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With the Author's Compliment.

THE RESULTS OF THE INOCULATIONS OF MILCH COWS WITH CULTURES OF THE BACILLUS DIPHTHERIÆ.

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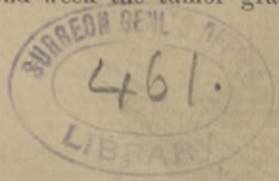
DURING the past twelve or fourteen years there have appeared at odd intervals several epidemics of diphtheria that are believed to have originated with the milk used by the persons affected. This circumstance has led to their being known as "milk epidemics" of diphtheria, and by some individuals the milk is believed to have been capable of producing the disease at the time it was obtained from the cow, and not, as is more generally the opinion, that it had become infected on its way from the cow to the consumers. In at least three outbreaks in England, there was no evidence that the milk had been infected by those through whose hands it had passed, and in consequence, suspicion was directed to the cows from which it had been obtained. With the idea of determining how far this suspicion rested upon a possible basis, Klein (1) endeavored to study the reaction of milch cows to the virus of human diphtheria. With this in view, he inoculated each of two healthy cows subcutaneously over the left shoulder with one cubic centimetre of a bouillon culture of the bacillus diphtheriæ. On the second and third days after inoculation the temperature in each rose a degree, centigrade, and there was diminution in the amount of food consumed, but by the fourth day both cows were again in an apparently normal condition.

On the second day following the inoculation there appeared beneath the skin at the seat of operation, a localized, soft, painful swelling, which continued so, and by about the eighth or tenth day was as large as one's fist. During the second week the tumor gradually



diminished in size, became harder and more circumscribed, and could be readily moved about over the underlying tissues. During this time (the second week) both cows became affected with a cough, and auscultation and percussion revealed the presence of decided thickening, and bronchial respirations in the upper portions of both lungs.

On the twefth day Cow No. 1 ceased taking food and died on the night of the fourteenth day. On the twenty-third day Cow No. 2 showed evidence of approaching her end, and she was killed on the twenty fifth day.

After the third day from the date of inoculation neither animal had a rise of temperature. Between the sixth and the tenth day after inoculation in Cow No. 1, and the fifth and the eleventh day in Cow No. 2, there appeared upon the udder and teats a most peculiar eruption, appearing at first as injected papules, which on the following day became surrounded by a small vesicle, which in time was limited by a zone of reddened skin. The skin beneath the vesicle was indurated. The vesicles contained clear lymph. In ten days after their first appearance the vesicular eruption had become pustular; in another day the pustules were covered with a brown scab. During the two or three days following, the scab enlarged and became brown-black in color. If at this time a scab was removed, a bloody indurated ulcer remained. After two or three days more the scab dropped away spontaneously, leaving a dry and healed scar; the entire process taking usually from five to seven days. The smallest of these points of eruption were about 0.5 cm. in diameter, the largest from 1.0 to 1.25 cm. in diameter. They were always round or roundish in outline, and in the vesicular and pustular stages some of them exhibited a dark centre. None of them were umbilicated. At the autopsies upon these cows essentially similar conditions were found in both.

In the neighborhood of the left shoulder, at the seat of inoculation, intimately attached to the skin, was found a dense mass which formed, with the subcutaneous and muscular tissues, a peculiar circumscribed tumor, freely movable over the underlying structures and surrounded by an œdematous and hemorrhagic zone. Upon transverse section it was seen to consist of numerous thin and thick lamellæ of necrotic tissue, between which transverse section of muscle fibres could be seen. In the neighborhood of this tumor was a swollen, œdematous

lymph-gland, which was somewhat hemorrhagic in its periphery. Adjacent to this gland were numerous smaller glands of a dark-red color. Upon microscopic examination both the peripheral and central portions of these glands contained much extravasated blood.

Both lungs in the upper portion, and also the middle lobe of the right, and the lower lobe of the left, were markedly hyperæmic. Groups of lobules were found that were of a deep-red color, injected, œdematous, and sank in water. The septa between the lobules were œdematous. The sub-pleural lymph canals were seen to be filled with clear (Cow No. 1), and with blood-stained (Cow No. 2) lymph. Upon section through the upper lobes the bronchi were everywhere seen to be surrounded by a dense gray exudate which faded off into the surrounding tissues. The bronchial glands were swollen and œdematous.

