THE ACTION OF GLYCERIN ON BACTERIA IN THE PRESENCE OF CELL EXUDATES.

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Glycerin has been employed for a number of years for the conservation of vaccine virus [1] and other materials of similar nature, *e. g.*, the virus of rabies, clavele, rinderpest, and foot and mouth disease. Its preservative properties for animal and vegetable tissues are also well recognized. It has, however, been employed more as a diluent, particularly in the case of bovine virus, than because of its other properties, as it seemed better adapted for this purpose than the other substances tested.

Not only does glycerin possess the property of conserving the activity of vaccine virus for a longer time than any other preservative agent tested, but it has also a marked influence on the bacterial content of the virus. Leoni [2] claims to have discovered this fact as long ago as 1889, and since then he has continually employed glycerin for destroying the bacteria in, as well as for the preservation of, the virus. Notwithstanding Leoni's claim of priority, we have until now given Copeman [3] the credit of pointing out the value of glycerin as a preservative and germicide when added to vaccine virus. Copeman's statements regarding the action of glycerin on the bacteria present in the virus have been accepted by nearly all, if not by all, as final, and have doubtless been the cause of many placing too much confidence in it as a purifier of vaccine, as Rosenau [4] as a result of his study of the subject has already stated.

[1] Cheyne, R. R., "On the Preservation of Vaccine Lymph," *Medical Times* (London), 1850, xxi, 267.

[2] Leoni, O., *Sui fattori dell' activita pattogena e specifica de vaccino. Trans. XIth. Session Internat. Medical Congress;* Section on Hygiene, 1894, p. 31.

[3] Copeman, *Vaccination*, 1899, pp. 151–182.

[4] Rosenau, *The Antiseptic and Germicidal Properties of Glycerin*, Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, Bulletin No. 16, 1902.

During the latter part of 1902, and as a part of a discussion of the subject before the Society of American Bacteriologists, I showed a few plate cultivations made from vaccine emulsion treated with glycerin and other substances. The observations made at this time suggested that perhaps there were other factors than the glycerin concerned in the destruction of the bacteria present in the glycerinated virus. With this possibility in view the writer has continued this study and has accumulated additional facts which would seem to add some little to our knowledge of the subject.

The addition of glycerin to vaccine virus in the proportion of from 50 to 60 per cent. of its volume will, as a rule, cause a gradual diminution in the number of the bacteria present, but the effect at best is variable. The glycerinated vaccine emulsion shows, as a rule, only a few living bacteria after being kept for two or three weeks at a low temperature; but if the mixture has been kept at room temperature or at a higher temperature, the diminution in number of the bacteria is proportionately greater.

On the other hand, it occasionally happens that another specimen of virus contains a comparatively small number of bacteria of a species which is usually readily destroyed by the glycerin, and yet these may persist for so long a time in a living state in the virus that its activity will be greatly impaired by the long contact with the glycerin and it thus be rendered unfit for use.

After encountering several such refractory vaccines, it became apparent that the employment of glycerin did not give uniform results, and, therefore, that the statement of Copeman regarding its action called for revision.

An emulsion of vaccine tissue, to which glycerin in the proportion of 50 to 60 per cent. of its volume has been added, will suffer a sudden drop in the number of bacteria it contains; but after this the further diminution in number may be slow and gradual as the following experiments show. Two specimens were employed, of which one (No. 1) only was tested against glycerin. The number of bacteria is given at 1-hour, 2-hour, and 24-hour periods. The temperature was 18–23° C.

	Vaccine emulsion in water. .0001 c.c.	Vaccine emulsion in 50% glycerin. .0001 c. c.	
	I	II	I
Immediate	480	Innumerable	12,000
1 hour	600		1,920
2 hours	—	Innumerable	1,600
24 "	Innumerable		

Low temperatures, as stated, exert a marked influence on the rapidity with which glycerin destroys bacteria. Emulsions of vaccine in glycerin of 60 per cent. strength, kept in a refrigerator at about 10° C., gave the following counts on the days mentioned:

	1st day.	7th day.	14th day.	21st day.
No. of Sample 1.....	1,800	2,000	150	78
" " " 2.....	12,600	2,100	215	380
" " " 3.....	33,000	1,050	370	62
" " " 4.....	3,000	965	670	650
" " " 5.....	30,000	1,500	960	370
" " " 6.....	20,000	725	210	81
" " " 7.....	3,500	1,050	700	—
" " " 8.....	51,000	1,800	180	—

On making closer study of this phase of the subject it appeared that, as a general rule, the several varieties of bacteria were destroyed much more rapidly in vaccine emulsions containing glycerin alone than in cultures to which glycerin was added, or their suspensions in distilled water. The following table shows the viability of several varieties of bacteria subjected to various percentages of glycerin in bouillon and distilled water (page 728).

