THE INFLUENCE OF TEMPERATURE, AND SOME OTHER PHYSICAL CONDITIONS, ON CALF VACCINE.

By ALAN B. GREEN, M.A., M.D., B.C. Cantab.
Bacteriologist-in-charge, Calf Vaccine Department, Lister Institute of Preventive Medicine.

1. Temperature.

The effect of temperature on calf vaccine is a matter both of scientific interest and of economic importance in preventive medicine. The latter point is of especial significance in hot climates, where rapid deterioration of the potency of lymph is not uncommon; whilst, irrespective of latitude, knowledge of the optimum temperature at which vaccine lymph can be stored is much to be desired.

Work on these lines with vaccine has been done by Blaxall (1902), Blaxall and Fremlin (1906), and Carini (1906); and with vaccine and various organisms by Macfadyen and Rowland (1900).

It seemed desirable, however, to extend the scheme of work as regards calf vaccine adopted by these observers, and to some extent to amend them. It seemed worth while, for instance, instead of exposing one vaccine in the form of dry powder at 60° C., and another as glycerinated suspension at 10° C., and so on, to take for each experiment vaccine collected from one calf; to prepare this in a number of ways, and to ascertain the behaviour of these preparations under different temperature conditions.

In the present experiments vaccine from one calf has been used in the following forms:—

(*a*) *Crude exuded lymph.* This is the exudate expressed from vesicles 120 hours after vaccination of the calf, stored in sealed capillary tubes. This lymph generally coagulates soon after collection, the coagulum adhering to the sides of the tube. The fluid portion, separated from the coagulum, sealed up in capillary tubes was used for experiment.

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TABLE I.

+ = Vesiculation. ? = Specific reaction not amounting to vesiculation. − = Absence of specific reaction.

No. of Experiment	Preparation of vaccine	59—60° C. Minutes							36—37° C. Days					10—15° C. Weeks					3—4° C. Weeks					−3—−4° C. Weeks	
		1	5	10	15	20	30	60	1	5	8	12	20	4	8	12	25	52	4	8	12	25	52	25	52
1	Exudate	+	+	+	+	?	−	−	+	+	+	+	+	+	+	+	+	?	+	+	+	+	?	+	+
	Glycerinated	+	+	+	?	−	−	−	+	+	+	+	?	+	+	+	?	−	+	+	+	+	?	+	+
	Chloroformed	+	+	+	?	−	−	−	+	+	+	?	?	+	+	+	+	−	+	+	+	+	?	+	+
	Dry powdered	+	+	+	+	+	+	?	+	+	+	+	+	+	+	+	+	−	+	+	+	+	?	+	+
	Lanolinated	+	+	+	+	+	+	?	+	+	+	+	+	+	+	+	+	−	+	+	+	+	?	+	+
2	Exudate	+	+	+	?	−	−	−	+	+	+	+	?	+	+	+	+	?	+	+	+	+	+	+	+
	Glycerinated	+	+	−	−	−	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+	+	?	+	+
	Chloroformed	+	+	?	−	−	−	−	+	+	+	?	−	+	+	+	?	−	+	+	+	+	?	+	+
	Dry powdered	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	?	−	+	+	+	?	−	+	−
	Lanolinated	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+
3	Exudate	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+
	Glycerinated	+	+	+	−	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	?	+	+
	Chloroformed	+	+	?	−	−	−	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+	?	+	+
	Dry powdered	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	?	+	−
	Lanolinated	+	+	+	+	+	+	?	+	+	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+
4	Exudate	+	+	+	−	−	−	−	+	+	+	+	?	+	+	+	+	−	+	+	+	+	+	+	+
	Glycerinated	+	+	−	−	−	−	−	+	+	?	−	−	+	+	+	+	−	+	+	+	+	?	+	+
	Chloroformed	+	+	?	−	−	−	−	+	+	−	−	−	+	+	+	+	−	+	+	+	+	?	+	+
	Dry powdered	+	+	+	+	+	?	?	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	−
	Lanolinated	+	+	+	+	?	−	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
5	Exudate	+	+	+	+	−	−	−	+	+	+	+	?	+	+	+	+	−	+	+	+	+	?	+	+
	Glycerinated	+	+	+	−	−	−	−	+	+	+	?	−	+	+	+	?	−	+	+	+	+	?	+	+
	Chloroformed	+	+	+	−	−	−	−	+	+	+	+	?	+	+	+	?	−	+	+	+	+	?	+	+
	Dry powdered	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	?
	Lanolinated	+	+	+	+	+	?	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	?	+	+

(*b*) *Glycerinated calf vaccine.* This is a suspension of one part by weight of finely triturated vaccine pulp in four parts by weight of a 50 % glycerine and water solution.

