A GEL DIFFUSION METHOD FOR THE QUANTITATIVE ASSAY OF DUCK HEPATITIS VIRUS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Michael G. Gabridge

1966

A GEL DIFFUSION METHOD FOR THE QUANTITATIVE ASSAY OF DUCK HEPATITIS VIRUS

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1966

Dedicated

to

my wife

ACKNOWLEDGEMENTS

The author would like to express his gratitude to Dr. John P. Newman, Professor of Microbiology and Public Health, for the guidance, patience and consideration extended over the past several years. His fine example served as a constant guide during the research and preparation of this manuscript.

I would also like to acknowledge the kind and helpful assistance of Mrs. C. M. Newth and Mrs. M. G. Shue, and express my appreciation to the staff of the Diagnostic Microbiology Laboratory of the M. S. U. Veterinary Clinic for their aid and advice.

Last, but not least, a special thanks to my family for their words of encouragement and sincere interest in this project.

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INTRODUCTION

Because of their minute size and unique properties, viruses can only be counted in an indirect manner. Methods range from negative staining in electron microscopy to hemagglutination and cytopathogenicity, but nearly all have drawbacks for routine diagnostic and small-scale research studies. When the virus is non-cytopathic in cell culture and does not agglutinate red blood cells, one must often resort to the inoculation of embryonating chicken eggs with a series of virus dilutions and the determination of titer with the aid of statistics. This is time consuming, expensive, and subject to error from a variety of sources.

The purpose of this study was to find a new method for quantifying such viruses, using duck hepatitis virus as the model system. The new procedure involved virus precipitation in gel with antiserum, with the position of the precipitate proportional to the virus titer. Using a standard curve, the distance in millimeters from the point of origin could be transposed to the number of particles or infectious units per amount of inoculum. The ratio of reactant volume of reaction area was the inverse of that used in older methods, assuming that this would result in increased sensitivity.

LITERATURE REVIEW

Duck Hepatitis

Duck hepatitis is a highly contagious disease specific for ducks. The morbidity and mortality rate is usually highest in ducklings less than three weeks of age. It was first recognized in New York state by Levine and Fabricant (1950), and has been reported in England (Asplin and McLauchlin, 1954), Canada (MacPherson and Avery, 1956), Germany (Reuss, 1959), and Egypt (Hagen and Bruner, 1961).

Clinically, the disease is manifested by a slit-like appearance of the eyes, squatting, general dullness and listlessness, convulsions, and opisthotonus (Hanson and Alberts, 1956). Death often occurs within one to two hours after the onset of symptoms (Fabricant et al., 1957), and the mortality rate may be as high as 95 per cent.

The most characteristic pathologic alteration is an enlarged, mottled liver (Hanson and Alberts, 1956) caused by hemorrhage and edema, and often accompanied by a jaundice-like discoloration (Fabricant et al., 1957). The spleen, heart and brain, though not outwardly affected, have been implicated since they contain detectable amounts of virus (Hwang and Dougherty, 1964).

Microscopically, the lesions resemble those associated with human hepatitis and include proliferation of the bile ducts, necrosis of hepatic cells, and paravascular infiltration with granulocytes and plasma cells (Hanson, 1958).

Duck Hepatitis Virus

This RNA virus is a sphere with a diameter of 20-40 mu (Reuss, 1959) and is ether stable (Pollard and Starr, 1960). Virus particles found in hepatic cells of infected ducklings may be dispersed in the cytoplasm, packed into crystals, or contained within membranes (Richter et al., 1964).

At 25°C, 37°C, and 56°C there is a definite reduction in the ID_{50} after 5 weeks, 48 hours, and 90 min, respectively (Hanson et al., 1961). The virus remains viable after treatment with ammonium sulfate or 30 per cent methanol for 24 hrs, and viral infectivity is stable from pH 4.8 to 7.8 (ibid.).

Chicken embryos are susceptible to infection with duck hepatitis virus, DHV (Asplin, 1958; Hwang and Dougherty, 1964). Characteristic lesions include a severe edema in the abdominal region, general dwarfing, and a reddening of the skin, with death occurring two to six days after inoculation (Hanson and Alberts, 1956).

Pollard and Starr (1959) reported multiplication of the virus without cytopathic effects (CPE) in chicken embryo explants. Trypsinized cells were not susceptible to the virus. This differs from the findings of Kaeberle et al. (1961) who successfully propagated DHV in monolayer cultures of chicken embryo liver cells dispersed with trypsin. Fitzgerald et al. (1963) was the first to report a marked CPE and necrosis in duck embryo kidney cells after the 24th virus passage.

Sazawa et al. (1963) tested a recently isolated strain of DHV for infectivity in duck kidney, chick kidney, chicken

embryo, and duck embryo cells. Multiplication occurred in the duck embryo cells and the CPE was indistinct and described as "hazy". No conspicuous CPE or cellular damage was reported by Hwang (1965) after twenty serial passages in duck embryo fibroblast cultures.

Quantitative Techniques

Methods for the quantitative assay of viruses may be grouped into three categories: quantal, enumerative, and graded.

The quantal type of assay involves an "all or nothing" response in which a host exposed to the virus is classed as infected or non-infected. If the host is a test animal, infection may be recognized by visible or microscopic lesions, paralysis, or death. If embryonating eggs are used, there may be hemorrhage of subcutaneous tissue, congestion of the epidermis, dwarfing of the embryo, or similar manifestations (Cunningham, 1966).

In cell culture systems, infection is evidenced by a CPE (Rubin and Temin, 1958), hemadsorption (White, 1963), hemagglutination (Fazekas de St. Groth and White, 1938), or a change in the pH of the medium (Huang, 1943).

Enumerative assays involve some type of direct count and results are expressed in real rather than statistical units. Actual counts of viruses may be made with the electron microscope, but the number of physical particles does not always correlate with the number of infectious particles (Smadel et al., 1939; Friedewald and Pickels, 1944; Issacs and Donald, 1955; Tyrell and Valentine, 1957).

The most widely used form of enumerative assay involves the counting of plaques or areas of necrosis in a monolayer cell culture (Dulbecco, 1952). This has been used with a number of viruses, including Coxsackie virus (Hsiung and Melnick, 1955), foot and mouth disease virus (Bachrach, 1957), and polyoma virus (Dulbecco and Freeman, 1959).

Dulbecco and Vogt (1953) proved mathematically the validity of assuming one viral particle produced one plaque, but there is an inordinate amount of variability in the method and a departure from the expected distribution could easily occur (Armitage, 1957).

Graded assays are based on some measure of the degree of response, such as survival time, size of an infected organ, or size of a lesion (Harris, 1964). Such methods are normally used with oncogenic viruses (Rubin, 1960), but have also been used with Newcastle disease virus (Cunningham, 1966) and agents of the psittacosis group (Golub, 1948).

Gel Diffusion

Immunologic precipitation involves a reaction which can best be expressed by the Marrack-Heidelberger hypothesis (Cruickshank, 1963): bivalent antibody molecules link up adjacent antigen-antibody complexes, and if optimal proportions exist, form a three-dimensional lattice network large enough to be visible. These combinations take place in a number of stages and are essentially reversible (Humphrey and White, 1964).

