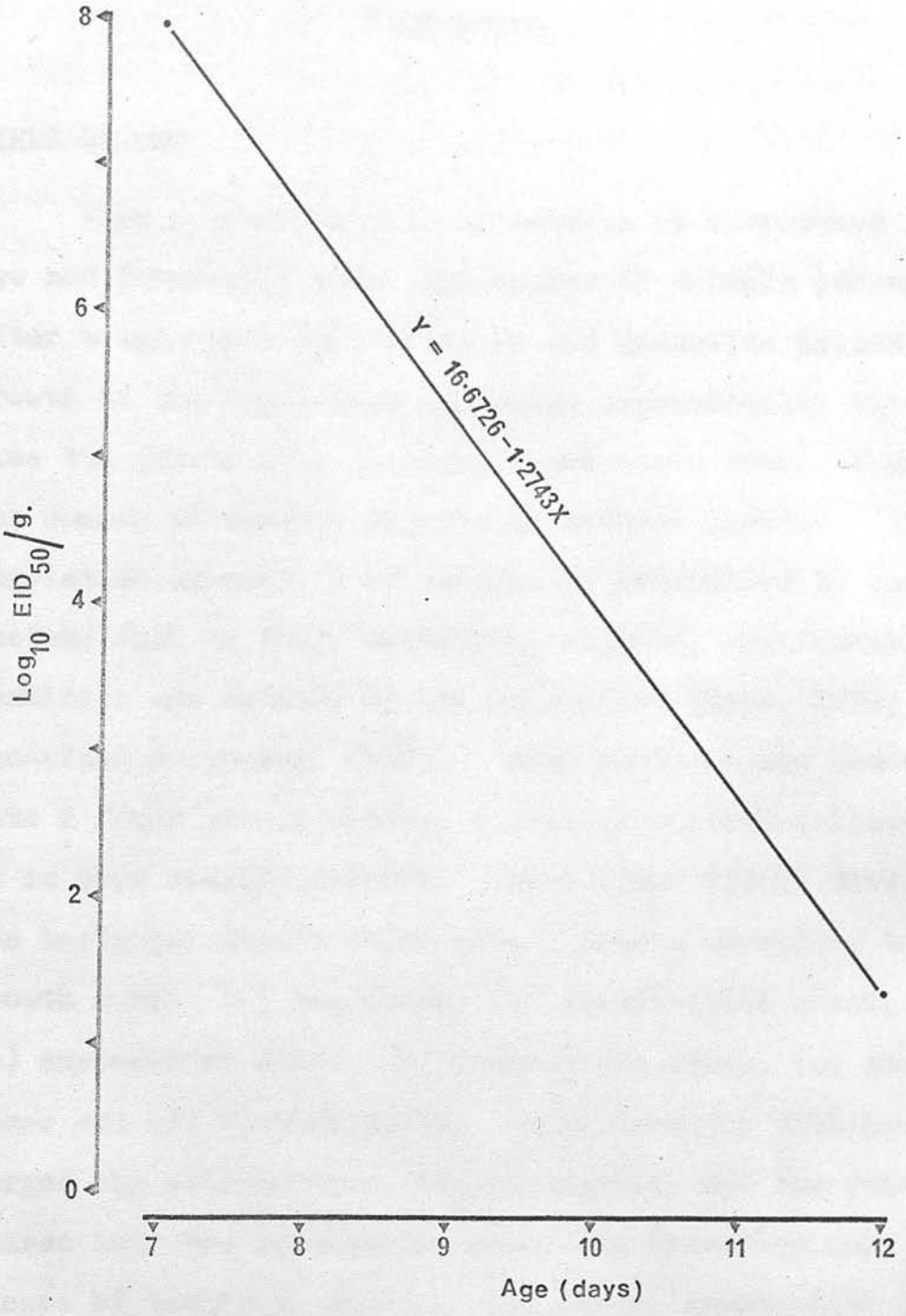


Figure 14. The linear regression of the titres of DHV (Houchin) in chicken embryos infected when 7 to 12 days old.

