HS

RINDERPEST

The Adaptation of the Virus to the Chick Embryo and its Use as a Vaccine

A THESIS

Presented to the Faculty of the Graduate School,
Department of Comparative Pathology and Immunology,
Medical Faculty of the University of Ottawa for the
Degree of Master of Science.

By

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August, 1954.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. Chas. A. Mitchell, Head of the Department of Comparative Pathology and Immunology, Chief of the Division of Animal Pathology, Canada Department of Agriculture, for his encouragement and very able guidance in making this work possible. He also wishes to express his thanks to Dr. J. Auer, Professor, Head of the Department of Anatomy, Medical Faculty, University of Ottawa and his staff for their help and cooperation.

The author also extends his most sincere thanks for the very able technical assistance provided by Mrs. M. McKercher and Mr. R. Belanger, G. I. E. S. staff.

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INTRODUCTION AND HISTORICAL REVIEW

Rinderpest or cattle plague a disease caused by an ultra-microscopic virus, is the most devastating illness to which cattle are heir. It has aided in determining the fate of military campaigns and has caused widespread havoc in times of peace. A few examples will indicate its power of destruction. The infection gained a foothold in mid Europe in the early part of the 19th Century. It brought about the destruction in one year of approximately three million cattle in Lombardy alone and contributed greatly to Napoleon's difficulties in his 1814 campaign. About 1845 the disease gained entrance to the United Kingdom where the destruction was so widespread that many tenant farmers were ruined and the whole agricultural industry depressed. Entering South Africa in 1897 it quickly involved many herds and caused a loss of approximately two million cattle. After the First Great War it spread out from a small focus of infection in Russia over a large part of the country and finally destroyed approximately eight million cattle.

The capacity to spread and the great mortality among infected animals has directed attention to the infection from early times. In fact, the rise of veterinary schools, which commenced about 1760, was directly related to a fear of rinderpest. It follows that efforts were made to devise means of inducing resistance in susceptible animals. The earliest of these were crude, fruitless and require no consideration.

Boer farmers were apparently the first to discover an agent having some capacity to protect animals when they found empirically that the bile of cattle which had died of rinderpest would induce a degree of protection in susceptible animals if given in large doses subcutaneously. Koch (1) was commissioned to investigate rinderpest in South Africa and he submitted the empirical method to experimental trial. The results indicated that while the product was far from satisfactory it would at least produce a temporary degree of resistance in the majority of animals inoculated.

Somewhat later, Kolle and Turner (2) having observed the protective action of serum taken from recovered animals attempted to combine this with an appropriate amount of live virus, with the objective of producing an immunity without the hazards of an acute infection when susceptible cattle were inoculated with the mixture. Boynton (3) experimented with vaccine which was made by grinding tissues taken from infected cattle and inactivating the virus contained therein with glycerin, phenol and heat. In 1926, Kelser (4) discovered that chloroform quickly inactivated the virus and used this chemical to treat the finely ground tissue taken from infected animals. This treated tissue proved capable of stimulating immunity, providing a large dose was given. The following year, Daubney (5) introduced a similar vaccine which however depended upon formalin to inactivate the virus. Except for the product of Kolle and Turner, all these

vaccines contained only inactivated virus and consequently were incapable of inducing a long term immunity. Moreover, they were relatively crude, subject to contamination and with the disavantage of requiring large amounts of infected tissue to produce a sufficiency of virus for the stimulation of immunity. In fact, it was found necessary to use the tissue taken from one infected cow to produce sufficient vaccine for the protection of 100 cattle. This precluded the employment of these products on an extensive scale because of the prohibitive cost.

A new approach to the problem grew out of the work of Schien (6) who in 1926 succeeded in passaging the virus of rinderpest in serial order through goats. This resulted in considerable attenuation. Edwards (7) made use of this adapted virus when he combined it with hyper-immune serum in a simultaneous method. One reason for using the goat virus was to avoid transferring piroplasmosis which was prevalent in India among cattle. It was left to Stirling (8) to employ the attenuated goat virus alone. Later Saunders and Ayyar (9) systematically passaged rinderpest virus in serial order through goats for a long period of time, studying the changes in its virulence for cattle. They noted a very considerable lowering of virulence by the time the 150th serial passage was reached. Following the publication of this report, Daubney and Hudson (10) of Kenya Colony commenced the serial passage of four different strains with the thought in mind that one might have more desirable properties. They finally fixed upon one strain, which was identified as 'O'. They found that by the 88th passage attenuation

had reached a point where the strain could generally be employed with a limited amount of danger to cattle. This goat adapted strain became widely used for the production of immunity against rinderpest in cattle in certain parts of Africa. However, a considerable number of all breeds were susceptible to a degree which resulted in serious illness or death and some breeds were so susceptible that the use of the virus was precluded.

Because of the danger of biological attack during the last War, the whole field of disease of man and animals was carefully surveyed by a Joint Canadian American Commission established by the two countries. Among other things the conclusion was reached that rinderpest virus was a potential agent of great danger. For this reason a project was established at the War Diseases Control Station, Grosse Ile, P. Q. The objective was to study the infection along broad lines but particularly to attempt propagating the virus by newer methods, such as in the developing chick embryo. The results of this work were reported in the open literature at the conclusion of hostilities. Shope, Griffiths, and Jenkins (11) had succeeded in adopting one strain of rinderpest virus to the chorio-allantoic membrane* of the developing chick embryo. Jenkins and Shope (12) also reported the successful adaptation of this strain from the C.A. membrane to the embryo proper by way of the yolk sac. Serial passage

* Hereinafter the chorioallantoic membrane will be referred to as the C.A. membrane.

in this tissue resulted in gradual loss of virulence to a point which permitted inoculating the adapted virus into cattle where it produced no visible illness except a slight rise of temperature. This exposure to the adapted virus resulted in the stimulation of a long term immunity. The embryo adapted strain was the basis of a vaccine which was produced on a large scale and formed a means of defence against a biological attack.

All viruses, except rinderpest, which propagate in the chick embryo or its appendages indicate their presence by some well marked effect; thus influenza, Newcastle disease and encephalomyelitis kill the embryo, while the virus of vaccinia and other pox diseases stimulates the formation of plaques on the C.A. membrane. Until lately it was generally accepted by those working in the field that rinderpest virus produced no effect on the chick embryo or membranes. This lack of marked effect on the chick embryo or its appendages made the precise determination of the presence of virus by the usual means impossible. Therefore more laborious methods have to be employed. The material to be examined must be inoculated into cattle - the natural host - where it either produces a fatal clinical infection or a non-clinical infection which results in immunity, the latter being determined by the inoculation of a virulent strain of virus.

Following the defeat of Japan, the War Diseases Control Station was discontinued. No other facilities were available in the Western hemisphere sufficiently safe to permit determining from time to time

whether the seed virus was viable. Therefore for many months the virus had to be left in the refrigerator. Later facilities became available which contained sufficient safeguards to permit passaging the virus in chick embryo "by faith", but not the inoculation of animals. Finally, the Grosse Ile Station was re-opened and the necessary examination carried out. The result indicated that the seed material was sufficiently active to confer immunity on cattle but had deteriorated to a point where invasion of the chick embryo failed. Using a number of methods, attempts were made to re-establish the parent strain. All these failed. Therefore it became necessary to retrace in their entirety the steps which had been taken during the War. Certain difficulties were encountered but these were overcome and finally Carter (13) succeeded in again adapting to the C.A. membrane of the chick embryo the virulent Kabete strain of rinderpest virus. Once established on the C.A. membrane it was not difficult to passage in serial order. This was carried out for 43 generations. The tests conducted indicated the virulence for the original bovine host had not been lost. At intervals attempts were made to introduce it into the yolk sac but except in one instance, when propagation took place for a short time, all these failed. About this time the Station had to be closed temporarily for repairs. This led to the material being frozen and held at -5° C. for a period of nine months. When the Station was again opened the work about to be described was commenced.

EXPERIMENTAL

At this point it may be desirable to enunciate the objectives of the study. These were as follows:-

(a) Propagation of the rinderpest virus on the C.A. membrane of the chick embryo,

(b) Transference from the C.A. membrane to the embryo, using the yolk sac route,

(c) Determining if serial passages in the embryo would again result in loss of virulence for the natural host,

(d) If virulence were lost to ascertain if the adapted strain would stimulate immunity when inoculated into susceptible cattle,

(e) If an adapted strain suitable for a vaccine was secured, to develop methods of retaining viability at room temperature so that transportation of the product could be carried out without loss of potency.

The above is intimately associated with the theoretical consideration of this special feature of the virus field. The adaptation to a new host and loss of virulence for the original host has occurred in a number of instances, sometimes without workers being conscious of what was taking place. Pasteur (14) produced what is termed a fixed virus by the serial passage of rabies virus through rabbits. Virulence for this animal was enhanced and a corresponding decline occurred for other animals. Theiler (15) deliberately set out to change the yellow fever virus by adaptation. It was first adapted to the central nervous system of white mice in which it became extremely virulent but lost practically all virulence for primates. Alexander (16) using a similar method brought about a change in the horse-sickness virus

which resulted in avirulence for the horse but marked virulence for mice. These examples indicate that frequently by establishing a virus in a foreign host there is a tendency for it to increase in virulence in this host and decrease in virulence for the original host. The reason for this has not been determined precisely but has received theoretical consideration from several workers. Burnet (17) points out that a virus is not a collection of uniform particles but a population of variants, moreover that mutation may take place among them and that mutants may favour certain environments thus changing the predominant particle population of a given virus. Mitchell (18) favours this view but adds that since viruses possess no enzyme systems and propagate only in living cells, using the enzyme systems of these cells, that their capacity for change must be greater than that of other forms of life. The growth of virus is apparently related to the metabolism of nucleic acid of the invaded cell and the development of virus protein is likely a several stage process, only the end stage being the infective particle while stages along the way are antigenic. Some colour to this view is given by the work of Gaylord and Melnick (19), also that of Hershey (20).