Oudin (1946) first developed the technique of forming antigen-antibody precipitate(s) in gels using the single

diffusion tube test. Antigen was placed on top of an antiserum-agar mixture and diffusion resulted in a precipitate appearing in the latter.

Elek (1948) and Ouchterlony (1948) modified this technique to one of double diffusion where both antigen and antibody migrate toward each other through a gel before reacting. Precipitation is affected by the salt concentration of the agar (Aladjem and Lieberman, 1952), the pH (Wilson and Pringle, 1954), and molecular weight of the antigen (Korngold and Van Leewen, 1957).

The first demonstration of a virus-caused precipitation in agar was made by Jansen and Francis (1953) using influenza virus. Belyavin (1955) first suggested its diagnostic application. Viruses of mumps and Newcastle disease have also been studied with gel diffusion (Belyavin, 1957).

Murty and Hanson (1961) did an analysis of DHV using a modified microgel diffusion method, and found three lines of precipitate when liver from infected duck embryos was diffused against unadsorbed hyperimmune rabbit serum. Only two of these lines were actually specific for the DHV.

Precipitation and gel diffusion tests are generally used for qualitative analyses, but attempts have been made to establish quantitative applications. Heidelberger and Kendall (1935) were among the first to work in this area, and used the difference in optical density before and after precipitation as a parameter related to virus concentration.

Adding different concentrations of antigen (Gussoni, 1964) or antibody(Bjorklund, 1952) and defining the inhibition

of visible precipitate as the endpoint in a titration may also be done.

Attempts have been made to develop a quantitative analysis based on the fact that the distance of a precipitate band from a well of reactant is commensurate with the concentration of the substance being diffused (Preer, 1956; Korngold and Van Leewen, 1957; Aladjem et al., 1959). Le Bouvier (1957) found the size and density of a band reflected both the absolute and relative concentrations of antigen or antibody.

Such methods are continually being refined and modified, with a resultant increase in sensitivity. Feinberg (1957) used an adaptation of the original Ouchterlony technique and found that visible precipitate could be formed by as little as 0.05 ug of protein.

Studies utilizing double diffusion are generally considered more accurate than those using single diffusion since the former does not depend on how much material was in the reaction zone initially, but on how much can be fed into it (Crowle, 1961). Single diffusion tests are also more susceptible to secondary precipitation or non-specific multiple bands which give false results (Leuker and Crowle, 1963).

Elek (1949) noted that in double diffusion with the migration of the two reactants taking place at an angle of 90 degrees to each other, conditions are produced for the interaction of antigen and antibody in all proportions. Expressions have been developed that relate the antigen's diffusion coefficient to the slope of the line (Neff, 1957;

Becker, 1959) and the tangent of the angle (Allison and Humphrey, 1959) formed in such a system.

MATERIALS AND METHODS

Virus

The duck hepatitis virus used in this study was obtained from Dr. E. Dougherty of the Duck Research Laboratory at Eastport, Long Island. It was contained in allantoic fluid from infected chicken embryos and labelled $L_2$75; L_2 signified the virus was adapted to growth in chicken embryos and "75" indicated the number of passages since the original isolation. Upon receipt it was stored at -70°C.

Prior to use, the allantoic fluid was thawed and centrifuged at 500g for 20 min. The sediment was discarded and the supernatant fluid was kept at 4°C.

Virus Purification

The anion resin used for the virus purification was the chloride form of Dowex 1-8X (200-400 mesh). To insure purity of the stock resin, it was washed in 3N sodium hydroxide for 4 hrs, rinsed once in distilled water, washed in 3N sodium chloride for 3 hrs, and given a final rinse in distilled, deionized water.

The virus suspension was combined with the washed resin in a ratio of 2:1 so that the final volume was 20-30 ml (Mallman, 1961). The resulting mixture was placed in a 50 ml centrifuge tube, shaken intermittently for 15 min, centrifuged at 20,000g for 15 min, and the supernatant fluid removed. The resin pellet was resuspended in 10 ml of 10% aqueous sodium phosphate, shaken, and centrifuged in the same manner.

The resulting supernatant fluid, now referred to as "purified DHV", was stored at 4°C. Its virus content was confirmed with electron micrographs and Ouchterlony tests.

Antisera

Several antisera were prepared using rabbits.

Antiserum A was obtained after five intraveneous injections of DHV $L_2$78. Amount of inoculum was 1.0 ml, administered at four-day intervals. The serum was collected one week after the fifth injection.

Antiserum B was produced in response to a stable suspension of 0.5 ml of Freund's Complete Adjuvant and 0.5 ml of DHV $L_2$78 injected subcutaneously into several places on the back of a rabbit. This was repeated in three weeks and the serum collected seven days later. Adsorbed antiserum B was obtained by combining normal allantoic fluid with antiserum in a ratio of 1:3, centrifuging, and discarding the sediment.

A series of subcutaneous injections similar to those just described were used in the production of antiserum C, but the virus had been purified on the anion exchange resin.

Diffusion Agar

The agar used in the Ouchterlony plates and diffusion block was similar to that suggested by Campbell *et al.* (1963). The trypan blue was included to increase the ease with which lines would photograph, and the pH was buffered to 7.8-8.0 to enhance precipitation. The composition of the agar was as follows:

Ionagar #2 *	0.425 gm
Saline	46.8 ml
Borate Buffer **	2.5 ml
Merthiolate (1%)	1.0 ml
Trypan Blue (1%)	0.2 ml

The Ionagar, saline, and buffer were mixed, and autoclaved at 121°C for 15 min. The merthiolate and trypan blue were added after cooling and the mixture was held at 50°C until it was allowed to solidify in the plate or reaction well.

* Available from Oxo Limited, London.

** Borate Buffer:

Boric Acid	6.184 gm
Borax	9.536 gm
NaCl	4.383 gm

Distilled water to 1 liter. Final pH = 8.5

Ouchterlony Plates

Ouchterlony plates were prepared from 100 x 15 mm plastic petri dishes filled with 20 ml of diffusion agar. An ink diagram with the desired arrangement of wells was placed under the dish and holes were cut with a cork borer previously cleaned with alcohol and flamed.

The wells, approximately 2 cm apart, were initially filled with 0.2 ml of liquid. Plates were held at room temperature and the wells refilled after 24 hrs. Photographs were made using a 35 mm camera with indirect fluorescent lighting.