DISCUSSION

VIRUS GROWTH

When a breeding pair of animals is introduced into a new and favourable area, the number of animals increases after a lag phase equivalent to the gestation period. The growth of the population increases exponentially but in time the growth rate is checked and slows down. Thereafter the number of animals fluctuates between limits. The regulation of numbers of animals is controlled by complex factors such as food, predation, disease, environmental condition and density of the population (Lack, 1954; MacArther & Connell, 1966). When bacteria are inoculated into a fresh growth medium, a similar pattern follows but it is more readily defined. Thus Monod (1949) divided the bacterial growth curve into 6 phases according to the growth rate: (a) lag phase, (b) acceleration phase, (c) exponential phase, (d) retardation phase, (e) stationary phase and (f) decline phase. More recently Wilkinson (1973) merged the acceleration, the exponential and the retardation phases into the exponential phase and described only 4 stages of bacterial growth; lag phase, exponential phase, stationary phase and decline phase.

The dynamics of the growth of viruses has interested many workers over the years and several reviews have been published, that by Fenner (1968) being outstanding and comprehensive. He divided growth cycles of virus in infected cells of cell cultures into a number of steps:

(a) attachment, (b) penetration, which may involve engulfing and uncoating, (c) eclipse and biosynthesis of viral components and (d) viral assembly and ultimate release as new virions.

After specific attachment of the virion to a virus receptor of a cell wall, penetration which includes engulfment and uncoating begins. Most animal viruses are believed to be taken up into the cell by a process of phagocytosis sometimes called viropexis. More recently Dales (1973) classified mechanisms of penetration into viropexis, viropexis and fusion, fusion, and other mechanisms. The viral nucleic acid is released into the cell by a process of uncoating which occurs as soon as the virion is enclosed within a phagocytic vesicle. In one example of a picornavirus, namely, poliovirus, the viral capsid is modified on contact with the receptors on the cell-membrane of susceptible cells, either before or after engulfment. In other words, viral nucleic acid escapes from the nucleocapsid at the cell membrane.

Completion of penetration is marked by the eclipse of infectivity of the virion and subsequent production of separate viral components before assembly and maturation. The eclipse of infectivity is judged by assays for the virions and not for infective nucleic acid which may be still demonstrable in the infected cells. Assembly and maturation of picornavirus occurs in the cytoplasm of infected cells. Release of picornavirus, after viral maturation, probably occurs through destruction of cytoplasmic membranes.

The release of new virus particles marks the beginning of the exponential phase during which the number of virus particles increases rapidly. Release is controlled by various factors such as the types of viruses and types and numbers of infected cells. Recent quantitative information on the number of new virus particles released by cells is scanty. Influenza B viruses had longer lag phases and lower virus yields than influenza A virus (Henle et al., 1947); when a dose of one ID_{50} of influenza A virus was inoculated into the allantoic cavity of a chicken embryo the average yield of virus was 63 ID_{50} after a lag phase of 6 hours whereas when a dose of one ID_{50} of influenza B virus was similarly inoculated the yield was 36 ID_{50} after a lag phase of 9 hours. About 100 to 200 infective doses of poliovirus were released per cell in monkey kidney cell cultures within half an hour after the end of lag phase (Lwoff et al., 1955 a, b).

The size of infected cells also influenced virus yield; Dunnebacke and Reaume (1958) studied the yield of poliovirus and found that the average virus yield per cell was higher in larger cells.

GROWTH OF DUCK HEPATITIS VIRUS

DHV was first described in 1950 by Levine and Fabricant. Surprisingly, there are no reports of the growth of wild virulent strains of the virus in ducklings. Hwang and Dougherty (1964) measured the concentration of

virus of two virulent field strains and two attenuated chicken embryo-passaged strains in seven tissues of ducklings inoculated intramuscularly when newly hatched. Ducklings infected with virulent viruses died about 48 hours later. Ducklings infected with the attenuated viruses were killed at 48 hours after inoculation. Virus concentrations in tissues from ducklings infected with the virulent strains were higher than those from ducklings infected with the chicken embryo-passaged strains. Virus titres of the virulent strains were highest in the liver and lowest in the brain. In our studies, similar DHV (Houchin) titres were attained from tissues of ducklings infected when one and 11 days old and killed at 48 hours after inoculation.