The objective of the work about to be reported centres around the adaptation of rinderpest virus to a new host, that is to the chick embryo; its loss of virulence for the original host and its maintenance of antigenicity permitting the production of a biological product which will profoundly stimulate the defensive mechanism of the normal host without bringing about clinical disease.

EXPERIMENTAL METHODS

Source of the Seed Virus.

The original virus was obtained from Africa during the War. It is known to have been carried in cattle for many generations but the record of its original source has been lost. It is usually referred to as the Kabete 'O' strain and is the only one ever propagated in the chick embryo, although many others have been tried. The virus which Carter (13) had adopted to the C.A. membrane had been left in the frozen state for several months. This was the seed material used in the initial experiment about to be described. The seed material for the later experiments was the virulent Kabete 'O' strain passaged in cattle, the spleens of which were harvested at fever peak and used as a source of seed material.

Attenuation of the Virus.

In initial passages from freshly harvested spleen, one gram of spleen was finely ground in a glass grinder with sufficient beef-infusion broth to give a 10^{-1} dilution. In serial passage, one C.A. membrane plus 1.0 ml. of egg fluid was used in this proportion to the required amount and finely ground in a glass grinder. Penicillin and streptomycin were added to the above material before it was used as inoculum. Eggs taken from a highly fertile and disease-free flock were incubated for ten days, carefully screened, and only those containing strong, active embryos selected. These eggs were prepared for inoculation as described below; then by displacing the air cell, the C.A. membrane was caused to drop leaving a depressed area to receive the inoculum on its surface. A volume of 0.2 ml. or 0.5 ml. of inoculum was used per egg depending on

the supply available and on the amount of passage material required; 0.5 ml. is the amount preferred. The inoculated eggs were carefully sealed with paraffin wax, incubated for 96 hours at 38° C; and the C.A. membranes carefully harvested for immediate use in serial passage or to be frozen as a source of seed material. Where possible each fifth serial passage was tested by animal inoculation.

Experiment 1: The egg-passage strain adapted by Dr. Carter was used in this experiment. Calves were inoculated with the 45th, 49th, 51st, 56th, 61st, 70th, 75th, 79th, 106th, and 116th passage. Fourteen days later they were challenged with 1 ml. of 10^{-1}, 1 ml. of 10^{-2} or 2 ml. of 10^{-2} dilutions of emulsions of a finely ground spleen infected with the Kabete strain of virus.

Experiment 2: An 0.5 ml. amount of a 10% emulsion in beef infusion broth of spleen harvested at the peak of fever from an animal infected with the Kabete strain of rinderpest virus was used as the initial inoculum in this and in the next two experiments. Tests of infectivity were made after 6, 15, 25, 30, 57, and 64 passages.

Experiment 3: In this experiment, the tests of infectivity were made after 6, 19, 51, and 56 passages of the virus on the C.A. membrane.

Experiment 4: In this fourth trial the tests of infectivity of the C.A. membrane material were made after the 6th and 43rd passages.

After inoculation with infected material, it was observed that the C.A. membranes became thickened, grey and edematous, presumably an indication of viral multiplication. When this did not occur live virus was no longer found to be present in that particular passage.

During some of the earlier passages contamination of the inoculated eggs with a mold became troublesome. The growth of this mold was not inhibited by the antibiotics employed, penicillin and streptomycin. To overcome this difficulty, the eggs and egg-trays were dipped in a 10% Roccal solution before incubation, the incubator regularly scrubbed out with Roccal solution; and germicidial lamps installed in the fume-hoods used for the egg passages. No further trouble with mold contamination was encountered when this procedure was adhered to.

Passage of the Virus in Yolk Sac.

Eggs from a fertile and disease free flock were incubated for seven days, carefully screened, and only those with strong, active embryos used. The yolk sac of each egg was inoculated with 0.2 ml. or preferably 0.5 ml. of viral suspension. The initial inoculum consisted of a suspension of a finely ground infected C.A. membranes mixed with egg fluid in the proportion of one membrane to 1.0 ml. of egg fluid. In serial passage the inoculum consisted of embryos, minus the heads, and egg fluid in the above proportions. As with the C.A. membrane inoculum, penicillin and streptomycin were added to the yolk sac inoculum. The inoculated eggs were carefully sealed with paraffin wax, incubated for 96 hours at 38° C, and embryos harvested for serial passage or for the freezing down for seed material.

Experiment 1:

(a) The 51st C.A. membrane passage of Dr. Carter's egg adapted strain was used for the first series of yolk sac inoculations. It was passaged serially for a total of 16 passages. The 5th and 14th passages were tested in calves for immunizing activity.

(b) The 56th C.A. membrane passage of the same strain was passed serially for 11 passages. The 10th passage was tested in a calf for immunizing activity.

(c) The 61st C.A. membrane passage of the same strain was passed serially for 51 passages. The 6th, 11th, 16th, 24th, and 51st passages were tested in calves for immunizing activity.

(d) The 71st C.A. membrane passage of the same strain was passed serially for 39 passages. The 8th and 39th passages were tested in calves for immunizing activity.

Experiment 2: The 44th passage of the first of the C.A. membrane adapted strains was passed serially in the yolk sac and the 13th and 26th passages tested in calves for immunizing activity.

Experiment 3: The 41st passage of the second of the C.A. membrane adapted strains was passed serially in the yolk sac and the 10th and 20th passage tested in calves for immunizing activity.

Experiment 4: The 33rd C.A. membrane passage of the third of the C.A. membrane adapted strains was passed serially in the yolk sac for 19 passages. The 10th passage was tested in a calf for immunizing activity.

A sufficient number of eggs were inoculated at each serial passage to ensure a stock-pile of material for future use. In the preliminary work serial passages were made at 4-day intervals but later only at monthly intervals with no apparent resultant change in the activity of the stored material.

Lyophilization of Vaccines.

The C.A. membrane and yolk sac material was dried for a period of 48 hours on a small Edwards centrifugal dryer. The finely ground infected egg material was prepared for drying in the same proportions as used in making up the inoculum. Six ampoules were dried at one operation and each vial contained two ml. of the above suspension.

Experiment 1: Six vials of the 67th passage of the first of C.A. membrane adapted strains was dried for 48 hours. One sample was tested in a calf for immunizing activity immediately. The second sample was tested after being stored at room temperature for 31 days.

Experiment 2: Six vials of the 117th C.A. membrane passage of Dr. Carter's egg adapted passage was dried for 48 hours. One sample was tested in a calf for immunizing activity after 11 days of storage in a dry-ice chest. The second sample was tested after being stored at room temperature for 31 days.

Experiment 3: Six vials of the 26th yolk sac passage, started initially from the 44th C.A. membrane passage of the first of the C.A. membrane adapted strains, were dried for 48 hours. The first sample was tested in a calf for immunizing activity after 24 hours storage in a dry-ice chest, the second sample after being stored at room temperature for 30 days.

Experiment 4: Six vials of the 22nd yolk sac passage, started initially from the 41st C.A. membrane passage of the second of the C.A. membrane adapted strains, were dried for 48 hours. The first sample was tested by

calf inoculation after holding for 48 hours in the dry ice chest, another sample after storage at room temperature for 16 days.

Experiment 5: Six vials of the 59th C.A. membrane passage, the second of the C.A. membrane adapted strains, were dried for 48 hours. The first sample was tested immediately by calf inoculation for immunizing activity. A second sample was stored at room temperature for 14 days before testing.

RESULTS

Attenuation of the Virus by C.A. Membrane Passage.

In the initial experiment with the Kabete strain of rinderpest virus adapted to the C.A. membrane by Dr. Carter, all of the calves inoculated with the 45th to 116th passage remained clinically well without temperature reactions (Table I). They were found fourteen days later to be immune to challenge with 200 to 2000 times the infective dose as estimated from the data of Walker, Griffith, and Shope (21). A number of inoculated animals were challenged with the most virulent strain of rinderpest virus to which they also proved solidly immune.

It appeared therefore that this egg-adapted strain of rinderpest virus had lost its capacity for producing a progressive and fatal infection but had retained its immunizing activity. It may be postulated that during storage in the frozen state for several months, the more virulent virus particles had died off, leaving the less virulent which were still capable of propagation. A somewhat similar observation was made by Walker (22) with Newcastle disease virus. Since the production of a non-virulent strain of virus that will still confer protection on

inoculated animals, is the desired goal in vaccination procedures against such agents, it seemed highly important to repeat this trial to see if such attenuation might be effected in any regular manner, that is to determine whether these results could be readily duplicated.

In the first of these attempts at attenuation by passage in the chick embryo the initial inoculation by the C.A. membrane route was made with an emulsion of spleen harvested at the peak of fever from an infected animal. Difficulties were encountered with mold contamination which necessitated reversion at the tenth passage back to the second, and at thirteenth back to the tenth. The 15th, 25th, 30th, 57th and 64th passages were tested by animal inoculation. The animals inoculated with the 30th and two later passages showed marked temperature reactions only, and were solidly immune to challenge. The other two passages, although still eliciting a definite temperature rise, did not cause any further symptoms.