Diffusion Block and Procedure

The apparatus used in the diffusion study was a specially constructed 12 x 4 x 3/4 inch block of plexiglass. The block contained eight separate reaction wells, each 1/2 inch in depth (Figures 1 and 2). A reaction well consisted of two

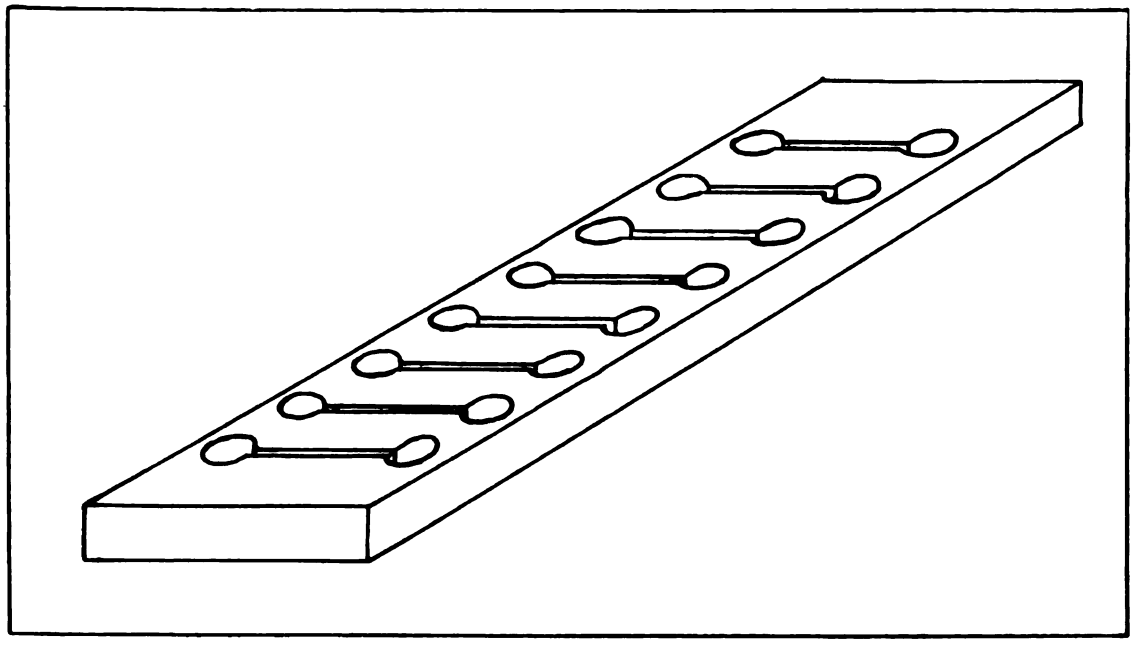


Figure 1. Sketch of plastic diffusion block.

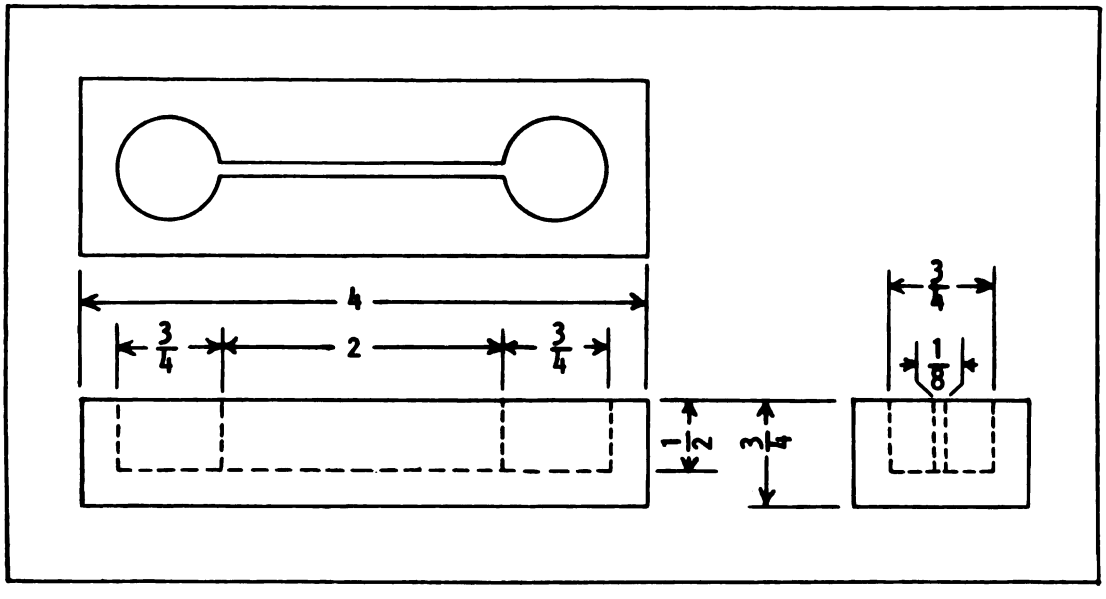


Figure 2. Diagram of one reaction well (measurements in inches).

circular reservoirs (3/4 inch diam.) for antigen and antibody connected by a 2 x 1/8 inch slot which contained agar and served as the reaction site.

Before introducing the agar, the slot was isolated from the reservoirs by a short piece of masking tape applied to the latters' inside surfaces. A plastic 1.0 ml serological pipette was used to deliver 1.0 ml of diffusion agar into the slot.

Once the agar solidified, the tape was removed and the agar strip cut to the desired length. This was done by placing the block over a ruled paper and making certain that the final agar strip would be equidistant from both reservoirs. After the excess agar was removed, the reservoirs were swabbed with 70% alcohol and the block was placed under an ultraviolet lamp until dry.

Reservoirs were filled with 1.0 ml of the proper dilution of either antiserum or virus. After all solutions had been introduced into the appropriate wells, the block was covered with a 12 x 4 inch piece of flexible plastic film (previously cleansed with 70% alcohol) on top of which was placed a 12 x 4 x 1/8 inch section of plexiglass.

Grading Precipitate Lines

Some degree of the relative intensity of precipitate lines was afforded by establishing an arbitrary scale as follows:

0 No visible reaction

- Line only visible when agar strip was removed from slot and examined from side

+ Light precipitate; barely visible in block; hard to measure since it has no definite boundries

++ Moderate precipitate; easily visible in block; easy to measure

+++ Heavy precipitate; very easy to discern and measure

This method is somewhat subjective but proved adequate since the results were only used for comparative purposes.

Reference Titration

Serial ten-fold dilutions of the virus were prepared using 4.5 ml of sterile nutrient broth as the diluent. A sterile 1 ml serological pipette was used to deliver 0.5 ml of virus to the first tube of broth. Using a clean, sterile 1 ml pipette, the mixture was aspirated and expelled 15 times prior to transfering 0.5 ml to the next tube of broth. This pipette was discarded and the process repeated in all of the remaining tubes.

Five 7-day old chicken embryos were each inoculated with 0.2 ml of a virus dilution via the allantoic cavity (Cunningham, 1963). After injecting five embryos, the last 0.1 ml of the dilution in the syringe was put into a tube of brain-heart infusion broth and incubated 48 hrs to check for bacterial contamination.

The eggs were incubated at 99.5°F and candled daily for 7 days. Death of embryos within the first 24 hrs was

attributed to trauma or contamination, and these embryos were not included in the final results. Embryos found dead after this period were examined for edema in the abdominal region, dwarfing, and hemorrhaging to ascertain viral infection. The titer was then determined using the method of Reed and Muench (1938).

Electron Microscopy

Electron micrographs were taken to insure the purity of the virus adsorbed to the resin. The purified virus suspension was sprayed with a pyrex nebulizer onto #2200 EFFA copper grids covered with formvar. After drying, the specimens were shadowcast with palladium at an angle of 60 degrees. These were examined in an RCA, type EMU-2 electron microscope, and exposures were made on 2 x 2 photographic plates. Negative plates were made from these and the images were enlarged 4-5 times prior to printing on Kodabromide photographic paper.