Data on the growth curve of a chicken embryo-passaged strain of DHV are available. Barinsky and Tsypkin (1966) examined liver, spleen, brain and bone marrow of the ribs of ducklings infected when one day old with a 13 chicken embryo-passaged DHV. Tissues were collected 6, 12, 24, 48 and 72 hours after inoculation with 0.5 ml of a liver suspension from infected chicken embryos containing 10^8 EID_{50}/0.1 ml. Virus titres in infected ducklings tissues were assessed in chicken embryos. Virus was found in tissues as early as 6 hours, but the lag phase was not clearly demonstrated because a large dose of 5×10^8 EID_{50} of virus was given to ducklings, and no tissue was titrated between 0 to 6 hours. The line drawn in their graph between 0 to 6 hours is therefore not valid. The virus

recovered from infected tissues at 6 hours might not be new virus, and is more likely to be a residual virus from the inoculum. From their graph, the lag phase was probably between 6 and 12 hours. Peak virus titres of $10^{5.33}$ EID_{50}/0.1 ml occurred in the liver 48 hours after inoculation; a titre still lower than the dose of 5×10^{8} EID_{50} given. Evidence of a decline in the virus titre at 72 hours after inoculation was observed but the growth curve was incomplete because further observations were not made. Moreover, the data reported might have missed the end of the exponential increase phase because the time interval used for the observation was relatively too long. In our studies, with a shorter interval between observations during the first 48 hours and using smaller doses, the virus disappeared in ducklings infected when one day old for at least 8 hours. Virus was recovered at 12 hours. The estimated plateau phase occurred between the 24th and the 168th hour. Thereafter the virus titres declined. The high titres attained during the plateau phase were the same or higher than the titres of the doses given to the ducklings.

Another difference between the studies of Barinsky and Tsypkin (1966) and ours was the pathogenicity for ducklings of the DHV used. Their virus killed infected ducklings after 4-5 days whereas the DHV (Houchin), we used, killed infected ducklings within 3 days despite a much smaller dose of $1 \times 10^{6.0}$ EID_{50}. The likely reason for the lowered pathogenicity of the Russian virus was the fact that this virus had been passaged in chicken embryos.

Others (Levine & Fabricant, 1950; Reuss, 1959; Hwang & Dougherty, 1962) have observed the development of lower pathogenicity for ducklings in strains passaged in chicken embryos.

We found that lower virus titres were attained in the livers of ducklings infected when older than 11 days old. In fact the titres declined with age and simultaneously the proportion of ducklings surviving increased. These findings suggest the occurrence of attenuated infection in older birds, which is comparable to the infection of young birds with attenuated virus noted by Hwang and Dougherty (1964) and Barinsky and Tsypkin (1966).

The growth of a cell culture-adapted strain of DHV in cell cultures (Fitzgerald & Hanson, 1966), and of chicken embryo-passaged strains in whole embryos (Toth, 1969a; Mason et al., 1972) have also been partially studied. The DHV growth pattern in the host to which the virus was adapted was similar to that of DHV (Houchin) in ducklings in our studies; after eclipse periods, the viruses multiplied quickly and all reached their peaks within 24 to 48 hours, and all the curves had plateau phases. The durations of the plateau phases of the viruses grown in cell cultures or chicken embryos were not determined because titres were not assessed beyond 96 hours after inoculation. Hwang and Dougherty (1964) measured the concentration of DHV in chicken embryos and found little difference in virus titres from embryos harvested at 48 and 144 hours after inoculation.