In the second of these attempts, the initial inoculation was again made with an emulsion of spleen harvested at the peak of fever from an infected animal. The 6th, 19th, 51st and 56th passages were tested by animal inoculation. The animal inoculated with the 19th passage died of rinderpest on the seventh day. Owing to repairs in progress in the testing units, no passages could be tested between 19th and 51st. This 51st passage induced a marked rise in temperature but no other symptoms and protected the calf against subsequent experimental infection with the virus. The animal inoculated with the 56th passage showed only a mild temperature rise but was nevertheless immune to challenge. A

third C.A. membrane passage series of the same virus strain also resulted in a successful attenuation of the agent. At the 5th serial passage, the virus produced rinderpest on animal inoculation, whereas the calf inoculated with the 43rd passage showed only a marked temperature rise and was immune to challenge.

In brief therefore, in three different serial C.A. membrane passages, the Kabete strain of rinderpest virus, derived directly from the spleen of infected cattle had become adapted to the chick embryo tissue. Even more important in doing so it had become attenuated while retaining its immunizing potency. That attenuation by the C.A. membrane route may be more readily accomplished than by the yolk sac route might be surmised from a review of the results of Jenkins and Shope (12) who required a much greater number of passages to effect attenuation than was necessary in the foregoing experiments.

Because of the cost of animals no attempt was made to titrate with precision the amount of virus present in the C.A. membranes. It was considered sufficient to determine the approximate amount required to stimulate an active immunity in animals inoculated. The strain used in this titration was the 70th passage of the egg-adapted strain of Dr. Carter, which gave no temperature reaction and produced no symptoms in animals. On injecting this material subcutaneously, it was found that a 1:10 dilution of membrane and egg fluids brought about protection in a calf, that is one C.A. membrane would be sufficient to protect ten cattle. This protection was well marked by the 14th day. Representative cattle were held for a duration of

six months and challenged when they proved to be solidly immune.

Serial Passage of Virus in Embryo by Yolk Sac Route.

Since the propagation of the virus on the C.A. membrane does not involve as much tissue as propagation through the yolk sac route, it seemed likely that the latter would yield a greater quantity of virus. If protection of the cattle population on a large scale were necessary, this would prove a factor of economic importance. For this reason the following attempts to adapt the C.A. membrane virus strains to the yolk sac were carried out as summarized in table II.

The first strain used was that adapted to the C.A. membrane by Dr. Carter. The 51st, 56th, 61st, and 71st C.A. membrane passages of this strain were passed through the yolk sac 11 to 51 times. The early passages were immunizing but beyond the 24th this activity had apparently been lost; in one instance it was lost at the 14th passage.

Numerous attempts to adapt the earlier serial passages of the three newly adapted C.A. membrane strains to the yolk sac were without success. When the 44th passage of the first of these new strains was used, success was attained. Both the 13th and 26th yolk sac passages of this strain produced solid immunity in calves, the former still causing a marked temperature rise similar to that of the parent strain: the 44th C.A. membrane passage. Although the 26th yolk sac passage still gave some temperature reaction in animals, it was of a milder nature. The 41st passage of the second C.A. membrane adapted strain, likewise on serial passage through the yolk sac furnished

Table I

Passage of Virus on C. A. membrane

No. of Expt.	Strain	Results of Tests in Calves		
		Passage Tested	Symptoms after Initial Inoculation*	Challenge with Live Virus**
1	Carter's egg adapted Kabete strain; stored, frozen	45, 49, 51, 56, 61, 70, 75 79, 106, 116	Clinically well no temperature reactions	45 - 116 immunized
2	Kabete strain infected spleen, 10% emulsion 0.5 ml. of inoculum. #1 of newly adapted strains	7th mold in 10th reverted to 2nd	rinderpest developed	
		6th mold in 13th reverted to 10th	rinderpest developed	
		15th	rinderpest developed	
		25th	rinderpest developed Animal recovered.	
		30, 57, 64th	From 30th onward marked temperature reaction only	30-immunized 57-immunized 64-immunized
3	Kabete strain infected spleen 10% emulsion, 0.5 ml. of inoculum. #2 of newly adapted strains	6th	rinderpest developed	
		19th	rinderpest developed	
		51st	marked temperature reaction	immunized
		56th	mild temperature reaction	immunized
4	Kabete strain infected spleen 10% emulsion, 0.5 ml. of inoculum. #3 of newly adapted strains	6th	rinderpest developed	
		43rd	marked temperature reaction	immunized
		47th	marked temperature reaction	immunized

* Temperature reactions: marked 104.5° to 105.5°,
mild 103.5° to 104.5°,
very mild 103° to 103.5°.

** Animal considered immune if it developed no symptoms when challenged with a 2 ml. dose of a 10^{-2} dilution of live virus 14 days to six months after the initial inoculation with the adapted strain.

material which after 10 to 20 passages produced only a mild temperature reaction in the test animal and a good immunity. The third C.A. membrane adapted strain at the 33rd passage was also passed in the yolk sac successfully for 19 passages when it was lost through improperly incubated eggs. At the 10th yolk sac passage it immunized inoculated animals but still gave a rather severe temperature reaction.

It appeared from the foregoing experiments that the rinderpest virus must become well adapted to the C.A. membrane before it is possible to adapt it to the embryo proper by the yolk sac route. This stage of adaptation seemed to coincide with the stage at which the virus had become attenuated to such a degree that it no longer caused fatal rinderpest but would still, like its parent C.A. membrane adapted strain, induce a relatively severe temperature.

Lyophilization of Embryonated Egg-adapted Virus.

Although the activity of virus preparations can be maintained if they are frozen and held at a sufficiently low temperature, this is obviously not a practical procedure for a vaccine designed for field use particularly in the warm countries in which rinderpest is a major cattle disease. Having developed a method of attenuating the virus and of obtaining it in reasonably large quantities, the next step was to devise a more practical method of maintenance. Of the various current methods of preservation, lyophilization, that is drying from the frozen state, appeared the most attractive and convenient. The success of this procedure depends upon the reduction of the moisture content to a low level; if this is too high, the virus may die and hence lose its immunizing capacity.

Table II

Passage of Virus in Yolk Sac

No. of Expt.	Strain	Total number of Yolk Sac passages made	Passage Tested	Results of Tests in Calves	
				Symptoms after Initial Inoculation	Challenge with Live virus
1	Carter's egg adapted strain, 51st C.A.passage	16	5th 14th	none none	immunized not immunized
	56th C.A.passage	11	11th	none	not immunized
	61st C.A.passage	51	6th 11th 16th 24th 51st	none none none none none	immunized immunized immunized immunized not immunized
	71st C.A.passage	39	8th 39th	none none	immunized not immunized
2	C.A. membrane newly adapted #1, 44th C.A.passage	26	13th 26th	marked temperature reaction mild temperature reaction	immunized immunized
3	C.A. membrane newly adapted #2, 41st C.A.passage	20	10th 20th	mild temperature reaction very mild temperature reaction	immunized immunized
4	C.A. membrane newly adapted #3, 33rd C.A.passage	19	10th	marked temperature reaction	immunized

A small Edwards centrifugal freeze dryer was the only machine for this purpose available at the laboratory and although not felt to be ideal for the purpose was utilized. Preliminary runs were made with material from uninoculated eggs which might be expected to have a consistency similar to that of the vaccines. Although an attempt was made to reduce the residual moisture content to 0.5% or less, the best achieved was 1.0% after 48 hours in the primary (24 hrs.) and secondary (24 hrs.) dryers.

The results with the first lot of material dried, six vials of the 67th C.A. membrane passage of the first of the newly adapted strains, were only partially satisfactory. The material when tested immediately after drying solidly immunized the animal and produced a temperature reaction corresponding to that of the control material before drying. However, after storage for 31 days at room temperature it had lost its immunizing potency, the inoculated animal succumbing to rinderpest on challenge. It was felt that the residual moisture content of this initial product may have been too high for storage under adverse conditions for such a period. The fact that the virus withstood the drying process showed, nevertheless, that the Edwards centrifugal dryer could be utilized for this purpose.

Much more encouraging results were obtained with the second lot of dried viral material, the 117th C.A. passage of Dr. Carter's egg adapted strain. Two dried samples were tested in animals, one after 11 days storage in the ice chest, the other after 31 days at room temperature. In both cases the test animal proved immune to challenge. Three

additional lots were dried in the same manner, (a) the 26th yolk sac passage from the 44th C.A. membrane passage of the first of the newly-adapted strains, (b) the 22nd yolk sac passage from the 41st C.A. membrane passage of the second of the newly-adapted strains and (c) the 59th C.A. membrane passage of the second of the newly-adapted strains. When the first of these was tested after storage at room temperature for 30 days, the second after 16 days, and the third after 14 days, all three were found to be actively immunizing. One attempt to lyophilize the third of the newly-adapted C.A. membrane strains had to be postponed because of operational difficulties.

In brief therefore, five lots of viral material in all were dried, three of which were C.A. membrane passages, and two were yolk sac passages. In all five cases the freshly dried material immunized the inoculated animals, indicating that the virus withstood the lyophilization procedure. Of the samples held at room temperature for periods up to 31 days, only one strain proved inert after storage for 31 days, the other four were active at 14 to 31 days.