(*c*) *Chloroformed calf vaccine.* This is a suspension of one part by weight of finely triturated vaccine pulp in two parts by weight of distilled water, subjected to the action of chloroform vapour and air for from 10 to 15 minutes at 20° C. with subsequent removal of all chloroform and the addition of two parts by weight of glycerine.

(*d*) *Desiccated calf vaccine.* This is vaccine pulp reduced to dry powder.

(*e*) *Lanolinated calf vaccine.* This is a mixture of one part by weight of calf vaccine pulp with four parts by weight of lanolin.

All these were placed in sealed capillary glass tubes except (*e*) which, on account of its consistency, had to be placed in a wider-mouthed vessel of about 0·25 c.c. capacity which was hermetically sealed.

These tubes were placed at 59°—60° C., 36°—37° C., 10°—15° C., 3°—4° C. and − 4° C. for varying intervals of time. The contents of the tubes were inoculated on a calf 24 hours after the exposures had finished, and the results of such inoculations were noted 120 hours later.

The results obtained in these experiments are shown in Table I.

Table II is obtained by an analysis of the results in Table I, and gives the average result of the five experiments, and shows that whereas glycerinated lymph, whether treated previously with chloroform or not, retained its infectivity in a very similar manner under the various conditions of temperature; the other three varieties of material behaved somewhat differently.

TABLE II.

	Average duration of infectivity at different temperatures				
Preparation of vaccine	60° C. Minutes	37° C. Days	10—15° C. Days	4° C. Days	−4° C. Days
Glycerinated	11	12	203	364	>364
Chloroformed	11·5	13	224	364	>364
Exudate	16	20	322	>364	>364
Lanolinated	39	20	301	>364	>364
Dry powdered	51	>20	252	287	301

I am indebted to Dr C. J. Martin for pointing out to me that the results in Table II indicate a certain regularity in the effect of temperature upon the durability of calf lymph. For example if logarithms of times as ordinates be plotted against degrees of temperature as abscissae, the results of the experiments form points on a straight line (Fig. 1).

In other words there exists a regular logarithmic relation between the duration of infectivity and the temperature at which the lymph is kept, which is expressed by the equation

$$\frac{1}{T_2 - T_1} \log \frac{t_1}{t_2} = \text{a constant},$$

where t_1 and t_2 represent the time of durability of lymph at temperatures T_1 and T_2 respectively.

From the above observations the temperature coefficient for the velocity with which glycerinated lymph deteriorates can be determined and is found to be about 3 for each 10°, so that lymph kept at 20° C. deteriorates three times as quickly as lymph kept at 10° C.

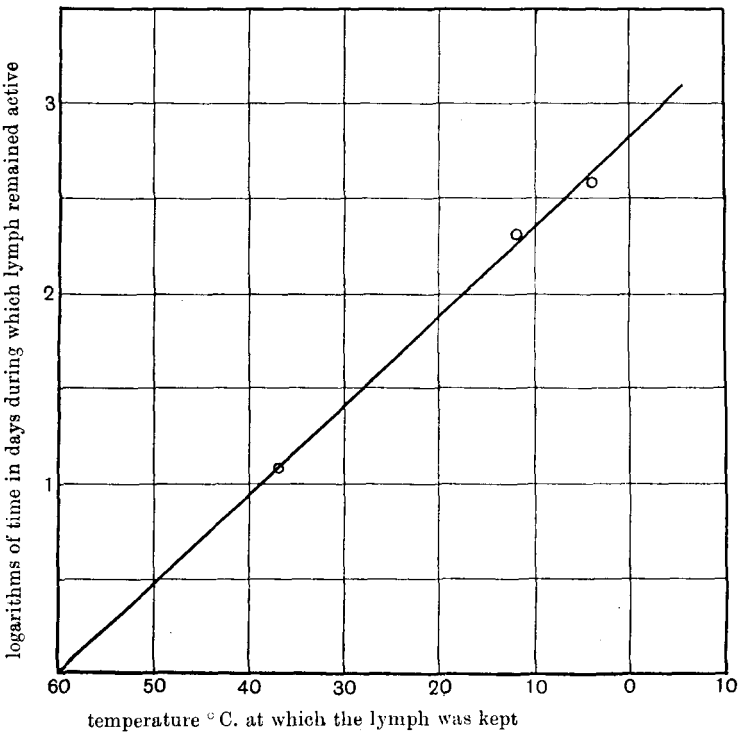

Fig. 1.

