Report on the influence of glycerine in inhibiting growth of micro-organisms in vaccine lymph / by S. Monckton Copeman and F.R. Blaxall.

GLYCERINATED CALF VACCINE LYMPH.

REPORT

ON THE INFLUENCE OF GLYCERINE IN INHIBITING GROWTH OF MICRO-ORGANISMS IN VACCINE LYMPH;

BY

DR. S. MONCKTON COPEMAN

AND

DR. F. R. BLAXALL.

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APPENDIX C.

REPORTS on the INFLUENCE of GLYCERINE, of LANOLINE, and of VASELINE, in INHIBITING the GROWTH of MICRO-ORGANISMS commonly found in VACCINE LYMPH; by Dr. S. MONCKTON COPEMAN and Dr. F. R. BLAXALL.

PART I., by Dr. COPEMAN.

Account of the Microbic Constituents of Vaccine Lymph, with History of the various Methods experimentally undertaken, in this and other Countries, for separating from Vaccine Lymph extraneous Micro-organisms, and for preserving indefinitely its Specific Property.

In a paper presented to the International Congress of Hygiene held in London in 1891, and subsequently published in the Transactions of that Congress, attention was called to a special method which I had devised for the bacteriological purification and for the preservation of vaccine lymph.* This method consisted in the intimate admixture of a given amount of lymph, or rather vesicle pulp, with twice its quantity of a 50 per cent. solution of chemically pure glycerine in distilled water, and in subsequent storage of the resulting emulsion in sealed capillary tubes for several weeks.

For some years antecedent to 1891, I had been engaged in investigating the nature and mode of action of the specific virus contained in vaccine lymph, and, in view of the earlier work on this subject by Chauveau† and Burdon Sanderson‡ having afforded presumption of the particulate nature of the specific virus of vaccinia, my special attention had been directed in the first instance to the bacteriological side of the inquiry.

With reference to the literature of the subject, the first account of the discovery of micro-organisms in vaccine lymph and in small-pox lymph appears to be that given by Keber§ of Danzig, who evidently regarded the bodies found by him as the carriers, if not the actual generators, of the virulent principle of these diseases.

Within the next two years the occurrence of similar bodies in vaccine lymph was described by Burdon Sanderson and by Klebs. In 1872 an important paper was published by Cohn‖ of Breslau, in which he treated the morphological aspects of the subject with much completeness. His observations, which related to both vaccine lymph and variolous lymph, have, as regards the lymph obtained from mature vesicles, at least, received general corroboration from all subsequent workers. He, however, apparently believed the micro-organisms found by him to be of one species only, to which accordingly he gave the name micrococcus vaccinæ or variolæ, as the case might be, whereas later observers have shown that organisms of more than one species are usually to be found in any given specimen of lymph.

Cohn called attention to the fact that in perfectly fresh vaccine lymph the "corpuscles" for the most part occur singly, but that others

* See also Proceedings of Royal Society, vol. 54, p. 189.
† Comptes Rendus, lxvi., 1868.
‡ Intimate Pathology of Contagion: 13th Report of the Medical Officer to the Privy Council.
§ Virchow's Archiv., xlii., 1868.
‖ *Ibid.*, lv., 1872.

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are joined together in pairs in a form resembling the figure 8; and he states that after the lymph has been kept for a time, the numbers of "the "double cells, increase, and soon, chains of four begin to be distinguish- "able. These chains are usually curved, or in zigzags; their attach- "ment one to another is evidently very slight, as they can readily be "displaced . . . . After a few hours' observation they are seen "to be all aggregated into irregular colonies or clumps, each consisting "of 16, 32, or more corpuscles." He also noted a point of importance in connexion with the opacity which is apt to occur in stored lymph, namely, that "in capillary glass tubes the multiplication of colonies "sometimes lasts a long time so that they acquire considerable size, "and present themselves as flocculi."

Just previously there had appeared a short communication by Weigert,* who investigated the histology of the vaccine vesicle, with the result that he found the lympathic vessels of the cutis plugged, during the early stages of the process, with granular masses of what appeared to be micrococci.

About twelve years later, Quist† published a series of experiments dealing with the possibility of cultivating, outside the animal body, the micro-organisms present in vaccine lymph. The culture fluid employed by him was composed of equal parts of blood-serum, glycerine, and distilled water, this mixture being rendered alkaline by the addition of $\frac{1}{300}$th part of carbonate of potash. Quist did not attain any great measure of success in the immediate object of this inquiry, but still, he showed that the specific contagium of vaccinia could exist, for a time at any rate, in a fluid containing a considerable proportion of glycerine.

Buist‡ of Edinburgh succeeded in isolating from vaccine lymph three varieties of micrococci which, when grown on nutrient media, gave rise to colonies of a white, yellow, or orange colour. All these Buist appears to have considered to be essential constituents of vaccine lymph, as is evidenced by the fact that he speaks of them as white, yellow, and orange vaccine respectively.

It was reserved for Pfeiffer§ to show that the various micro-organisms isolated by his predecessors, although to be found constantly in lymph, were identical with certain definite species with which he was familiar as occurring also in various tissues and body fluids under circumstances which had no relation either to vaccinia or variola. As a consequence, none of these organisms are, he pointed out, to be regarded as concerned in the specific action of the lymph.

Mention may also be made of the fact that Crookshank in his evidence before the Royal Commission on Vaccination, and also in a paper communicated by him to the International Congress of Hygiene, in 1891 (vol. iii.), states that he has succeeded in isolating from vaccine lymph, by the method of plate cultivation, an immense number of bacteria, including micrococci, bacilli, torulæ, &c., of which he set out a detailed list. All of these he recognises as well-known saprophytic bacterial forms, associated, some of them, with processes of suppuration, but none of which, he says, can be regarded as the contagium of vaccinia, seeing that no single one of them is constantly present in vaccine lymph, human or bovine. As regards the latter statement, I find myself entirely in agreement with him. For the rest, his formidable list of bacteria to

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* Centralb. f. Bakt., 1871, p. 609.
† Berlin, Klin. Woch., 1883, No. 52.
‡ Vaccinia and Variola, Edinburgh, 1886.
§ Zeitschrift. f. Hygiene, iii., 2, p. 189.

be obtained from vaccine lymph is discounted, as regards danger incurred in the operation of vaccination, by the fact that he is silent as to whether any, and if so what, precautions had been taken by him with regard to the collection of the lymph which he tested. Nor did he attempt to distinguish between those bacteria which are commonly to be found in lymph and those whose presence therein is exceptional.

As the result of my own work in this connexion, it would appear that in addition to a small bacillus described elsewhere both by Klein and myself, and believed by us to be essential to vaccinia, there are at least three species of micro-organisms, one or more of which species are almost universally to be found in every sample of human or calf vaccine lymph examined. These are, staphylococcus albus epidermidis, S. pyogenes aureus, and S. cereus flavus; and they correspond probably to Buist's white, orange, and yellow vaccine respectively. Of these, the staphylococcus albus epidermidis is usually to be found in the upper layers of healthy skin of unvaccinated persons. In addition, I have been able to satisfy myself as to the occasional presence of the streptococcus pyogenes. I myself have never detected the streptococcus of erysipelas; but it is on record that, in certainly one instance at least, this microbe has been isolated from vaccine lymph.

Early in the course of my experiments it struck me that the exuberant growth commonly manifested by these evidently "extraneous" organisms, might very possibly be interfering with, if not superseding, the more important and essential organism that I was seeking. I therefore turned my attention to the discovery, if possible, of some means of so treating vaccine lymph as to inhibit the multiplication in it of "extraneous" organisms without at the same time injuring its potency for vaccination.

Another consideration which directed my research work at that time in the direction indicated, was, that my attention had been called to the opacity which commonly occurs, after a longer or shorter period, more particularly in human lymph which has been stored in sealed capillary tubes, as well as to the deterioration in activity of the lymph which is found to be a concomitant of such opacity. As to this, I have in the course of my investigations been able to show that the opacity of old stored lymph may be quite independent of any coagulation in the lymph, no coagulum being found on breaking many of the tubes in which opacity was most marked. Further, I have shown that if cultivation experiments are carried out with the contents of tubes which have become opaque, and also simultaneously, for purposes of control, with samples from tubes of comparatively fresh lymph, many more colonies are likely to result in the plates established from the old tubes than in those established from more recently stored lymph. There can hardly be question, therefore, that the opacity of old stored lymph is, in the main, the outcome of the multiplication in it of aërobic bacteria, the ancestors of which were present in the lymph when first collected, although their numbers were then so comparatively small as not to render it in any way turbid. These bacteria evidently find in the lymph, especially when removed from the body, a suitable medium for their subsequent multiplication, while at the same time it would appear that growth and multiplication of them has the result of gradually inhibiting the specific effect proper to the vaccine virus itself.