Bovine vaccine virus always contains a variety of bacteria. The species most commonly met with are the pyogenic cocci, the colon bacillus, B. vaccinalis variabilis, streptothrices, moulds, unidentified endogenous spore-bearing, bacilli, and, more rarely, the common air varieties.

All the experiments with vaccine and other substances enumerated in this paper were made at room temperature, which ranged between 12° and 23° C. The estimation of the number of bacteria in plate cultivation was made in fractions of a cubic centimetre. When an emulsion of vaccine tissue is made with distilled water, and the estimation of the bacteria is delayed a day, the number of bacteria increases so rapidly that it is next

to impossible to enumerate the colonies after that time, although dilutions of $\frac{1}{1,000,000}$ of a cubic centimetre are employed. On the other hand, if glycerin be added in quantity from 25 to 50 per cent. of the emulsions, the number of bacteria falls in a very short time to one half or less of the original number present.

		Dist. Water.	Bouillon.	Dist. Water.	Bouillon.	Dist. Water.	Bouillon.	Dist. Water.	Bouillon.	Dist. Water.	Bouillon.	Dist. Water.	Bouillon.
Percentage of Glycerin.....		60	60	50	50	40	40	25	25	12½	12½	6¼	6¼
Staph. Pyog. Albus.....		— 4	— 6	— 4	— 8	— 12	— 12	+ 28	+ 28	+ 28	+ 28	+ 28	+ 28
Strep. Pyog.....	"	— 2	— 2	— 4	— 4	— 4	— 4	— 4	— 6	— 18	— 24	+ 28	+ 28
B. Pyocyaneus.....	"	— 4	— 4	— 4	— 6	— 4	— 6	— 8	— 16	+ 28	+ 28	+ 28	+ 28
B. Prodigiosus.....	"	— 4	— 4	— 4	— 6	— 6	— 6	— 8	— 12	+ 25	+ 28	+ 28	+ 28
B. Swine Plague.....	"	— 2	— 2	— 2	— 2	— 2	— 2	— 2	— 4	— 2	+ 25	— 2	+ 25
B. Diphtheriæ.....	"	— 6	— 6	— 6	— 6	— 6	— 6	— 6	— 10	— 24	— 16	— 24	+ 28
B. Typhosus.....	"	— 4	— 4	— 6	— 6	— 6	— 6	— 8	— 12	— 14	+ 28	— 16	+ 28
S. Choleræ.....	"	— 2	— 2	— 2	— 2	— 2	— 2	— 2	— 6	+ 4	+ 8	+ 8	+ 10
B. Anthracis.....	"	— 135	— 135	— 157	— 140	— 157	— 157	— 160	— 160	— 200	— 160	— 164	— 200
B. Tetani.....	"	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27	+ 27
Rose Yeast.....	"	— 6	— 6	— 6	— 6	— 4	— 6	— 6	— 8	— 22	— 22	+ 27	+ 27
Aspergillus Niger.....	"	— 2	— 2	— 2	— 2	— 2	— 4						

NOTE.—The figures indicate days, the plus and minus signs indicate whether growth was or was not obtained on the corresponding day.

In view of the fact, which the previous table exhibits, that certain bacteria in pure culture are destroyed more slowly by glycerin than in vaccine and tissue emulsions, the notion occurred to me that the tissue fluids or cell contents might in some way be concerned in producing the sudden reduction in number, and also that this reaction might occur through some combination between

the glycerin and the constituents of the emulsion, or by virtue of the glycerin extracting from the cells some peculiarly active bactericidal substance.

Denys and LeClef [5] have shown that exudates caused by pus cocci are bactericidal to these organisms, and that the fluids extracted from such exudates also possess this destructive property; and Simon [6] claims to have proven that leucocytes obtained from aleuronat injections are decidedly bactericidal to streptococci and much more active than the fluid part of the exudate freed from cells, or the blood serum of the animal.

Emulsions of vaccine tissue made with water were taken as stock solutions. To these solutions were added equal quantities of immune sera as follows: Serum from a vaccinated calf, antidiphtheritic, antitetanic, antistreptococcic, antistaphylococcic, antipneumococcic, antidysenteric, and antityphoid sera; and also serum from a goat in which leucocytosis had been induced, and normal horse serum. The bacterial counts of 0.0001 cubic centimetre of the mixture were made at stated intervals, and they showed a reduction of the bacteria in nearly all the experiments during the first twenty-four hours, after which time rapid increase in number occurred. The reduction was greatest in those tests in which freshly drawn sera were used, and little or no reduction occurred when sera a week or more old were employed. The immune sera, except when fresh, did not exert as great bactericidal action as fresh normal serum; and their action was still weaker when the nomal serum was added to the emulsions before the immune sera. The removal of the cells from these sera, either by prolonged centrifugalization or by filtering through a Berkefeld filter, affected somewhat their bactericidal qualities.