A point of interest is that the effect of temperature in hastening the deterioration of glycerinated lymph obeys the same law as is followed by an ordinary chemical reaction and the effect is moreover quantitatively of the same order, viz. two to three times for a rise of 10° C. A similar

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law for the influence of temperature upon the germicidal action of mercuric chloride and other disinfectants has been found by Madsen and Nyman (1907) and H. Chick (1908) to apply in the case of anthrax spores and vegetative forms of bacteria.

This logarithmic law is obeyed up to the temperature of 37° C. If however from the data below and at this temperature, the time of durability at 60° C. is calculated from the above formula, it is found to be about one day, or one hundred times that found by experiment. Somewhere between 37° and 60° temperature *per se* begins to influence the vitality of the lymph unfavourably. If Fig. 1 is reconstructed, plotting logarithms of velocities of destruction of lymph (reciprocals of durability) as ordinates against temperature as abscissae, the points obtained for temperatures 4° C., 12·5° C., 37° C. lie nearly on a straight line, but the point corresponding to the temperature of 60° C. is reached by making the slope much steeper. This indicates that an added effect due to temperature alone, is, somewhere between 37° C. and 60° C., superposed upon the gradual loss of vitality which proceeds at all temperatures.

A practical advantage which follows from the discovery of this regular temperature coefficient for this process of destruction of the virus in calf lymph is that one can foretell the results of experiments which are in themselves very tedious to make. For example the length of durability at − 4° C. was found to exceed one year but was not determined in any instance (see Tables I and II), owing to the extreme slowness of the process of destruction at that temperature. By an application of the above formula it appears that at a temperature of about − 4° C. glycerinated lymph would still be active for nearly three years (1000 days). This figure can be obtained also by continuing the straight line drawn in Fig. 1, until the logarithm of the corresponding time can be read off for a temperature of − 4° C. (log time at this point = about 3·00 and time = 1000).

In some further experiments portions of lymph pulp derived from six calves 120 hours after vaccination were dried, powdered and sealed up in glass capillary tubes. In order to ascertain the limit of resistance of the potency of dried vaccine to a temperature of 99° to 100° C., these capillary tubes were immersed in boiling water, one tube of each of the six experimental lymphs being kept at room temperature as a control. A few hours after such exposure these lymphs were inoculated on a calf, and the results noted 120 hours later.

These results are shown in the following table III.

TABLE III.

\+ = Vesiculation. ? = Specific reaction not amounting to vesiculation.
− = Absence of vesiculation.

Number of experiment	Potency of control	Potency of dried powdered calf vaccine in sealed capillary tubes after immersion in boiling water. Minutes 1	2	3	4	5	10	15	20	30	60
1	+	+	+	+	+	+	?	−	−	−	−
2	+	+	+	+	+	+	−	−	−	−	−
3	+	+	+	+	+	+	−	−	−	−	−
4	+	+	+	+	+	+	−	−	−	−	−
5	+	+	+	+	+	+	+	?	?	−	−
6	+	+	+	+	+	+	?	−	−	−	−

That is to say, dried powdered calf vaccine after exposure in the manner described to a temperature of 99° to 100° C. gave rise to vesiculation in every case after an exposure of five minutes, in one case after exposure for 10 minutes, and in one case showed distinct signs of specific reaction by marked induration and redness along the line of inoculation after exposure for 20 minutes.

Expressed calf lymph and lanolinated calf lymph have shown no signs of potency after exposure for one minute to a temperature of 99° to 100° C.

This resistance of dried calf vaccine to a temperature of 99° to 100° C. has apparently not been investigated before, and I am indebted to Dr C. J. Martin for suggesting it.

2. Osmotic pressure.

Negative osmosis.

It is well known that the specific virus of vaccinia is able to withstand for many weeks association with either pure glycerine or with a solution of glycerine in distilled water in various proportions.

The result of such association is very slight progressive loss of potency on the part of the specific virus.

Similarly, association of calf vaccine with a saturated watery solution of some of the sugars, notably cane sugar, results in but slight loss of potency to the vaccine.

From some experiments which I have made, this resistance is shown to be as marked at room temperature when freshly expressed fluid vaccine is admixed with either glycerine or with a saturated watery

solution of cane sugar, as when calf pulp is admixed with these substances.