Because of the number of animals required and the time involved it was not possible to conduct as many or as frequent tests as would have been desirable. For example, the time required for one animal inoculation test is 28 days, 14 days being allowed to elapse after inoculation before the challenge with virulent material is made, and another 14 days for observation of rinderpest symptoms before the animal can be considered immune. Also, owing to the cost and extensive facilities required, no attempt was made to determine quantitatively whether there had been any decrease in the immunizing titre of the

dried material, but as stated the qualitative test indicated that they had retained a high degree of immunizing potency.

These preliminary lyophilization experiments show, however, that this egg adapted material is capable of being processed by a commercial machine to provide a product that can be easily handled and stored. It is proposed in the future to improve the process further by keeping the virus-containing egg material frozen throughout the entire drying process. With a larger machine it might be possible also to reduce the length of the drying period and provide an even more active product.

Discussion

Rinderpest virus has been successfully propagated on the C.A. membrane of the chick embryo. Once the virus commences to propagate on the C.A. membrane it gradually becomes adapted and henceforth is relatively easy to propagate. During the earlier passages the virus retains its pathogenicity for the natural host and it is only after a considerable number of serial passages that it commences to decrease in virulence. The adapted strain, after it has decreased in virulence to the point where it no longer causes the death of the inoculated animal, still remains active antigenically and solidly immunizes the inoculated animal. At no time in the preceding experiments have any of the adapted strains, once they had become attenuated, reverted to the previously virulent form and caused the death of the inoculated animals. All animals inoculated with the actively immunizing strains have remained immune up to a period of six months beyond which they have not been tested.

Table III

Lyophilized Egg-adapted Viral Material

No. of Expt.	Strain	Storage Time	Results of Tests in Calves	
			Symptoms after Initial Inoculation	Challenge with Live Virus
1	67th C.A. passage of newly adapted strain #1	none	Mild temperature reaction	Immunized
		31 days R.T.	none	not immunized
2	117th C.A. passage of Carter's egg adapted strain	11 days I.C.	none	Immunized
		31 days R.T.	none	Immunized
3	26th Y.S. passage from 44th C.A. passage of newly adapted strain #1	1 day I.C.	Mild temperature reaction	Immunized
		30 days R.T.	Marked temperature	Immunized
4	22nd Y.S. passage from 41st C.A. passage of newly adapted strain #2	2 days I.C.	none	Immunized
		16 days R.T.	none	Immunized
5	59th C.A. passage of newly adapted strain #2	none	Very mild temperature reaction	Immunized
		14 days R.T.	Very mild temperature reaction	Immunized

R.T. = room temperature 72° to 75°F.

I.C. = ice chest - 60°C.

It would appear, therefore, that once the rinderpest virus has become well-adapted to the C.A. membrane it may be established quite readily in the embryo proper via the yolk sac route. In all of the preceding experiments, this yolk sac adaptation was successfully accomplished only after the C.A. membrane strain had become sufficiently attenuated so that it immunized but did not cause the death of the inoculated animal. Moreover, as mentioned previously, the yolk sac passages retained the properties of their parent C.A. membrane strain and were equally as active in their immunizing potentialities. With serial passage of both the C.A. membrane and yolk sac adapted strains, the virus became gradually attenuated and the temperature reactions elicited in the inoculated animals became less severe. Later passage strains giving very mild or no temperature reaction in inoculated animals, nevertheless, appeared equally as active in their immunizing capacities as earlier adapted strains. The possibility that continued serial passage might eventually reduce the virulence of the virus to such a degree that it would also lose its immunizing activity could not be overlooked, however. This point has been considered with regard to the strain adapted to the C.A. membrane by Dr. Carter. This particular strain, which in its early passages from the 45th onward caused no or very little temperature reaction, is now in its 117th C.A. passage. For the last 50 passages it has induced no temperature reaction when inoculated into a susceptible animal and has not decreased in any way in its immunizing activity. Indeed, with all adapted strains worked with to date,

although the attenuation increases as judged from the temperature reactions of inoculated animals, there has not as yet been any demonstrated change in the immunizing activity with serial passage. In brief the strains of rinderpest virus now adapted to the C.A. membrane and the embryo proper by the yolk sac route appear to be relatively stable and thus suitable for use in a vaccine preparation.

Consideration was next given to the problem of the final preparation of a rinderpest virus vaccine that would retain its immunizing activity and also be in a suitable form for transportation and storage. The preliminary experiments with lyophilization showed this method of vaccine preparation to be very promising, and that these egg-adapted strains of the virus may be processed by a commercial machine to provide a product that can be easily handled and stored. Although the small Edwards centrifugal dryer employed in these experiments was satisfactory for small lots, to prepare the product on any large scale would require a bigger machine, and preferably one in which the viral material could be kept frozen throughout the whole drying process. Such vaccines could be prepared at a relatively low cost, and stock piled for emergencies without undue difficulty. Furthermore, lots of seed material could be safely stored in a dry ice chest for long periods of time and be available for rapid and large scale production of such vaccines should the need arise.

SUMMARY

The Kabete strain of rinderpest virus has been successfully adapted to the chorio-allantoic membrane of the chick embryo. The adapted virus when serially passaged for a considerable number of passages became attenuated until it no longer caused a fatal infection but immunized the inoculated animal.

This C.A. membrane adapted strain was then adapted to the embryo proper by the yolk sac route. When once established in the embryo proper, the virus could be passaged serially like the C.A. membrane adapted strain and retained the properties of the parent C.A. membrane adapted strain. Further attenuation continued with serial passage as judged by the decreasing temperature reactions obtained in animals inoculated with later passages. All of the adapted strains appeared equally active in their immunizing potentialities in the limited number of test animals used.

Both the C.A. membrane adapted strain and the strain adapted to the embryo proper were lyophilized, that is dried from the frozen state, without loss of potency. After lyophilization these strains retained their immunizing activity and withstood storage at room temperature for periods up to 31 days. Such lyophilized, avianized rinderpest vaccines could be produced at a very reasonable cost and in a relatively short time should the need to control an outbreak of the disease suddenly arise.

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APPENDIX

Technique for Chorioallantoic Membrane Passage.

Technique for Dropping C.A. Membrane.

Ten-day embryonated eggs were used and the procedure was as follows:

(1) An x was marked on the shell where it was desired to drop the membrane (as close to the embryo as possible) and where no major blood vessel could be damaged.

(2) The area on and around x, and the area of the shell over the air space was sterilized with an iodine or alcohol swab.

(3) A drop of hot paraffin was placed over the x.

(4) The shell was drilled through at the sterilized spot over the air sac and at the point marked x, just down to the membrane in each instance. A circular abrasive wheel was used for exposing the shell membrane under x. There was then a grooved depression in the paraffin over x.

(5) A split was rubbed in the shell membrane at x, with a blunt hypodermic needle.

(6) A drop or two of sterile saline was delivered at x with a sterile syringe, this saline disappeared and facilitated the dropping of the membrane.

(7) The membrane at the air sac was pierced.

(8) Suction was applied to the air sac and the membrane usually dropped to the desired position.

(9) The eggs were candled to check the dropped membranes.

(10) The membranes were then inoculated with the desired amount.

(11) The openings at x and at the air sac were then sealed with hot paraffin.

Inoculation

(a) The procedure for inoculation from a frozen sample was as follows: the membranes were placed together with 1 ml. of sterile saline per membrane in a sterile glass grinder. After grinding, the prepared eggs were inoculated. If the amount of material was limited 0.2 ml. of the ground material was inoculated on each C.A. membrane. When possible 0.5 ml. per egg was the preferred amount; (Results were more satisfactory with the latter amount). Penicillin and streptomycin were used with all inocula as follows: 1000 to 10,000 units of penicillin to 1 ml. of inoculum and 1 to 10 mgm. of streptomycin to 1 ml. of inoculum.

(b) If the inoculation was with fresh material, the C.A. membranes were harvested as follows:

(1) The eggs were candled to see if the membranes were still dropped.

(2) Each egg was placed on a 1% phenol swab (3"x3").

(3) The area of the shell which was uppermost was covered with a 2% iodine-alcohol solution, extending past the edge of the present air space.

(4) Commencing at the site of injection with fine forceps (sterile) the shell was removed with a lifting motion exposing all of the dropped membrane.

(5) With a sterile syringe and needle the membrane was punctured and 1 to 1½ ml. of egg fluid drawn off (this latter step was omitted when the membranes were to be frozen).

(6) Then with sterile forceps and scissors the membrane was picked up, loosened around the edges, snipped free, and placed in a covered sterile petri dish. The amniotic membrane was then grasped and removed.

(7) If the next passage was immediate the membranes and egg fluids were ground together (1 ml. of fluid to a membrane) in the quantity required. The prepared eggs were inoculated as explained in the opening paragraph of Inoculation. After the eggs were inoculated and sealed, they were incubated for 96 hours at 38° Centigrade. All egg inocula were placed on culture media to test for bacteriological sterility. The inoculum was tested by animal inoculation after 5 to 7 serial passages. The test dose consisted of 1 to 3 ml. of a finely ground emulsion consisting of 3 membranes plus 3 ml. of egg fluids. Animals used were calves aged 3 to 12 months, and occasionally older animals.

Yolk Sac Inoculation

A standard yolk sac inoculation was used, using all care and precaution as in the above C.A. membrane passages.

(1) 7-day embryonated eggs were used.