PATHOGENESIS

After oral administration into one- and 11-day old ducklings DHV (Houchin) apparently disappeared and the virus was first recovered in the liver; at this time no virus was found in the other tissues. Virus in the liver also reached the plateau earlier than in the other tissues. Moreover, the highest titres of virus occurred in the liver. Our findings, therefore, confirmed those of Fabricant and his colleagues (1957) who studied the gross and histopathological lesions of DHV-infected ducklings. They found that extensive liver damage was evident within 24 hours at which time no significant lesions were found in the other tissues. Adamiker (1969), using electron microscopy, observed marked cyto-hepatic necrosis at 24 hours. The evidence, therefore, strongly suggests that the liver is the primary target organ of DHV..

Bile duct proliferation, one of the characteristic microscopic lesions of DHV, was reported by many workers using both light microscopy (Fabricant et al., 1957; Hanson, 1958) and electron microscopy (Richter et al., 1964). Such changes were also reported in the livers of infected mallard ducklings (Friend & Trainer, 1972) and in infected chicken embryos (Fitzgerald etal., 1969). Our analysis of the exponential phase of growth of DHV revealed that both the rates of virus increase and the times at which the virus increase occurred in the liver and bile were the same; the complete curve of DHV in the bile was directly related to that of DHV in the liver. Because the formation of the bile takes place in the liver (Clarkson & Richards, 1971) DHV

in the bile probably came from the liver. Other viruses are known to be excreted in bile; poliovirus type I was found excreted in the hepatic bile of cynomolgus monkeys within an hour of intravenous injection; large amounts of T_7 bacteriophage were also found excreted into the bile of mice 2 hours after intravenous infection (Mims, 1964).

DHV was found in pancreas, kidney, spleen, small and large intestines before being found in the blood but after the virus was found in the liver. The maximum virus titres in these tissues were lower than that in the liver but were higher than that of blood which suggested multiplication of DHV in these tissues at the early stage of infection but at less favourable level than that in the liver. Supportive evidence of this hypothesis is contained in the studies of Levine and Fabricant (1950), Asplin and McLauchlan (1954) and Strel'nikow (1970) who reported pathological changes in kidney, spleen and pancreas of affected ducklings respectively. These changes were also noted in the course of our studies. In contrast, the complete curve of DHV in the heart, lung, brain, thymus, bursa of Fabricius and leg muscle was directly related to that of DHV in the blood, both in regard to the virus concentration and to the time. In other words, DHV was found in these tissues during the viraemic stage and, when DHV was not found in the blood, virus was not recovered from these tissues. The evidence therefore suggests that no multiplication occurred in these tissues.

Virus was excreted in the faeces of infected ducklings (Reuss, 1959; Asplin, 1961; Rispens, 1969; Anon., 1974)