This persistence of pathogenicity implies marked ability of the vaccine virus to withstand negative osmotic pressure. For one of the chief characteristics of both glycerine and of cane sugar is their hygroscopic influence.

Positive osmosis.

Novy and Knapp (1906) found that *Tr. brucei* and *Tr. lewisi* and *Sp. obermeieri* in rats' blood were affected injuriously in varying degrees by dialysis against running distilled water for different lengths of time, extending from a few minutes to several hours.

Following on Novy and Knapp's work Ballah (1906) described some experiments on calf vaccine and concluded that the potency of vaccine was not affected by dialysis against distilled water for 18 hours. I have been able to confirm Ballah's statements.

Ballah's work, however, differed in technique from that of Novy and Knapp in an important particular, on which Ballah makes no comment. For, whereas Novy and Knapp placed blood containing their organisms in the dialyser, Ballah dialysed "vaccine emulsion." If this emulsion was a glycerinated suspension, as must be concluded in the absence of a definite statement to the contrary, then Ballah probably subjected vaccine organisms to positive osmotic pressure after they had been already subjected to negative osmotic pressure by glycerine.

It was with a view to the avoidance of such a complication that the lymphs of the present series of experiments were selected in a condition as analogous as possible to the bloods of Novy and Knapp.

In the present series vaccine lymph was expressed from the vesicles of a calf 120 hours after vaccination.

This lymph was collected in glass capillary tubes which were then sealed, and coagulation was then allowed to occur. After the coagulum had shrunk somewhat—20 hours after collection—the fluid contents of the tubes were removed to fine collodion sacks. These sacks were sealed, and were suspended in running distilled water for from 2 to 24 hours at room temperature, and the potency of their contents was subsequently compared on calves with that of a control (undialysed) portion of each vaccine.

Out of a series of 37 experiments no evidence was obtained to show that dialysis had any injurious action on the specific vaccine virus.

3. Filtration.

During the past few years renewed attempts have been made to investigate the filterability of calf vaccine. Earlier efforts pointed to the inability of the vaccine virus to pass through bacteriological filters. But in 1905 Negri published an article which indicated that, under certain conditions, the virus was capable of passage through a Berkefeld filter. The chief difference between the technique of Negri and that of former investigators lay in the fact that Negri worked with vaccine emulsion, which, after trituration to a degree of extreme fineness, had been stored in the cold for two weeks or more after preparation. The theory advanced for the filterability of vaccine organisms of such an emulsion was that this storage led to changes in the size of the organism, analogous perhaps to the formation of spores, whereby they could pass through the filter channels while the larger sized organisms could not. Previous investigators would appear to have worked with coarser and more recent emulsions.

Remlinger and Nouri (1905), and Siegel (1905), published papers in 1905 confirming Negri's results.

In 1906 Carini (1906) published his work on the subject with a carefully reasoned argument. This last named investigator appears to think that, working on the lines laid down by Negri, a very small number of specific organisms may pass through Berkefeld V and N filters. He is however careful to mention (*a*) that his experimental inoculations of these filtrates were made on animals which were used simultaneously for the routine production of vaccine, and therefore, that accidental contamination of his experimental incisions could not be absolutely excluded; and (*b*) that the character of such vesicles as he obtained was poor, and indicated inoculation with a degenerated virus. It is important to note, though Carini does not point it out, that vesicles of this type might easily result when a partly dried incision is insufficiently inoculated with normal vaccine; such a condition in fact as might be afforded were a minute quantity of vaccine from a normally vaccinated surface to be carried into another incised, but previously uninoculated, surface of the same animal.

During the last two years I have carried out a number of experiments on the filtration of calf vaccine emulsion. During the earlier part of this work I was unacquainted with Negri's procedure, but after the publication of his technique great care was taken to follow this closely in the case of over forty experiments. In many cases the filtrates were

centrifugalised and the upper layers were pipetted off to try and concentrate any organisms which might be present.

All the filters were Berkefeld's N or V. These were new filters of the smallest laboratory pattern, sterilised before use.

In order to test the filters for possible defects, emulsions were examined, previous to passage, for extraneous micro-organisms and the filtrates were examined subsequently to ascertain whether these had passed through, and if so, in what quantity.

The filtrates were inoculated in some instances on guinea-pigs and in all cases on calves. A control, or unfiltered portion of emulsion, in each instance was inoculated on the same animal. The calves were used at the same time for the routine production of vaccine.