(2) The inoculum for the initial passage consisted of ground C.A. membranes and egg fluids in proportion of 1 C.A. membrane to 1 ml. of egg fluid, 0.5 ml. of inoculum being inoculated into the yolk sac of each egg.

(3) Inoculum for serial passage consisted of 1 embryo ground with 1 ml. of egg fluid to the desired amount and 0.5 ml. of this material was inoculated into the yolk sac of each egg.

(4) The inoculated eggs were incubated for 96 hours at 38° Centigrade.

(5) In harvesting all of the embryo was used with the exception of the head.

(6) All other procedures follow in step with that outlined in the procedure for C.A. membrane inoculation.

Lyophilization Procedure.

(1) Machine used was the Edwards centrifugal freeze dryer model L.B.5. - this is the small table model.

(2) Two Cenco Megavac pumps were used to maintain the vacuum. The minimum vacuum required for operation of this machine was 70 to 80 microns of mercury. 60 microns were obtained at the start of a run and 25 microns at the end of the secondary drying.

(3) 12 cc. of virus containing egg material was prepared in proportion of 1 C.A. membrane to 1 ml. of egg fluids or 1 embryo to 1 ml. of egg fluids. This material was finely ground in a glass grinder and 2 ml. was added to each of six ampoules. The maximum load for this machine was 16 ml., - a maximum load of 12 ml. per run was used.

(4) Ampoules were placed in the primary dryer for 24 hrs. - their necks being covered by sterile gauze sleeves.

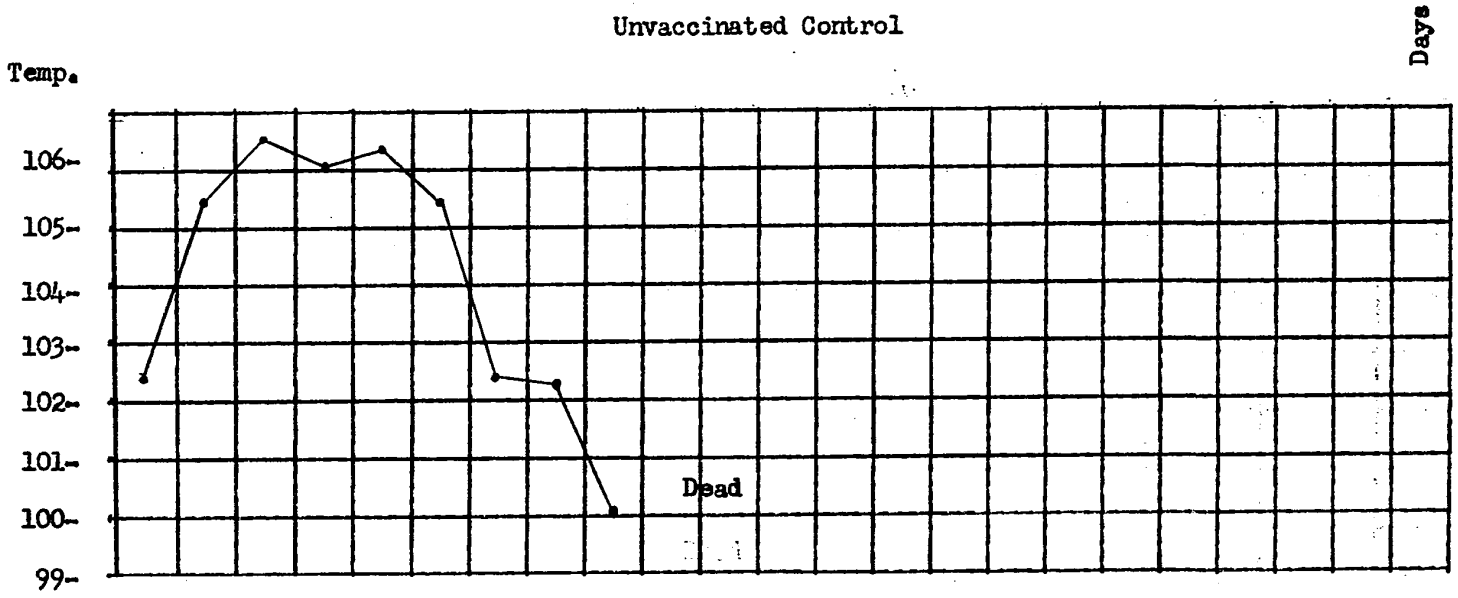
(5) At the end of 24 hrs. - these ampoules were removed from the primary dryer - the gauze sleeves removed - sterile cotton inserts placed loosely in the neck of the ampoules and the necks drawn out in a hot flame to facilitate sealing off on the secondary dryer.

(6) Phosphorous pentoxide is the chemical drying agent used in this machine. The trays were filled before the primary drying and cleaned and refilled before the secondary drying was commenced.

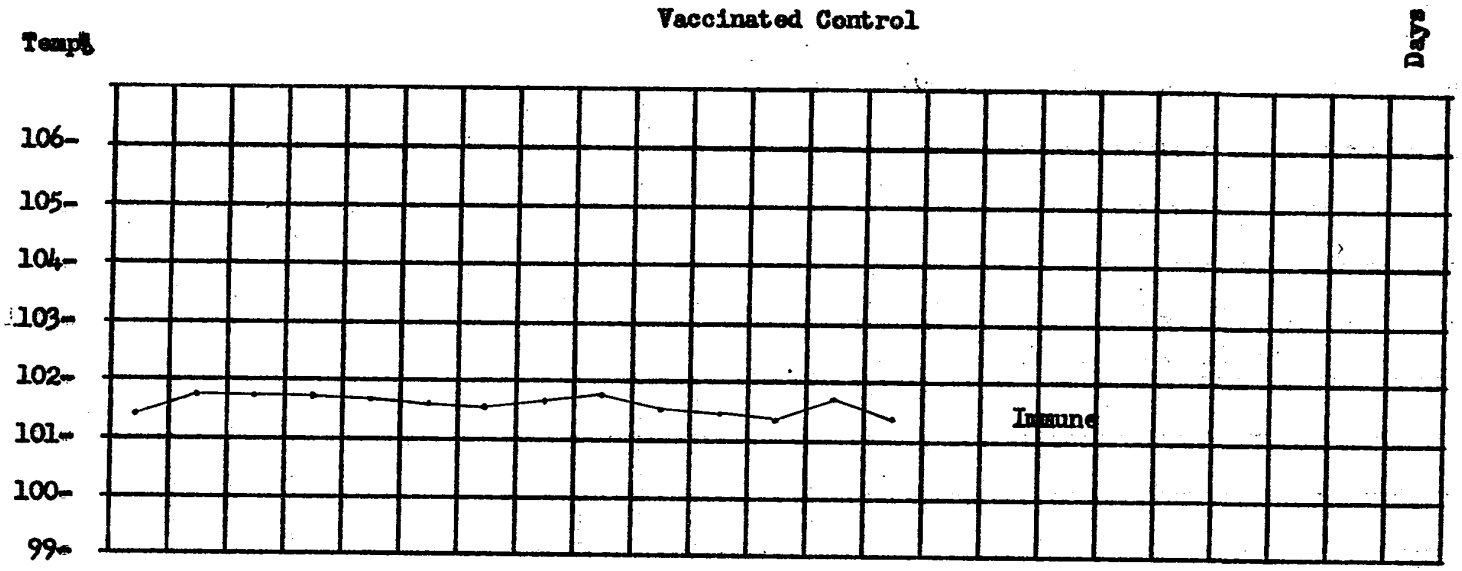
(7) The ampoules were placed on the secondary dryer for 24 hrs.

(8) At the end of 24 hrs., dry nitrogen was allowed into the system in sufficient quantity that only a slight vacuum remained.

(9) The ampoules were sealed off by a sealing torch and were ready for labelling and storage.

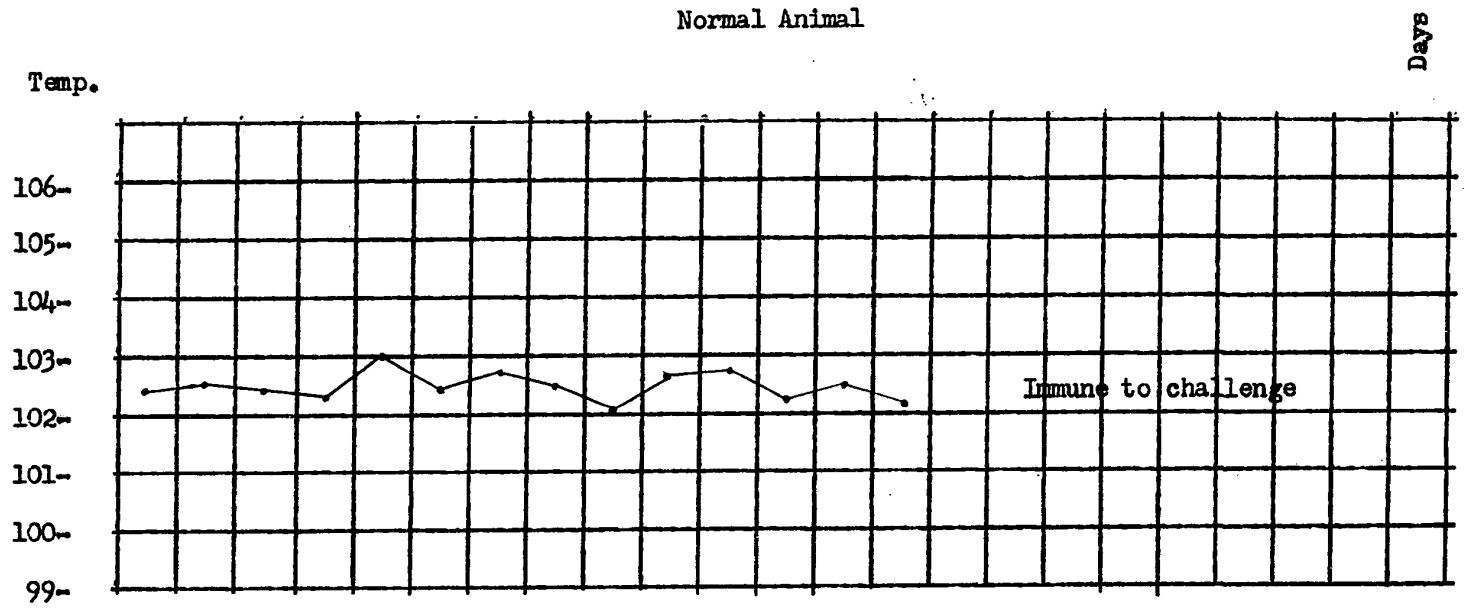


Inoculated subcutaneously with 2 cc. of 10^{-2} dilution of virulent virus.