Upon all grounds, then, the obvious indication for my guidance was not only to prevent such multiplication of "extraneous" organisms subsequent to storage in the usual manner, but, if possible, to remove

them altogether, as soon as the lymph was taken from the living subject, without injury to the actual contagium of vaccinia.

Without detailing the various experiments adopted with this object in view, a full account of which has been published elsewhere, it is necessary in this place to state briefly the lines on which the work was carried out. In the first place, trial was made of the method of exposure of lymph, for definite periods, to a temperature considerably above blood-heat, which had, in the hands of Kitasato, met with such brilliant success in the isolation of the bacillus of tetanus. Proceeding in this manner, and in every experiment observing the precaution of making control cultures, I presently arrived at a temperature, exposure to which is apparently incompatible with the continued existence of those micro-organisms which can ordinarily be grown when vaccine lymph is inoculated into nutrient jelly. The temperature in question ranged between 38° C. and 42° C. But the method did not in practice prove advantageous. Thus, if plate culture were made of lymph, after exposure for an hour at the lower register, a few points of growth occasionally, after the lapse of a day or so, made their appearance; and, on the other hand, the higher temperature, though it inhibited all extraneous microbes, appeared sometimes to exert an injurious effect on the lymph, as far as regards the normal vesiculation which should result from its inoculation in the living animal. Some method of readier application and requiring less delicate manipulation was, therefore, obviously desirable. This I at length found in the addition to the lymph, or rather to the vesicular pulp obtained from a vaccinated calf, of a sterilised 50 per cent. solution of chemically pure glycerine in distilled water prior to storage of the mixture in capillary tubes, which had been themselves previously sterilised by heat.

Admixture of glycerine and vaccine lymph is, of course, no new device. Many years ago, Müller* showed that vaccine lymph might be diluted with three times its bulk of glycerine, and still retain its properties unimpaired; a fact which has been taken advantage of at many of the Continental vaccine stations, and by more than one purveyor of trade lymph. Curschmann, writing on the subject of small-pox in Ziemssen's Encyclopedia, refers to this method as follows:—

"Müller has the great credit of having discovered the fact that by mixing vaccine matter with glycerine in certain proportions, the activity of the former is not diminished, so that we have here a means of *increasing the volume of the lymph* when the quantity is small, or when there is an unusual demand for it. . . . . The lymph and glycerine mixture *appears to keep as well as the unmixed lymph*."

But from this statement it is quite obvious that the sole object of employing glycerine in the manner described was to increase the amount of material available for vaccination. With this same end in view, glycerine was used by Dr. Stephen Mackenzie, at the London Hospital,† in the epidemic of 1870–71, the mixture being made up immediately before it was required for a large series of re-vaccinations. Similar means for increasing their amount of available lymph have been frequently employed by public vaccinators and others in times of stress, but, until recently, altogether without appreciation of the inhibitory action exerted by the glycerine in bringing about bacteriological

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* Vierteljahrschr f. gerichtl. und öffentl. Med., 1869, Bd. II.

† Vaccination with Lymph diluted with Glycerine, "Lancet," February 18th, 1871.

purification of the lymph when the mixture is stored, for some time prior to use, under conditions preventing access of air and light.

When, however, a glycerine emulsion is properly prepared after the method I have advocated, it is found that the growth of extraneous aërobic bacteria is at once greatly inhibited, while, after a longer or shorter period, they are practically all killed out. This effect is best demonstrated by making a series of plate cultivations from tubes of glycerinated lymph, at gradually increasing intervals of time, a control plate being poured in each instance from a specimen of the lymph material prior to the admixture of glycerine.

These observations of mine, since their publication in 1891, have received ample corroboration from a number of observers in various parts of the world, as follows :—

In 1892, a paper dealing with this question was published by MM. Chambon and St Yves Ménard,* in which they relate their experience of the use of glycerinated calf lymph when kept for a considerable period in capillary glass tubes (previously sterilised) closed by the blow pipe. Not only were the results they obtained with originally active lymph highly satisfactory, but lymph which, in its fresh state, had given mediocre results, produced after 15 days' admixture with glycerine a passable vesicle, and, after 40, 50, or 60 days, a typical one. The improvement in the activity of such lymph seemed to them to be due to the gradual extinction of extraneous microbes under the combined influence of glycerine and time. Professor Straus, who made plate cultures with their glycerinated lymph, found that when fresh it gave rise to numerous colonies of various microbes, especially staphylococcus pyogenes aureus and staphylococcus albus ; but that when it had been stored 50 to 60 days, plate cultures therefrom remained absolutely sterile as regards these extraneous microbes. Samples tested at intervals between these two extremes presented fewer and fewer microbes as they became older. These experiments were repeated many times, and invariably with similar results.

This evidence, so entirely corroborative of my own work, is the more important as it appears certain, from a perusal of their original paper, that the authors were ignorant that similar results had been previously arrived at, and that these had been published nearly 12 months before the appearance of their article.

The value of glycerine in this connexion is also strongly advocated by Leoni in a paper read before the Medical Congress held at Rome in April 1894, and afterwards published in the "Revue d'Hygiène."† He finds that vaccine lymph as freshly collected is apt to contain large numbers of micro-organisms, some of which are capable of exerting pathogenic properties when inoculated into the system along with the true vaccine virus. And he states that these microbes disappear completely from, or that at the least their number is vastly decreased in, vaccine which, having been prepared with glycerine, is preserved for a period of from one to four months before use.

His conclusions may perhaps be best given in his own words :—

"Le vaccin récemment recueilli est un vaccin contaminé" ;

"Les agents de la contamination s'épuisent dans le vaccin conservé pendant quelque temps dans la glycérine" ;

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* "Gazette des Hôpitaux," December 15th, 1892, p. 1346.

† "Revue d'Hygiène," August 20th, 1894, p. 692.

"Le vaccin conservé dans la glycérine pendant 1 à 4 mois après la récolte représente le type du vaccin *pur*, d'une *virulence uniquement spécifique*";

"C'est de cette qualité de vaccin que l'hygiéniste doit aujourd'hui tenir compte dans la prophylaxie de la variole."

Klein* also bears testimony to the power exerted by glycerine in freeing vaccine lymph from bacteria. In speaking of the organism specific to vaccinia as being probably a spore-bearing bacillus, he says: ". . . . it is established that the active principle of vaccine is "preserved in glycerine, although, as is also known, pure glycerine "acting for long times is a germicide for cocci and sporeless bacilli."

In 1896, a Commission under the presidency of Dr. Schmidtmann, and including Drs. Koch, Pfeiffer, and Frosch, together with the directors of the Vaccinal Institutes of Berlin, Cologne, and Stettin, was appointed by the German Government to inquire and report as to the best methods for the collection, preservation, storage, distribution, and use of vaccine lymph. In their report, which has been recently published, the Commissioners arrive at the conclusion (among others) that fresh lymph contains numerous microbes, the number of which, on the addition of glycerine, diminishes as the age of the mixture increases. The microbes that they isolated included mould-fungi, torulæ, sarcinæ, different varieties of proteus, and numbers of bacilli and cocci. Most of these bacteria were of a saprophytic nature, but single colonies of staphylococci pathogenic for rodents† were found in 5 out of 18 samples of lymph examined. To determine to what extent glycerine is efficacious in destroying the vitality of various definitely *pathogenic* microbes, numerous streptococci and diphtheria bacilli were mixed with specimens of lymph. As a result, the streptococci were killed in 11 days and the diphtheria bacilli in 20 days. Dr. Schultz, the director of the Berlin Institute, found that keeping for a few days, after admixture of the lymph with glycerine, sufficed to destroy most of the bacteria usually present, and that their destruction was more rapid when glycerine was present to the extent of 60 per cent. than when the proportion reached 50 per cent. only.

Attempts by these experimenters to make other chemical agents serve for rendering vaccine lymph free from bacteria led to no results of value. Although the lymph could thus be rendered free from extraneous bacteria it was found to be inefficacious as vaccine.‡ Their next procedure was to determine the amount of glycerine that could be added to lymph so as to exert a powerful action in purifying it from extraneous microbes, without in any way interfering with its specific action when employed for the vaccination of children or calves, and they found that addition of glycerine with distilled water, to the extent of from 15 to 20 times the weight of the vesicle pulp collected, in no way interfered with the value of the material for the purpose of the protection which can be afforded by vaccination.