Of the results of the inoculations on guinea-pigs every one was negative, while the controls showed specific activity.

Of the results on calves every filtrate, with two exceptions, gave a negative result; while the controls on the same animals gave well-marked vesiculation.

In the case of the first of the two exceptions distinct vesiculation showed 120 hours after inoculation. The site of the inoculation was on the inner side of the right hind leg, about 5 cm. from the ilio-inguinal fold and at right angles to it. The inoculation was a line about 5 cm. long. The site in question had been ill-chosen for an experimental inoculation, for when the calf was standing up this surface of skin was in contact with a portion of a surface of skin which was being used for routine vaccination; although when the calf was on the vaccination-table the two surfaces were separated considerably.

In this case, the possibility that this experimental incision was inoculated accidentally from the skin surface with which it came in contact, cannot be excluded.

In the case of the second exception there was no question of direct contact between the experimental incision and the normally vaccinated surface. But at the site of inoculation, 120 hours after inoculation with a filtrate of a Berkefeld V filter, there was one spot of an apparent attempt at vesiculation tailing off into a slightly raised red papule. The appearance in fact was that of very feeble specific reaction. The control of the filtrate produced well-marked vesiculation, though not of first-rate quality, on the same animal.

Apart from the proximity of the control the whole abdomen of the calf was used for routine vaccination as well as a small portion of the thorax somewhat adjacent to the site of the experimental incision,

and it is impossible therefore to exclude entirely the possibility of accidental contamination, though it is quite possible that no such contamination occurred.

The results of these experiments as a whole cannot be said to afford confirmation of Negri's work. On the other hand they appear to be parallel with Carini's results, and to show that if the specific virus of vaccinia is capable of passage through a Berkefeld V such passage is of rare occurrence, and is limited to a very small number of germs.

It has been stated earlier that my first experiments were not carried out according to Negri's technique. With these a 1 in 5 emulsion was usually filtered through a Berkefeld V or N, and occasionally through a Pasteur-Chamberland by means of a partial vacuum, without a preliminary passage through filter paper or cotton wool. A result was then commonly obtained which I have not seen noted elsewhere.

When the filtrate was examined, after negative pressure had been going on for an hour or more, small white masses of organic matter were noticed, and often in considerable amount. These masses were not uncommonly so large as to point to a defect in the filter. But *B. subtilis* and staphylococci failed to pass through the filter, while the white particles were present in the filtrate in numerous large fragments.

With fractional filtration it was noted that while all fractions might be clear for some minutes after passage, they gradually became clouded and the white precipitate appeared, the fragments of which increased in size for a time.

This precipitate appeared in the filtrates of Berkefeld's V or N, to a lesser extent in the filtrate of a Pasteur-Chamberland, and not at all in the filtrates of a Martin gelatin filter. The precipitate generally appeared least dense in the earliest fractions.

Hitherto this precipitate, separated from the bulk of the fluid filtrate by centrifugalisation, has failed to give any specific lesions on calves, while the control (unfiltered portion of the same vaccine) has yielded typical vesicles.

In view of this fact, if for no other reason, the microscopical appearance of the precipitate is of interest. I hesitate at the present time to say that any of the microscopic elements of the precipitate resemble definitely the body or bodies described as the specific cause of vaccinia by authors, although it seems to me that in a hanging drop preparation they are not unlike some published descriptions of the vaccine organism.

SUMMARY OF CONCLUSIONS.

1. Temperature influences the rate at which glycerinated lymph deteriorates in accordance with the law of Arrhenius, expressing the effect of temperature upon chemical action; the rate of deterioration increasing three times per 10° rise in temperature.

2. Dry powdered calf vaccine in sealed glass capillary tubes still gave rise to typical vesicles after exposure to a temperature of 100° C. for from 5 to 10 minutes.

3. The vaccine virus can withstand positive osmosis for 24 hours or more, and negative osmosis for 8 weeks or more, at room temperature.

4. It appears doubtful whether the vaccine virus will pass through a Berkefeld V filter even when a stored vaccine emulsion is used for filtration. If any virus does pass through it only does so exceptionally and in small quantities.

5. When a glycerinated vaccine emulsion is filtered, white flocculi frequently form in the filtrate. These are at first minute, but subsequently adhere and form a macroscopic sediment. This sediment, when inoculated on calves, does not cause vesiculation.

Microscopically these flocculi, in hanging drop preparations, resemble the vaccine bodies described by one or more observers.

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