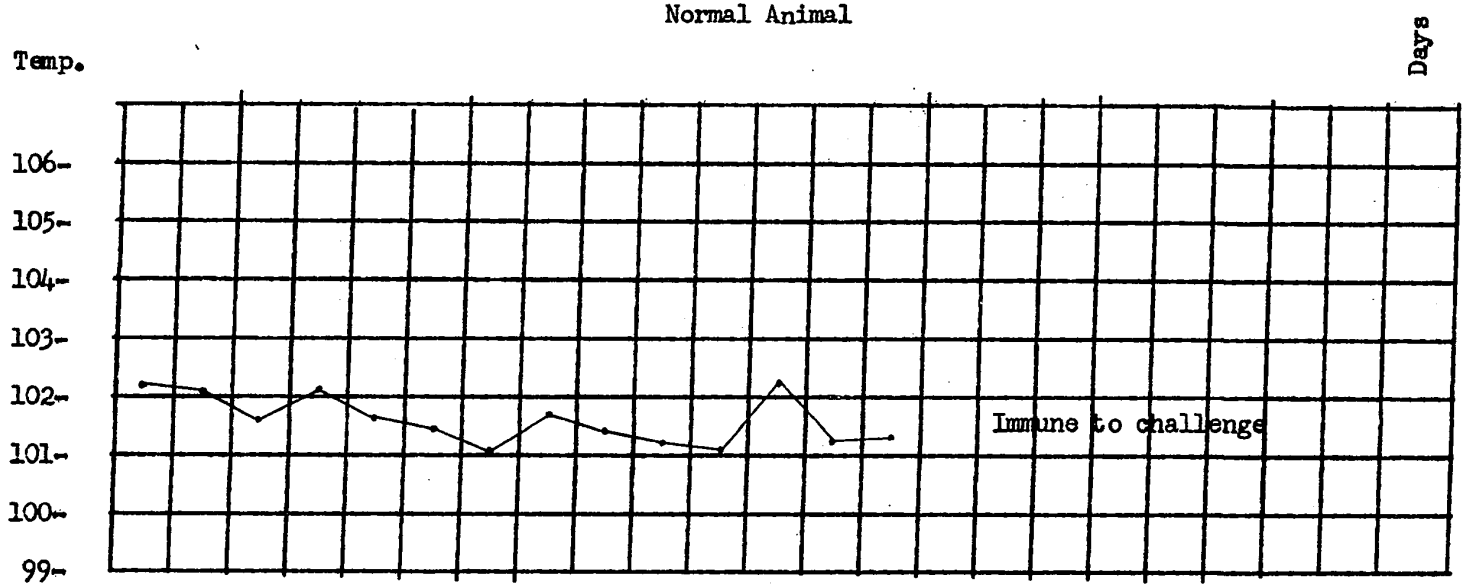
Vaccinated subcutaneously with 2 cc. of adapted virus.

Inoculated subcutaneously 14 days later with 2 cc. 10^{-2} dilution of virulent virus.

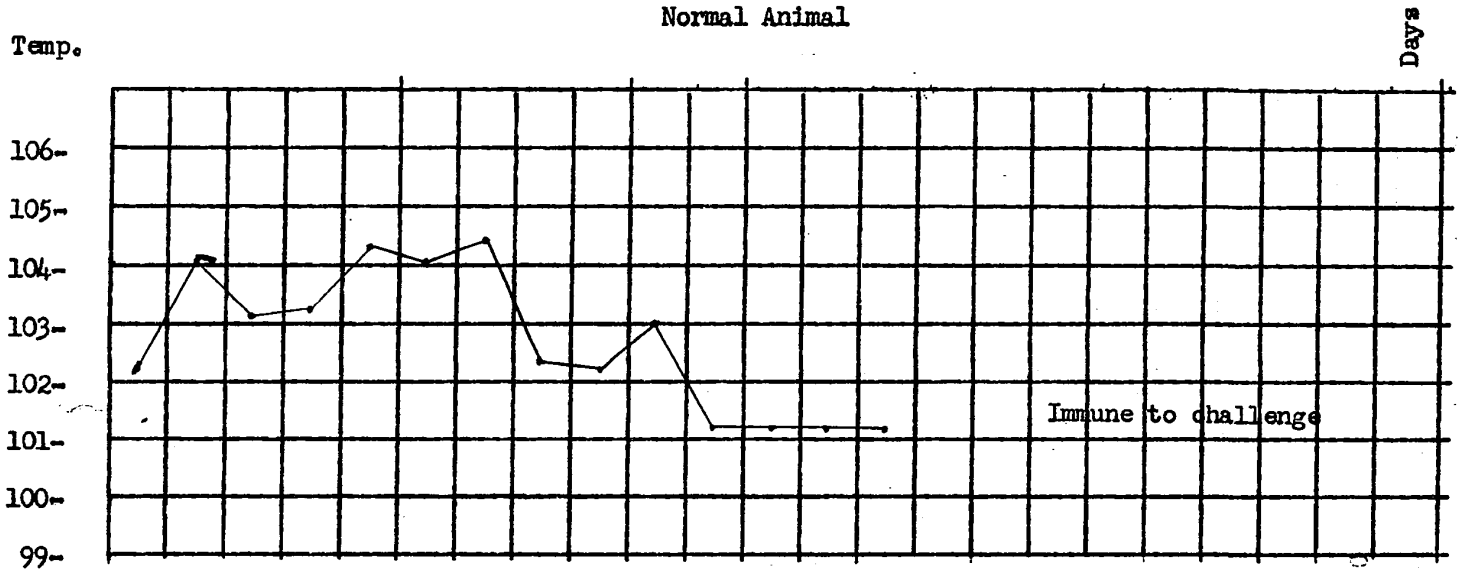


Inoculated subcutaneously with 2 cc. of Carter's adapted strain, 106th C.A. passage.

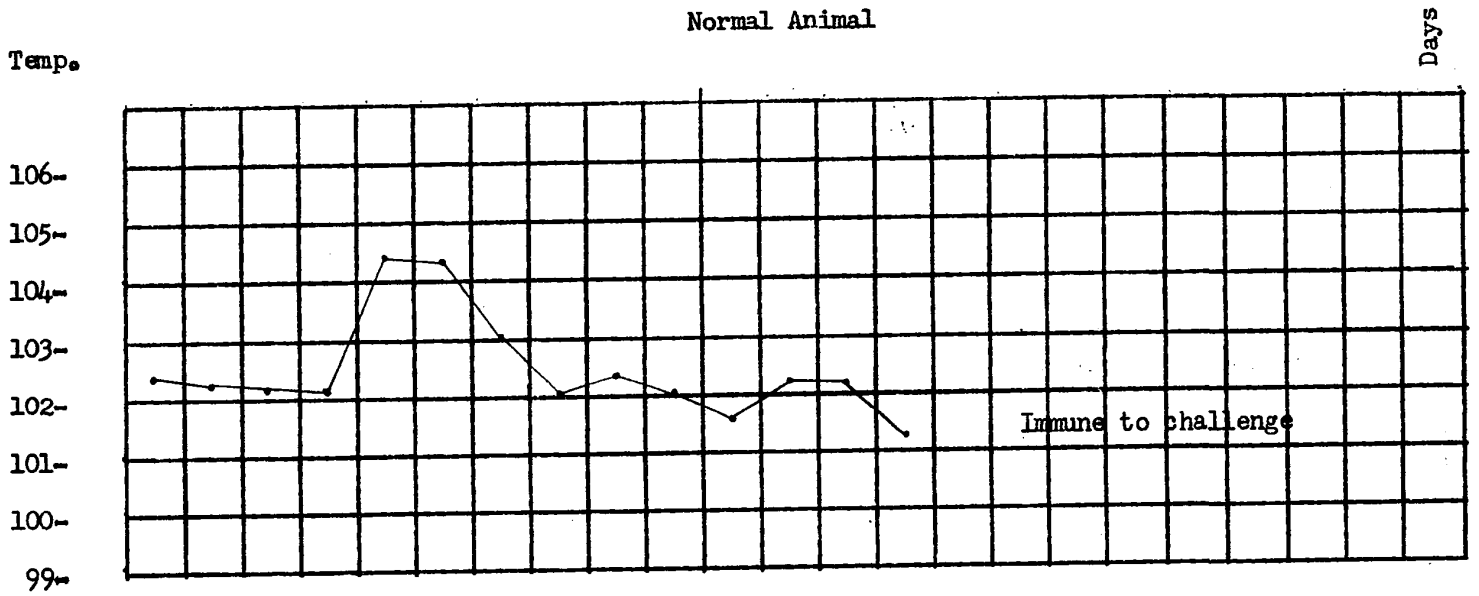
Normal Animal



Inoculated subcutaneously with 2 cc. of Carter's adapted strain, 117th C.A. passage, dried 48 hours.