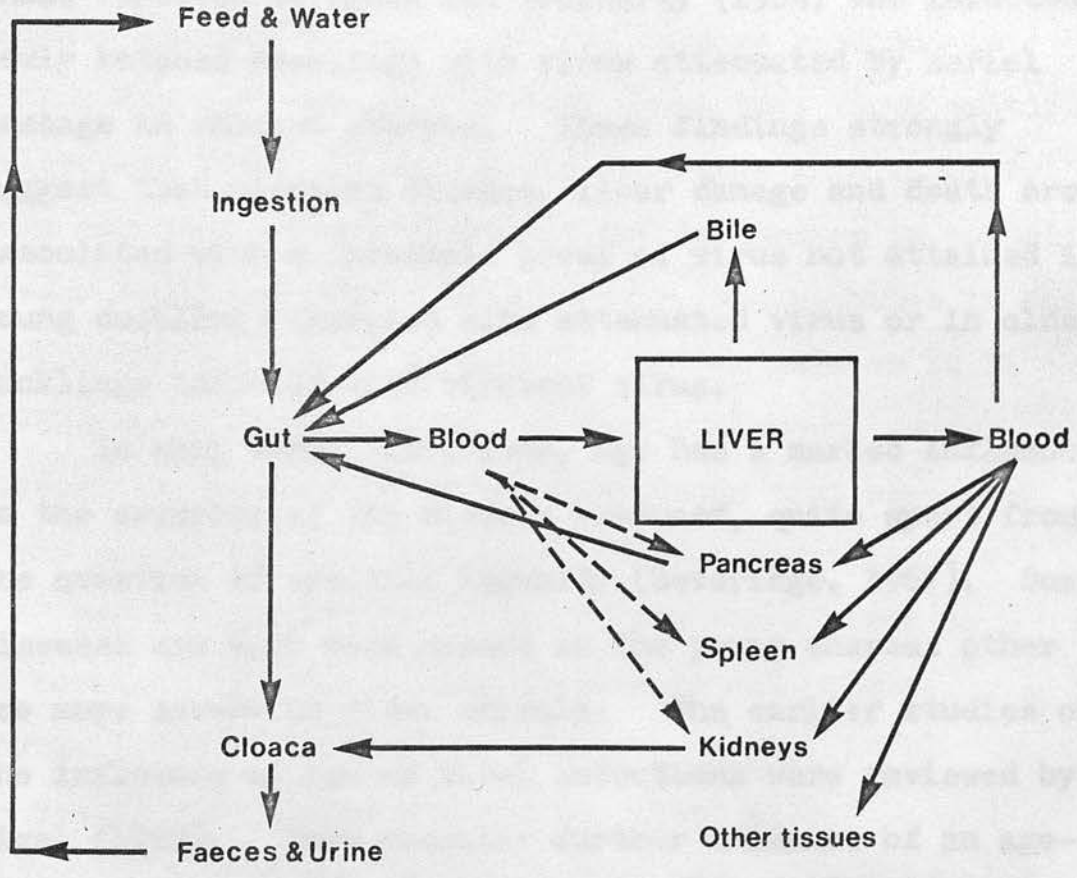
and of chickens (Asplin & McLauchlan, 1954). DHV (Houchin) in the cloacal swabs of infected ducklings probably came from the bile; the virus did not persist longer than 2 weeks. The period of recovery of DHV from cloacal swabs was shorter when older birds were infected. Our findings confirmed those of Rispens (1966) but conflict with those of Reuss (1959) who claimed excretion in ducklings still occurred 6 and 8 weeks after infection. His opinion, however, was based on findings from one duckling infected experimentally. Asplin (1961) found latent infection to be uncommon and recovered ducklings did not appear to remain carriers (Asplin, 1958).

From our experiments, the incubation period was 1 to 2 days and the mortality reached a peak within 3 days when the virus concentration in the tissues of infected ducklings, particularly the liver, was maximal. The clinical signs occurred when viraemia approached the maximum with generalisation of the virus throughout the body. Severe liver damage was prominent during this period.

A schema summarising our views on the pathogenesis of DHV is shown in figure 15.

Figure 15.

Suggested Pathogenesis Of DHV In Ducklings



AGE EFFECTS

When our older ducklings were infected with DHV, few showed clinical signs and most were inapparently infected. Most survived. Liver damage was less than in younger birds and the DHV titres in the livers and other tissues were lower than that in the livers or tissues of younger ducklings. The titres in the livers of older ducklings infected with our virulent virus were of the same order as those reported by Hwang and Dougherty (1964) who infected newly hatched ducklings with virus attenuated by serial passage in chicken embryos. These findings strongly suggest that clinical disease, liver damage and death are associated with a threshold level of virus not attained in young ducklings infected with attenuated virus or in older ducklings infected with virulent virus.

In many viral infections, age has a marked influence in the severity of the disease produced, quite apart from the question of specific immunity (Beveridge, 1967). Some diseases are much more severe in the young whereas other are more severe in older animals. The earlier studies on the influence of age on viral infections were reviewed by Sigel (1952). More recently further evidence of an age-related resistance to various viral infections has accumulated; the viruses implicated include coxsackie virus (Heineburg et al., 1964), sindbis virus (Vilček, 1964; Reinarz, et al., 1971), dengue virus (Cole & Wisseman, 1969) and Marek's disease virus (Anderson et al., 1971; Calnek, 1973; Sharma et al., 1973). Foot-and-mouth

disease caused a higher mortality in calves (Shahan, 1963) and young pigs (Callis & Shahan, 1970) than in adults. In Rift Valley fever mortality was high in newborn lambs and low in adult sheep (Daubney et al., 1931). In chickens, Newcastle disease virus was more virulent in the young (Fabricant, 1950).