By consent, therefore, of a large number of observers fully qualified to judge of the matter, we have, in glycerinated calf lymph properly prepared, a vaccine material which, while even more efficient as vaccine than the original lymph, can be produced practically free from

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* Micro-organisms and Disease, 1896, p. 399.

† It is pointed out by the Commission that no comparison can be drawn between the virulence for the human being of lymph-bacteria and that evidenced by their action on the lower animals.

‡ *Cf.* Kitasato, "Sei-i-kwai Medical Journal," October 17th, 1896.

the extraneous organisms which, at one time or another, have been isolated from fresh or stored lymph by the method of plate cultivation.*

Of not least importance is the fact that, as shown by Dr. Blaxall and myself,† nutrient material may, by this method of preparation with glycerine, be rendered free also from pathogenic bacteria, such as those of tubercle and erysipelas, even when these have previously been added in considerable quantity, for experimental purposes.

The question of quality of vaccine lymph naturally engaged the attention of the English Royal Commission on Vaccination at the time they were taking the evidence of witnesses, and among the recommendations contained in their final report is one to the effect that no person should be required to submit to vaccination by means of any other lymph than that derived from the calf. The reason for this recommendation appears to be avoidance of even remote risk of inoculation, in the process of vaccination, of infections other than cow-pox, such, for instance, as those of syphilis and leprosy (section 433); as also the desirability of minimising the opportunity for infection by erysipelas, by obviating the necessity for opening vaccine vesicles involved in arm-to-arm vaccination (section 447). The succeeding section of the report, which deals more especially with the subject discussed in the present paper, may be set out in full. It is as follows:—

> 448. "We think that safety would be increased by preserving the lymph in tubes instead of on 'dry points.' There is some difference of opinion on this matter amongst those with whose opinions we have been furnished. On the whole, however, we think the weight of experience as well as reason is in the direction we have indicated.
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> "In connexion with this subject, our attention has been drawn to the experiments recently made by Dr. Copeman, as to the effect of the storage of vaccine lymph in glycerine. The conclusions at which he arrives are that the addition of glycerine, whilst it leaves the efficacy of the lymph undiminished, or even increases it, tends to destroy other organisms. If it be the fact that the efficacy of the lymph remains unimpaired, its storage in glycerine would largely diminish the difficulties connected with the use of calf lymph, which are inseparable from *calf-to-arm* vaccination. The investigation has not reached a point at which it is possible to pronounce with certainty whether the anticipated results would be obtained. And it was at one time suggested that the introduction of glycerine was likely to be mischievous. The question is one a further investigation of which is obviously desirable.
>
> "If lymph is to be preserved in glycerine, due care would be requisite to ensure its purity, and the absence of contamination in its introduction."

To the particular sentence in the section 448 of the Royal Commissioners' Report quoted above, "And it was at one time suggested that "the introduction of glycerine was likely to be mischievous," some special reference is desirable.

The Commissioners had in view an outbreak of disease, having the clinical characters of impetigo, which occurred in the summer of 1885 in

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* An exception is bacillus subtilis—the common hay bacillus—the spores of which are very resistant to the action of glycerine. This microbe, however, possesses no pathogenic properties, and as it is probably derived from the surface of the skin of the calf it is compartively easy to eliminate it by a proper method of lavage of the animal's abdomen prior to collection of the lymph.

† Paper read before Physiological Section of the British Association: Liverpool Meeting, September 1896.

villages situated on the island of Rügen in the Baltic, among 79 children vaccinated in the latter part of June of that year. This outbreak forms the subject of a paper by the late Sir George Buchanan in the Transactions of the Epidemiological Society for 1885–86. He states that for the vaccination of these 79 children only two tubes of lymph (of human origin) were available, and that consequently their contents were further mixed with glycerine ("glycerinum purissimum") before use. Thymol, to the extent of one-third, had been added to the lymph previous to its having been sent out from the Government establishment at Stettin, and before its admixture with glycerine. The German Commission appointed to inquire into the outbreak appears to have suspected the added glycerine as having contaminated the lymph, but Sir George Buchanan notes certain points in the circumstantial evidence, not inconsistent with the Stettin mixture having, *before glycerine had been added to it*, contained deleterious matters. However this may have been, I was asked, in giving evidence before the Royal Commission on Vaccination in 1893, whether the facts respecting this vaccinal impetigo at Rügen did not discount my suggested use of glycerine in connexion with lymph supplies. I pointed out, however, that in the first place, dilution of lymph with an indefinite quantity of "glycerine" of unknown composition, immediately before employing the mixture for vaccination, was a very different matter from storing, for a length of time before use, lymph with chemically pure glycerine in definite amount and of definite strength, and duly protecting the mixture from light and air.* Further, I pointed out that what, at the time of the outbreak in question, may have been termed "glycerinum purissimum," would, in all probability, at the present day be considered by no means deserving of that term. This latter statement I made on the authority of Messrs. Price, the chief manufacturers of glycerine in this country, whose manager, at the request of his directors, was good enough to put at my disposal his extensive practical knowledge of the details and the literature of this subject. The information thus obtained has been included in the appendix to the last volume of evidence shortly to be issued by the Royal Commission on Vaccination.

The glycerine employed by me in my investigations has been that manufactured in this country by Messrs. Price; but in Germany, where my method of purification and preservation of lymph is now universally adopted in the Government establishments for the supply of vaccine lymph, an Austrian glycerine made by Sarg of Vienna is used. This is said by Dr. Schultz, the director of the Berlin establishment, to be of a less "drying" nature than English glycerine.

In order to obtain an authoritative judgment on the degree of chemical purity secured in the manufacture of glycerine at the present time, and for the purpose also of accurately determining the difference, if any, in the nature of the English and Austrian brands, I induced Dr. Wilson Hake to analyse samples of glycerine manufactured by Messrs. Price and Sarg, respectively, and samples also of a new English brand which has just been placed on the market by Messrs. Lever Brothers. The results of the chemical examination of these samples, which through the kindness of Dr. Hake I am able to append, prove incontestably that all three samples, especially that of Messrs. Price, exhibit a high degree of purity. In all there is complete absence of metallic

* As the result of a recent visit to a number of Continental Vaccine Establishments I have learnt, in addition, that, even at the present day, it is not customary to sterilise the mixture of glycerine and water prior to admixture with the vaccine material. Such sterilisation is in my opinion, of considerable importance.

contamination of any kind, while the amount of organic impurity is insignificant. The main difference observed is the slightly greater degree of concentration obtained in the English products as compared with that of Austrian manufacture.

In accordance with the suggestion contained in section 448 of the Commissioners' Report, the Board made arrangements with Dr. Blaxall, the Lecturer on Bacteriology at Westminster Hospital, to carry out a series of experiments, with the object of elucidating more fully the comparative values of the glycerine method and other methods for the purification and preservation of vaccine lymph. In Dr. Blaxall's report, which is appended, it will be found that he has investigated the action on vaccine material, not only of glycerine, but also of lanoline and vaseline, substances which within quite recent years have been introduced by two officers of the Indian Medical Service, Surgeon Lieut.-Colonel King* and Surgeon Major Bamber,† respectively, as being superior to glycerine as agencies for the preservation of vaccine. Neither of these observers bring forward any bacteriological evidence as to the germicide action of the substances they advocate, as will be seen on reference to their original papers; a point of view from which, as Dr. Blaxall shows, lanoline and vaseline are to be regarded as possessing little if any value. Indeed it would appear that the "extraneous" organisms originally present in the lymph with which they are admixed, so far from exhibiting, as time goes on, any diminution in number, tend on the contrary to become largely increased, as shown by the test of plate cultivation.

Lanoline and vaseline are thought, by their advocates, to possess superiority over glycerine mainly for the following reason. These officers note that a glycerine emulsion tends to become mouldy after a short period, whereas this they do not find to be the case when either lanoline or vaseline is employed. That glycerinated lymph should have suffered deterioration in the way they indicate suggests either that it was not properly prepared or that it was left exposed to the air. That the occurrence of a similar accident is not an impossibility with lanoline and vaseline preparations is demonstrated by Dr. Blaxall's experience, as set out by him in his report.

Addendum.

Report by Dr. Wilson Hake on three Samples of Glycerine submitted to him by Dr. Copeman.

Sample A.—Glycerine dest: chem: pur. F. A. Sarg (Patent), Wien.
Sample B.—Glycerine, Price's pure, London.
Sample C.—Glycerine, Lever Brothers' pure, Birkenhead.
} Baird and Tatlock, Glasgow.

	A. (Sarg.)	B. (Price.)	C. (Lever.)
Specific gravity	1·2547	1·2600	1·2590
Water	2·84 per cent.	0·64 per cent.	1·74 per cent.
Ash	0·00 „	0·00 „	0·00 „
Glycerine	97·16 „	99·36 „	98·26 „
	100·00	100·00	100·00

* Madras Government Order, No. 2406 L., September 20th, 1890, and "Indian Medical Gazette," December 1896.

† Vaseline Vaccine, Rawalpindi, 1896.

All three samples may be regarded as very pure examples of commercial glycerine which has evidently been refined by distillation. They are colourless and of high specific gravity (sp.gr. of pure anhydrous glycerine = 1·264). They are free from mineral impurities of any kind and are neutral in reaction. Sample B. is the best, and C. stands intermediate between A. and B. There is a slight trace of organic impurity in all, but more pronounced in A. and C. than in B.

H. Wilson Hake.

Westminster Hospital Medical School,
Caxton Street, S.W., March 17th, 1897.

Part II., by Dr. F. R. Blaxall.

A Record of Experiments on the Action of Glycerine, Vaseline, and Lanoline, on Vaccine Material derived directly from Calves.

The glycerine used was Price's "Pure Glycerine," having a sp.gr. of 1·260. It was thoroughly mixed with sterilised distilled water to 50 per cent., by weight, and the resulting mixture was again sterilised.

The vaseline used was "Pure Vaseline," which, when bacteriologically tested, had been found to be sterile. The lanoline used was "Pure Lanoline," and this also had been found to be sterile.

The vaccine material was collected from calves which had been vaccinated in the ordinary course at the Animal Vaccine Establishment, Lamb's Conduit Street, W.C. Lymph from these same calves was being used as the current supply at that station. In the first three experiments, the vaccine material was collected 120 hours after vaccination of the calf; in the fourth experiment, 96 hours after vaccination.

The vaccine material of experiment was procured in the following manner:—The vaccinated surface of the calf was well cleansed with water, and the crusts on the vesicles wiped off with a clean towel. Next the vesicles were scraped with a sterilised knife, and the scrapings thus obtained were received into a sterilised stoppered bottle of known weight.

This bottle, with its contents, was then immediately taken to the laboratory and the experiment proceeded with.

Experiment I.—The vaccine material (120-hour lymph) from the bottle was divided into three equal parts by weight; division being effected expeditiously, under cover, and with sterilised instruments, so that no contamination of the vaccine might take place. Each separate portion of lymph was then rubbed up in a sterilised mortar (as before, under cover), and was mixed with the chosen vehicle for its dilution. Complete distribution of the vaccine material throughout the vehicle was obtained in each instance by prolonged rubbing. In this way:—

1 part was mixed with 4 times its weight of 50 per cent. glycerine in distilled water; so that the resulting emulsion contained 40 per cent. of glycerine;

1 part was mixed with 4 times its weight of vaseline undiluted, so the resulting emulsion contained 80 per cent. vaseline;

1 part was mixed with 5 times its weight of lanoline undiluted, so that the resulting emulsion contained 83·3 per cent. of lanoline.

Immediately satisfactory emulsions had been in each instance obtained, a definite amount of nutrient agar-agar contained in a test tube, and

which had just previously been liquefied and then allowed sufficiently to cool, was inoculated with emulsion, was well shaken up, and poured on a plate. Inoculation of the agar was effected by means of a looped platinum needle ; the same needle with the same loop being used for all the experiments. The number of loopfuls thus taken from any one emulsion was intended to be equivalent to one loopful of the undiluted vaccine material. Thus of the diluted glycerine emulsion, 5 loopfuls were taken ; of the vaseline emulsion, 5 ; and of the lanoline emulsion, 6.*

Three plates of nutrient agar-agar were established in the above fashion from each emulsion ; the second plate being set up through the medium of three loopfuls of material from the test tube whence the first plate was poured, and the third plate being established in like manner from the test tube for the second plate.

The stock vaseline and lanoline emulsions were then placed in sterilised bottles duly stoppered. The stock dilute-glycerine emulsion was placed in a sterilised test tube plugged with cotton wool. All were stored in a cool cupboard in the dark.

The several agar plates were, after solidifying, placed for 24 hours in an incubator at 37° C., and then for 6 days in an incubator at 20° C. At the expiration of that time the several plates were photographed.

In this first experiment, it was found, after 6 days incubation at 20° C., that the *first*, or original agar plates in all three series, were so densely crowded with colonies as not to promise good pictures. Wherefore the *second* plates, or "first dilutions" of the original agar tubes were alone photographed. A rough counting of the colonies on these second plates, which had been established immediately after emulsification was effected, gave the following numbers :—

Glycerine (plate II.)	1,000	colonies	per plate.
Vaseline (plate II.)	400	„	„
Lanoline (plate II.)	700	„	„

One week after making the emulsions further plates were established in the same manner, with the same number of loopfuls from the stock emulsions. These plates showed an increase in the number of colonies in samples from the vaselinated and lanolinated emulsions, but a decrease of colonies in the sample from the glycerinated emulsion. Similarly with plates made a *fortnight* and *three weeks* after making the emulsions, the colonies from the glycerinated material were fewer, whilst those from the vaselinated and lanolinated sources much more numerous than before.

At the end of *four weeks* none of the plates established from the glycerine stock emulsion showed any colonies at all ; but at this date plates from the vaseline and lanoline emulsions still showed increase of colonies.

At the end of *six weeks*, plates from the glycerine stock emulsion were again found free from extraneous organisms, whereas plates from the lanoline and vaseline stock emulsions were crowded with colonies. A rough counting of the plates gave at this date—

Glycerine (plate I.)	0	colonies	per plate.
Vaseline (plate II.)	8,000	„	„
Lanoline (plate II.)	10,000	„	„

With this glycerinated lymph, 5 weeks after making the emulsion, Dr. Cory vaccinated six children, each by five insertions. All the children's arms "took" ; one in five places, three in four, one in three, and one in two places. This rather poor result was in all probability

* A "loopful" may be taken to represent about 0·005 of a cubic centimetre.

due to the fact that the glycerinated lymph had been stored in a test tube to which air had access through the cotton wool plug; moreover, the emulsion in the test tube was freely movable when the latter was handled, thereby causing undesirable exposure to air. Further experiments have led to the conclusion that the glycerinated material preserves its activity best when stored, as originally suggested by Dr. Copeman, in capillary tubes, or in small pipettes, where the fluid can have very little air in contact with it.

Figs. 1, 3, and 5 are reproductions of photographs of the *second* agar plates incubated for seven days subsequent to inoculation with material from glycerine, vaseline, and lanoline emulsions which had not been stored after manufacture. All three plates show a copious growth of extraneous microbes.

Fig. 2 is a reproduction of a *first* agar plate derived from the stock glycerine emulsion after the latter had been stored 4 *weeks*. No growth whatever has occurred. Figs. 4 and 6 are representations of *second* plates of the vaseline and lanoline stock emulsions after they had been stored for *six weeks*.

These are seen to be crowded with colonies of extraneous microbes.

Experiment II.—Scrapings of vesicles were collected from a calf 120 hours after vaccination, with the same precautions as in Experiment I. As before, the vaccine material was weighed, rubbed up in a mortar, and thoroughly mixed with four times its weight of 50 per cent. sterile glycerine and distilled water. The emulsion was then drawn up into capillary tubes and into small pipettes, and stored.

Fig. 7 is a reproduction of a photograph of an agar-agar plate (after incubation for 24 hours at 37° C. and for six additional days at 20° C.) which had been inoculated with one loopful of the rubbed up vaccine material prior to its admixture with the glycerine. Fig. 8 represents a similar plate inoculated with five loopfuls of the same emulsion immediately after glycerination. The number of colonies on the two plates will be observed to correspond closely.

Fig. 9 represents an agar-agar plate (as before, after incubation for seven days) which had been inoculated with five loopfuls of the stock glycerinated emulsion *one week* subsequent to its manufacture. The number of colonies is observed to be slightly diminished.

Fig. 10 represents a similar plate set up a *fortnight* after glycerination. A further decrease in the number of colonies is now very conspicuous. Such decrease is even more marked after a lapse of *three weeks*, as is shown by Fig. 11.

At the end of *four weeks*, the agar-agar plate inoculated from the stock glycerine emulsion was found free from extraneous organisms, Fig. 12.

Some of this particular emulsion, contained in a capillary tube, was now used by Dr. Cory to vaccinate three infants. All three "took" well, each in five places. Fig. 13 is a reproduction of a photograph of one of these children's arms, taken on the 8th day.

Experiment III.—Scrapings of vesicles were collected from a calf 120 hours after vaccination, with the same precautions as before. These were weighed, rubbed up, and diluted with 15 times their weight of 50 per cent. sterile glycerine and distilled water; so that the per-centage of glycerine present became 46·9. One loopful of this emulsion, immediately after glycerination, was inoculated into nutrient agar-agar, and plates were established therewith. Plates established from the stock

glycerine emulsion a week, a fortnight, three weeks, and four weeks, respectively, from commencement of storage, showed as regards the first three, week by week, a rapid diminution in the number of colonies which appeared after incubation in the manner described, while the fourth was found to be quite free from extraneous organisms.

Some of this glycerine emulsion, four weeks after manufacture and storage in a pipette, was used by Dr. Cory and by Mr. Stott, at the Animal Vaccine Establishment, Lamb's Conduit Street, for the vaccination of 65 infants. All these children's arms took, Dr. Cory obtaining, in his series of 36 cases, 100 per cent. of insertion success. Of the total number, 54 were successful in five places, two in four, two in three, and one in two places. Fig. 14 is a reproduction of a photograph of one of these children's arms, taken on the tenth day of vaccination. Some of this emulsion was also used to re-vaccinate a groom at the Brown Institution. In this instance also the arm "took" excellently.

Experiment IV.—Scrapings of vesicles were obtained from a calf 96 hours after vaccination, the same precautions being taken as before. This material was weighed and divided into three equal parts. One part was emulsified with four times its weight of 50 per cent. glycerine and distilled water; one part with four times its weight of vaseline undiluted; and one part with five times its weight of lanoline—as in Experiment I. Agar-agar plates were established from these emulsions antecedent to their storage, and the numbers of colonies which appeared on the several plates at this stage were found in close agreement. The three emulsions were then placed in sterile stoppered bottles, some of the glycerinated material being also stored in capillary tubes and in pipettes. It was designed to repeat No. 1 Experiment with these emulsions, and sample plates were again established from them after they had been stored a week. Again the vaseline and lanoline plates showed at this stage an increase in the number of colonies, whereas the glycerine plate showeda diminution. The vaseline plate, moreover, after four days, became covered with colonies of a green mould (penicillium glaucum), and was useless for photographic purposes.

At the end of the second week of this experiment it was found impossible to continue it as regarded the vaseline and lanoline emulsions. These had become, in their separate sterilised stoppered bottles, both covered with a luxuriant crop of the above green mould. No such growth appeared in the bottle containing the glycerine emulsion. On the contrary, plates established from it, after two and three weeks storage, showed further diminution in the number of colonies; and at the end of the fourth week the comparison plate was altogether free from extraneous organisms.

Thus, Experiment No. IV. proved, as did Experiment No. I., the superiority of glycerine (dilute) over vaseline and over lanoline in eliminating the extraneous organisms which are commonly present in vaccine material.

In the foregoing experiments it will have been noted that the plates used for comparison were, in nearly every instance, plates established from agar test tubes directly inoculated from the emulsion. The exception to this rule was Experiment I., in which the plates used for comparison were established from agar tubes inoculated, not directly from the emulsions, but from the primary agar tubes in which loopfuls of emulsion had been, in the first instance, distributed. In that experiment, the sterile plate obtained at the end of the fourth week of storage of vaccine material *in glycerine* was the only plate of the series established

directly from the original emulsion which has been photographed. It remains to be stated in this connexion that, in the majority of the instances, the results were "controlled" by duplicate plates established at the same time; and, furthermore, that material which in plate culture showed no colonies was always tested again two or three times in similar fashion.

The Bacteriology of Vaccine derived from Vaccinated Calves.

As has been repeatedly shown, calf lymph ordinarily contains a large number of organisms which are in no way concerned with its specific action. Also it has been shown that lymph, if carelessly collected, may contain many or any of the numerous saprophytes common to dust, dirt, &c., as well as, perhaps, certain pathogenic microbes. Finally, it has been shown that, by the use of the glycerine method, these bacteria may be greatly reduced in numbers if not altogether eliminated.

In the above experiments, and in a very large number of other examinations of samples of calf lymph, the following organisms were found. They are arranged in the order of their prevalence and predominance.

1. { Staphylococcus cereus flavus / „ „ albus } (Passet).
2. { Large yeast, orange coloured. / Small yeast, light brown colour. / Small yeast, pale salmon colour, and very slow growing.
3. Staphylococcus pyogenes albus (Rosenbach).
4. Staphylococcus pyogenes aureus (Rosenbach).
5. Staphylococcus pyogenes citreus (Passet).
6. Bacillus mesentericus vulgatus.
7. Bacillus subtilis.
8. Moulds, *penicillia*, *mucors*, *aspergilli*: Sarcinæ, *lutea*, *aurantiaca*.

In all series of experiments here dealt with, groups 1 and 2 were always present. The slow growing yeast was not, however, generally visible as a distinct colony till the seventh or eighth day.

Staphylococcus pyogenes albus was frequently present in lymph, but generally in small numbers.

Staphylococcus aureus was rather less frequently observed, and S. citreus was only occasionally found.

Bacillus mesentericus may be regarded as accidental.

Bacillus subtilis rarely occurred if precautions were taken; its presence seeming to depend entirely on the care exercised in collecting the lymph. The same is true of group 8.

It must be stated, however, that all the samples of calf lymph examined were derived from the same station, namely, from the Animal Vaccine Establishment, Lamb's Conduit Street. Here calf-to-calf vaccination is continuously practised; so that the adventitious saprophytes found in the lymph at one and another time are apt to be similar in kind, the extraneous organisms originally present having been, so to speak, cultivated *pari passu* with the vaccine virus itself. Very possibly, therefore, a strain of lymph from another station might present a "flora" differing in kind and in amount, as indeed has been found to be the case with certain foreign lymphs. In this connexion it may be noted that lymph taken directly from calves vaccinated with vaccine material rendered free from extraneous organisms, as for instance with glycerinated lymph a month after glycerination, often presents remarkably few

colonies of such organisms. In one such sample only four colonies appeared on a plate culture of the lymph; one of staphylococcus cereus albus and three of the light brown yeast.

Experiments on the action of Glycerine upon the Life and Growth of Yeasts.

As a sequel to former experiments with glycerine upon the vitality of various bacteria, recorded in a paper read before the British Association at the Liverpool Meeting, in 1896, the action on yeasts of differing per-centages of glycerine in a culture medium was tested on several different samples. Test tubes containing equal quantities of peptone beef broth, but mixed with amounts of glycerine ranging from 20 to 30, 40, and 50 per cent., were, after sterilisation, inoculated with five different yeasts. Care was, of course, taken that the amounts of yeast inoculated in the tubes were approximately equal; and, at the same time, tubes of peptone beef broth without glycerine were similarly inoculated with the yeasts to serve as control experiments.

The yeasts used were—

Common pink yeast (Rosa Hefe), not found in vaccine lymph.
Orange coloured yeast, common in vaccine calf lymph.
Dark brown yeast, isolated from garden mould.
Saccharomyces glutinis.
Light brown yeast, isolated from small-pox crusts.

Rosa Hefe resisted the action of 50 per cent. glycerine for four months, and still continued to grow freely.

The orange coloured yeast succumbed to 40 per cent. glycerine in a fortnight, and to 20 per cent. glycerine in four weeks.

The dark brown yeast resisted the action of 40 per cent. glycerine for eight weeks, and 20 per cent. glycerine for sixteen weeks.

Saccharomyces glutinis was still alive after a fortnight in 20 per cent. glycerine, but it failed to grow in peptone beef broth containing higher per-centages of glycerine.

The "small-pox crust" yeast succumbed to 40 per cent. glycerine in a month, and even 20 per cent. glycerine inhibited its growth after eight weeks.

All the control experiments which were carried on simultaneously with each periodical examination of the glycerinated tubes gave positive results.

There is here shown a marked variation in the power of resistance to the action of glycerine possessed by different yeasts. The prolonged resistance of some kinds is of interest; but it is specially noteworthy that this power of resistance to the action of glycerine is not characteristic of those yeasts which have, on one or another occasion, been isolated from vaccine lymph.

PLATE-CULTURE TEST OF VACCINE LYMPH

PRESERVED IN ONE AND ANOTHER WAY.

PLATE I.

FIG. 1.

Photograph of *second* agar plate, incubated for seven days subsequent to inoculation with GLYCERINE vaccine emulsion which had *not* been stored after preparation.

Numerous micro-organisms, chiefly staphylococci, are seen to have developed.

FIG. 2.

Photograph of *first* agar plate, incubated for seven days subsequent to inoculation with GLYCERINE vaccine emulsion which had been stored for *four weeks* after preparation.

No growth whatever has occurred.

PLATE I.

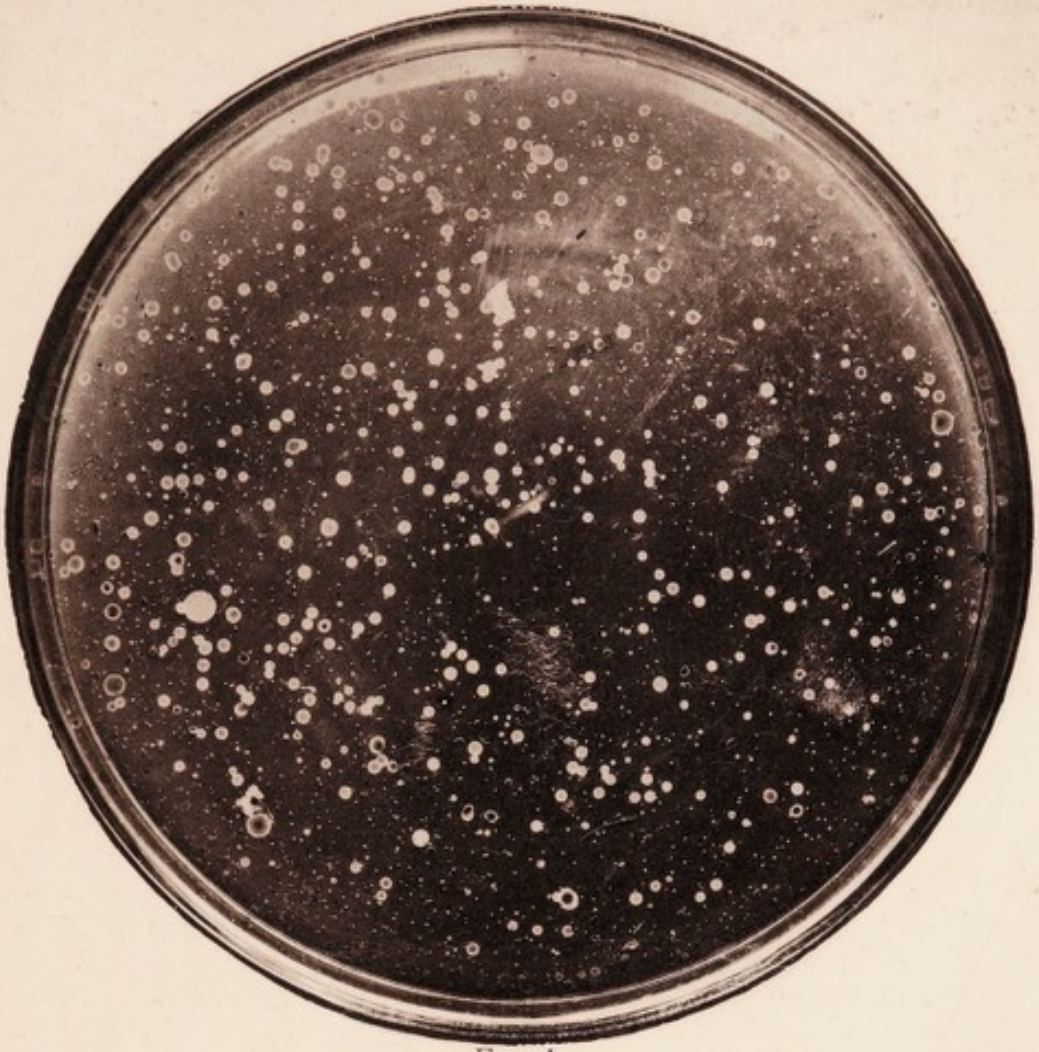

FIG. 1.

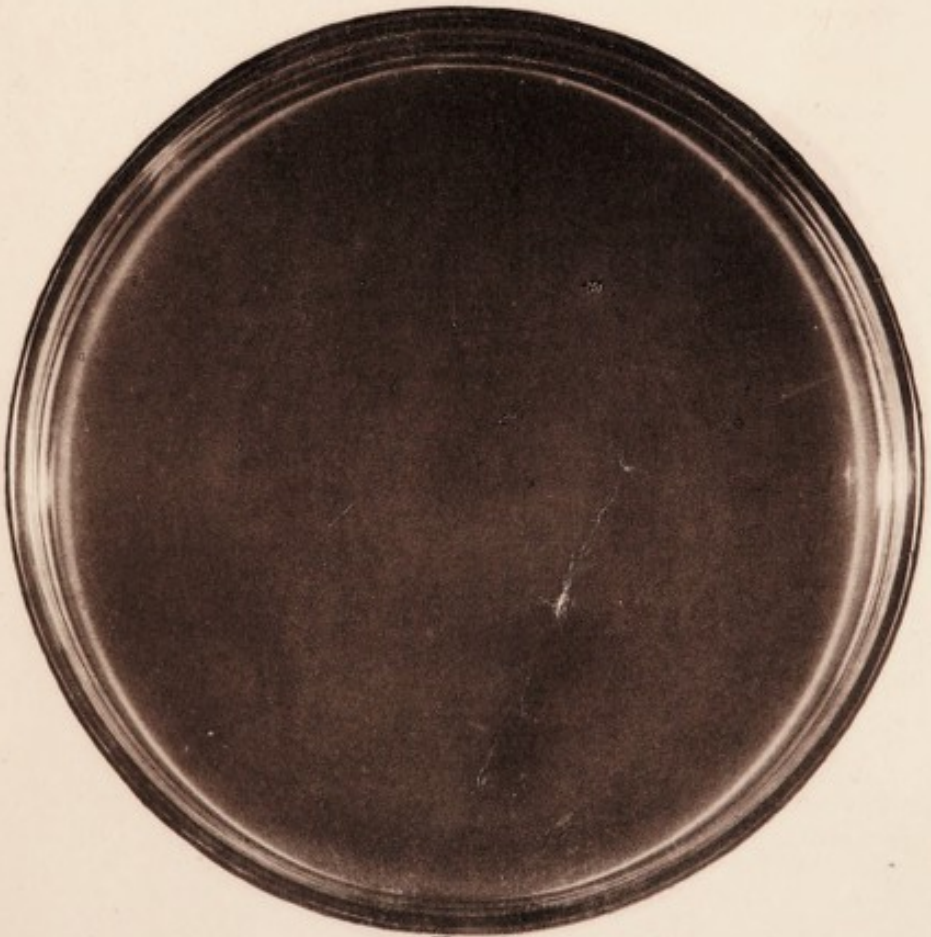

FIG. 2.

PLATE II.

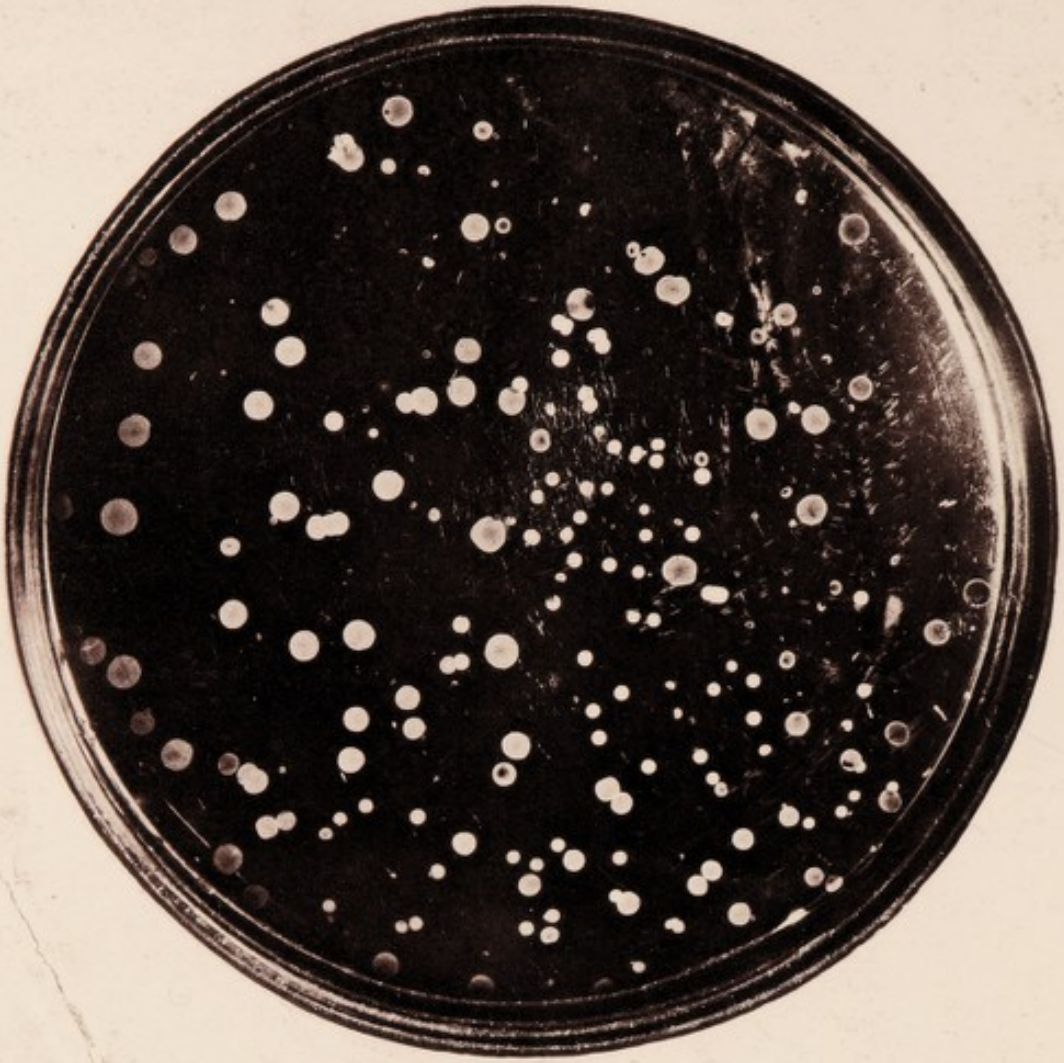

FIG. 3.

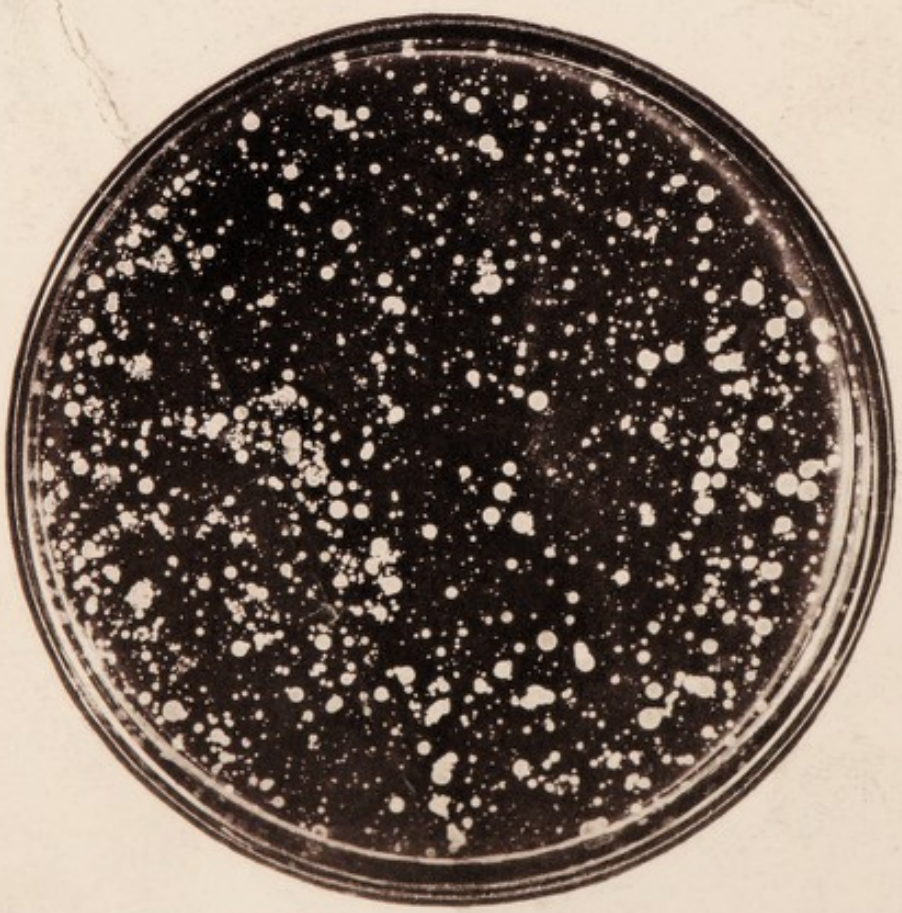

FIG. 4.

PLATE III.

FIG. 5.

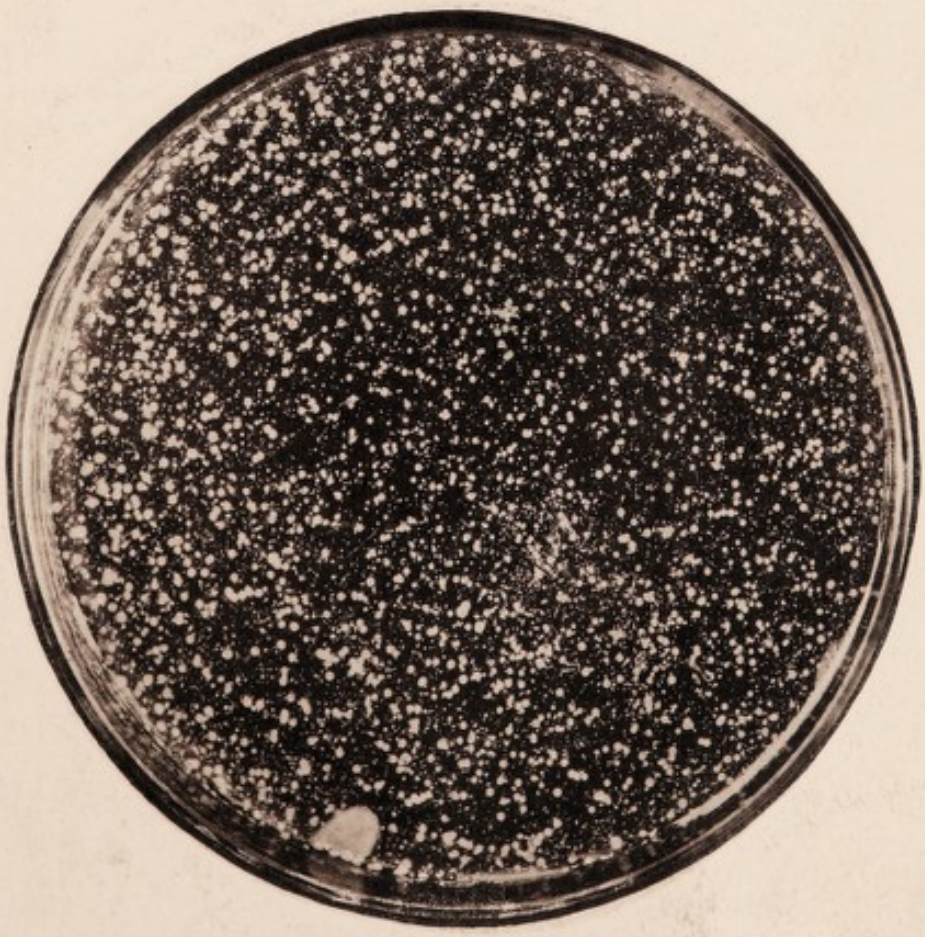

FIG. 6.

FIG 7.

FIG. 8.

PLATE-CULTURE TEST OF VACCINE LYMPH

PRESERVED IN ONE AND ANOTHER WAY.

PLATE II.

FIG. 3.

Photograph of *second* agar plate, incubated for seven days subsequent to inoculation with VASELINE vaccine emulsion which had *not* been stored after preparation.

The plate shows a copious growth of extraneous microbes.

FIG. 4.

Photograph of *second* agar plate, incubated for seven days subsequent to inoculation with VASELINE vaccine emulsion which had been stored for *six weeks* after preparation.

The plate is seen to be crowded with colonies of extraneous microbes.

PLATE-CULTURE TEST OF VACCINE LYMPH
PRESERVED IN ONE AND ANOTHER WAY.

PLATE III.

FIG. 5.

Photograph of *second* agar plate, incubated for seven days subsequent to inoculation with LANOLINE vaccine emulsion which had *not* been stored after preparation.

The plate shows a copious growth of extraneous microbes.

FIG. 6.

Photograph of *second* agar plate, incubated for seven days subsequent to inoculation with LANOLINE vaccine emulsion which had been stored for *six weeks* after preparation.

The plate is seen to be crowded with colonies of extraneous microbes.

PLATE-CULTURE TEST OF VACCINE LYMPH

PRESERVED IN ONE AND ANOTHER WAY.

PLATE IV.

FIG. 7.

Photograph of agar plate, incubated for seven days subsequent to inoculation with *one loopful* of rubbed-up vaccine material *prior to its admixture* with glycerine.

FIG. 8.

Photograph of agar plate, incubated for seven days subsequent to inoculation with *five loopfuls* of the same material *immediately after* glycerination.

The number of colonies on these two plates will be observed to correspond closely.

PLATE-CULTURE TEST OF VACCINE LYMPH

PRESERVED IN ONE AND ANOTHER WAY.

PLATE V.

FIG. 9.

Photograph of agar plate, incubated for seven days subsequent to inoculation with *five loopfuls* of the stock glycerinated emulsion *one week* after preparation.

The number of colonies is somewhat diminished.

FIG. 10.

Photograph of similar agar plate, inoculated *a fortnight* after glycerination.

A further decrease in the number of colonies is now conspicuous.

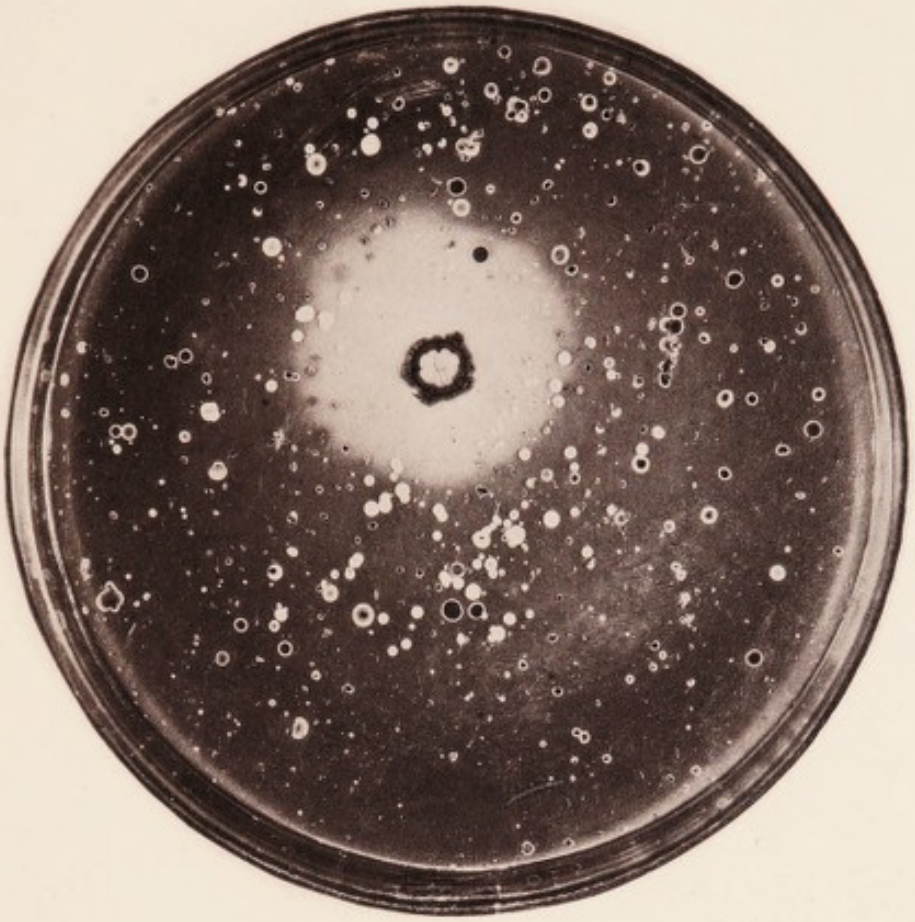

FIG. 9.

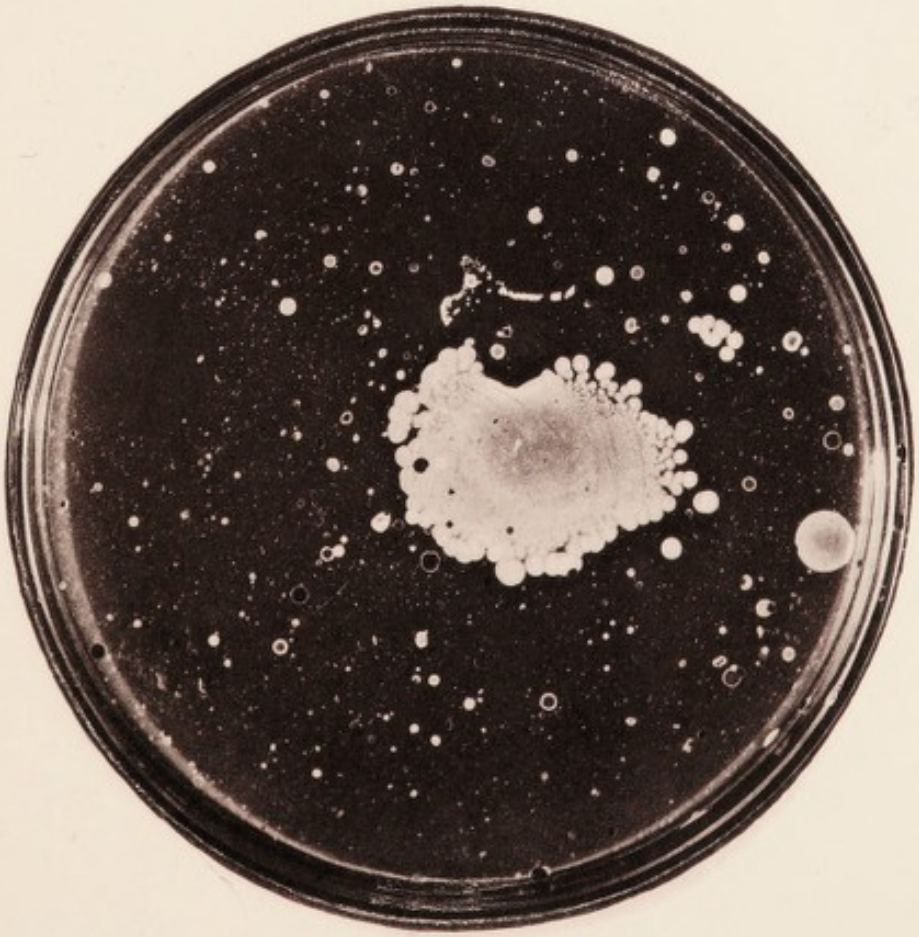

FIG. 10.

FIG. 11.

FIG. 12.

PLATE-CULTURE TEST OF VACCINE LYMPH

PRESERVED IN ONE AND ANOTHER WAY.

PLATE VI.

FIG. 11.

Photograph of agar plate, incubated for seven days subsequent to inoculation with *five loopfuls* of the stock glycerinated emulsion *three weeks* after preparation.

The decrease in the number of colonies is still more marked than in the former plates.

FIG. 12.

Photograph of similar agar plate, inoculated *four weeks* after glycerination.

No growth whatever has occurred.

VESICLES PRODUCED ON INFANT VACCINATED WITH GLYCERINATED LYMPH.

PLATE VII.

FIG. 13.

Photograph of child, taken on the eighth day of vaccination, with glycerinated vaccine lymph, which, as shown in Fig. 12, was found, when tested by the method of plate cultivation, to be free from extraneous micro-organisms.

PLATE VII.

FIG. 13.

PLATE VIII.

FIG. 14.

VESICLES PRODUCED ON INFANT VACCINATED WITH GLYCERINATED LYMPH.

PLATE VIII.

FIG. 14.

Photograph of child, taken on the tenth day of vaccination, with glycerinated vaccine lymph, which, after storage for four weeks, was found, when tested by the method of plate cultivation, to be free from extraneous micro-organisms.

This child formed one of a series of 36 cases, all vaccinated (in five insertions each) by Dr. Cory on the same day and with the same sample of glycerinated lymph. In this series Dr. Cory obtained 100 per cent. of insertion success.