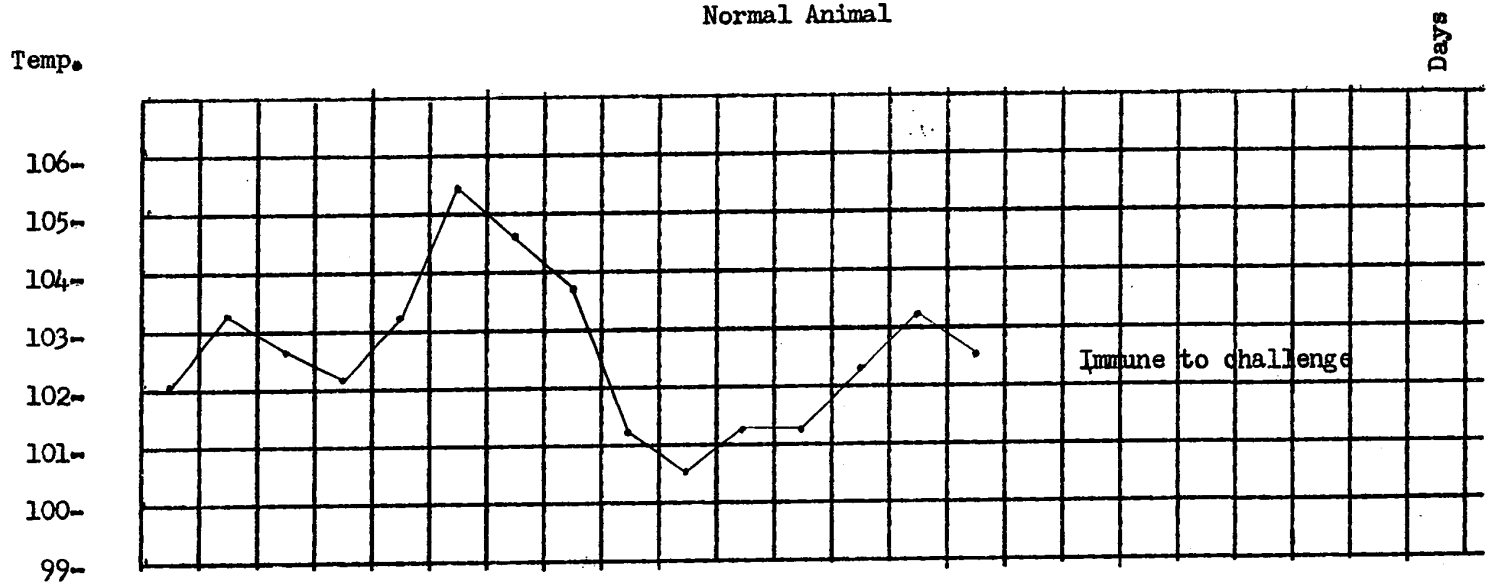


Inoculated subcutaneously with 2 cc. of #1 adapted strain, 57th C.A. passage.

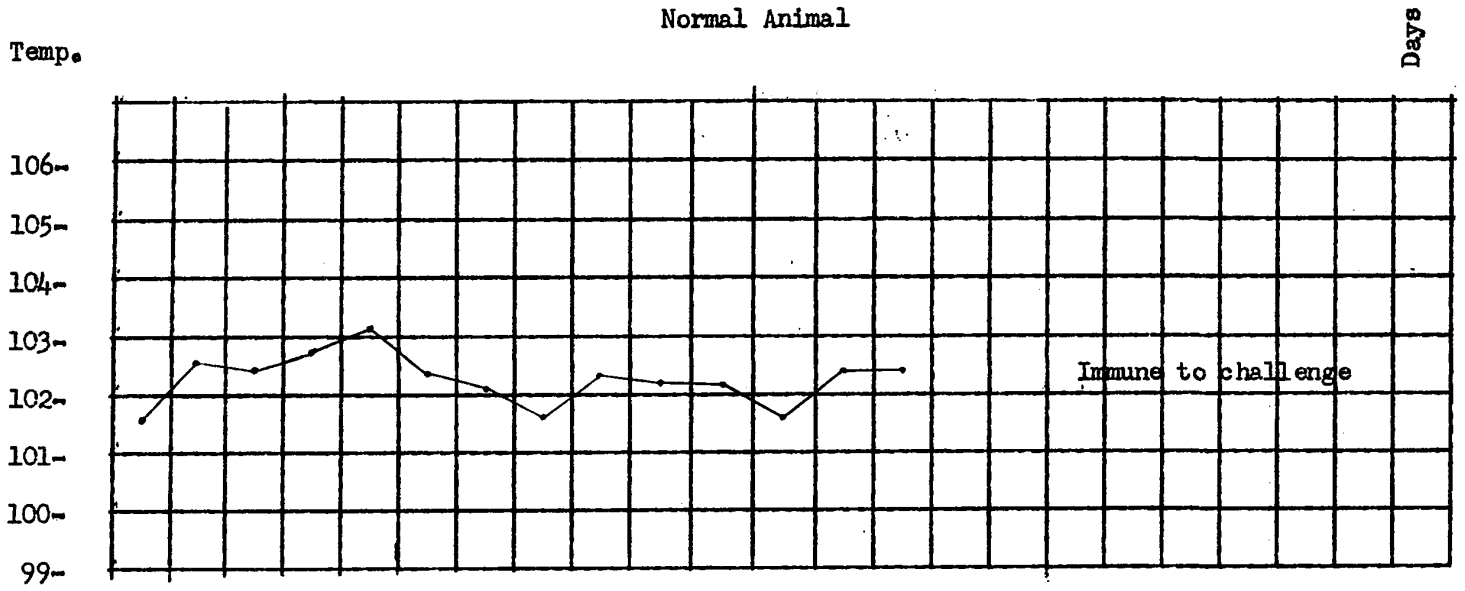


Inoculated subcutaneously with 2 cc. of #1 adapted strain, 67th C.A. passage, dried 48 hours.

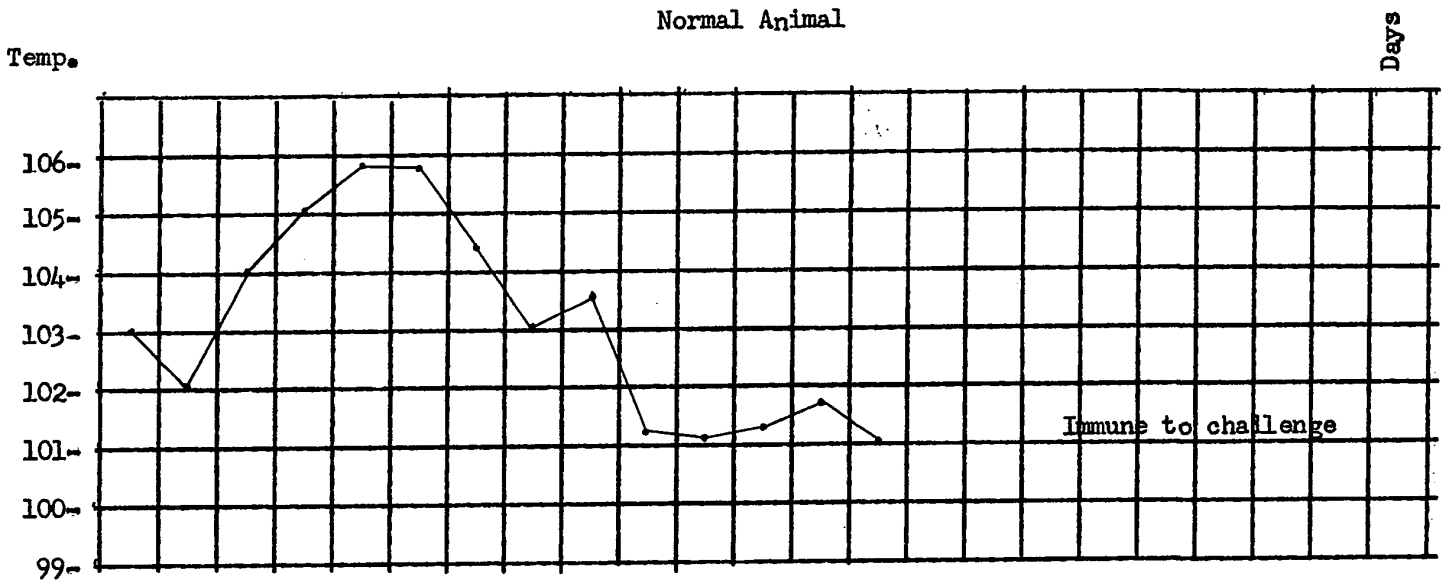
Normal Animal



Inoculated subcutaneously with 2 cc. of #2 adapted strain, 51st C.A. passage.