Age-related resistance in ducklings to DVH is striking. In general, mortality was high among ducklings under 3 weeks of age and very low in older birds. As yet, there is no adequate explanation as to why DHV is more virulent in younger birds. Our studies have shown that virus multiplied earlier and better in 11 day-old ducklings than in older birds and this probably explains the difference in mortality between young and old. Unexpectedly, the response in one day-old ducklings was, apparently, not as good as in 11 day-old ducklings; analyses of complete growth curves, however, failed to support the hypothesis that responses in one and 11 day-old ducklings differed significantly.

Why DHV multiplies better in the young birds is not known. The phenomenon, however, is not unique. Sindbis virus (Vilček, 1964; Reinarz et al., 1971) and dengue virus (Cole & Wisseman, 1969) also multiplied better and reached higher peak titres causing fatal infection in suckling mice whereas peak virus titres and mortality were lower in older mice. The workers concerned examined the role of interferon and concluded that

interferon production was not responsible for the resistance in the older mice. In contrast, Heineburg and colleagues (1964) concluded that the age-related resistance of mice to coxsackie virus infection was directly related to interferon production because they found more interferon produced in tissues of the older infected mice.

Rapid dividing cells are known to be more favourable for cultivation of viruses (Fuccillo & Sever, 1973). Physiological changes of the host cells while the animal is growing may be one of the factors. Kaňtoch and Kuczkowska (1964) found that the susceptibility of newborn mice to infection with coxsackie virus depended on the presence of susceptible embryonic muscle cells; older mice were not susceptible. Mouse hepatitis virus infected liver cell cultures prepared from newborn but not old mice (Bang, 1972). Using electron microscopy, Kapp and Balázs (1970) reported that major changes of cellular structures of normal ducklings' liver took place during its first 20 days after hatching. This period coincides remarkably well with the development of resistance and decline in ability of the virus to multiply in our studies.

Cellular defense mechanisms may be one of the important factors. Macrophages are able to ingest and destroy many viruses (Mims, 1964; Jandásek et al., 1969). Antimacrophage serum was found to enhance many viral infections including infections due to encephalomyocarditis virus, yellow fever virus (Panijel & Cayeux, 1968) and vesicular stomatitis (Hirsch et al., 1969). Inhibition of the activity of lymphocytes using antilymphoid sera also

enhances many viral infections including vaccinia, herpes simplex, Rauscher leukaemia, Moloney leukaemia, adeno-12 and vesicular stomatitis virus infections (Hirsch & Murphy, 1968) and reovirus in mice (Ida & Hinuma, 1971). Macrophages of adult mice produced more interferon and more effectively prevented release of complete infectious virus than those of suckling mice (Hirsch et al., 1970). Moreover, adult mouse macrophages also more effectively destroyed phagocytosed herpes virus. Jandásek and his colleagues (1969) studied the rôle of macrophages in age-related resistance of mice by injecting vaccinia virus intraperitoneally and concluded that older mice were more resistant than younger mice because the peritoneal macrophages more effectively phagocytosed and localised the virus than the macrophages of young mice. These aspects have not, as yet, been studied in ducklings infected with DHV.

Infection may not have occurred in adult ducks infected with DHV. Hanson and Alberts (1960) found that oral administration of DHV produced neutralising antibodies in 2-week-old ducklings but not in adult ducks. Asplin (1961) confirmed that a better antibody response was observed in young ducklings, 2 to 4 weeks of age, than in mature birds; adult ducks infected or dosed with DHV failed to produce passively resistant ducklings implying that adult birds were not infected. Parenteral injection of larger doses of DHV or repeated doses usually induced an antibody response probably because the virus acted as a non-

replicating antigen (Hwang et al., 1962; Hwang, 1972; Rispens, 1969). Rispens (1969)also compared the responses of young and older birds and found that the progenies of birds first vaccinated when 4 weeks old possessed a stronger passively acquired resistance than the progenies of birds first vaccinated when older. Vindel (1962), on the other hand, reported natural egg-transmission of the virus implying that adult birds were infected with DHV and passed the virus on into their eggs.