RESULTS

Diffusion Plate Tests

To test the various antisera for the capacity to precipitate with the virus, several Ouchterlony plates were prepared. Figure 3 depicts such a plate in which antiserum B was tested against both DHV and normal allantoic fluid. Two lines of heavy precipitate are prominent, with a third line barely visible behind the peak under the middle well.

Testing antiserum C in a similar manner served as a check on the relative purity of the resin-adsorbed virus suspension. One heavy line of precipitate along with two very light lines were formed (Fig. 4). One of the light lines was triangular in shape, while the other was much less distinct and its exact shape could not be easily determined.

Antiserum A repeatedly failed to give any evidence of precipitate when diffused against virus used as the antigen.

A tendency for two lines of precipitate to lie almost in the same position was frequently observed. It was noted for both antiserum B and C, and is illustrated in figure 5. The fainter of the two heavy lines appears single at the top well, but separates into two lines opposite the lower wells.

When diluted antisera were placed in the wells opposite a constant amount of virus, a continuous line encircling the center well was formed. All of the dilutions tested (1:1 to 1:10) gave some sort of visible reaction, but those higher than 1:2 were very indistinct.

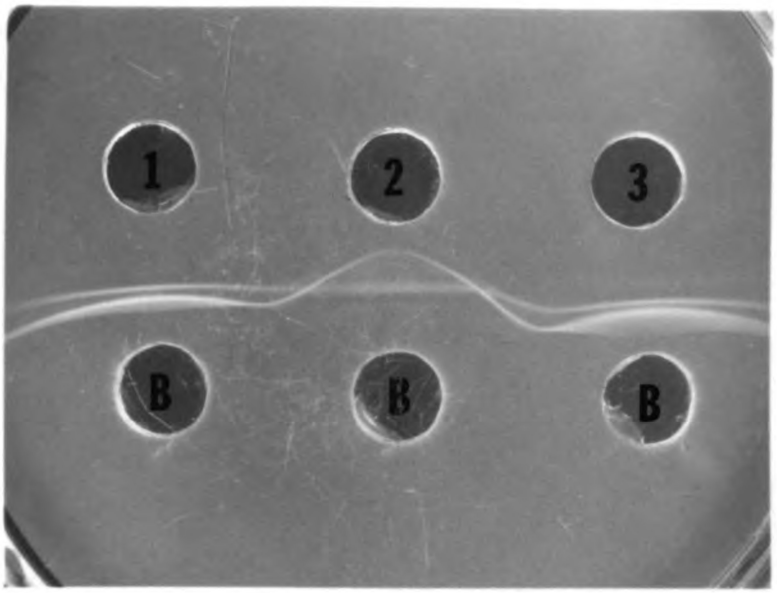


Figure 3. Diffusion of DHV $L_2$78 and normal allantoic fluid against antiserum B.

1 and 3 = DHV; 2 = allantoic fluid; B = antiserum B

Figure 4. Diffusion of DHV $L_2$78 and normal allantoic fluid against adsorbed-virus antiserum.

1,3,5 = DHV; 2,4,6 = allantoic fluid; C = antiserum C

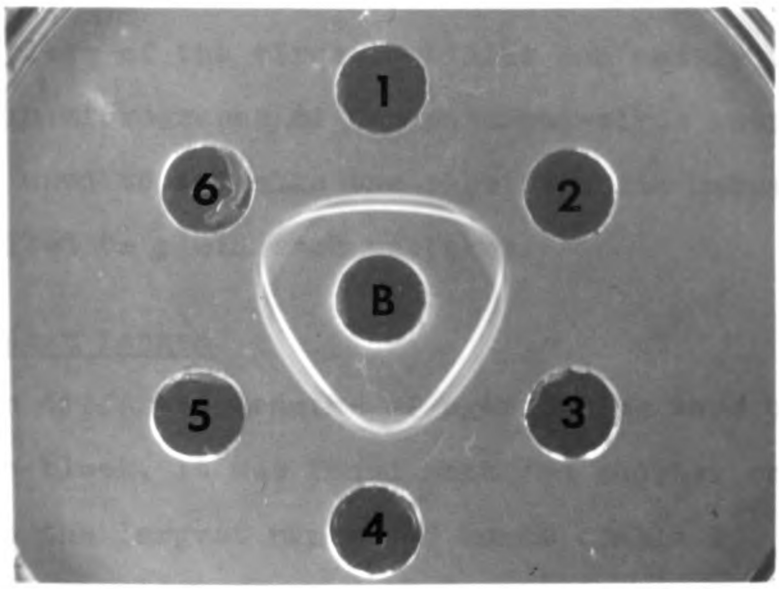


Figure 5. Illustration of the tendency for two lines of precipitate to appear as one.
1,3,5 = DHV; 2,4,6 = allantoic fluid; B = antiserum B

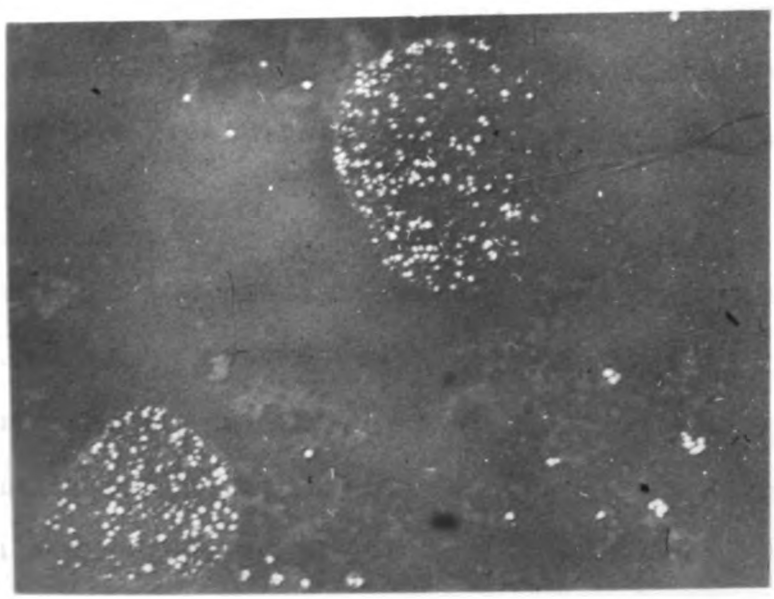


Figure 6. Electron micrograph of shadowed preparation of purified duck hepatitis virus (X 48,000).

The homogeneity of the purified virus suspension and antiserum C was also checked using electron microscopy. Microdroplets of the virus particles can easily be distinguished in micrographs of the adsorbed-virus suspension (Fig. 6) used to immunize the rabbit, while normal allantoic fluid failed to yield such patterns.

Optimal Agar Length

When different lengths of agar strips were used in the diffusion block, it was found that the shorter ones (½", 1", 1½") gave the largest number of bands (Table 1). The 2 inch strip failed to display any signs of precipitate after six days, while the three shorter ones all had some evidence of reaction. The visibility or degree of reaction (see Materials and Methods) was best in the ½ inch strip, and the 1½ inch strip had the least distinct lines. The distance given in this and remaining tables represents the distance of the leading edge of the precipitate band in mm from the antigen well.