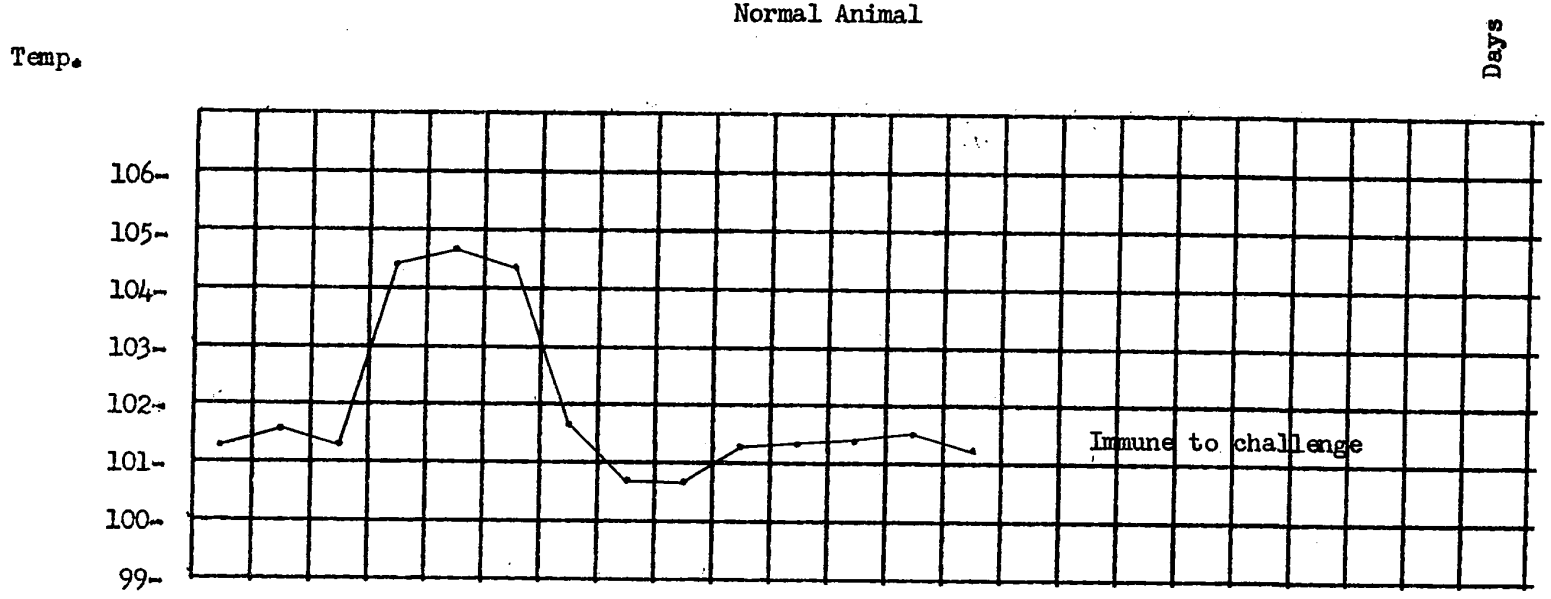


Inoculated subcutaneously with 2 cc. of #2 adapted strain, 59th C.A. passage, dried 48 hours.



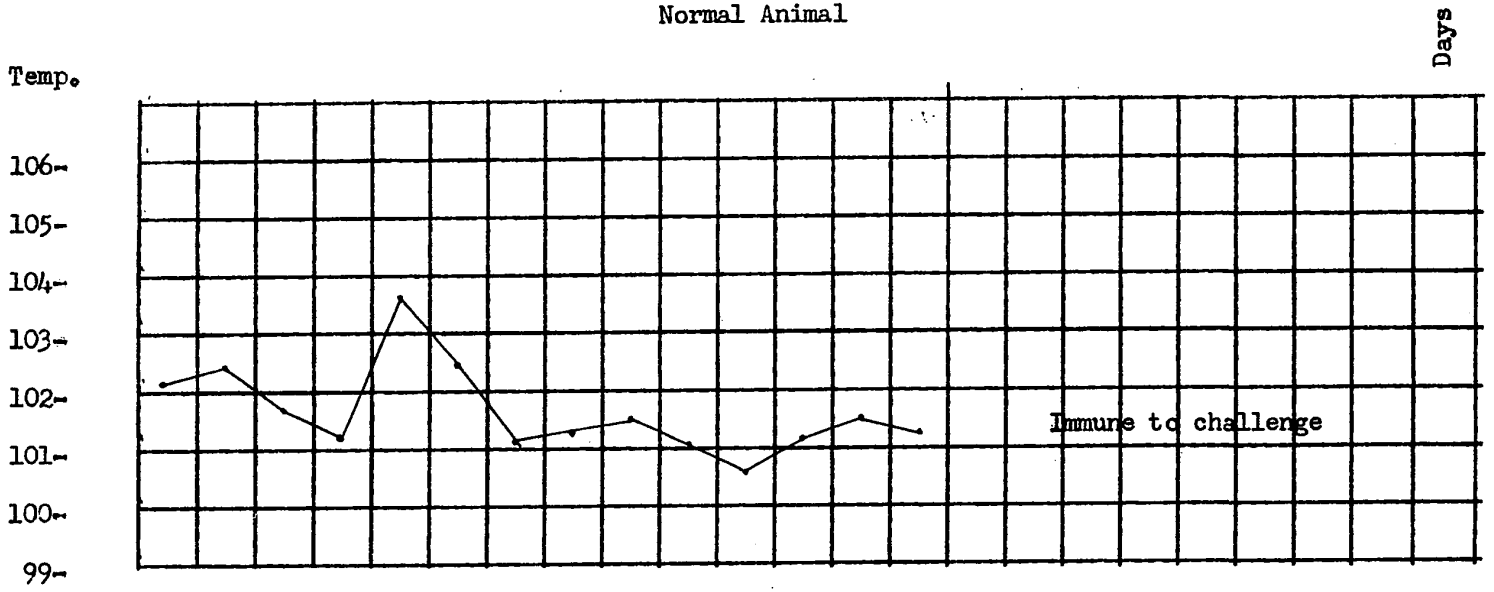
Inoculated subcutaneously with 2 cc. of the 13th yolk sac passage from the 44 C.A. passage, #1 adapted strain.

Normal Animal



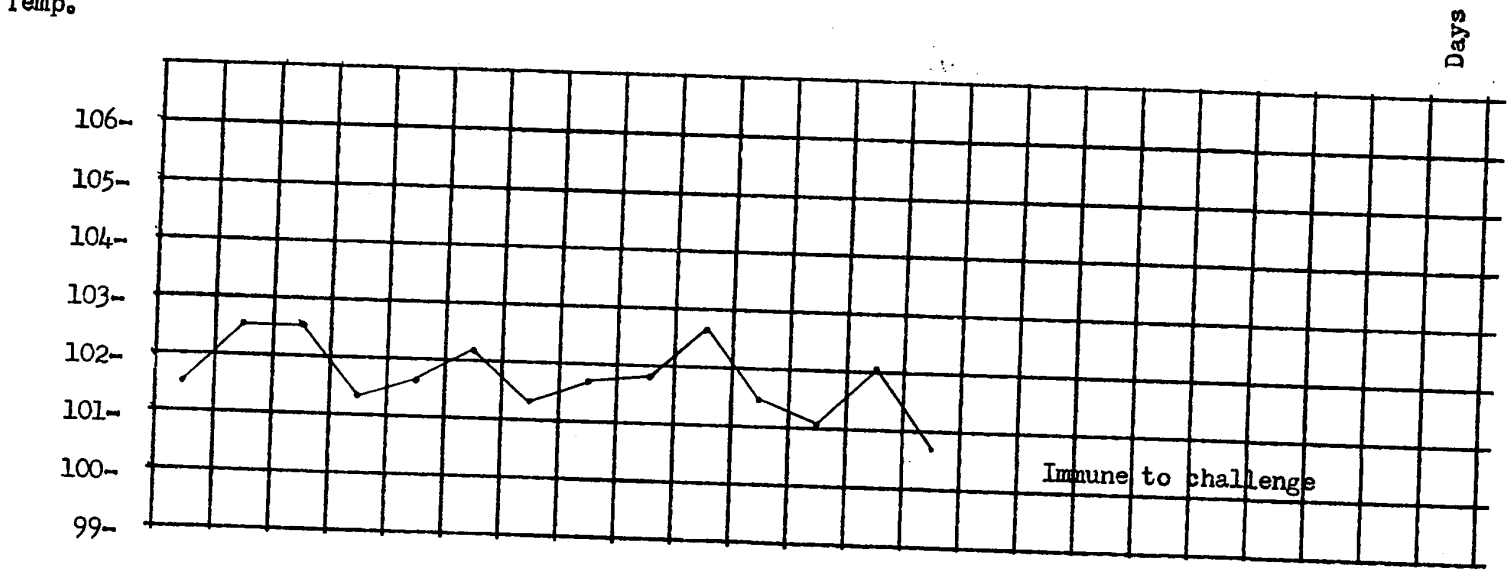
Inoculated subcutaneously with 2 cc. of the 26th yolk sac passage dried 48 hours, from the 44 C.A. passage #1 adapted strain.

Normal Animal



Inoculated subcutaneously with 2 cc. of the 10th yolk sac passage from the 41st C.A. passage, #2 adapted strain.

Normal Animal



Inoculated subcutaneously with 2 cc. of the 22nd yolk sac passage dried 48 hours, from the 41st C.A. passage, #2 adapted strain.