In the titration of DHV (Houchin) in duck embryos when 8 to 18 days of age, we found that embryos up to 14 days old were equally susceptible, but the mortalities of duck embryos infected when 16 and 18 days old were lower. When one-day-old ducklings were infected with DHV (Houchin), the LD_{50} was even lower than that in duck embryos infected when 16 and 18 days old. The evidence, therefore, indicates that the age-related resistance to DHV began in embryonic life.

Age-related resistance to DHV also occurred in chicken embryos. In our studies, the titres of DHV (Houchin) markedly decreased as the age of chicken embryos at the time of infection increased from 7 to 12 days old. Similarly, when Sazawa and colleagues (1963) titrated DHV in 6 to 7 and 10 to 11 days old chicken embryos, the titres attained from the older chicken embryos were about 50 per cent lower than the titres attained from the younger chicken embryos. Asplin and McLauchlan (1954) and Vindel (1963) also stated that the characteristic lesions

of DHV in chicken embryos depended upon the ages of the embryos at the time of inoculation.

In contrast when we titrated the chicken embryo-adapted strain of DHV, the H_{56}, in chicken embryos 7 to 12 days of age, the virus titres calculated from deaths of chicken embryos were similar. This phenomenon is not unique to DHV infection. Cole and Wisseman (1969) have shown that low-mouse-passaged dengue virus caused high mortality in suckling mice and low mortality in weanling and adult mice, whereas high-mouse-passaged virus caused high mortality in mice of all age groups. In other words, the outcome of many virus infections including DHV does not depend entirely on the host alone; it is possible to select virus mutants which are not affected by age factors.

VIRUS IDENTIFICATION

Identification of DHV is usually based on some or all of the following criteria: (a) the reproducible and characteristic clinical signs in affected young ducklings, (b) death with characteristic liver lesions in affected ducklings, usually under 3 weeks of age, (c) the reproducible and characteristic lesions in inoculated chicken embryos, and (d) neutralisation tests in ducklings or chicken embryos using specific antisera. These criteria are all subjective. There is therefore a need for objective criteria preferably based on specific serological reactions other than the

neutralisation test in chicken embryos in which the end-point is determined subjectively.

Several groups (Murty & Hanson, 1961; Kaeberle et al., 1961; Gabridge & Newman, 1971) have reported success with agar gel tests but others (Wachendörfer, 1965; Toth, 1972b; Anon., 1973a) have failed to produce specific antibody despite use of various methods of antigen preparation including purification, and different methods of immunisation in different species of animals. Likewise we failed to consistently produce a specific antiserum.

Two groups in Russia reported success with fluorescent antibody techniques and recommended the test in diagnosis (Vertinskii et al., 1968; Maiboroda, 1972). Others have still to assess the technique.

TITRATION ERROR

The subjective assessment of the presence or absence of DHV infection decreases the accuracy of dilution end-points. Moreover, titrations are open to error; the magnitudes of the error are large. For example, a titre with a standard error of 0.4 has 95% confidence limits 2.1 logs apart; i.e. $10^{6.0 \pm 0.40}EID_{50}$ will be in a range of $10^{4.90}$ to $10^{7.10}EID_{50}$. Despite the errors, reproducibility was evident and typical growth curves were readily defined. In other words, isolated titrations were not as reliable as

sequential titrations which yielded titres that were related to each other.

LOGISTIC CURVE

The logistic curve is empirical (Waksman, 1949). Moreover, to obtain the line of best fit, the upper asymptote, K, is arbitrarily chosen (Andrawartha & Birch, 1954). The logistic curve also fails to depict the complete cycle of virus growth. Nevertheless, the logistic curve gave the line of best fit which best described the exponential phase of DHV growth.

When the time units were transformed into logarithms the skewness was eliminated and the curves approached normality. The new curves thereafter were amenable to conventional analysis enabling comparisons of complete growth curves and avoiding arbitrary decisions as to what data be included.

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