Reactant State

Changes in the precipitation pattern were observed when the physical state of the reactants was altered (Table 2). Having the reactant in the form of a gel was achieved by mixing the liquid reactant with a specified volume of melted diffusion agar prior to placing it in the well. Table 2 also contains results from an analogous experiment where the reactants were in the liquid form but diluted with the appropriate amounts of saline so the two sets of data might be compared. A very uneven, complex pattern of bands was

Table 1. Results from diffusion tests with different lengths of agar strips.

Length of Zone	Number of Bands	Time to Appear	Visibility (6 days)	Distance (6 days)
½ inch	4	a. 36 hrs	+++	12.5 mm
		b. 36 hrs	++	12.4 mm
		c. 36 hrs	+++	8.0 mm
		d. 48 hrs	+	4.0 mm
1 inch	2	a. 60 hrs	++	18.0 mm
		b. 96 hrs	+	15.0 mm
1½ inch	1	a. 144 hrs	-	24.5 mm
2 inch	0	0	0	0

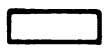
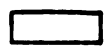
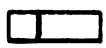
Antigen: DHV $L_2$78

Antibody: Antiserum C

Temp.: 37°C

Reactant State: Gel

Table 2. Effects of the reactants' physical state on the precipitation pattern.

	LIQUID			GEL		
Time (hrs)	Bands	Distance (mm)	Pattern	Bands	Distance (mm)	Pattern
12	0	0		0	0	
24	1	a. 4.0		1	a. 4.0	
36	2	a. 2.5 b. 4.0		3	a. 3.0 b. 4.0 c. 4.5	
48	3	a. 2.5 b. 4.0 c. 6.0		4	a. 2.5 b. 4.3 c. 4.5 d. 8.0	
60	5	a. 1.0 b. 2.0 c. 3.0 d. 5.5 e. 7.0		4	a. 2.0 b. 4.4 c. 4.5 d. 8.0	
72	5	a. 1.0 b. 2.0 c. 3.0 d. 3.5 e. 7.0		5	a. 1.5 b. 2.5 c. 3.0 d. 5.0 e. 8.0	

obtained when a gel-liquid interface existed, whereas a gel-gel interface resulted in a more orderly arrangement of bands. The overlapping of lines in the former became less of a hindrance in measuring as time increased since the migration tended to separate the bands. When the reactants were in the liquid form, 72 hrs was the optimal time for measuring, but measurements could be made any time after 36 hrs when they were in the gel form. Liquids in the reactant reservoirs were also undesirable since they were easily spilled when the block was removed from the incubator to be inspected.

The number of individual bands observed at the specified time intervals remained comparable in both cases, as did the distance between the two bands farthest apart. Once the first band appeared, precipitate formed in both directions with the migration toward the antibody well being the more prominent.

There was a linear relationship between the time and the distance of the band closest to the antibody well when the gel phase was used. The line was noted to move approximately 0.5 mm per 12 hr period.

Temperature Effects

The effects of temperature of the diffusion patterns involved both the number and the visibility of the precipitate bands (Table 3). At 4°C, only one or two lines appeared, depending on the length of reaction strip, whereas 4 or 5 appeared at temperatures of 21° and 37°C. The distinctness or visibility also increased with an increase in temperature. As in the previous experiments, the shortest agar strip had

Table 3. Effect of temperature on the quality and quantity of precipitate bands.

Temperature	Length of Agar	Time to Appear	Visibility	Distance
4°C	½ inch	a. 144 hrs	+	7.5 mm
		b. 168 hrs	-	3.5 mm
	1 inch	a. 168 hrs	+	3.5 mm
21°C	½ inch	a. 42 hrs	++	9.4 mm
		b. 96 hrs	+	4.0 mm
		c. 120 hrs	+	9.0 mm
	1 inch	a. 120 hrs	+	18.5 mm
37°C	½ inch	a. 36 hrs	++	8.0 mm
		b. 36 hrs	+	9.0 mm
		c. 36 hrs	++	9.5 mm
		d. 48 hrs	+	4.5 mm
		e. 60 hrs	+	8.0 mm
	1 inch	a. 60 hrs	++	17.0 mm
		b. 108 hrs	+	15.0 mm

the greatest number of bands.

Bands in the ½ inch strip held at 4°C presented a problem in measuring since they were curved, with one side of the curve almost parallel to the surface of the agar when viewed from the side. A top view only revealed diffuse areas of gray with very few distinct lines. In this case, the measurements taken were those where the band met the bottom of the agar strip. With the higher temperatures, the bands were essentially vertical and could be measured without removing the strip from the block.

Single Diffusion

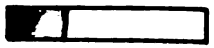
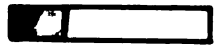
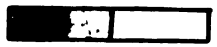
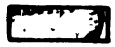
One set of reaction wells was prepared with the antiserum incorporated directly in the agar (Table 4). This was a form of single diffusion since the antibody concentration remained essentially constant compared to the gradient established when the virus diffused into the agar strip.

Two types of single diffusion were tested: one with equal volumes of antiserum and agar, and the other with twice as much agar. The results were similar for both, but the latter developed an extra line within one wide band after 48 hrs.

Broad areas of precipitate were formed when only the virus was allowed to migrate, while single and distinct bands usually resulted from double diffusion. The latter was more desirable because of the ease in measuring.

One should note that in the single diffusion experiment it was necessary to have the virus contained in a liquid

Table 4. Results from diffusion test with antiserum incorporated in the agar.

Diffusion:	Single		Single	
As/Agar Ratio:	1:1		1:2	
Agar Length:	½ inch	1 inch	½ inch	1 inch
Appearance at 24 hrs				
Visibility at 24 hrs	+	+	+	+
Appearance at 48 hrs				
Visibility at 48 hrs	++	++	++	++
Appearance at 72 hrs				
Visibility at 72 hrs	+	+++	+	+++

since the use of a gel would result in antiserum diffusing into the antigen reservoir, thereby creating double diffusion.

Reference Titration

In order to establish a standard curve for the virus titration using precipitation, it was necessary to determine the quantity of virus in the allantoic fluid mixtures. This was accomplished using the method of Reed and Muench (1938). Five embryos were used with each dilution, and all embryos found dead after 24 hrs had noticable hemorrhage, dwarfing, and edema. With $L_2$79, dilutions of 10^{-4} or lower killed all of the embryos, while those of 10^{-7} and higher did not affect any. The titer was $10^{5.5}$ embryo lethal doses per 0.2 ml, or $10^{6.2}$ ELD_{50} in 1.0 ml. The titer for $L_2$78 was $10^{4.5}$ ELD_{50} per 1.0 ml.

The same results were obtained using the Spearman-Karber method (Harris, 1964), and with the Pizzi (1950) formula the standard error of the log ELD_{50} was $\pm$ 0.109.

Experimental Titration

When various concentrations of virus were placed in the wells of the diffusion block and allowed to react against a constant amount of antiserum, precipitate formed at various points along the agar strip (Table 5). The more concentrated the virus, the farther the bands were from the antigen well. In the higher concentrations, several bands were evident, while the diffusion of less concentrated virus resulted in only a single band.

Table 5. Final composition of reactants, and results from diffusion block titration.

Well	Antiserum (ml)	Agar (ml)	Saline (ml)	Virus (ml x dil)	Agar (ml)	Saline (ml)	Distance (mm) 72 hrs	132 hrs
1	0.5	0.5	-	-	0.5	0.5	0	0
2	-	0.5	0.5	0.5×10^{0}	0.5	-	0	0
3	0.5	0.5	-	0.5×10^{0}	0.5	-	14.5	18.5
4	0.5	0.5	-	0.25×10^{0}	0.5	0.25	12.5	16.0
5	0.5	0.5	-	0.5×10^{-1}	0.5	-	9.5	10.5
6	0.5	0.5	-	0.25×10^{-1}	0.5	0.25	8.0	7.5
7	0.5	0.5	-	0.5×10^{-2}	0.5	-	0	6.5
8	0.5	0.5	-	0.25×10^{-2}	0.5	0.25	0	6.0

Virus: DHV $L_2 79$ ($10^{6.2}$ ELD_{50}/ml)

Antiserum: B

Temp: 37°C

Length: ½ inch

To insure that none of the bands used in the calculations were due to allantoic fluid, a control was established in which normal allantoic fluid was added to agar in a ratio of 1:1 and tested against the various antisera. The only visible reaction after 5 days was a very faint (+) line approximately 1 mm wide and located 5.5 mm from the antibody well. This was observed with both antiserum B and C and was narrower in the latter. Since the leading edge of the precipitate was always more than 7.0 mm from the antigen well, non-specific bands should not interfere in a titration.

When the virus was diffused, the distance of the leading edge of precipitate was checked at various time intervals. When plotted in a semi-log fashion against the titer, a linear relationship was observed (Fig. 7). All of the lines tended to converge in one area which represents the "equivalence point". This is where the antigen and antibody first meet and the dilution at which they are in optimal proportions. The area to the right of this point is the region of antigen excess, as evidenced by the band moving toward the antibody well.

The area between the top and bottom lines in this graph simultaneously represents a distance and a time interval, and is thus equivalent to velocity. By measuring the vertical distance between two lines and then dividing by the time difference, a velocity value can be determined. When these are calculated for several concentrations and then plotted against titer, another straight line relationship is observed (Fig. 8).

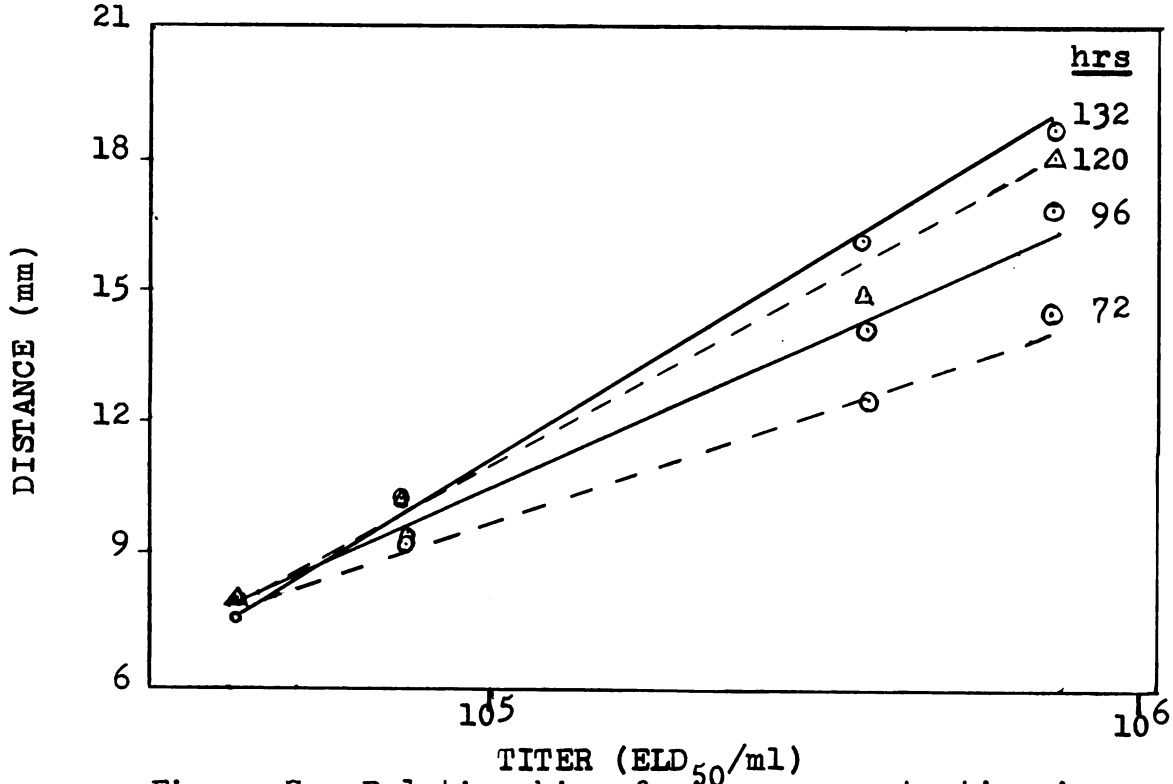


Figure 7. Relationship of virus concentration in reservoir to distance of leading edge of precipitate.

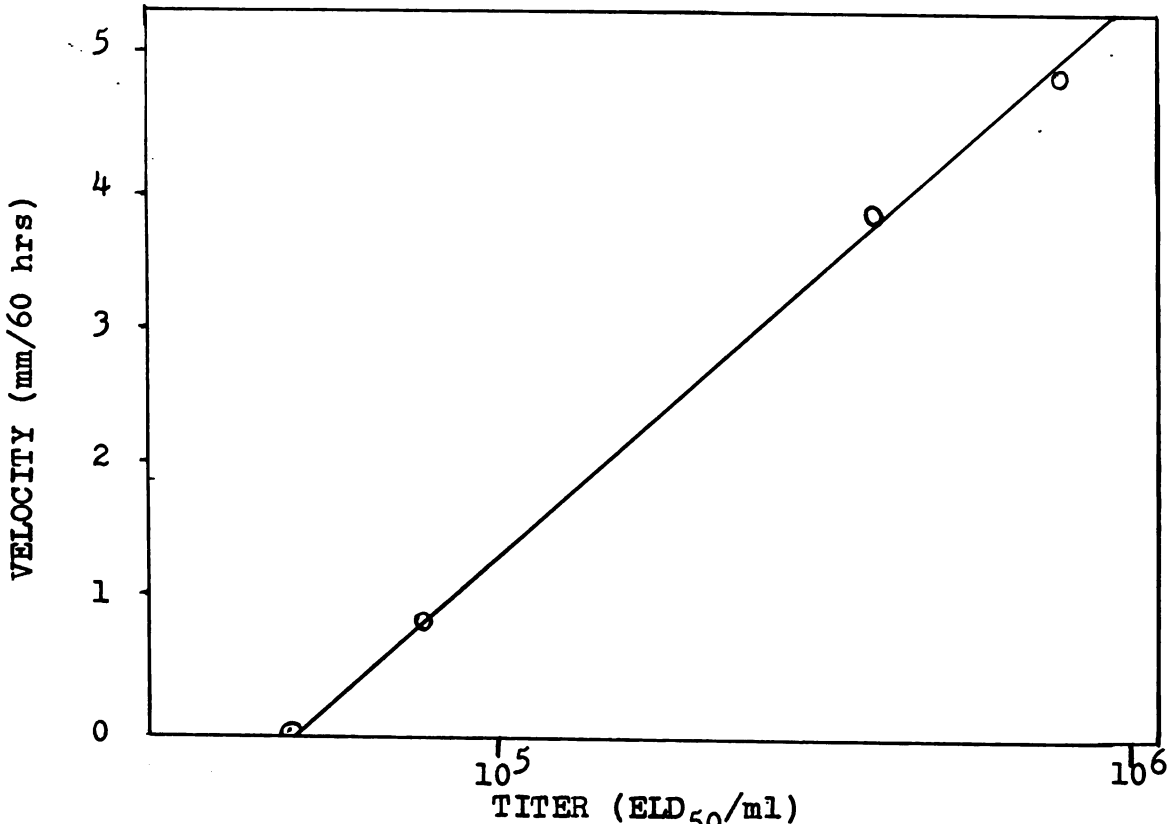


Figure 8. Effect of virus concentration on velocity of precipitate migration.

Statistical Analysis

To determine the relative precision of the block titration method, one virus sample was tested 15 times. The antigen was a 10^{-1} dilution of $L_2 79$ with a final titer of $10^{5.2}$ ELD_{50}/ml. Tests were run in a ½ inch agar strip incubated at 37°C for 75 hours prior to measuring (Table 6). A standard millimeter rule was used to determine the distance of the leading edge of precipitate from the antigen well, measured to the nearest 0.2 mm.

Results are graphically represented in Figure 9. The mean of the 15 tests was 9.57 mm, while the mode and median were 9.4 mm. Sample standard deviation was 0.4 mm (Parratt, 1961).

Table 6. Tabulation of the results from replicate titration experiment.

Value (X_i)	Frequency (f)	Deviation (dev.)	(f)(dev.)	$(f)(dev.)^2$
9.0	1	-0.57	0.57	0.3249
9.2	1	-0.37	0.37	0.1369
9.4	7	-0.17	1.19	0.2023
9.6	3	+0.03	0.09	0.0027
10.0	1	+0.43	0.43	0.1849
10.4	2	+0.83	1.66	1.3778

Mean = $\Sigma X_i/n$ = 143.6/15 = 9.57

Mode = 9.4

Median = 9.4

Sample Variance = $\Sigma (X_i-\bar{X})^2/n-1$ = 2.2295/14 = 0.1593

Sample Standard Deviation = (sample variance)$^{\frac{1}{2}}$ = 0.4

Mean Deviation = $\Sigma |dev.| /n$ = 0.29

95% Confidence Interval = $9.37 \leq \bar{X} \leq 9.77$

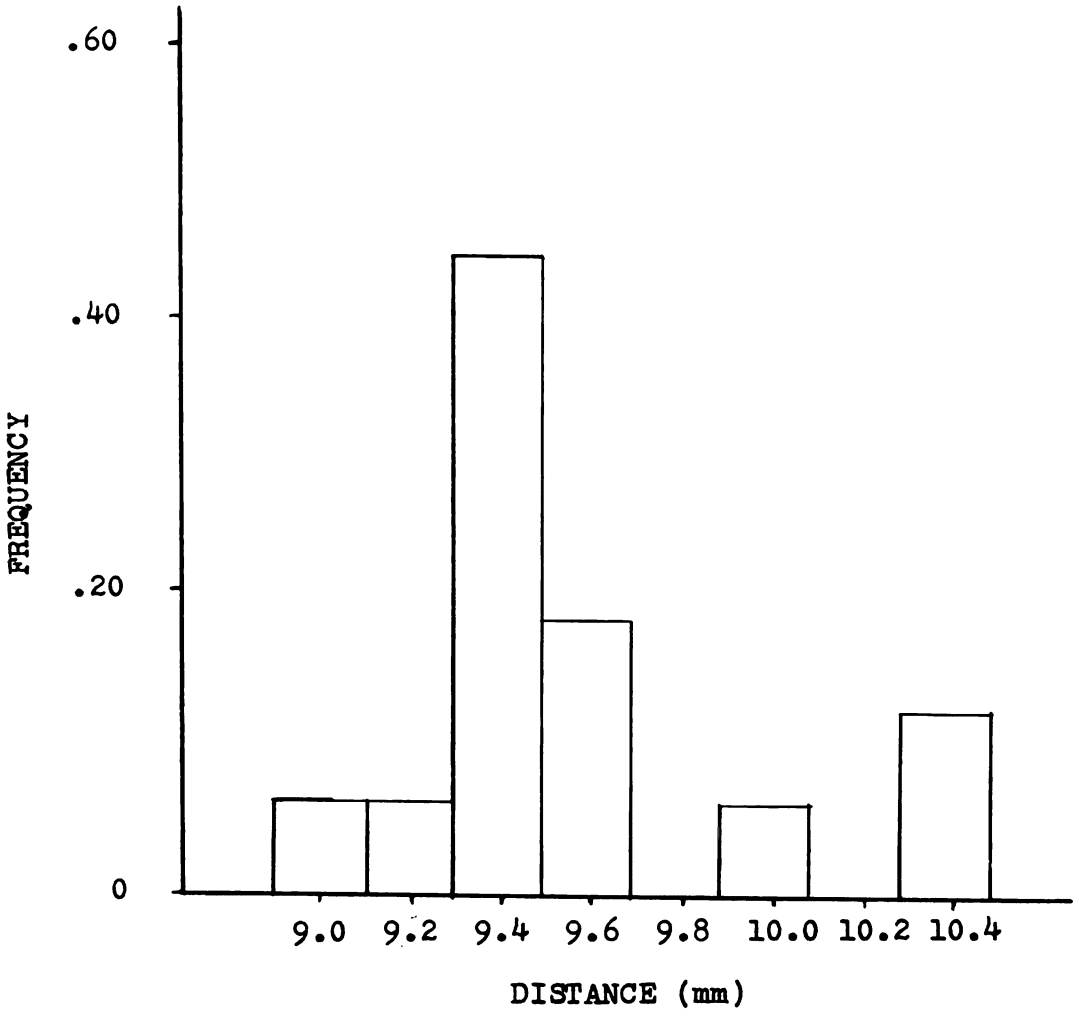


Figure 9. Histogram of frequency versus distance of the leading edge of precipitate from the antigen well.

DISCUSSION

The results from diffusing duck hepatitis virus against various antisera essentially confirmed the findings of Murty and Hanson (1961). Two lines of precipitate were specific for the virus, with a third appearing occasionally. Since the third line lay on or immediately adjacent to one of the specific lines, this may actually have been a resolution of one line into two. However, it was noted that the intensity of this third line was inversely proportional to the degree of purity of the antiserum, and since the majority of particles in normal allantoic fluid are about the same size as the virus (Nazerian, 1960), the third line most probably was due to embryonic impurities.

Though the original gel precipitation study of DHV was done several years ago, no attempt has been made prior to this time to apply the data quantitatively. In all of the published works on DHV where a titer had been determined (Hanson and Alberts, 1956; Rao et al., 1958; Pollard and Starr, 1959; Kaeberle et al., 1961; Hwang and Dougherty, 1962; Sazawa et al., 1963; Fitzgerald and Hanson, 1966), the only method used was that of quantal titration in chicken embryos. Doing this for each phase of an experiment involves a very large number of embryonating eggs, an egg incubator, complete sets of virus dilutions, and daily candling for 6-7 days. The diffusion method suggested here only requires a plastic diffusion block, one set of dilutions, and antiserum.