With a view of ascertaining whether the addition of a heterologous exudate might influence the number of bacteria in watery vaccine emulsions, an exudate was secured from a rabbit twenty-four hours after injecting it subcutaneously with a sterilized suspension of aleuronat, and, after grinding to a fine paste, was

[5] Denys and LeClef, Sur le mécanisme de l'immunité chez le lapin vacciné contre les streptococoque pyogène. *Le Céllule*, etc., 1895, vol. xi, 188–190.

[6] Simon, *Centralblatt für Bakteriologie und Parasitenkunde*, 1901, xxix, 116–119.

added to the emulsions with an equal amount of water. The resulting mixture was rich in saprophytic and other bacteria. When equal quantities of these emulsions were mixed there followed, within twenty-four hours, a decided diminution in the number of bacteria. The controls of 0.0001 cubic centimetre gave innumerable colonies, whereas the number of colonies developing in the plates of the mixture was only from 300 to 750. The addition of a small quantity of fresh normal or immune serum caused a still greater reduction amounting, in extreme cases, to a fall to even as low as five colonies. The exudate obtained with aleuronat seemed to have a special action upon the pus cocci, as these were the first bacteria to disappear from the mixtures.

It was considered desirable to ascertain whether the serum from a sample of blood which contained an excess of leucocytes was more effective than normal serum in destroying the bacteria in vaccine emulsions. A medium-sized goat was, therefore, injected intravenously with 1.5 grams of thiosanimine, following which the leucocyte count rose in twenty-four hours from 6500 to 21,000 per cubic millimetre. One part of vaccine emulsion in water was now mixed with an equal quantity of serum obtained by bleeding this goat. The bacterial counts were as follows: Immediately after the mixture, 9736; twenty-four hours later, 4564; then a progressive increase. When, however, to this combination an equal amount of normal serum was added, rapid diminution in the bacterial count took place. Thus, immediately after mixing the count was 9700; two and one half hours later it was 2520; after twenty-four hours it was 873, and in one hundred and forty-four hours the plates were sterile. From this experiment it is safe to conclude that when a sufficiency of normal serum is used all the bacteria in vaccine emulsions can be destroyed; but there seems no reason to assume that serum from blood containing many leucocytes is more effective than normal serum. On the other hand, it would appear that normal serum is capable of destroying various bacteria, including the pyogenic cocci, in the presence of the constituents of the vaccine emulsion.

This result was extended by testing the exudate from a human

lung in condition of red hepatization against normal serum. The exudate, owing to the fact that the lung tissue was not perfectly fresh, contained besides pneumococci and staphylococci certain bacilli, possibly of putrefactive origin. As was to have been expected from the results of previous experiments, the serum had no marked influence on the bacterial count. And yet, as has been shown, when normal serum is mixed with vaccine emulsion, the pyogenic bacteria contained in the latter are markedly reduced in numbers. I was therefore led by these results to test the combined action of glycerin and serum upon vaccine emulsion.

I have already pointed to the difficulty with which glycerin alone destroys certain pathogenic bacteria, and I have shown that while normal serum can effect this end, the process is in its nature a slow one. Now I have found that when normal serum and glycerin are permitted to act together they bring about the reduction more quickly than either fluid alone; and I have also noted the peculiar result that immune sera are less effective in this respect than normal serum, or, at least, with the latter the reduction proceeds with considerably greater celerity.

This last series of results can be more or less altered by modifying the conditions of the experiment. For example: A watery suspension of vaccine tissue when mixed with normal serum and glycerin will lose bacteria more quickly than with the serum alone. But if an immune serum and glycerin are first added to the emulsion and a mixture of normal serum and glycerin be subsequently introduced, then the loss takes place still more quickly and completely. If, again, a sterile exudate be developed by means of aleuronat and emulsified in an immune serum and glycerin before being added to the vaccine emulsion, and if, a little later, a mixture of normal serum and glycerin be introduced, the vaccine emulsion will fail to give any growth at the end of forty-eight hours.

I studied a pneumonic exudate containing innumerable bacteria in every loop. This exudate, when mixed with glycerin, suffered progressive diminution of bacteria, but all growth did not cease until after thirty-two hours. The addition of normal serum, on

the other hand, to the mixture of glycerin and exudate brought about sterility in the short space of five hours.

It was found that the bactericidal action of various immune and normal sera was increased by the addition of glycerin, and the most favorable mixtures were those containing twenty-five per cent. of this substance. And it was also found that in order to destroy bacteria by means of mixtures of serum and glycerin, the most striking results were obtained when the glycerin was mixed with the immune or normal serum before being added to the material containing the bacteria.