On the surface of the liver were numerous irregular, gray or brownish spots, which proved to be necrotic areas of liver tissue. Both kidneys were hyperæmic, with also gray areas of fatty degeneration in the cortical substance. The visceral pericardium contained areas of extravasated blood, both punctiform and conglomerate.

The microscopic examination of horizontal sections of the tumor from the seat of inoculation showed a process characterized by white, necrotic septa, which formed a picture similar, in all essential respects, to that seen in sections of membrane from human diphtheria. The muscle bundles between the septa were, at many points, in advanced necrosis. Everywhere in the septa and necrotic tissues appeared masses of diphtheria bacilli, either singly in small or, as in the tumor from Cow No. 2, in large ball-like masses.

Muscle fibres near the necrotic tissues were here and there, either in part or entirely, supplanted by masses of bacilli. In many points the gradual penetration of the dying muscle-substance by the bacteria could be readily made out.

From the tissues of the tumors from both cows cultures were made. In not one of twenty-four smear tubes was anything but pure cultures of a bacillus obtained, which in morphological and cultural peculiarities could not be differentiated from the bacillus diphtheriæ obtained from man or from guinea-pigs. Cultures from the lungs, kidneys, and heart's blood were negative. From the lymph of the vesicles on the udder, it was possible to demonstrate

the bacillus diphtheriæ, not only in cover-glass preparations, but also by culture methods.

Bacteriological examination of the milk made on the fifth day after inoculation, the date at which the eruption first appeared upon the udder, resulted in the appearance of colonies of the bacillus diphtheriæ on three out of four slanted gelatin tubes, upon the surface of each of which $\frac{1}{16}$ c.c. of the milk from Cow No. 2 had been deposited. On three subsequent occasions, the tenth, eleventh, and twenty-fifth days after inoculation, the milk was again examined, but no colonies of the bacillus diphtheriæ could be found.

In order to demonstrate the contagious nature of the eruption on the udders of these cows, two calves were inoculated with the contents of the vesicles and pustules. The inoculations were made in the way commonly practised in vaccinating calves, viz.: into a small pocket in the skin of the abdomen.

Between the sixth and the seventeenth days both calves developed an eruption in all essential respects identical with that upon the cows from which they had been inoculated. Calf No. 2 developed a cough, and Calf No. 1 a mucous discharge from the nostrils. On the twenty-fourth day both calves were killed. No evidence of the eruption could at this time be found. The upper lobes of both lungs of both calves, and the upper part of the adjacent lobes were dark-red in color, solid, and sank in water. Upon section the bronchi were surrounded by a dense gray tissue. The medullary substance of both kidneys was hyperæmic, while the cortical substance was grayish-white and conspicuously fatty.

This is, in short, the description given by Klein of the results of his inoculations of milch cows with cultures of the bacillus diphtheriæ. The particular object of Klein's experiments was apparently less to study the pathological lesions produced in these animals by the inoculations, than to determine if it was possible, through the inoculation of cows with the virus of human diphtheria, to demonstrate its presence in their milk. The experiments were suggested to him by an opinion then extant in England, that in several of the milk epidemics of diphtheria (particularly those in the north of London in 1878, in Yorktown and Camberwell in 1886, and in Enfield and Barking in 1888) the infective agent had reached the milk through the cow, and had not been introduced into the milk through human agency—an

opinion of sufficient gravity to call for all light that experiment is capable of throwing upon it. This opinion is probably influenced by the erroneous impression that the pseudo-membranous process in the throat, with which calves are at times affected, is identical with diphtheria in the human being.

In an address made by Löffler, at the International Medical Congress, in Berlin, 1890, in the Section on Hygiene, he expressed the opinion that the results of these experiments were of a doubtful nature, and that the subject was of such importance as to require confirmation from other sources. It was through this address that my attention was called to the experiments of Klein, and as opportunity has presented during the past winter to repeat these inoculations, I have done so. It is the results of these experiments that I propose to embody in this paper.

Cow No. 1. *Inoculated March* 8, 1892; *died March* 24, 1892.—On March 8, 1892, I inoculated into the tissues of the right shoulder of this cow, 1 c.c. of a bouillon culture of bacillus diphtheriæ, the virulence of which was at the same time tested upon a guinea-pig, the latter dying after the usual time and with the tissue changes common to these animals when under the influence of the virulent bacillus diphtheriæ. This cow had for some time prior to inoculation been tuberculous, and at the date of inoculation was markedly so.

Before inoculation careful examination was made of her udder and teats, and they were found to be normal, no excoriations, blebs, ulcers, or anything else of an unusual character being present.

At this time her temperature was 102° F., and she had been having a tolerably regular evening exacerbation of about a degree higher. The daily range of temperature after inoculation will be seen by reference to temperature chart No. 1.

At no time while under observation did this cow show any marked constitutional disturbances, beyond a rise of temperature, that might have been looked upon as a result of the inoculation. She was never "off her feed," and beyond a cough showed no signs of discomfort. Her udder and teats were frequently examined, but at no time did we discover anything in the form of an eruption.

For three or four days after inoculation there was tenderness over the point at which the needle had been introduced; this was accompanied by a slight swelling which rapidly diminished in size until ultimately it gave to the hand the sensation of a dense, flat nodule of about 3 x 5 cm. It was firmly attached to both the skin and underlying tissues, so that it could not be easily moved about. Beside these points no noticeable alteration in the condition of the animal could be made out. She died November 24th, sixteen days after having been inoculated.

At autopsy the tuberculous process was found to be very widespread. The lungs were little more than caseous masses and conglomerate tubercles. The bronchial glands presented a condition of caseation. The pleuræ were studded with tuberculous masses, likewise the peritoneum. The tuberculous process in the pleuræ and lungs was so advanced that no attempt was made to study the changes described by Klein in these organs, as resulting from his inoculations of cows with the bacillus diphtheriæ. The udder was carefully examined externally for evidences of pre-existing eruption, and internally by means of parallel incisions made at a distance of three-quarters of an inch from one another throughout the entire gland, but no abnormal condition was detected.

TEMPERATURE CHART 1.

Cow No. 1. Inoculated with bacillus diphtheriæ November 8, 1892; died November 24, 1892.

At the seat of inoculation was found a flat, tough mass, more or less adherent to both skin and underlying tissues, though with a little effort it was easily separated from both. This mass measured approximately 2 x 3 x 0.5 cm. This oval bit of tissue was unusually dense, and it was only with much difficulty that the point of a scalpel could be introduced into it. It presented nothing characteristic to the naked eye. It was simply a white, fibrous-looking mass.

Under proper precautions an incision was made into the tumor with a knife that had been sterilized in the flame and allowed to cool. From the faces of the incision scrapings were made, and with these scrapings blood-serum tubes were inoculated; Petri plates of glycerin-agar were made, and slant tubes of glycerin-agar were also inoculated. By none of these methods could we demonsrtate the presence in the tumor of living diphtheria bacilli.

The tumor was preserved in absolute alcohol, and when properly dehydrated, a portion was imbedded in celloidin, while from another portion sections were cut without imbedding. Examination of these sections revealed a most interesting condition.

Upon *microscopic examination* the tumor was seen to consist of two portions, a peripheral zone of newly formed connective tissue, and an irregular central

area advanced in a condition of necrosis. In this central area, which formed the bulk of the tumor, could be seen muscle fibres and connective tissue in various stages of death. At many points the destructive process had advanced to such an extent that, beyond a reticulum of fibrin with here and there fragments of cell nuclei, but little of the original structure could be made out.

The epidermis, where preserved, was quite normal. Just beneath it was a layer consisting of connective tissue and muscle containing an excess of nuclei. The fibres and bundles of connective tissue were pressed apart, evidently the result of œdema. Beneath the layer of muscle was a thicker band of connective tissue which also presented an excess of nuclei. The nuclei in the layers just beneath the epidermis were of two kinds, polyform leucocytes and connective-tissue nuclei. The leucocytes were sometimes aggregated into masses which lay between and in the tissue elements. The lymph spaces of the tissues were dilated and contained numerous leucocytes. The small veins were sometimes almost plugged with them, yet many still contained a few red blood-corpuscles. Where the leucocytes were present in greatest numbers the tissue elements about them did not stain. The increase in number of connective-tissue nuclei was particularly in the intermuscular septa in the subcutaneous tissues; they were round, elongated, or slightly irregular, and some were fragmented. There were, moreover, polynuclear leucocytes in these septa.

The muscle fibres themselves in these situations were swollen and presented a hyaline aspect; striations could not be found in them, and in a few instances an appearance was obtained that suggested the entrance of leucocytes into these hyaline fibres. The sarcolemma nuclei were also increased, sometimes several fold. At one point in the muscle there was an exaggeration of nuclei, presenting the appearance of an abscess. Here the muscle fibres were most disintegrated, and many cells of the connective-tissue type were discernible. Adjacent to this the tissues contained very few normally staining nuclei, and the tissues were quite without stain and presented a finely granular or reticulated appearance, suggestive of fibrin. Such nuclear stain as was obtained here was taken by fragments of nuclei, and these presented the greatest variety of form, being small, point-like, drawn out, and usually distorted, but agreeing in the common property of staining intensely with fuchsin. This is the area of most profound activity of the irritant. The fragmented nuclei in this situation came from two sources: the fixed cells and the emigrated cells or leucocytes. Just at the edge of the tumor where the necrotic tissue passed into the more nearly normal tissue, the participation of the fixed cells in the process of nuclear fragmentation was more readily seen. As the deeper parts of the tissue were reached the extensive nuclear fragmentation grew less and less, but here and there were small and larger areas of cells with round nuclei. These were sometimes only three or four in number, while again they were more numerous. They were the fixed cells. Leucocytes were not always found in these areas; they were often absent from the smaller, but were usually present in the larger. The small round cells, as stated, were regarded as derived from the fixed cells; and while they were most frequently not associated

with cells which had fragmented nuclei, yet occasionally the reverse was the case.

The lymphatics in this locality contained an increased number of leucocytes. Moreover, the endothelial cells lining their walls were sometimes necrotic and contained fragmented nuclei. In a section of a lymphatic, for example, in which several elongated endothelial cells were seen, perhaps one-half showed a distinct fragmentation of their nuclei. The smaller veins, also, as before mentioned, were often almost full of leucocytes, and in these too, at times, the endothelial cells of the intima had suffered this form of cell necrosis.

The further removed the tissues were from the point of most intense action of the irritant the less the alteration in their structure; but even in the most peripheral portion of the tumor mass there was cell death, as indicated by the fragments of nuclei in the tissues. A single nuclear figure was found in the fixed cells about the middle of the specimen, in a place where there was apparently active proliferation of the fixed cells.

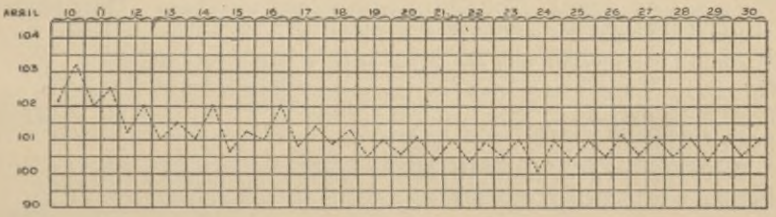
The specimen stained by Weigert's fibrin method showed a focus containing many leucocytes and clumps of diphtheria bacilli. This corresponds with the abscess-like area above described.

The conspicuous alteration in the tissues generally at this point was the changes in the nuclei of the fixed and wandering cells, a nuclear fragmentation in all respects identical to that described by Oertel (2) in the tissues of the human subject dead of diphtheria, and by Welch and Flexner (3) in the tissues of animals that have succumbed to the experimental form of the disease. Under a low magnifying power this condition is seen as islands of irregular granules that stain intensely with the ordinary nuclear dyes and lie scattered through a tissue that either does not stain at all or very imperfectly. In addition to these granular areas, that could readily be seen to consist of nuclear detritus, were other irregular masses that stained also with the basic aniline dyes, but which were much less coarsely granular than the groups of nuclear fragments. Under a higher magnifying power these latter resolved themselves into masses of bacilli in all morphological and staining respects identical with the bacillus diphtheriæ, except that in most instances the irregularity of outline and staining peculiarity was more marked than common. (See Plate: Fig. 1.)

In certain sections these groups of bacilli appeared as irregular rosette-like patches of varying size, while again in sections made from a different portion of the tumor, or running in another direction; they appeared as irregular, broken, granular lines. The tissue about them was in all cases nearly completely destroyed (see Plate), so that it was impossible to make out definitely the relation of the organisms to the surrounding tissue; but as a result of examination of a number of sections, it is probable that they had developed in and filled lymphatic spaces. In the irregular, roundish or rosette-like patches of bacilli the staining was sometimes less intense at the centre than at the margin of the clump, while in the long, irregular linear masses the color was more homogeneous. None of these masses of bacilli could be detected in the peripheral zone.

Cow No. 2. *Inoculated April* 10, 1893; *killed May* 1, 1893.—On April 10, 1893, at 10.30 A.M., Cow No. 2 was inoculated. She was thin but otherwise healthy; was suckling a calf at the time of inoculation. Prior to inoculation her temperature range had been from 100° to 103° F.; there was no eruption upon the udder nor was there anything to indicate a condition other than normal. She was inoculated in the soft tissues of the right shoulder with 1 c.c. of a bouillon culture of bacillus diphtheriæ, the virulence of which was at the same time tested upon a guinea-pig. The guinea-pig died in about sixty hours after having been inoculated with 0.5 c.c. of the same bouillon culture; the autopsy revealed the conditions commonly seen under these circumstances. The culture employed was about forty-two hours old and was but a few generations removed from a case of genuine, primary diphtheria.

TEMPERATURE CHART 2.



Cow No. 2. Inoculated with bacillus diphtheriæ April 10, 1893; killed May 1, 1893.

At the time of inoculation the temperature of the cow was 102.1° F.; on the evening of that day it went up 1.1° F., and fell a degree on the following morning. This was the highest rise of temperature after inoculation, as can be seen by the accompanying chart. She evinced no constitutional effects of the treatment, other than that she showed signs of weakness after the fifth day; and though careful watch was kept for an eruption upon the udder similar to that described by Klein, nothing of the kind appeared. For a few days after inoculation there was some swelling and considerable tenderness about the seat of operation, but after about seven or eight days the tenderness had apparently disappeared, and the swelling was gradually diminishing in size. This continued until the twentieth day after inoculation, when she was killed. At autopsy the following conditions were observed:

At the seat of inoculation there was a small subcutaneous nodule firmly adherent to the overlying skin, and freely movable over the underlying tissues. Viewed from the external surface this nodule was about 3 cm. in diameter, but when dissected out was not over 1 x 2 x 2.5 cm. in volume. It was *very* hard; upon section it was laminated, and tolerably sharply circumscribed; it did not fade away into the surrounding tissues. At one side of it the neighboring tissues were marked by minute hemorrhages.

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The axillary and cervical lymphatics were not enlarged, nor were any of the other subcutaneous lymphatic glands. The lungs were normal, containing no hardened areas, nor any spots indicative of either a recent or a remote diseased condition. The parietal pleura was smooth and normal. The pericardium contained about 50 c.c. of clear fluid; both the pericardium and the endocardium were free from hemorrhagic points; no fluid in either the pleural or peritoneal cavities. On the lower surface of the left lobe of the liver, which was otherwise apparently normal, were grouped together three irregular yellowish spots, which extended a short distance into the substance of the organ. The smallest of these spots was about 1 cm. in diameter, and the other two were about 1 x 2.5 cm. in area. Beyond a few scattered irregular whitish points, there were no other markings upon the liver.

The adrenal bodies were apparently normal. On the surface of the left kidney there was at one point a small whitish area, evidently due to fatty change, besides two or three minute cysts; the kidneys were otherwise normal. The spleen was normal. The other viscera showed no change. There was no change either upon the surface of the udder or in its substance, as was seen by parallel sections through the organ made at about one inch apart. From the centre of the tumor mass, at the seat of inoculation, five blood-serum slants were inoculated; after ninety-six hours at 37° C., they all remained sterile.

Microscopic examination of the seat of inoculation revealed a condition that was in general identical with that described in the same locality in Cow No. 1, but differed from it in certain details. The process, while everywhere conspicuous for cell death, as evidenced by fragmentation of the nuclei, was, nevertheless, not so far advanced as in Cow No. 1. In many of the lymphatic spaces between the tissue bundles masses of leucocytes could be made out, some of which had undergone complete necrosis, while others were less affected. Throughout many of these masses of leucocytes bacilli were scattered about in an irregular way, and in some instances small clumps of from four to six bacilli could be seen, having the appearance of being located within the bodies of leucocytes. In none of the sections could the tangled clumps of long, irregular bacilli, as seen in Cow No. 1, be detected, but where the organisms were found they were always scattered about irregularly, as described above. Where muscle fibres were present they were in an advanced condition of hyaline degeneration, and were frequently to be seen penetrated by leucocytes. Though bacilli in all morphological and staining respects identical with the bacillus diphtheriæ were present in large numbers, it was nevertheless impossible to obtain cultures of them from scrapings from the centre of the tumor. As stated, five blood serum tubes were inoculated, but in none of them did the organisms develop. The yellowish points that were found by macroscopic examination of the lower surface of the left lobe of the liver, were found microscopically to extend some distance into the substance of the organ, and to represent areas of fatty metamorphosis.

Bacteriological Examination of the Milk.

The point upon which special stress is laid in the experiments of Klein, is the discovery of the bacillus diphtheriæ in the milk of the cows that he had inoculated. For this reason particular care was taken with this portion of our work.

The investigation of the milk of Cow No. 1 began on the day of inoculation and continued every day for the four succeeding days, when a day was skipped; then two days were missed, after which the samples were collected at somewhat longer intervals. The teats were washed clean and the sample of milk for study was collected only after milking had been in progress for a short time. The milk was received in sterilized test-tubes provided with sterilized cotton plugs, and the bacteriological study began in, at most, ten minutes after it was collected.

In the majority of cases the blood-serum mixture of Löffler was employed in these analyses, but in a few instances slanted glycerin-agar and Petri plates of glycerin-agar were substituted. For each inoculation approximately $\frac{1}{20}$ c.c. of the milk was used. The results of the bacteriological study of the milk on nine different days succeeding the inoculation were negative in all cases, in so far as the presence of the bacillus diphtheriæ was concerned, and in general the tubes and plates contained fewer organisms of any character than one would have anticipated.

The results of these studies in detail are as follows:

November 8, 1892. One-half hour after inoculation about $\frac{1}{20}$ c.c. of milk was smeared upon the surface of each of three slanted glycerin-agar tubes; from each of these a blood-serum (Löffler's) slant was inoculated. At 37° C no colonies of bacillus diphtheriæ developed. This milk was placed at 37° C. for twenty-four hours, and again two blood-serum slants were inoculated, but no colonies of the Löffler bacillus appeared. Similar results were obtained with a set of glycerin-agar Petri plates.

9th. Two samples of milk were examined—one at the beginning and the other at the end of the milking. From each sample two smears upon slanted blood serum, and one set of glycerin-agar Petri plates were made, but no colonies of bacillus diphtheriæ appeared. Both samples of milk were then placed at 37° C. for twenty-four hours, after which two blood-serum tubes were inoculated and one set of glycerin-agar plates were made from each of them, but still no colonies of Löffler's bacillus developed.

10th. Again samples were collected from the beginning and end of the milking. From each sample two blood-serum slants and two sets of glycerin-agar plates were prepared. No colonies of the bacillus diphtheriæ appeared.

11th. From samples taken at the beginning and at the end of the milking two blood-serum slants and one set of glycerin-agar plates were prepared All failed to reveal the presence of colonies of bacillus diphtheriæ.

12th. Two blood-serum slants were prepared from the milk at the beginning and two from a sample from the end of the milking. No colonies of bacillus diphtheriæ developed.

At this date the samples from November 9th and 10th, that had been standing at the room temperature since they were collected, were examined by means of the blood-serum slants, but no diphtheria bacilli could be found.

14th. Two blood-serum slants and one set of glycerin-agar plates were made from a sample of milk collected a short time after milking had been in progress. No colonies of bacillus diphtheriæ appeared.

17th. Two blood-serum slants were prepared from the milk at the beginning, and two similar tubes from that at the end of the milking. Both negative as regards bacillus diphtheriæ.

December 3. