

STUDIES ON KFD VIRUS WITH REFERENCE TO IMMUNISATION

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**SUBMITTED BY
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Master of Science
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By
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C o n t e n t s.

CHAPTER I	Page
General Introduction.....	1 - 12
CHAPTER II	
Approach to the problem.....	13 - 21
CHAPTER III	
Material and Methods.....	22 - 35
CHAPTER IV	
Results.....	36 - 53
CHAPTER V	
Discussion.....	54 - 69
CHAPTER VI	
Summary and Conclusions	70 - 71
CHAPTER VII	
Bibliography.....	72 - 82

CHAPTER I.

GENERAL INTRODUCTION.

GENERAL INTRODUCTION

Arthropod borne viruses are defined as viruses which have the capacity to infect certain vertebrates, mammals and birds and to multiply in the body of arthropod vectors. The arthropod is infected by feeding on the host having viremia. The virus undergoes multiplication in the body of the arthropod and after an extrinsic incubation period is capable of infecting a susceptible host by the bite of the arthropod. The arthropod is not affected by the multiplication of the virus in its body and can transmit the disease throughout its life once it gets infected. Some arthropods such as ticks have been known to transmit the infection to their progeny and thus act as a reservoir of virus. This definition excludes viruses which are transmitted mechanically by an arthropod.

The participation of arthropods in the transmission of certain diseases had been known for a long period. The first instance of transmission of a virus disease by an arthropod vector, a mosquito, was conclusively proved by the members of the Yellow Fever Commission (Reed, Carroll and Agramonte 1911). They showed that the mosquitoes are essential in the natural cycle of the yellow fever as it infects man. Isolation of sandfly fever virus from the midge *Phlebotomus* and its role in transmission was later proved by Doerr, Franz and Taussig(1909). The first evidence of transmission of

Dengue fever by *Aedes aegypti* mosquito was shown by Bancroft in 1906 and was conclusively established by Cleland in 1919.

The role of ticks in the transmission of virus disease was shown by Gordon (1932) in connection with louping ill in sheep in Scotland. Silber and Soloviev (1946) considered tick as the vector of RSSE. Ticks are not only the vectors but are reservoirs also, as evidence of transovarial transmission has been shown in the case of tick borne encephalitis group of viruses.

About 50 viruses have been isolated in the last 30 years and more and more viruses are being added to the list of arthropod borne viruses every year. Many of the viruses in the Arbovir (Arthropod borne) family have been classified into groups by Casals & Webster (1957), members of which can be more or less closely related to each other serologically and are sharply separable from belonging to other groups. This grouping was possible on the basis of antigen antibody relationships detected by immunologic reactions such as haemagglutination and haemagglutination inhibition tests, complement fixation tests, neutralisation tests and cross protection tests. However this classification excludes other biological properties of viruses such as host range, routes of inoculation, incubation period and type of disease produced in man.

Studies on arbor virus diseases in India were started in the year 1952 at Virus Research Centre, Poona. It was felt by the investigators that a preliminary survey of the sera of Indian population would throw light on the presence of immunity to certain of the neurotropic viruses from other parts of the world. The immunity pattern of 576 sera collected from different parts of the subcontinent to ten viruses has been presented by Smithburn, Kerr and Gatne(1954). They showed that dengue and West Nile virus predominated in most parts of Southern and Central India. Antibodies, to JBE were also shown to be present in the sera of individuals residing in Madras State and Madhya Pradesh. The authors, however, cautioned about considering presence of neutralising antibodies to JBE as definite proof of the presence of that virus because of the known antigenic relationship between JBE, Murray valley encephalitis, Saint Louis encephalitis and West Nile viruses. Later studies by Work and Shah(1956) Webb and Pereira (1956) Lopeyssonnie L and Gobalkichenin S (1958) have proved the presence of JBE virus in Southern India by clinical diagnosis, confirmed by serological tests.

All the three viruses mentioned above have mosquitoes as transmitting agents, eight sera however, were shown to have antibodies to RSSE, a group of virus which is transmitted by ticks. Six of these eight sera were from Saurashtra, one from Bombay State and one from Madras. The significance of these findings was not understood and remains an obscure riddle to this day.

In March 1957 the attention of the investigators of Virus Research Centre, Poona, was drawn to the reported deaths of monkeys in a forest area of Shimoga District (Work & Trapido 1957b). The investigators at first suspected it to be due to yellow fever virus. They found that the common symptoms in human patients were sudden onset of headache, fever, low back and limb pains and severe prostration, often associated with inflammation of the eyes, diarrhoea and vomiting by 3rd or 4th day of illness. Bleeding from the nose, gums, stomach and intestines was noticed in a few cases. Temperature exceeded 39.4°C but seldom rose higher (Work et al 1957a).

The isolation of KFD virus was accomplished in the year 1957 from the acute serum collected on 1st and 5th day of illness from human patients as well as from the post mortem specimen of a langur, *Presbytis entellus* and two bonnet macaques, *Macaca radiata* monkeys. Isolation was effected by intracerebral inoculation of serum, suspensions of heart muscle, skeletal muscle, lung, liver, spleen, kidney and brain of monkeys into two day old infants and three week old white mice. The incubation period in infants was 3-4 days and in adults 4-6 days and the mouse brain suspension titer exceeded 10^{-8} to 10^{-9} even in the earliest passages. The virus titer of mouse brain suspension reached 10^{-6} by subcutaneous route of inoculation in subadult mice after a prolonged incubation

period of 6-13 days. Rhesus, *Macaca mulatta*, when inoculated with infected langur sera by IC and IP routes, circulated virus from 4th through 7th day and 6 and 7th post inoculation days respectively and their sera collected three weeks after inoculation neutralised more than 3 logs of KFD virus. The sera of some monkeys from KFD area neutralised one hundred LD₅₀ mouse doses, indicating a survival from natural infection or inapparent infections (Work 1958).

KFD virus was first suspected to be an arbor virus because of the epizootic involvement of forest monkeys and the presence of a visceral disease in man similar to that of yellow fever virus. Crude saline antigens were tested by complement fixation (CF) against pools of Casals group A and B arbor virus hyperimmune sera. A group hyperimmune sera showed no fixation while B group showed fixation. Hyperimmune RSSE mouse serum fixed complement to high titer with KFD virus strains and RSSE antigen. Serum and antigen dilution by box CF titration confirmed that KFD isolates were similar to RSSE virus (Work 1958). These findings were further supported by isolation of KFD virus from ticks *Haemaphysalis spinigera* (Trapido et al 1959) and confirmed by chick tick chick cycle by Varma H.G.R. Webb H.E. and Pavri (1960), a behaviour similar to that of RSSE, which, incidently is a virus belonging to the tick borne encephalitis group.

Isolation of KFD virus in March 1957 opened a new field in the study of tick borne (RSS complex) viruses in India. The tick borne complex of viruses were not shown to have caused human disease in the tropics till then. This group of viruses has an extensive geographical distribution. An important feature to be noted is its marked variation in pathogenicity and tissue tropism.

In Great Britain a disease known as Louping ill involving CNS in sheep has been described by Gordon (1932). It is primarily a disease of sheep though human infections of shepherds and laboratory infections have been reported. Russian tick borne or spring summer encephalitis, blundulant meningo encephalitis or 'diphasic milk fever, and Omsk haemorrhagic fever have been encountered in Soviet Union from Far East to Western Russia in Europe. Many workers have shown the presence of this group of viruses responsible for causing diseases involving CNS in Central Europe extending from Sweden and Finland in the North to Balkan Peninsula in the South (quoted Olitsky and Casals 1959). A virus strain was isolated from ticks in Malaya by Smith CEG (1956) but no cases in humans have been reported so far.

The most common feature of the viruses of this group is transmission by ticks of the family Ixodidae. This has not been shown in the case of KFD. Repeated isolation of KFD virus from ticks of the *Haemaphysalus* species and its role in trans-

mission has been shown by Varma et al (1960). Transmission by mosquitoes has never been demonstrated. These viruses are grouped together on comparative antigenic analysis by Pond et al (1953).

The viruses of this group show marked tissue tropism.. Louping ill has been known for over a century in Scotland as a disease of sheep capable of producing CRS manifestations. In 1930 Pool et al established its etiology. It has never posed a serious problem in Great Britain but for the infection of laboratory workers and sheep attendants in enzootic area. In Russia it was first recognised by Panov in 1934 and Silber (quoted Olitsky and Casals 1959) isolated the virus in 1937. However it was traced back at least to 1880 when Kozhevnikov described a peculiar form of epilepsy now believed to be a sequel to tick borne encephalitis. It was also found in Siberia and European Russia as well as Western Europe, where, however it was relatively mild as compared to the Far Eastern variety. In Central Europe it had posed an important public health problem.

Though the viruses belonging to this group have been shown to be transmitted by tick bite, the transmission has also been attributed due to the drinking of milk from infected goats. The disease has been designated either as Bivulent meningo encephalitis or diphasic milk fever. The goats however do get infected through the tick bite.

The area of KFD from which virus isolations from humans were made in 1957, covered 70 sq.miles. Since then the cases in humans have been occurring centrifugally, moving mainly towards South and South West of the original focus. The most important feature of this disease is its great pathogenicity to two species of monkeys the langur *Presbytis entellus* and bonnet *Macacus radiata*. There has been a regular mortality in these two species in the infected area since 1957. The monkey mortality is less during monsoon than the rest of the seasons. It is interesting to note that the distribution of monkey death parallels with that of human cases. The infected areas appear to be increasing every year and other foci of this disease might be found in India, Work (1958).

KFD virus has been repeatedly isolated from post mortem specimens of monkeys *Presbytis entellus* and *Macaca radiata* sera of patients as well as ticks from all the three stages, viz. nymphs, larvae and adults. It has been isolated from milk of an infected woman by Shah and Murthi (1960) but its transmission through milk has not been demonstrated. The maximum number of cases are encountered in the months of April and May. Controls of this disease had to be considered before it became a major public health problem.

Review of literature on control and prevention of tick borne complex of viruses shows that the measures vary from the destruction of vectors on the one hand to the immunisation of the population by a suitable vaccine on the other.

Chumakov (quoted Olitsky and Clarke) has suggested the use of one acaricide aerosol bomb to be used within 5 to 7½ acres of forest to exterminate tick population and inhibit its repopulation for a period of 5 to 7 days. Extensive pasturage to reduce forage reserves and number of rodent shelters which support supply of foods to larvae and nymphs, affecting detrimentally the tick population has been suggested by Shumakov et al (1961).

A formalised mouse brain vaccine against RSSE has been used in the Soviet Union for some years and has given favourable results in a number of field trials (Sinorodintsev 1958). The vaccine was prepared by inactivating a 5% suspension of infected mouse brain by formalin in a final concentration of 1:600 at 0-4°C in 20 days. White mice vaccinated 3 times intraperitoneally with 0.25 ml were able to withstand against 10^5 logs of virus but they did not develop immunity to intracerebral challenge. In humans, three doses of 3 to 5 ml subcutaneously given at intervals of 7-10 days resulted in immunity for a limited time (1-2 years). He has reported that in 1939 there were 2 mild cases among 925 vaccinated wood cutters whereas 27 severe cases with 7 deaths among 1185 unvaccinated persons. In 1940 there was only 1 case out of 9578 vaccinated persons but 28 cases with 9 deaths in 6180 unvaccinated persons. A dried killed vaccine preparation was found to be stable for a long period.

Seroprophylaxis has been suggested by Chumakov (quoted by Yavia 1959) who showed its efficacy. Serum injected 24 hours after infection in mice protected mice in a number of cases. If serum was injected after 6 days it was active only in case of small doses. Serum prophylaxis carried out early after the tick bite, decreased the tick borne encephalitis morbidity by 3-4 times and if the disease developed serum prophylaxis prevented its development to severe form (Yavia 1959).

Hyperimmune serum was prepared in horses by inoculating 120-150 ml of mouse brain antigen subcutaneously at intervals of 7-8 days. Serum was collected 12 days after the 8th dose. Antiserum was purified by the diatherm 3 method. Use of antiencephalitis gamma globulin had been used for serum prophylaxis.

The efficacy of mouse brain RSSE vaccine and the urgency of the problem were taken into consideration. The "Walter Reed Army Medical Centre Washington" at the instance of the Rockefeller Foundation, undertook the production of RSSE vaccine for protecting the population from KFD area. Preliminary investigations on animals suggested that RSSE vaccine showed better protection as compared to KFD vaccine. A 10% mouse brain suspension with RSSE virus, inactivated by 0.2% formalin at 4°C was used in the extensive vaccination programme from Jan 1958 (Aniker et al 1962) Webb (1961) found that the disease was no less severe in vaccinated cases as compared to the unvaccinated ones. There were six cases in vaccinated individuals out of the total of 13 patients observed. The vaccinated persons were shown to have failed in the production of antibodies. (Shah et al 1962, Pavri et al 1962).

In the meantime, the Government of India, after taking into consideration the apparently recent introduction of KFD virus in India, and the high percentage of mortality (10%) due to KFD virus, undertook the decision, in consultation with the representatives of the Government of Maharashtra, Government of Mysore and I.C.M.R., to take all measures including preparation of vaccine to combat KFD. Rockefeller Foundation was kind enough to provide the necessary equipment for the establishment of a well equipped laboratory at Haffkine Institute, Bombay. It was decided that some experiments should be undertaken to lay down the requirements before going on to a large scale manufacture.

As KFD virus was found to behave in a similar manner in mice as RSE virus the usefulness of mouse brain, as a host for virus growth and vaccine production seemed to be a definite answer to the problem of preparation of KFD vaccine. However, in view of the fact that mouse brain vaccine might cause severe demyelinating lesions and at times show fatal reactions when repeated courses are given, it was considered advisable to simultaneously attempt other types of host systems also, such as tissue culture and fertile eggs. Since chick embryo has proved a suitable host for some arboviruses, it was decided to study its suitability as a host system for preparation of KFD vaccine.

The purpose of this study, therefore, was to determine the utility of the chick embryo as a host for virus growth; to find out an effective and safe method of inactivation and

Also, since vaccine produced should be stable under field conditions of storage, i.e. refrigerated temperature, to assess the stability of the vaccines prepared.

CHAPTER II

APPROACH TO THE PROBLEM

Approach to the Problem.

Yellow fever virus has played an important role in the development of our knowledge of Arbo Group of viruses. Since the findings of the Yellow fever Commission Reed, Carroll and Agramonte (1911) incriminating the mosquitoes as the vector of yellow fever, more and more data was obtained showing arthropods to be the vectors of various diseases due to virus. The list is increasing year after year and about 50 viruses have already been established as belonging to this group.

Arbo viruses of major public health importance are yellow fever, Japanese B encephalitis and tick borne encephalitis group of viruses. Data on production and testing of vaccines on large scale is available with these group of viruses. The only other viruses for which experimental vaccine lots have been prepared and tested are the Saint Louis encephalitis (SLE) and the Dengue fever virus. Vaccines against Western equine encephalitis, Eastern equine encephalitis and Venezuelan equine encephalitis have been prepared but used for vaccinating animals and laboratory personnel only. Yellow fever virus vaccine is an attenuated live virus vaccine and approach in that direction needs prolonged study. In the present case, early preparation of vaccine was desirable to eliminate KFD infection from that locality. More concentration was paid, therefore, to killed virus vaccine. Our approach to the problem of preparation of vaccine was, therefore, based on the experience of the production of Japanese B encephalitis and tick borne encephalitis group of virus vaccines.

Sabin(1943) has presented basic data on the production and testing of Japanese B encephalitis and Saint Louis encephalitis virus vaccines. He studied chick embryo, syrian hamsters, new born lambs and young sheep as an alternative source for mouse brain and found that except for syrian hamsters, other sources were unsuitable as a good source either for virus or immunising antigen. Syrian hamsters showed almost as high a titer as the 2-3 weeks old mice but were not considered useful as they do not breed as easily and their brains are only double the size of mouse brain. Mouse brain was found to be the most suitable host for this virus. A 10% mouse brain vaccine was prepared from JBE inoculated 2-3 weeks old mice and inactivated by various physico chemical methods. A formalin inactivated vaccine was, however, used for vaccination of the Japanese population for a period of at least 1-2 years. Casals and Olitsky (1945) showed that the RSSE mouse brain vaccine, when inoculated 0.2 ml intraperitoneally in mice on 0 and 2nd day, resulted in the development of immunity in 2 days as tested by intraperitoneal challenge, and that immunity lasted for at least 430 days, the period of testing. Levkovich has used 22 days old mice for preparation of JEE virus vaccine. A mention may be made here that Svetovidavsky et al (1960) have suggested the preparation of vaccines from suckling mice and rats instead of subadult mice, in order to obtain allergen free vaccines against transmissible encephalitides and rabies. They found that 6 weeks old mice and 16 day old rats develop encephalitogenic substance causing neuroparalytic accidents in guinea pigs.

Use of suckling mice for production of vaccines on a large scale have not been reported so far.

A number of investigators have studied use of chick embryo as a host for obtaining a high yield of virus for preparation of vaccine using Japanese B encephalitis virus. As mentioned above, Sahin reported chick embryo as unsuitable as a source for JBE virus. Smith and Herring (1956) reported results on cultivation of JBE virus in chick embryo by using embryos of age varying from 5 to 14 days. They found that eggs were most susceptible by yolk sac route of inoculation regardless of age of chick embryo and that 10 days old embryos showed uniform deaths. Titration in eggs gave reliable and comparable results. Dead eggs showed a higher titer than live ones. Howitt (1946) has carried out experiments, such as finding an optimum route of inoculation, optimum age of chick embryo, optimum amount of inoculum, optimum day of harvest, effect of passages in eggs and temperature of incubation of eggs after inoculation. Similar, though not exactly similar, experiments using tick borne encephalitis virus have been carried out by Fedorov (1960) who prepared vaccines from these different sources and tested them in animals. He too found the whole chick embryo as the most useful tissue for preparation of vaccines. Yolk sac route of inoculation was preferred by all these workers as higher titers were obtained by this route. Warren J and Hough R.G. (1946) however have obtained good titers by inoculating into the allantoic fluid of 7 days old chick embryo and have reported that

the chick embryo vaccines were sufficiently immunogenic to be tried for mass vaccination.

With the development of tissue culture methods and the successful adaptation of most of the arbor group of viruses to different cell lines a number of investigators were attracted towards using tissue culture method for cultivation of virus for vaccine. Bhatt and Work (1957) and McCollum and Foley (1957) have reported adaptation of JBE virus to tissue culture but no report has so far been made regarding the preparation of JBE virus vaccine from tissue culture. Danes and Benda (1960a) and Ilyenko (1959) have used chick embryo fibroblast tissue for growing virus of tick borne encephalitis and have prepared vaccine from the virus grown in this tissue. They have used the monolayer of chick embryo fibroblast tissue as a host and differ only in seeding the virus. Thus Danes and Benda have seeded the virus one day after cell culture; Ilyenko has seeded virus 3-4 days after the growth of the cells. The vaccines obtained after inactivation at 4°C with formalin were highly immunogenic. Polnar (1959) has grown the Hungarian strain Koni of tick borne encephalitis in Hattland type of chick embryo tissue culture and claims to have obtained a strain which has lost its pathogenicity for mice after 40th passage in tissue culture. He has, however, not reported its use as a vaccine in humans.

Thus we have seen that the main hosts being used for preparation of vaccines from arbor group of viruses are the mouse

brain, the chick embryo and the tissue culture. In our studies, on KFD vaccine preparation at the Haffkine Institute, all the three systems have been tried. The present work deals only with the studies on chick embryo vaccines.

Methods of inactivation:

Studies conducted on RSSE and Japanese B encephalitis vaccines are mostly on virus suspension inactivated by formalin. Sabin(1943) has reported use of ultra violet light, soft X rays and ozone for inactivation of JBE virus for vaccine preparation. Smorodintsev (1958) has used ether, phenol and heat for inactivation of RSSE virus. Their investigations led them to the conclusion that formalin not only inactivated the virus but caused no appreciable loss in the antigenicity of the vaccines produced. This could not, however, be said with the other inactivating agents mentioned above. Many workers like Casals & Olitskey 1945, Danes and Benda (1960), Loukovich(1940) Pond et al (1953) Schubladze(1959) have used formalin for inactivation of this group of viruses though with varying concentrations and temperatures. The final concentrations of formalin vary from 0.5% - 0.05% depending upon the tissue used for the preparation of vaccine. Inactivation was carried out at 4°C as Smorodintsev's findings showed a loss in antigenicity when inactivation was carried out at 37°C. Similar results were obtained by Sabin for JBE virus vaccines. Fedorov, however, has inactivated tick borne encephalitis virus using a final concentration of 1:750 of formalin at 18°C for 3 days, without loss of antigenity as studied by potency tests in mice.

Andonov(1957) has described various physical and chemical methods of inactivation of tick borne encephalitis viruses. Thus he found that heating 10% virus suspension of Ias 8 strain at 60°C for 1 hour, Ix-10 strain at 70°C and Im strain at 60°C for 30 minutes failed to produce complete inactivation. Heating at 100°C for 1-2 min. did not always cause full inactivation. Ultra violet irradiation for 30 minutes caused reliable inactivation. He used alcohol, 5% phenol, 2-6% HCHO, 1 and 3% chloramine, 0.02% $K_2Cr_2O_7$, 5% lysol and 2% NaOH to inactivate the viruses. All his studies were however directed against decontamination rather than for preparation of vaccines. Ananov (1959) has used H_2O_2 of various concentrations to inactivate Ix-10 strain of tick borne encephalitis. He has also tried methylene blue to elicit photodynamic effect on tick borne encephalitis virus but he too has not described whether inactivation studies were carried out to prepare vaccines.

LoGrippe in 1954 started studies in search of a suitable chemical for inactivation of viruses while retaining their antigenicity. On screening about 600 chemicals he came across two agents a) Nitrogen mustard gas and b) Beta-propio-lactone (BPL) as the suitable chemicals; however of the two he found Beta-propio-lactone was the chemical of choice. He has done a comparative antigenic analysis of the vaccines produced by inactivating virus suspension of 111 strain of murine encephalomyelitis(encephalomyocarditis group of viruses), Eastern equine encephalitis and rabies.

A comparison was made between live virus vaccines, vaccines prepared by inactivating virus suspension with BPL, formalin or phenol. He was able to show that BPL inactivated vaccines were almost comparable to live virus vaccines. In fact he found that BPL inactivated vaccines were superior to those prepared by formalin or phenol. Polley and Guerin (1957) have used BPL for inactivation of influenza virus and observed that vaccines thus prepared were antigenic. BPL has also been used for preparation of noninfectious haemagglutinins (Il'enko V.I Semenov B.P & Rezapova A.I. 1961).

BPL has certain advantages over formalin or phenol in that, that the concentrations of BPL required for complete inactivation is comparatively less than that of formalin and that the rate of inactivation is faster. It is active at a wide range of pH. All the viruses (viz. EEE, LCM, polio, rabies, influenza, etc) so far used have been inactivated by BPL. It has a high penetrating power, 1% BPL used for tissue sterilisation was capable of inactivating LCM, MM and ERF viruses from quantities titrable to non-detectable if the tissue did not exceed 3 mm in diameter. LoGrippe (1958) has further modified his method of inactivation of viruses by coupling the action of BPL with that of ultra violet light.

Virus vaccines have been generally found to be low in antigenicity when inactivated by heat. Smorodintsev showed that heat inactivated vaccines of tick borne encephalitis virus were of low potency. EFD virus failed to get inactivated even after two hours of incubation at 56°C when preinactivation titer of the

suspension was high and the vaccines produced were of lower potency as compared to the formalin inactivated mouse brain vaccines.

As live virus was isolated from polio vaccine prepared by reputed pharmaceutical concerns in U.S.A. in 1954, more and more attention was paid to the rate of inactivation of polio virus by formalin by a number of authors such as Charney et al (1959) Salk and Gori (1959), Sven Gard (1959) Timm et al 1956. Salk has given various reasons for failure of these vaccines in getting inactivated by formalin; the most important being that filtration of material was not carried out by some concerns. On studies of kinetics of inactivation he found that log of infectivity usually diminished in a linear relation to time of exposure to formaldehyde i.e. the inactivation ran the course of 1st order reaction. This conclusion was however not substantiated by other investigators Charney et al 1959 Svengard 1959 Timm et al 1956 Danes and Benda (1960b) have reported the studies on rate of inactivation of tick borne encephalitis virus by formalin. The results of the inactivation studies on EFD virus by BFL and formalin are presented in Chapter IV.

Killed vaccine produced by any method should be highly antigenic i.e. there should be no appreciable loss in antigenicity with the loss of infectivity and the preparation should be stable for a long time when stored under conditions available in the field. A study was undertaken to determine the stability of vaccines produced.

There have been a number of potency tests described by various authors (Danes and Benda 1960, Pond et al 1953, Sabin 1943) The present study was limited to one method which has been found to be satisfactory at our laboratory. Serological tests such as haemagglutination (HA), haemagglutination inhibition (HI) and neutralisation tests (NT) were also undertaken for identification of virus from passages. The procedure followed for these tests has been described in Chapter III.

To summarise, the approach to the problem consisted of a) study of the chick embryo as a host system for high yield of virus, variations in various factors, like inoculi, age of embryo, incubation temperature, etc. were studied for this purpose, b) a comparison of virus inactivation by BPL and formalin and the potency of the resulting vaccines and c) the stability and keeping qualities of the vaccines so prepared.

CHAPTER III.

MATERIAL AND METHODS

Materials and methods.

Three strains of KFD virus were used; two of them were isolated from human cases and one from ticks. Of the two human isolates, one was isolated in the year 1957 and the other in 1960.

Strains isolated from human cases:

- 1) P9605: This strain was isolated at the Virus Research Centre, Poona in 1957. It was isolated from an acute serum of a patient who had a severe attack of KFD. Material of the 3rd mouse brain passage was provided by the Director, Virus Research Centre, Poona. A stock of this strain was made and used for different tests.
- 2) KF31: This strain was isolated from a mild case of KFD at the Virus Diagnostic Laboratory, Shimoga. It has undergone two passages in mouse brain, and was kindly supplied by the Special Officer for KFD, Mysore State, in the form of infected mouse brains in glycerine. It was passed in one litter of mice and a stock was prepared by making one more mouse passage. The fourth passage material was used for further tests.
- 3) G11338: This strain was isolated from a pool of 169 nymphs and 63 larvae of *Haemaphysalis* spp. collected on April 15, 1957. (Trapido et al 1959). This was kindly supplied to us as a 10% mouse brain suspension by the Director, Virus Research Centre, Poona. A stock of 5th mouse brain passage of this strain was prepared for further study.

Mice: Swiss albino strain of mice were used for isolations. infectivity titrations and potency tests. 2-4 day old litters were used for isolation of virus, 4-5 weeks adults for infectivity titrations and mice weighing between 12-14 gms were used for potency testing of the vaccines.

Eggs: Fertile eggs of white leghorn breed were used for passaging and preparation of vaccines. The supply of these eggs was from two sources i) from a commercial firm and ii) from the poultry maintained at Pimpri by the Institute. The eggs were incubated at the Institute and the age of chick embryos used in the tests ranged from 5 days to 13 days.

Chicks: One day old chicks were bled from the heart for the erythrocytes used in the haemagglutination(HA) and haemagglutination inhibition(HI) tests. In later experiments fowl cells were used.

Rabbits: Rabbits bred at the Institute were used for collecting serum to be used as diluents in the test.

The composition of the various diluents used in the test were as follows:-

1) Rabbit Serum Saline(RSS):

Rabbits were bled either from the ear vein or through the heart. The serum was separated and was inactivated at 66°C for 30 minutes. It was then diluted to 10% in 0.9% NaCl solution. This diluent was used for suspending mouse brain and chick embryo and as diluents in infectivity titrations. 25% rabbit serum saline was used for preparing stock virus suspensions.

ii) Normal saline:

A stock of 9% NaCl was prepared and stored in the refrigerator. It was diluted ten fold in demineralised distilled water to obtain a 0.9% NaCl solution and the same was autoclaved at 10 lbs pressure for 10 minutes. This was used for preparing 10% and 25% RSS and for washing of chick and fowl cells.

iii) Phosphate buffer pH 7.6.

Solution I.

0.1M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	71.64 g
NaCl	170.0 g
Distilled water.	2 litres.

Solution II:

0.1M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	15.6 g
NaCl	85.0 g
Distilled water.	1 litre

84 ml of solution II was added to 2 litres of solution I. It was diluted 10 times with distilled water; resultant pH was 7.6. The buffer was sterilised by autoclaving at 15 lbs for 15 minutes.

iv) Borate buffer pH 9.0 and 9.3:

Stock solutions of following concentrations were made:

a) NaOH 1M

4g of NaOH was dissolved in 100 ml of distilled water in a volumetric flask.

b) 9% NaCl

90 g of NaCl powder (Merck brand) was dissolved in 1 litre of distilled water.

c) 0.5 M Boric acid solution:

30.92g of boric acid (Merck brand) was dissolved in 1 litre of hot water.

To prepare 1 litre of Borate saline buffer pH 9.0

Solution a:	1 M	NaOH	24 ml
Solution b:	9%	NaCl	80 ml
Solution c:	0.5M	Boric acid	100ml

were added and diluted to bring up the volume to 1 litre with demineralised water.

To prepare Borate saline buffer pH 9.3

Solution a:	1M	NaOH	34 ml
Solution b:	9%	NaCl	80 ml
Solution c:	0.5M	Boric Acid	100 ml

were added and volume made to 1 litre with demineralised water.

v) Virus adjusting diluents (V.A.D)

These were used for suspending erythrocytes used for HA and HI tests. These were prepared from stock solutions as follows:

Stock solutions of 0.2 M dibasic phosphate and 0.2M NaCl and 0.2M monobasic phosphate and 0.2M NaCl were mixed in the proportions described below. To the mixture an equal volume of borate buffer pH 9.0 was added to produce the final pH mentioned.

Dibasic phosphate 0.2M $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ <u>0.2M NaCl</u>	Monobasic phosphate 0.2M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ <u>0.2M NaCl</u>	V.A.D. to obtain <u>final pH</u>
12.5	87.5	6.0
22.0	78.0	6.2
32.0	68.0	6.4
45.0	55.0	6.6
55.0	45.0	6.8
64.0	36.0	7.0

VI) Beta-propiolactone:

The chemical agent BPL- $\text{CH}_2 - \text{CH}_2 - \text{C} = \text{O}$ is a colourless



stable liquid in its concentrated state but unstable in aqueous solutions. It is satisfactorily stored in neutraglass sealed ampoules at -20°C to -30°C . BPL has a specific gravity of 1.1460 and is 37.5% (by volume) soluble in water at 25°C . It reacts readily with hydroxyl, amino, carboxyl, sulphhydryl and phenolic groups, all of which are associated with proteins. BPL was received in a polyethylene bottle which was transferred in ampoules. Ampoules were sealed thereafter. It was stored in deep freeze at -20°C and removed at the time of use only.

vii) Formalin:

Merck's formalin containing not less than 35% HCHO was used. A dilution of this in phosphate buffer pH 7.6 giving a final concentration of 0.2% was used in all the preparation of formalised vaccines except mentioned otherwise.

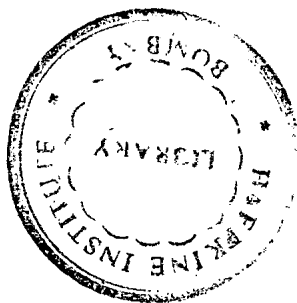
Methods:Inoculation of eggs:

Quite a number of methods have been described for inoculation of eggs. The methods described by Cabasso(1957) were followed.

- a) Yolk sac route of inoculation: 5-8 day old eggs were used. After marking the air sac, the egg was kept vertically with the air sac pointing upward. A small hole was made in the centre and a 38 mm 25g needle was inserted to a depth of 30 mm(the vertical height of the eggs generally varied between 55-60 mm). Inoculum was introduced into the Yolk sac. In order to make sure that the needle had reached yolk sac, a little quantity was aspirated back into the syringe. This practice was abandoned after getting sufficient practice.
- b) Chorion allantoic membrane inoculation: 11-13 days old eggs were used for inoculation. After displacing the air sac, the inoculum was introduced on the membrane. The eggs were kept horizontally and examined for pocks on CAM and for virus on the third day of inoculation.
- c) Inoculation into allantoic cavity: 9-10 days old chick embryo was used for inoculation into allantoic cavity. The eggs were held vertical, air sac was marked and 5 mm from the edge of the air sac, inoculum was introduced into the allantoic cavity by inserting the needle to a depth of 10 mm. Eggs were chilled overnight on 3rd day and allantoic fluid was collected. Further passages were made in eggs and the 5th passage material was inoculated in 3 days old litters to test the presence of virus.

Infectivity titrations:

Infectivity titrations were carried out in 4-5 weeks old mice. Serial ten fold dilutions of the virus suspension to be titrated were made in 10% rabbit serum saline. Separate pipettes were used for each dilution. The dilutions were inoculated in 4-5 weeks old mice, using a different syringe for each dilution, by intracerebral route with 0.03 ml or 0.2 ml by intraperitoneal route. Mice dying due to KFD virus were only considered for the calculation of titers. Non-specific deaths such as deaths occurring within the first three days were not considered for calculations. LD₅₀ titers were calculated by the Reed and Muench formula (1938).



Serological tests:

A) Neutralisation tests

Neutralisation test was carried out by using undiluted serum and ten fold dilutions of virus. Serum was inactivated at 56°C for 30 minutes. Serum virus mixture was incubated in water bath at 37°C for 1 hour and inoculated in 4-5 wks old mice intracerebrally. Though inoculation of serum virus mixture by intraperitoneal route is more sensitive (Casals & Olikaky 1945) it was not used as Hyperimmune sera having high values of Neutralisation Index (NI) were used for identification. Known positive and known negative sera were used as control. In one test, a fixed concentration of about 2 logs of virus dilution was used for neutralisation by serum. Observation period was of 14 days.

Survival ratio (SR) and Average Survival Time (AST) was determined for interpretation. A survival ratio of 5/5, 4/5 or 6/6, 5/6 was considered as positive; a SR of 3/5, 2/5 or 4/6, 3/6 was considered as equivocal and SR less than that as negative. A prolongation of AST by 4 days as compared to controls was considered as positive. Test was considered satisfactory if known positive and negative sera showed satisfactory results. Virus titer of serum virus mixture and virus suspension alone was calculated and the difference between the reciprocals of two was called Log Neutralisation Index (NI). A neutralisation index of 1.5 logs or more was considered as positive.

- B) Haemagglutination(HA) and Haemagglutination Inhibition test(HI).
The technique followed for HA and HI was similar to as described by Clarke and Casals(1955).

Haemagglutination test was routinely used to show the presence of virus in sick mice. A control normal mouse brain antigen was used in each test to show the specificity of agglutination. The test was carried out as follows:-

Preparation of antigen

Sick mice were harvested, brains removed and a 10% suspension was prepared in borate buffer pH 9.3. Stock suspension of protamine sulphate(Nutritional Biochemical Corporation make) prepared by suspending 50 mg powder in 1 ml of 0.9% NaCl was added to 10% mouse brain or chick embryo suspension to give a final concentration of 2.5 mg per ml of suspension. The mixture was kept in ice for 30 minutes and centrifuged in cold at 2500 RPM for 15 minutes. Supernate was used as antigen.

Normal mouse brain antigen was prepared in a similar manner.

Erythrocytes:

Fowl cells were first tested for haemagglutinability.

Subsequently cells from the same fowls were used.

Either fowl cells or one day old chick cells were used.

Cells preserved in Alsever's solution for not less than one day or more than eight days were washed in saline and centrifuged for 6 min, 3 min, 3 min, and finally for 6 minutes at 2000 RPM. A 10% suspension in 0.9% NaCl was further diluted to 0.25% in virus adjusting diluent(V.A.D).

Procedure.

Antigen was diluted 1:10 in borate saline buffer pH 9.0 containing 0.4% bovine albumin powder in solution. It was diluted two fold in eight tubes. To 0.5 ml of antigen dilutions 0.5 ml of 0.25% cells in V.A.D. were added. The usual pH was 6.4. The mixture was shaken and incubated in water bath at 37°C for 1 hour. Two tubes agglutination or more was considered as positive. The highest dilution showing haemagglutination was called the titer of the antigen.

C) Haemagglutination Inhibition test (HI)

HI test was done to confirm the isolation by using hyperimmune known positive serum. It was carried out as follows:-

Preparation of antigen:

Antigen for identification was prepared by the same method as that described for HA test. It was diluted, in prechilled borate buffer pH 9.0 containing 0.4%, bovine albumin, one hour before titration for uniform dispersal of the antigen. Dilution was effected to obtain 16 HA units in 0.5 ml of antigen, i.e. 8 HA units were used in the test since 0.25 ml of antigen was added to 0.25 ml of serum. It was retitrated after overnight incubation at 4°C to confirm the requisite number of HA units used in the test.

Preparation of sera for the test:

Sera collected for the test were diluted 1:5 in borate buffer pH 9.0 and were inactivated at 56°C for 30 min. They were

treated with 25% kaolin for 20 minutes at ^{room}~~100°~~ temperature (to absorb nonspecific agglutinins), centrifuged at 2500 RPM for $\frac{1}{2}$ hour and the supernate (1:10 dilution of serum) was used in the test. Hyperimmune sera, in mice, were prepared by immunising mice with 0.2 ml dose of formalised vaccines (inactivated by 0.5% formalin at 4°C for 7 days) 0 and 3 days apart and giving a live virus dose with infected mouse brain suspension on 10th, 16th and 20th day. Mice were bled on 28th-30th day. The titer was calculated by using known KFD antigen.

Procedure.

Two fold dilutions of serum were made in borate buffer pH 9.0, having 0.25 ml serum in each tube. To this 0.25 ml of antigen having 8HA units (16 HA units in 0.5 ml) was added. To control tubes 0.25 ml of diluent was added and the mixture shaken and kept in refrigerator overnight. It was retitrated on next day morning and cells were added in the same manner as described in HA test. The mixture was incubated in water bath at 37°C for 1 hour and the test was read. If there was inhibition by hyperimmune serum but no inhibition by control normal mouse sera the tentative diagnosis of virus was KFD. None of the other antigens viz. JSE, MVE, etc. were handled in this laboratory; hence the inhibition was considered to be specific.

Methods of inactivation:

A) By Beta-propiolactone (BPL):

A 10% solution of BPL (v/v) was first prepared in prechilled distilled water. This was done to slow down the rate of hydrolysis of BPL as well as to detect whether any deterioration or polymerisation of BPL has taken place on storage. On addition to cold water the drug forms globules which are miscible on vigorous shaking. Crystallisation or cloudiness on addition to ice cold water is an indication of deterioration of the drug. The dilution of BPL also obviates the effect of concentration on the protein. Concentrations varying from 0.2% to 0.01% were used for carrying out inactivation. Equal proportion of a solution of BPL in prechilled distilled water was added to 20% chick embryo suspension. The final concentration of chick embryo suspension was reduced to 10% and of BPL to the desired concentration. The suspensions were kept at 37°C in water bath or at + 4°C in refrigerator. Aliquots were removed at intervals and inoculated in 3-4 days old litters for isolation. Mortality with specific symptoms of KFD substantiated by HA, were considered as positive isolations. Further passages were made in those cases where specificity of illness due to KFD was in doubt. Controls without addition of BPL were also kept for incubation along with the test material and inoculated in mice to confirm the presence of virus after incubation. On the basis of preliminary screening a concentration of 0.05% BPL was chosen for studies on the rate of inactivation of KFD virus at 37°C.

A 10% suspension of chick embryo was prepared in phosphate buffer pH 7.6 and 10% BPL was added to give a final concentration of 0.05%. The suspension with and without (i.e. control) BPL were incubated at 37°C in water bath. Aliquots were removed at intervals of 5 min, 10 min, 20 min, 30 min and 1 hr and titrated immediately in 4-5 weeks old mice. Pre-inactivation titer and post incubation titers of control were also determined and results were plotted on the graph. 2-3 days old litters were inoculated for isolation of virus.

B) Rate of inactivation of KFD virus by 0.2% formalin at 4°C.

Formalin as an inactivating agent has been in use for a long time. 0.2% formalin of a concentration of 35% formaldehyde was used for inactivation of all the three strains. 10% chick embryo suspension (virus suspension) was prepared in phosphate buffer pH 7.6 and an aliquot was taken for preinactivation titer.

To the virus suspension, 1:10 dilution of formalin in phosphate buffer, was added to give a final concentration of 0.2%. The suspensions were incubated in refrigerator and occasionally shaken. Studies on rate of inactivation were carried out by removing aliquots of suspension at intervals and titrating them in 4-5 weeks old mice.

Methods of preparation of vaccines:

After preliminary tests have been completed, the following methods were adopted.

Optimum conditions for each strain of virus were determined and accordingly egg inoculations were carried out. Chick embryos

were collected three days after inoculation. The lens and retinal cap were dissected and discarded. Remaining chick embryo was rinsed in 0.9% NaCl, weighed and a 10% suspension was prepared in phosphate buffer pH 7.6 for inactivation by formalin and BPL. An aliquot was taken for preinactivation titer. Inactivation was carried out either at +4°C for 14 days with 0.2% formalin or for 1 hour at 37°C with 0.05% BPL. The pH in the latter case was brought to 7.0 by addition of 1.0N NaOH. The vaccines were tested for safety and potency after relevant inactivation period.

Safety test for vaccines:

Safety testing of the vaccines was carried out by inoculating vaccines undiluted and diluted 1:10 in phosphate buffer in 3-4 days old litters. Mice were observed for sickness for 21 days and sick mice were passed to another group of mice in order to confirm isolation of KFD virus from vaccines. Further passages were made till isolation whether positive or negative was confirmed.

Potency tests:

The test employed was a modification of the test described by Pond et al (1953). 12-14g mice were vaccinated with a 1:10 dilution of vaccine intraperitoneally with 0.5 ml on 0 and 3rd day. Control mice were not vaccinated. The mice were challenged on 7th day with graded dilutions of virus in both vaccinated and control mice. The difference between the reciprocals of the titers was called as Log Protective Index (Log Pr.I). All the titers in these tests were calculated by Reed and Muech method (1938).

CHAPTER IV

RESULTS

RESULTS

Three strains of KFD Virus were used for studies on adaptation to chick embryo. One strain G11338 was used for adaptation by different routes of inoculation. For the other two strains (P9605 and KF31) only yolk sac route of inoculation was followed.

I. Adaptation of KFD Virus to chick embryo.

A) Yolk Sac route of inoculation:

a) Strain G11338:

Serial passages in 7 days old chick embryo were made by inoculating 0.1 ml of 10% suspension (mouse brain for initial passage and chick embryo for further passages).

5th and 15th passage material was shown to be KFD by HI test (table I and II) and 10th passage by the more specific neutralisation test (table III). Virus yields at 5th and 10th passage were $10^{-8.7}$. Mouse LD₅₀ units when titrated by I.C. route were $10^{-6.4}$ and $10^{-7.4}$. Egg lethal doses 50 (ELD₅₀) units respectively. HI titer of antigens prepared from either chick embryo suspensions or mouse brain suspension (these were obtained by inoculating chick embryo suspension in 3-4 days old mice) have been shown at appropriate passage levels. (fig.1).

b) Strains P9605 and KF31.

Figs. 2 and 3 give details about the passages of P9605 and KF31 strains in eggs respectively. Identification was carried out at 20th passage level. KF31 was confirmed by HI test (table IV) and P9605 by HI test (table IV) and neutralisation test (table V).

B) Inoculation of Chorioallantoic membrane.

Fig.5 gives details about the passages of an egg adapted strain inoculated on Chorioallantoic membrane. The titers in mice of allantoic fluid, chorioallantoic membrane suspension and suspension of whole chick embryo have been presented. Whole chick embryo shows the highest titers followed by that of chorioallantoic membrane and the least of allantoic fluid.

C) Allantoic route of inoculation.

Results of five serial passages in eggs of a mouse adapted strain (G11338) are shown in fig.4. Allantoic fluid was used for inoculation from 2nd egg passage. 5th egg passage was harvested and chorioallantoic membrane suspension chick embryo suspension and allantoic fluid were inoculated in one litter of 3-4 days old mice. Only one mouse died on the 5th post-inoculation day when allantoic fluid was used as inoculum. Whether the death of this mouse was due to KFD virus infection was not confirmed. Mice inoculated with chick embryo suspension and chorioallantoic membrane suspension did not show any sickness.

Identification of Virus passaged in chick embryo.

Identification of virus was carried out by HI test using mouse hyperimmune and normal mouse serum as control. The titer of hyperimmune serum was 1:160. 5th and 15th egg passage material was identified as KFD virus by HI test run 7 and 15 as shown in table No. I and II.

The 10th egg passage material was shown to be KFD by the more specific neutralisation test, table III using convalescent human serum. Preparation of hyperimmune serum in rabbits was tried and pre and 14th post inoculation day rabbit sera were included in the same test. Human convalescent serum neutralised 2.4 logs of virus used for the test. Strains P9605 and KP31 were identified by HI (table No. IV and neutralisation test table IV). Mouse hyperimmune sera against Japanese B encephalitis, Dengue and KFD virus were used. Inhibition was shown only with KFD hyperimmune serum. Immune serum in monkeys and convalescent human serum was used in neutralisation test and all the sera neutralised the chick embryo adapted virus suspension, thus showing to be KFD.

II. Determination of optimum conditions for maximum yield of virus.

Experiments were carried out to determine optimum condition such as age of chick embryo, day of harvest, amount and dilution of inoculum and temperature of incubation, to obtain high yields of virus.

a) Age of chick embryo.

Chick embryos of age varying from 5 days to 8 days were used. The embryos were washed in 0.9% NaCl and a 10% suspension was titrated in 4-5 weeks old mice. All the eggs were harvested on 3rd day. Results are presented in table VI. It may be seen that there is no significant difference in the titers of the embryos of different ages and the titers vary from test to test.

b) Day of harvest.

From the results presented in table VII it may be seen that

the embryos harvested on the 3rd day gives the highest yield of virus.

c) Amount and dilution of inoculum.

Table 13 and 14 shows the mouse LD₅₀ titers of harvested material. 1.0 ml of inoculum was found to be unsuitable. 0.2 - 0.5 ml of inoculum was found to be suitable.

d) Temperature of incubation.

After inoculation of eggs a set of eggs were incubated at 35°C and the other at 37°C. All the eggs were harvested on 3rd 4th inoculation day except in test No.10. The results are presented in table. (15). $\times$ (p. 51).

III Inactivation of KFD virus (G11338 at 37°C and 4°C with varying concentration of BPL).

Concentrations varying from 0.2% to 0.01% BPL were tried. The results are presented in table 16 and 17. Deaths due to KFD virus only are included in the table. A summary of the minimum time required for complete inoculation for each concentration and temperature is presented in table No.18.

IV. Rate of inactivation of KFD virus by 0.05% BPL at 37°C.

Rate of inactivation of KFD virus by 0.05% BPL at 37°C is shown by graph, fig.19. Ordinate shows the reciprocals of log numbers of mouse LD₅₀ titers and Abscissa denotes intervals at which titrations were carried out. Isolation of virus from BPL inactivated suspension after incubation of 1 hour at 37°C was not possible in any of the reactions. Titration of control mice was carried out only on two occasions as shown in the fig.

V. Rate of inactivation by 0.2% formalin at 4°C. Only one concentration, 0.2% formalin, was used for studies on the rate of inactivation of KFD virus by formalin.

Fig. 70 represents the rate at which inactivation of KFD virus takes place at 4°C. All the 3 strains were studied for the rate of inactivation. All show a smooth curve.

VI. A comparison of potencies of BPL inactivated and formalin inactivated vaccines.

0.05% BPL was used for carrying out inactivation at 37°C in one hour and then stored at 4°C. 0.2% formalin was used with the same material for inactivation at 4°C. Vaccines thus prepared were included in the same test every time. fig. 8. shows the comparison of potencies of BPL inactivated and formalin inactivated vaccines.

VII Stability of vaccines.

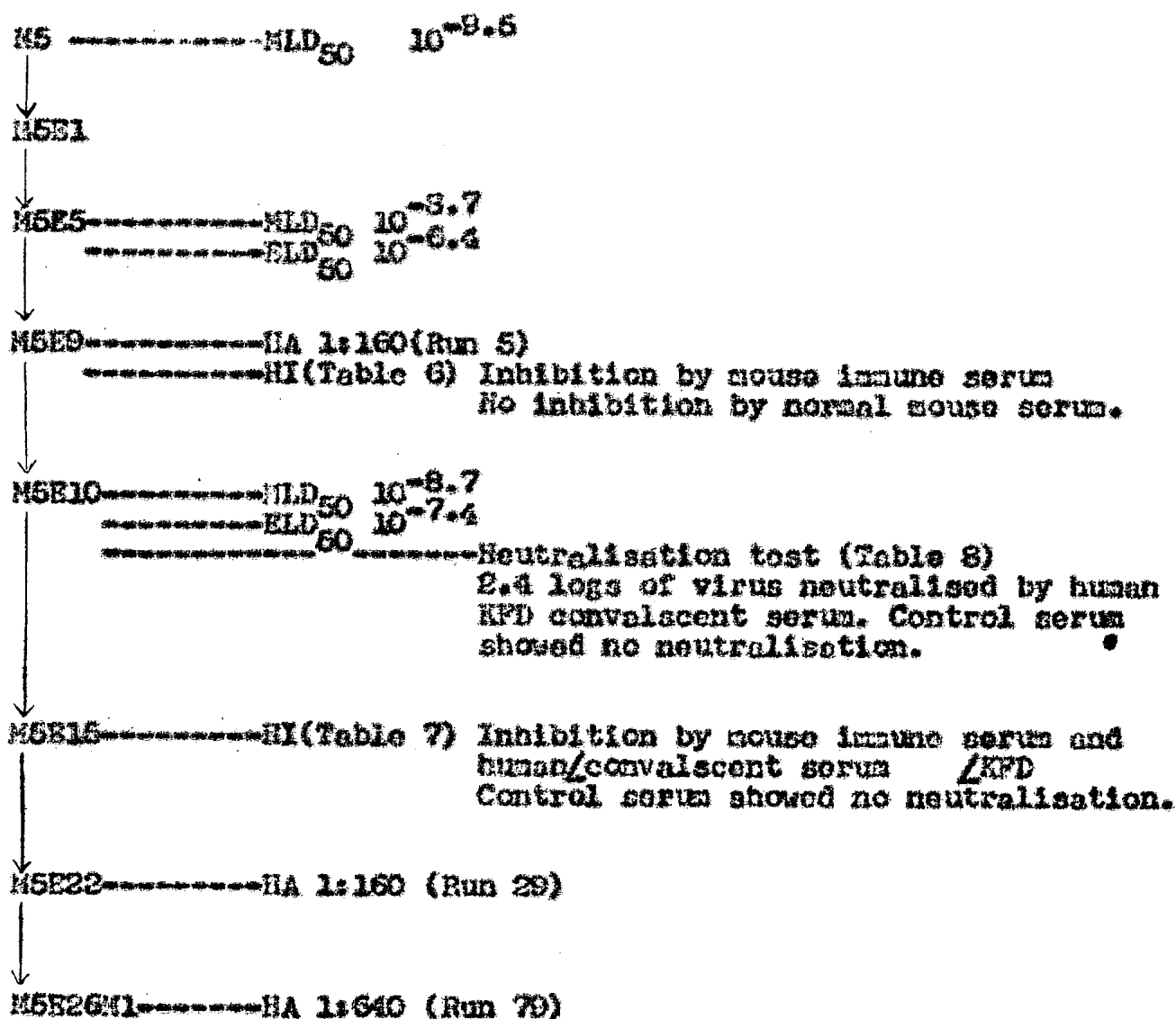
Formalin inactivated vaccine of each strain was tested for stability. Fig. 92 shows the values of protective indices upto a period of 238 days. Similarly BPL inactivated vaccines of each strain were tested but upto a period of 8 weeks only. The results are presented in fig. 92. Vaccines prepared by using KF31 inactivated by either chemical appears to be stable for a long time period and a drop of 1.4 logs was observed with formalin inactivated vaccine lot 80 into 238 days.

Adaptation of KFD Virus to Chick embryo.

A) Yolk sac route of inoculation

Fig. 1.

Strain G11338



M = passage in mice.

E = passage in eggs.

MLD₅₀ = mouse lethal dose 50%.

ELD₅₀ = Egg lethal dose 50%.

Fig.2.

42

Strain P96C5M4 ----- MLD₅₀ $10^{-9.6}$ ↓
M4E1

↓
M4E20----- HI(Table 9) a) Inhibition by KFD hyperimmune serum,
JBE, Dengue immune and normal mouse
serum did not show inhibition.

b) Neutralisation Test(Table 10).
Neutralised by KFD mouse immune serum
and monkey immune serum.

Fig.3.

Strain KF31M4 ----- MLD₅₀ $10^{-9.4}$ ↓
M4E1↓
M4E20

↓
M4E20M1----- HI(Table 9) Inhibition by KFD hyperimmune serum.
JBE, Dengue immune and normal mouse
serum did not show inhibition.

Fig.4

B. Inoculation of chorioallantoic membraneStrain G11339M5E26-----MLD₅₀ $10^{-8.0}$ ↓
M5E27

Allantoic fluid

Chorioallantoic
membrane suspension.

Amniotic fluid

↓
M5E27M1
SR=0/6
AST=5.7

↓
M5E27M1
SR=0/6
AST=3.8

↓
M5E27M1
SR=0/6
AST=3.7

↓
M5E31

Allantoic fluid

C.A.M. Suspension

Chick embryo suspension

MLD₅₀ $10^{-3.3}$ MLD₅₀ $10^{-5.2}$ MLD₅₀ $10^{-6.6}$

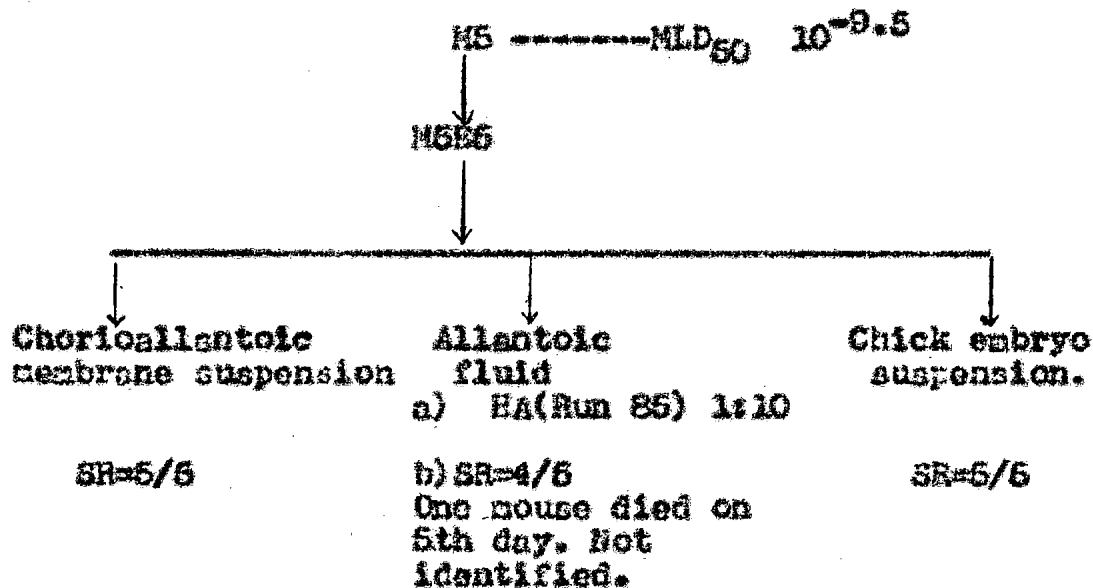
C. Allantoic route of inoculationStrain G11338

Table. -1

Identification of virus passaged in chick embryo.Strain: G11338.HA test on Strain G11338 M5E9

Sera: P 9605 hyperimmune serum was tested for
inhibition against 8 HA units.

Erythrocytes: One day old chick cells used.

S E R A	'Initial' dilution 37°C G11338 antigen 6.4 of serum										Control	
	1	2	3	4	5	6	7	8	9	1	2	
Normal Mouse Serum	1:10	+	+	+	+	+	not done				0	0
P9605 hyperimmune Serum	1:10	0	0	0	0	0	+	+	+	+	0	0

• = Haemagglutination.

0 = Not agglutinated.

Table. II

44

HI test on strain G11338 H5N15M1 and normal mouse brain antigen.SERA

P9605 Hyperimmune Serum and human KFD convalescent Serum was tested for inhibition against 8 HA units.

Erythrocytes: One day old chick cells used.

S E R A	Initial Dilution of serum	37°C G11338 Antigen 6.4								'H.N.B.' Antigen Control			
		11	2	3	4	5	6	7	8	1	2	1	2
P9605 mouse immune serum.	1:40	0	0	0	+	+	+	+	+	0	0	0	0
Human K.F.D. Convalescent.	1:10	0	0	0	0	0	+	+	+	0	0	0	0
Normal mouse serum.	1:10	+	+	+	+	not done				0	0	0	0

* Normal mouse brain antigen.

-----0-----

Table. III.

Neutralisation test

Materials: G11338 strain H5N10

Sera: Pre and 14th day post KFD inoculated rabbit sera and human convalescent serum.

Age of mice: 4-5 weeks

Dose: 0.03 ml. Route i.c.

Serum No	Category	Final dilution	SR	AST	Titer of control
KF41	Rabbit serum	$10^{-6.3}$	0/5	6.4	
	Pre inoc.				
KF42	-do-	-do-	0/4	6.0	
KF130	14th post inoc day.	-do-	0/5	6.6	
KF131	-do-	-do-	0/5	5.6	
KF79	Human conv.	-do-	6/5	14.0	
10/2005	Control.	$10^{-6.3}$	0/5	6.4	
-do-	-do-	$10^{-7.3}$	0/5	7.2	
-do-	-do-	$10^{-8.3}$	1/5	9.2	
-do-	-do-	$10^{-9.3}$	5/5	14.0	

$10^{-8.7}$

Table.IV

HI Test on strains P9605 and KF31 H4E20M1

SERA: P9605, JBE, Dengue hyperimmune sera and normal mouse serum against 8 HA units.

Erythro-
cytes: One day old chick cells washed & used.

Normal mouse brain antigen showed no HA activity.

S E R A	'Init' dil. of ser.	KF31 antigen						P9605 antigen						'Con- N.M.B trol			
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	1	2
Dengue hyper immune	1:10	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
JBE hyper- immune.	1:10	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
KFD hyper- immune.	1:40	0	0	0	+	+	+	0	0	0	+	+	+	0	0	0	0
Normal mouse serum.	1:10	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0

N.M.B. = Normal Mouse Brain antigen.

Table.V.

Neutralisation test on strain P9605.

Material: P9605 H4E20

Sera: P9605 House immune serum and Monkey immune sera.

Mice: 4-5 weeks Dose 0.03 ml Route I.C.

Table.Vf.
Virus dilution

S E R A	10 ^{-3.3}		10 ^{-4.3}		10 ^{-5.3}		10 ^{-6.3}		10 ^{-7.3}		10 ^{-8.3}		10 ^{-9.3}		Remarks.	
	SR	AST	SR	AST	SR	AST	SR	AST	SR	AST	SR	AST	SR	AST	Titer*	NI
Mouse immune serum	1/5	7.3	4/5	11.0	5/5	14.0	5/5	14.0	5/5	14.0					3.8	2.1
Monkey immune serum (Monkey No.2)	1/5	6.3	4/5	8.0	4/5	7.0	5/5	14.0	5/5	14.0					3.9	2.0
Monkey immune serum (Monkey No.3)	5/5	14.0	5/5	14.0	5/5	14.0	5/5	14.0	5/5	14.0					2.8	3.1
Control					0/5	8.2	4/5	7.0	5/5	14.0	5/5	14.0	5/5	14.0	5.9	

SR = Survival ratio - number surviving/number inoculated.

AST = Average survival time.

NI = Neutralisation Index.- difference in titers of control and hyperimmune serum.

* = Titers are expressed as reciprocals of log numbers.

Table VII
Determination of optimum age of chick embryo

Sr. No.	Test No	Material inoculated.			5 days		6 days		7 days		8 days	
		Strain	Pass	Titer*	Titer	Average wt.**	Titer	Average wt.	Titer	Average wt.	Titer	Average wt.
1	23	G11338	M5E26	8.0	8.5	0.61	7.3	1.08	7.3	1.80	6.5	2.06
2	31	G11338	M5E26	8.0	7.6	1.00	8.0	2.14	7.7	2.82	7.2	3.24
3	45	G11338	M3E27	8.3	Not	done	6.8 5.0 [@]	1.60 1.50	8.4	2.77	7.3	3.35
4	52	G11338	M5E27	8.7	5.5	0.87	7.3	1.36	7.6	2.4	8.2	3.49
5	56	G11338	M5E27	8.7	7.4	0.82	8.0	1.34	7.8	1.88	8.3	2.60
6	77A	P9606	M4E20	8.0	7.3	0.74	8.4	1.11	7.8	1.87	7.3	2.05
7	77B	KF31	M4E21	8.2	7.8	0.88	8.6	1.42	8.2	1.72	6.5	2.86
8	77C	KF31	M4E21	8.3	Not	done	5.5	1.25	5.7	1.80	7.7	2.80
9	77D	KF31	M4E20	8.3	8.3	0.44	6.6	1.60	7.3	1.86	7.7	2.61

* Titers of harvested material are expressed as the reciprocals of log numbers.

** Average weight of embryos recorded in grams.

@ From dead eggs.

Table VII

Determination of optimum day of harvest.

Test No.	Material inoculated			1st day		2nd day		3rd day		4th day	
	Strain	Pass	Titer*	Titer	Average wt.**	Titer	Average wt.	Titer	Average wt.	Titer	Average wt.
18	G11338	M5E25	N.D.	2.5	0.75	3.5	1.00	7.5	1.58	6.6	N.R.
25	P9605	M4E19	N.D.	N.D.	N.D.	7.3	N.R.	8.4	N.R.	7.8	N.R.
29	G11338	M5E26	8.0	5.4	1.00	6.7	1.50	7.4	1.50	7.8	3.06
54	G11338	M5E27	8.7	N.D.	-	7.1	1.93	7.8	1.34	②	-
77B	KF31	M4E21	7.4	N.D.	-	5.6	1.30	8.2	1.72	7.8	2.81
78A	P9605	M4E21	8.6	N.D.	-	6.3	1.33	8.3	2.08	7.8	1.93
78C	KF31	M4E21	8.3	N.D.	-	7.4	1.08	7.9	2.18	7.5	2.77

X * Titers of harvested material are expressed as the reciprocals of log numbers.

** Average weight of embryos recorded in grams.

② All embryos died - not titrated. ND = Not done H.R. = Not recorded.

All the eggs were inoculated with 0.1 ml of 10^{-1} dilution of virus suspension.

Table VIII ~~8~~ viiDetermination of optimum amount of inoculum.

Test No.	Material inoculated [@]			0.1 ml		0.2 ml		0.5 ml		1.0 ml	
	Strain	Pass	Titer*	Titer	Average Wt.**	Titer	Average wt.	Titer	Average wt.	Titer	Average wt.
8	G11338	M5E25	N.D.	6.8	N.R.	7.0	N.R.	7.2	N.R.	6.6	N.R.
26	G11338	M5E26	8.0	8.5	2.34	8.5	2.30	8.8	2.35	8.5	2.00
30	P9605	M4E19	N.D.	7.8 7.6	2.00	8.4 10.5	2.00	N.D.	-	N.D.	-
43	G11338	M5E26	8.0	8.0	2.07	8.5	2.74	8.8	2.53	4.5	1.52
75A	P9605	M4E21	8.5	8.5	1.64	8.6	1.65	8.2	1.65	7.5	1.51
75B	KF31	M4E21	7.4	8.3	1.70	8.0	1.90	7.3	1.73	N.D.	-

✕ * Titers of harvested material are expressed as the reciprocals of log numbers.

** Average weight of embryos recorded in grams.

N.D. Not done.

N.R. = Not recorded.

@ All the eggs were inoculated with 10^{-1} dilution of virus suspension and harvested on 3rd post inoculation day.

Fig. 14
Table VIII
Determination of optimum dilution of inoculum.

Test No.	Material inoculated ^①			10 ⁻¹			10 ⁻²			10 ⁻³			10 ⁻⁴		
	Strain	Pass	Titer*	Titer	Average wt.**		Titer	Average wt.		Titer	Average wt.		Titer	Average wt.	
35	G11338	M5E26	8.0	N.D.	-		8.3	2.01		8.7	2.06		N.D.	-	
39	G11338	M5E26	8.0	8.6	2.40		8.4	2.54		8.0	2.22		N.D.	-	
46	G11338	M5E27	8.7	7.4	1.71		7.5	1.61		8.4	1.77		N.D.	-	
73	F9605	M4E21	8.5	N.D.	-		8.3	1.71		8.5	1.65		7.4	1.72	
76A	KF31	M4E21	8.0	N.D.	-		7.0	1.77		7.5	1.79		7.77	1.73	
76B	F9605	M4E21	8.5	N.D.	-		7.8	1.43		8.2	1.70		7.6	1.55	

× * Titers of harvested material are expressed as the reciprocals of log numbers.

** Average weight of embryos recorded in grams.

N.D. Not done.

① All the eggs were inoculated with 0.1 ml virus suspension and harvested on 3rd post inoculation day.

Table X.

Determination of optimum temperature of incubation.

Test No.	Material inoculated [©]			35°C		37°C	
	Strain	Pass	Titer	Titer [*]	Average Wt. ^{**}	Titer	Average Wt.
10	G11338	M5E25	N.D.	6.8 ["]	N.R.	6.8 ["]	N.R.
21	G11338	M5E25	N.D.	7.6	1.01	7.9	1.23
53	G11338	M5E27	8.7	8.6	1.90	8.6	2.25
56	G11338	M5E27	8.7	7.7	1.31	7.8	1.88
76A	KP31	M4E21	7.4	8.2	1.16	7.5	1.79
76B	P9605	M4E21	8.6	7.8	1.14	8.2	1.70
76C	KP31	M4E21	7.4	5.7	0.90	8.0	1.63

† * Titers of harvested material are expressed as the reciprocals of log numbers.

** Average weight of embryos recorded in grams.

N.D. Not done.

N.R. Not recorded.

" Harvested on 4th day.

© All the eggs were inoculated with 0.1 ml of 10^{-1} dilution of virus suspension and harvested on 3rd post inoculation day.

Table XI.

Inactivation of KFD virus (G11338 strain) at 37°C with BPL of varying concentrations. @

Test No.	Conc. of BPL	Pre-inact. titer.	Dil. of inoc.	Virus inactivation at intervals of								Con-trol (No. / ml)
				10 min.	15 min.	20 min.	30 min.	45 min.	1 hr.	2 hrs.	4 hrs.	
9	0.2%	$10^{-8.0}$	10^{-1}	-	-	-	16/16	-	16/16	16/16	-	0/6
			10^{-2}	-	-	-	8/8	-	8/8	8/8	-	
14	0.1%	$10^{-6.6}$	10^{-1}	-	-	-	6/6	-	6/6	6/6	6/6	0/6
19	0.05%	$10^{-7.7}$	10^{-1}	-	0/6	-	6/6	-	5/5	6/6	5/5	0/6
32	0.05% 0.025%	$10^{-8.5}$	10^{-1}	0/9	-	0/9	0/5	-	7/7	6/6	-	0/5
			10^{-2}	0/6	-	0/10	5/6					
			10^{-3}	0/9	-	1/6						
37A	0.05%	$10^{-7.7}$	10^{-1}	-	-	4/4	15/15	15/15	17/17	-	-	0/6
37B	0.05%	$10^{-7.7}$	10^{-1}	-	-	0/6	15/15	11/11	16/16	-	-	0/8
37C	0.05%	$10^{-7.7}$	10^{-1}	-	-	4/6	14/14	15/15	16/16	-	-	0/5
32	0.025%	$10^{-7.8}$	10^{-1}	-	0/6	-	0/5	-	0/6	5/5	6/6	0/5
22	0.01%	$10^{-7.5}$	SR AST	0/5 3.2	- -	0/5 3.2	0/5 3.4	-	0/4 3.7	0/5 5.0	0/5 4.6	0/5 3.4
49'	0.05%	$10^{-6.5}$	10^{-1}	-	-	-	6/6*	-	11/11*	-	-	0/6
			10^{-2}	-	-	-	8/15	-	12/12*	-	-	

* Blind passages made on 6th post inoculation day.

Isolations were not successful on from these passages. SR: Survival ratio.

" Mother in one box ate all the babies. AST: Average survival time.

@ The different concentrations of BPL were not used with the same material. Different

': Test carried out by mouse brain material.

Table.XII.

Inactivation of KFD virus (G11338 strain) at 4°C with BPL of varying concentrations. ^③

Test No.	Conc. of BPL	Pre inact titer.	Dil. of inoc.	Virus inactivation at time intervals as shown by SR of inoculated mice.							'Control (No BPL)
				1/2 hr	1 hr	2 hrs	4 hrs	7 hrs	24 hrs.	48 hrs.	
14	0.2%	10 ^{-6.6}	10 ⁻¹	2/6	5/6	5/6	6/6	-	6/6	-	0/6
19	0.05%	10 ^{-7.7}	10 ⁻¹	0/6	0/6	0/6	5/5	-	6/6	-	0/6
41A	0.05%	10 ^{-7.7}	10 ⁻¹	-	-	-	4/6	12/18	17/17	-	0/8
41B	0.05%	10 ^{-7.7}	10 ⁻¹	-	-	-	3/4	10/10	18/18	-	0/6
41C	0.05%	10 ^{-7.7}	10 ⁻¹	-	-	-	6/6	16/16	11/11	-	0/6
32	0.025%	10 ^{-7.8}	10 ⁻¹	-	0/6	0/6	0/6	-	7/7	7/7	0/6
22	0.01%	10 ^{-7.5}	SR	-	0/5	0/5	0/5	-	0/5	0/5	0/5
			AST	-	3.2	3.2	4.2	-	4.4	5.6	4.2

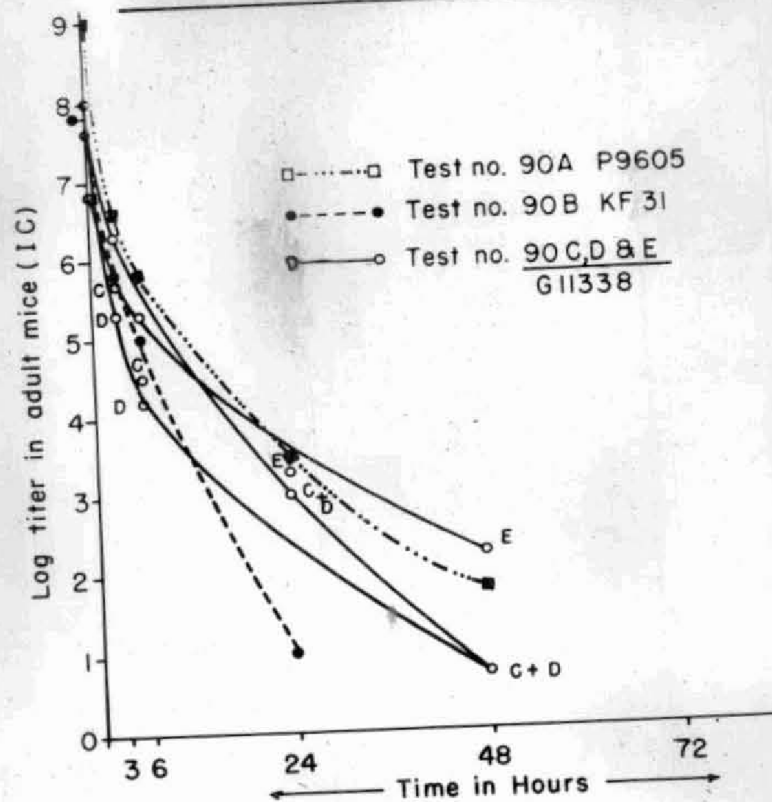
SR = Survival ratio.- Number surviving/Number of mice inoculated. AST= Average Survival time.
 ③ = The different concentrations of BPL were not used with the same material. Different material in different tests was used for each concentration.

Fig.1B

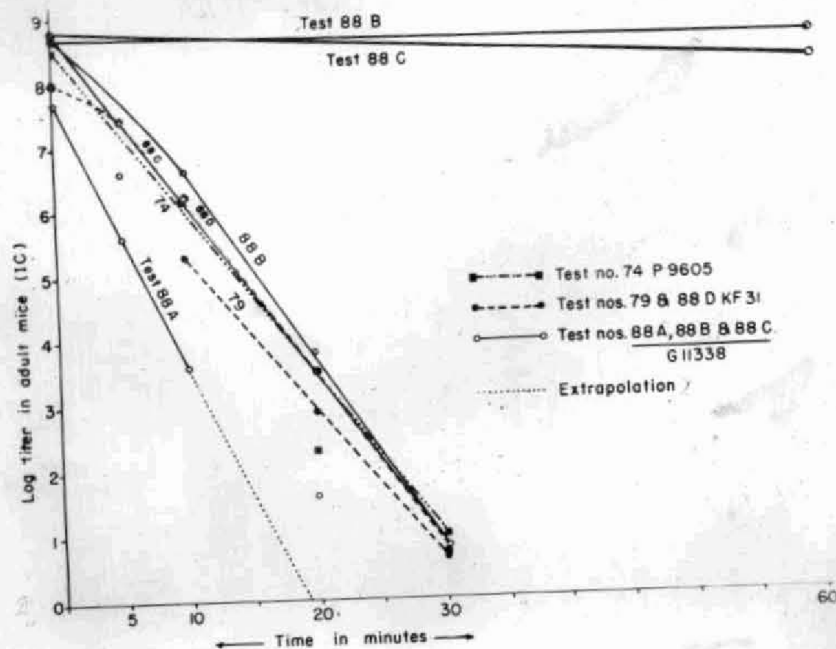
A summary of minimum time required for inactivation at 37°C & 4°C with varying conc. of BPL.

Conc. of BPL v/v.	Minimum time required for inact. at	
	37°C	4°C
0.2%	30 min. or less	2 hrs.
0.1%	30 min. or less	4 hrs.
0.05%	45 minutes.	24 hrs.
0.025%	2 hours.	24 hrs.
0.01%	Not inactivated in 4 hours	Not inactivated in 48 hrs.

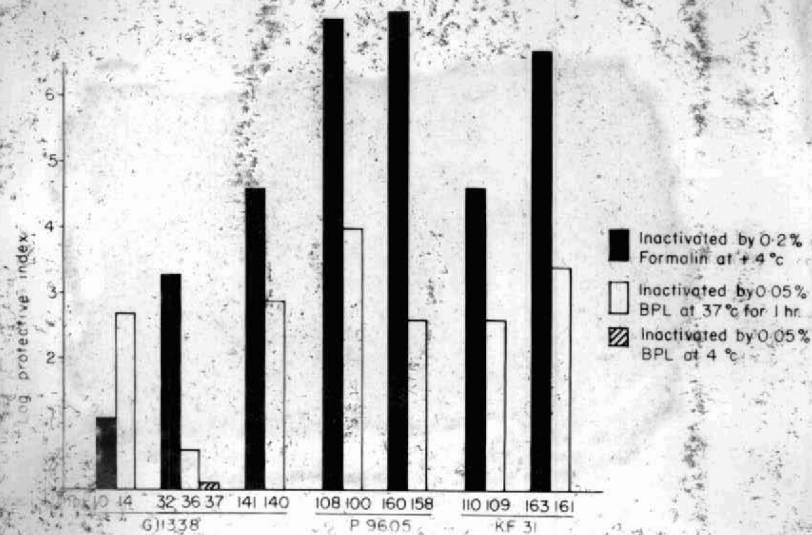
FORMALIN INACTIVATION CURVE TEST.



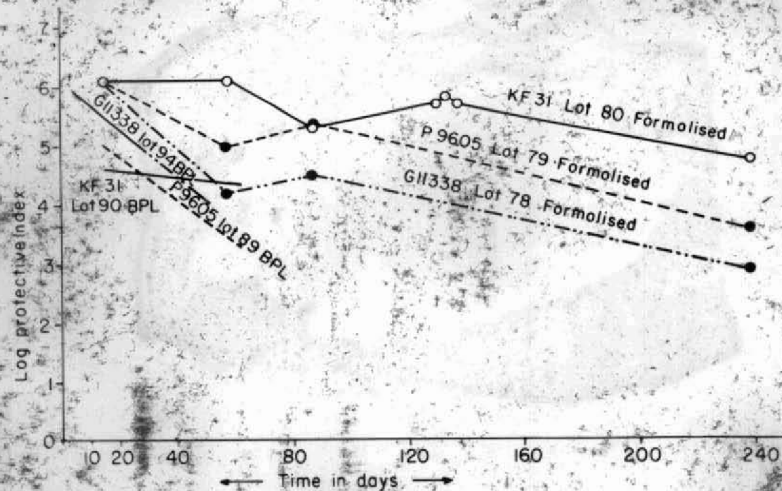
B.P.L. INACTIVATION CURVE TEST



COMPARISON OF B.P.L. & FORMALIN INACTIVATED VACCINES



STABILITY OF VACCINES



CHAPTER V.

DISCUSSION

DISCUSSION

The feasibility of the chick embryo as a host for the growth of virus was considered for the following reasons:

- 1) Quite a number of Arbo viruses have been adapted to chick embryo (Taylor et al 1952) and chick embryo vaccines against Japanese B encephalitis (Warren Hough R.G. 1946) and tick borne encephalitis (Fedorov U.V. 1960) have been prepared and showed to be effective.
- ii) Easy availability of fertile eggs throughout the year.
- iii) It is more economical to use chick embryo as yield of vaccine per embryo is more and cost of production is less in comparison to the production of mouse brain vaccine. The maintenance of a large mouse colony is not essential if fertile eggs are used as a source.
- iv) Harvesting eggs under sterile conditions is easier whereas harvesting mouse brains is a tedious procedure with greater chances of bacterial contamination.
- v) The eggs are comparatively free from intrinsic infections.
- vi) Last but not the least important is the safety of the vaccines from causing neuroparalytic reactions due to the repeated doses of vaccinations.

How far the use of the chick embryo was feasible will be discussed here.

It has been shown by a number of workers that the arbor group of viruses is easily adaptable to chick embryo by the yolk sac route of inoculation, and that the maximum concentration of virus is in the whole embryo. (Taylor et al, 1952 Howitt D.F. 1943). Three strains of KFD virus were used for studies on adaptation to chick embryo. One strain, G11338, was used for adaptation by different routes of inoculation. For the other two strains (P9605 and KF31) only yolk sac route of inoculation was followed.

Adaptation to chick embryo:

From Fig.1,2 and 3 it may be observed that adaptation of KFD virus to chick embryo by yolk sac route of inoculation was very easy. In fact isolation of KFD virus from an acute serum of a patient directly in chick embryo has been successful. The titer of virus at 5th egg passage itself reached as high as $10^{-8.7}$. However, adaptation by allantoic route of inoculation from mouse passage was not successful. A chick embryo adapted strain could be propagated on chorio allantoic membrane. Irrespective of the route of inoculation followed, the yield of virus from chick embryo tissue, however, was higher than from that of extra embryonic tissue. It was observed, as shown in fig.5, that the highest concentration of virus was in the whole chick embryo, followed by chorioallantoic membrane, and the least in the allantoic fluid. These findings are in accordance with those of Fedorov (1960) who noticed that the highest amount of tick borne encephalitis virus is concentrated

in the body of the embryo than in the yolk and chorio allantoic membrane with the least amount in the amniotic and the allantoic fluids. Failure of adaptation of KFD virus by allantoic route of inoculation, therefore, is not surprising. Warren & Hough (1946) however, have obtained a good yield of JBE virus by inoculation into allantoic cavity of 7-8 days old eggs. They concluded that the route of inoculation of virus(JBE) inoculated had no marked effect upon the yield, provided the agent was beyond chorio allantoic membrane. These findings were not applicable to KFD.

Yolk sac route of inoculation was found to be suitable both for the high yields of virus and for the simplicity of inoculation.

The yield of virus for preparation of a good vaccine is an important criterion to be considered. Attempts were made to obtain good yields by varying different conditions such as age of the chick embryo, amount of inoculum, dilution of inoculum, day of harvest, and temperature of incubation. As mentioned earlier, the titer of the 5th passage material was found to be equal to $10^{-8.7}$. Further passages in eggs did not either reduce or increase the titers and the titers of the 20th passage level ranged between $10^{-8.0}$ to $10^{-9.0}$. 25th to 27th passage material was used for studies on maximum yield of virus for G11238 strain but 20th passage level was used for studies with other strains. However, 20th passage material of all the three strains, was used for the preparation of chick embryo vaccines.

Results of the experiments on the determination of optimum conditions for maximal yield of virus.

A) Age of the chick embryos:

Embryos of the age varying from 6 to 8 days were used. From table VI it may be seen that there was no significant difference in the titers of the embryos of different ages and the tiers varied from test to test. The weight of the embryos did not seem to bear any regular relationship to the yield of virus. Since the total yield of suspension was more because of the higher weights of older embryos and also the consistency in the titers, eggs of 7-8 days old were preferred for vaccine preparation. Fedorov (1960) used embryos of 7-10 days age for comparison on yield of virus and found 8 days old as the most suitable for tick borne encephalitis group of viruses.

B) Day of harvest:

Table VII shows that the embryos harvested on the 3rd day gave the highest yield of virus. Harvesting the eggs on the 4th day was also unsuitable as most of the eggs would die on the 4th day. The titer of the virus in dead eggs would depend upon how soon the eggs were harvested after the death of the embryos. Thus more the time elapsed for harvesting of the dead eggs, the lower the titer of the virus in the chick embryo would be observed and hence the inconsistency in the yield of the virus would

be seen. Dead eggs would also result in the bacterial contamination of the embryos. So harvesting of live eggs was preferred. Harvesting on 3rd post inoculation day seemed to be the choice. This can however be said only when the higher concentration of virus of inoculum (4-0 - 7.0 logs of virus) was used. Concentrations less than these were not used.

C) Amount of inoculum:

In table VIII the titers of the virus of the embryos do not show any significant increase as the amount of inoculum was increased. In fact 1.0 ml of inoculum not only yielded the lower titers but also arrested the growth of the embryos. 0.2 - 0.5 ml of inoculum seemed to be suitable.

D) Dilution of inoculum:

These experiments were carried out to find out whether the dilution of the inoculum had significant effect on the final yield of virus. By referring to the table No. IX it seems that though there was no significant difference in titers, a dilution of 10^{-3} of the inoculum having a titer of 10^{-8} or above gave a comparatively higher yield. The dilution of 10^{-4} was not used for the strain G11338 but, for $\frac{9}{605}$, it suggests that with further reduction in concentration of inoculum there was reduction in titer. Whether this was applicable to G11338 or not is not known. KF31 strain did not show any significant difference with 10^{-3} or 10^{-4} dilution of inoculum.

E) Temperature of incubation:

The eggs incubated at 37°C and 35°C after the inoculations did not show any significant difference in the yield of virus (as shown in table X). The discrepancy in the results of test No.10 can be explained by the fact that the eggs were harvested on the 4th post inoculation day and as seen from the results on the determination of the optimum day of the harvest, table VII the titer of 4th day harvest material was less by 0.4- 0.9 logs than the 3rd day harvest material. This reduction in the titer on the 4th day might be due to the partial inactivation of the virus at 37°C. As the average weight of the embryos from the eggs incubated at 37°C was higher than the eggs incubated at 35°C the yield of embryo suspension for the preparation of the vaccines was more when the eggs were incubated at 37°C. Hence use of that temperature for the incubation of the eggs after inoculation was preferred.

The findings about the various optimum conditions, however, do not lead us to any significant or consistent increase in the yield of the virus as compared to the routine method, i.e. 7 days old eggs inoculated with 0.1 ml of 10^{-1} dilution, harvested on 3rd day from the eggs incubated at 37°C. There were considerable variations in the titers obtained by inoculation of the same material in eggs at different times or even from egg to egg. (as seen in Test 30, table VIII). Hence it was difficult to judge when the titer of the harvested material would be higher than 10^{-8} . Higher titers were found

to be essential for preparation of a good vaccine. It will be seen that only on one occasion was the titer of chick embryo suspension higher than the titer of mouse brain material (Test 30 table VIII) by 0.8 logs or more. In all other experiments it remained 0.7 - 1.0 log less than the latter. On the whole it may be said that 7-8 days old eggs when inoculated with 0.2 - 0.5 ml of 10^{-3} dilution gave a reasonably high titer when harvested on 3rd post inoculation day. These findings are in accordance with the findings of Fedorov U.V.(1960) on tick borne encephalitis virus.

Inactivation of KFD virus by Beta-propiolactone and formalin:

Formalin has been in use as an inactivating agent for a number of virus vaccines. Formalin inactivated tick borne encephalitis virus vaccines are already in use in Soviet Russia. (Levkovich E.N & Snorodintsev A.A. 1941). A duration of atleast 14 days is required for complete inactivation of tick borne encephalitis group of viruses at 4°C and a properly controlled inactivation is to be carried out. The injection of 0.2% formalin is a painful experience and volunteers are reluctant to undergo the complete schedule of 3 doses of 1-2 ml each (Aniker et al)(1962). Since hydrolysis products of BPL are not toxic (Kelly A.R. 1961) it was thought worthwhile to test it as an agent for inactivation of KFD virus.

A number of workers have reported the use of Beta-propiolactone for inactivation of viruses for vaccine preparation (LoGrippe G & Hartman 1965, Polley & Guerin 1967).

LoGrippe has listed a number of viruses such as EEE, WEE, An strain of encephalomyocarditis, Theilers encephalomyelitis, rabies(CVS), poliomyelitis(I,II and III), ECHO(4 & 9), Coxsackie (B3 and B5), herpes simplex, New Jersey vesicular stomatitis, Adenovirus 3, measles, mumps, hog cholera, influenza (A and B), hepatitis and E coli T3 phage, which have been successfully inactivated. BPL is also bactericidal and fungicidal(Hartman et al 1951). BPL hydrolyses in water but the virus is inactivated before the half life of BPL (LoGrippe 1959). The hydrolysis products of BPL have no effect on the inactivation of the viruses with the result that further deterioration in vaccine due to the presence of the inactivating agent is avoided. Dilution of the inactivated material, prolong storage or immediate neutralisation by sodium thiosulphate does not reactivate the virus, in short the action of BPL is irreversible. Because of the excellent penetrating power of BPL, the chances of the presence of a few viable residual particles from inactivated material are reduced. All the above mentioned properties of BPL have been enumerated by LoGrippe(1959).

LoGrippe(1956) has compared the efficacy of BPL inactivated vaccines with live virus vaccines and formalin inactivated vaccines. BPL inactivated vaccines were almost as effective as live virus vaccine, formalinised vaccines being less efficacious. He clearly showed the superiority of BPL inactivated vaccines over formalin inactivated vaccines prepared from M strain of encephalomyocarditis.

(murine encephalomyocarditis group of viruses). It had to be ascertained whether KFD virus also behaved in a similar way.

Inactivation of the KFD virus, can be carried out with various concentrations of the chemical. Hence the optimum dilution of BPL for inactivation at optimum temperature had to be determined. Concentrations varying from 0.2% -0.01% BPL were tried. The results of inactivation of KFD virus by BPL of various concentrations at 37°C and 4°C are presented in table XI & XII and a summary of these results in table XIII. It is desirable to use the minimum concentration possible of the inactivating agent, without sacrificing the safety of the vaccine. Unlike formalin, which is present throughout, BPL undergoes hydrolysis. The half life of BPL at 37°C is 28-32 minutes, and at 4° is 16 to 20 hours. The hydrolysis products of BPL are incapable of inactivating viruses. Although the bulk of virus is inactivated by a relatively small concentration of BPL, a disproportionate amount of drug is required to inactivate the residue and ultimate trace quantities of active virus. This is known as tailing effect. A concentration of 0.05% BPL seems to be the ideal. A concentration lower than 0.05% BPL is also capable of inactivating virus. Hence the traces of virus particles which might have escaped inactivation in the beginning may get inactivated by the lower remaining concentration of BPL. 0.01% BPL at 37°C was incapable of inactivating virus even in 4 hours duration, and comparison of average survival time(AST), which is prolonged when the titer of virus is low, showed that there was hardly any inactivation

by BPL at that concentration. It was the same case at 4°C. This point had to be taken into consideration while using 0.025% BPL. Though it was possible to inactivate KFD virus by 0.025% BPL at 37°C in two hours time (refer to test No. 32, table XI & XII.) It was thought rather risky to use this concentration which may be ineffective in countering the tailing effect. This was in fact the experience with vaccine lot No. CE162, wherein live virus was isolated. So a concentration of 0.05% BPL was chosen for detailed studies on inactivation of KFD virus and the assessment of the potency of the resultant vaccine.

Arboz viruses are generally unstable at 37°C or room temperature. Sabin (1943) has reported that when JBE virus was inactivated at 37°C by formalin, the vaccine was low in potency. Vaccines prepared against tick borne encephalitis virus, with one exception, (Fedorov 1960) have been inactivated by formalin at 4°C. Fedorov has used room temperature, 23°C, for inactivation. Thus temperature at which inactivation is carried out might have some effect on the potency of the formalinised vaccine. This is, however, not the case with BPL inactivated vaccines. From the results of potency of vaccine lot 36 and 37, Fig. 8 it is apparent that there is no significant difference whether inactivation of KFD virus by BPL is carried out at 37°C or at 4°C. This finding was also corroborated in case of mouse brain vaccines inactivated by BPL. Hence the studies were undertaken on the rate of inactivation of KFD virus ~~at 37°C~~ by BPL at 37°C.

A number of investigators have studied the kinetics of the rate of inactivation of Polio virus by formalin. So far only Danes and Benda(1960b) have presented results on the rate of inactivation of tick borne encephalitis virus by formalin. No such results with respect to BPL as an inactivating agent and using tick borne group of viruses have been encountered by us. LoGrippo(1955) has however studied rate of inactivation of EEE virus which belongs to A group of arbor viruses (Casals 1957). Studies on the rate of inactivation are important for the fact that the behaviour of the virus towards the chemical is known. The rate of inactivation of KFD virus by BPL as shown by Fig.6 appears to be linear regression with time. This is true with respect to all the three strains of KFD virus. On the basis of these results, it seems justifiable to extrapolate the lines to the base. The graph thus obtained indicates that, starting with a virus suspension having a titer as high as 10^{-9} , complete inactivation can be achieved in about 35 minutes. Whether there is a tailing effect or not cannot be judged from these results. Fig.6 (test No.88A) however shows that titratable virus was not detected at 20 minutes interval. Since the aliquot at 20 minutes inactivation period was not inoculated in the more sensitive infant mice, it is difficult to say whether it contained any viable particle. Isolation of live virus particles from 30 minutes onwards was, however, not possible. The sudden drop in titer in this test cannot be explained.

Studies on determination of a desirable concentration of formalin at different temperatures were not carried out as a concentration of 0.2% formalin was found to be suitable for RSSE virus vaccine and some experimental lots of KFD virus vaccines. It was considered as standard for comparison and studies on the rate of inactivation, potencies of vaccines etc.were carried out.

Rate of inactivation of KFD virus by 0.2% formalin does not show a similar linear relationship with the chemical. Danes and Benda(1960b) did not get smooth curves with the tick borne encephalitis virus. We have on the other hand obtained smooth curves. One can almost predict the titer of virus at a definite interval. The curve shows a sudden drop in virus titer during the first 6 hours with a subsequent gradual slowdown of the process. It is, however, difficult to say whether all the three strains behave in a similar manner. By referring to Fig.7 it may be noticed that the strain G11338 seems to show a slower rate of inactivation between 10 and 24 hrs and then suddenly show a drop in titers. At no time of the inactivation process is a 1st order reaction suggested. It is therefore difficult to assume that for any given lot of virus suspension, over treatment by formalin, as determined by extrapolation of the curve showing the rate of inactivation, may result in complete inactivation of virus. Since the suspension used for preparation of the vaccine contains a large amount of non viral proteins from the chick embryo tissue, deviations in the tail end of the reactions

cannot be overruled completely. It is difficult to decide whether any reactivation of inactivated particles take place. Rarely, isolation of viable particle on 10th and 14th post inactivation days has been observed.

Inactivation by BPL is a quicker process as compared to that by formalin. The rate of inactivation by BPL shows a linear relationship wherein there is no tailing effect. Formalin on the other hand tends to show tailing effect so that safety of the vaccines has to be carefully determined before issue of the vaccines. However, efficacy of the vaccine depends upon the potency and not on the inactivation alone. So a comparison of potency of formalin inactivated and BPL inactivated vaccines had to be done before recommending the suitable agent for inactivation.

Comparison of the vaccines prepared by inactivation with 0.05% BPL at 37°C for 1 hr and 0.2% formalin at 4°C.

Potency tests on the various lots of the vaccines were carried out by slight modification of the test suggested by Pond et al (1953) and found suitable for KFD virus vaccines as studied by Krishnamurthy at our laboratory. Krishnamurthy compared the methods of Pond and of Benda and Danés (1960) and came to the conclusion that the vaccine which was potent enough by the test carried out by following Pond's method was also good enough if the method of Benda and Danés.

was followed. In fact Casals and Olitsky(1945) have shown that immunity to RSSE virus is developed by the two doses of vaccines within 4 days and lasts throughout the life of the mice. Vaccines of the following lots 10 & 14, 32, 36 & 37, 140 & 141 (G11338) 100 & 108, 158 & 160(P9605) and 109 & 110, 161 & 163(KF31) had the same source of material. A 10% suspension of the chick embryo was divided into two parts one being inactivated by BPL and the other by formalin. Vaccines from the same source were tested in the same test. The results are presented in Fig.8. With the exception of vaccines Lot Nos.10 and 14 all the strains conclusively proved that formalin inactivated vaccines were far superior to BPL inactivated KFD vaccines. Similar results have been observed using mouse brain and tissue culture vaccines.

Stability of the vaccines.

Stability of the vaccines is another criterion which the choice of the inactivating agent can be determined. The formalinised vaccine should be able to maintain its potency for a long period, otherwise the vaccine which shows a lower potency at the initial stages but maintains it for a longer time is to be preferred. Formalin inactivated vaccines show a gradual drop in potency(Fig.9). Besides two of the three ^{inactivated} BPL / vaccines wherein potency tests were repeated at the interval of 45 days showed a drop almost equivalent to or more to that of formalin inactivated vaccines. Vaccine Lot 80(inactivated by formalin) showed a drop of only 1.4 logs in potency after

238 days. Thus the stability of the formalinised vaccine is as good as or better than that inactivated by BPL. Further its initial potency is so high that almost 4-6 months elapse before its potency reaches the level of the fresh BPL vaccines.

Three strains of KFD virus were chosen for obtaining a suitable antigenic strain for the preparation of vaccine. Two strains were isolated from human patients and one from a tick pool. The choice of a suitable strain for vaccine depends upon the protective index of the vaccines as shown by potency tests and upon the stability of the vaccine. Since all the vaccines were produced and tested under identical conditions the log protective indices could vary only once a range of test variability. The challenge strains used was homologous with one of the vaccine strains. It is rather difficult to interpret results upon only one challenge strain especially as a number of new strains are being continuously isolated from the infected area. However there is very little difference in the protective indices shown by vaccines prepared from heterologous and homologous strains. Also the results are sufficiently consistent to permit of certain general conclusions on their efficacy being drawn. These conclusions, however, must be accepted with reserve, until further work is done.

After determining the optimum conditions for obtaining a good yield of virus from chick embryo a comparison of BPL inactivated and formalin inactivated vaccines was made.

The latter mode of inactivation was found to be suitable both for potency and stability of the resultant vaccine. However, the potencies of chick embryo vaccines did not show the same consistency and high values of protection as the mouse brain and tissue culture vaccines.

It may be stated in general, therefore, that vaccines prepared from all the three selected strains and inactivated with formalin showed good protective capacity and stability, when tested in adult mice against challenge by a single virulent strain.

CHAPTER VI.

SUMMARY AND CONCLUSIONS.

Summary and conclusions.

1. All the three strains G11338, P9606 and KF31 were adaptable to chick embryo by Yolk sac route of inoculation. 27 serial passages with G11338 strain and 20 passages with the remaining two strains were made in eggs.
2. Adaptation of mouse brain passage material using G11338 strain of KFD virus by allantoic route of inoculation was not successful. Adaptation, by inoculation of chorioallantoic membrane of an egg adapted G11338 strain was possible. The maximum titer of harvested material by this route of inoculation was that of whole chick embryo followed by chorioallantoic membrane suspension and the lowest of allantoic fluid.
3. The effect of variation in conditions such as age of chick embryo, day of harvest, amount of inoculum, dilution of inoculum and temperature of incubation was determined. 7-8 days old eggs inoculated with 0.2 - 0.5 ml of 10^{-3} dilution gave a high yield of virus when harvested on 3rd day with the eggs incubated at 37°C.
4. Various concentrations of Beta-propio-lactone were tried for inactivation of KFD virus at 37°C and 4°C. 0.05% BPL concentration, found suitable from these results, was used for studies on the rate of inactivation at 37°C. The rate of inactivation showed a linear relationship to time of exposure to BPL.
5. Kinetics of inactivation with 0.2% formalin at 4°C were studied. It showed a gradual drop in titer of virus with time.

6. A comparison of formalin inactivated and BPL inactivated vaccines was carried out for immunizing activity. Except on one occasion, formalin inactivated vaccines were found to be far superior to BPL inactivated vaccines.
7. Stability of formalin inactivated and BPL inactivated vaccines at 4°C (approximating to the field conditions) was studied. The former were found to be superior. These studies were continued for a period of 238 days only with formalin inactivated vaccines.
8. The results presented in the previous chapter suggest that the formalin inactivated KFD virus vaccines may be the most suitable for human vaccine trials.

CHAPTER VII

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