The incubation time would be shortened to 5 days and the block could be kept in a standard laboratory incubator.

The principle behind the apparatus used in this study is that diffusion results from a difference in concentrations. In any closed system, molecules will migrate from one area to another until they are in equilibrium, or their concentrations are equal. In an Ouchterlony plate, about 0.1 ml of a substance is placed in a depression in the agar. If there is 20 ml of agar, diffusion will occur because the well concentration, C_w, is greater than the concentration of the substance in the agar, C_a. At equilibrium, $C_w = C_a$, and the final concentration, C_f, will be 0.1/20 x C_w, or 5% of the original concentration in the well. A similar ratio would exist for most macro and micro plate tests. In a tube test with equal volumes of antiserum, agar, and antigen, C_f would be 33% of C_w.

In the diffusion block method, C_f = 1/2.25 x C_w or 44%, since there was 1.0 ml in each well plus 0.25 ml of agar in the slot. These figures only represent approximations, since as a precipitate forms, the molecules involved are no longer available for diffusion; more molecules are then fed into the area, with a proportionate decrease in C_a. Comparatively, however, the concentration available for reaction is the highest in the block design and it should thus have the greatest sensitivity.

This sensitivity could easily be increased by changing the proportions in a block of the same general design. A much narrower reaction slot could be made, and it could fill

itself with agar by capillary action. Smaller reservoirs would result in the use of less serum, one of the limiting factors in this series of experiments.

Examining and recording the positions of lines could be done with the aid of a low power microscope, which could also be used to photograph the lines so that readings could be taken from an enlarged print. A diffusion titration in such a block would thus have the advantage of increased sensitivity and decreased reactant volume and incubation time. Dilutions would cover a space of several logs, instead of 1/2...1/4... 1/8...etc. as in the plate and tube tests.

To determine the titer, two alternatives are available. One can either measure the distance of the leading edge of precipitate and compare it to the standard curve for that specific time, or take measurements at two different times and calculate the velocity for comparison with another curve (Figures 7 and 8). Both velocity and distance exhibited a linear relationship to concentration as expected (Kabat and Mayer, 1964). The velocity curve, however, represents the average of several distance curves and should be more reliable.

Standard curves such as these would have to be established for each antiserum. If enough serum were available, one curve would be sufficient for up to hundreds of tests.

Of the antisera tested here, B was the most efficient with regards to distinctness of precipitate and antibody titer. Absorbing it with normal allantoic fluid minimized

the non-specific lines, but simultaneously exerted a diluting effect on the specific portion of the antiserum resulting in decreased intensity. Since the non-specific lines appear between those which are specific, they should not interfere in a test of this nature and absorption is not necessary.

The antiserum prepared using virus purified on the anion exchange resin gave the same type of curve for titer versus distance, but the lines were not as distinct. Trying a number of different resins and using a more concentrated virus suspension should give an antiserum equal to or better than those now in use.

Several of the environmental variables for the diffusion block reaction have been tested. The optimal combination involved double diffusion in a ½ inch agar strip with the reactants in the form of a gel. This should be allowed to react at 37°C for a period of 5 days.

Using this information plus the data from antiserum B given in figures 7 and 8, a hypothetical titration would be conducted in the following manner. The sample would be combined directly with agar in a 1:1 ratio and placed in well #1. A measurable line would form if the titer in the well was $10^{4.5}$ to $10^{6.0}$ ELD_{50}. The next three wells would have 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the sample plus agar to detect a titer greater than 10^{6}. The next well would contain agar plus equal volumes of the sample and a known virus suspension having a titer of $10^{5.0}$. If the sample titer were $10^{4.0}$, the minimum titer of this mixture would be 5.5×10^{4} and would be detectable. The remaining wells could then serve

as controls if they contained antiserum versus saline, virus vs. saline, and a known virus concentration vs. the antiserum used in the titration. The measurable limits of such a system would be approximately $10^{4.0}$ to 10^{10} ELD_{50} per ml of sample.

If one had an estimate of the titer or desired a more accurate determination after the initial results, a series of dilutions within one or two logs of the estimate would be prepared and the findings from all of them used to find the average.

In the preparation of the standard curves it should be noted that any acceptable system could be used for the reference titration. The method of Reed and Muench and that of Spearman-Karber were used here because they are the most widely used. The former, however, has been the object of a number of critical reviews such as that of Finney (1959), who observed that "no satisfactory measure of precision is available" and doubts that their basic assumptions are all valid.

Basing the standard curves on a more accure reference system would eliminate some of these objections. This would be feasible even if the better method were more difficult since it need be done only once for a large number of titrations.

In the statistical analysis of replicate titrations, results tended to cluster about a single point. Results and calculations are given in table 6. It will be noted that the

mean deviation is the average deviation before squaring, and is thus less sensitive to large fluctuations than the commonly used standard deviation. A small mean deviation, such as the calculated 0.29, indicates measurements are closely grouped and the distribution has a sharp peak (Parrat, 1961).

Two measurements (both 10.4 mm) were questionable since they were so far from the average value (9.57±4 mm) and their pattern of lines was much less distinct than the others. This could have been caused by putting too much virus in the reaction well, or having too little agar in the slot. In either case, the two values lie outside of the 2σ range where P = .95, so there is at least a 95% probability that a normal measurement will not be equal to or greater than 10.4. These two values may also be rejected on the basis of the 2.5d (Skoog and West, 1966), "Q" (Dean and Dixon, 1951), and "t" (Moore et al., 1951) tests for the significance of outlying values.

If these two measurements were omitted, the remainder of the data would have the following parameters:

Mean = 9.45

Variance = 0.07

Standard Deviation = 0.27

Mean Deviation = 0.20

These values are more precise than those given previously and indicate that the deviation could be much lower than expected. One way to obtain more accurate calculations would be to increase the number of observations substantially.

There are some tests reported to have a higher sensitivity than that in the block test, but these use nitrogen determinations (Heidelberg and Kendall, 1935; Rappaport, 1957) or optical density readings (Schmidt, 1957). They have disadvantages in time, equipment expense, complex purifications and chemical analyses, and the fact that non-specific precipitation will give erroneous results.

The apparatus and method presented in this study constitute an attempt to avoid some of these difficulties while achieving high sensitivity with some degree of precision. Its application should be especially pertinent when used in conjunction with viruses for which the quantitative methods are quite limited, such as with duck hepatitis virus.

SUMMARY

1. Two antigenic components specific for duck hepatitis virus, strain $L_2$78, were detected using gel diffusion.
2. Duck hepatitis virus was adsorbed to an ion exchange resin in an attempt at purification. Presence of the virus was confirmed with precipitation tests and electron microscopy.
3. Apparatus necessary for a new form of gel diffusion was designed and constructed.
4. Several variables were tested for the reactions occurring in the apparatus, including temperature, time, length of reaction well, type of diffusion, and physical state of the reactants.
5. A new method for the quantification of duck hepatitis virus was established in which the position of precipitate in a reaction well was proportional to the virus titer. Its principle and reliability were discussed, and suggestions offered for possible improvements.

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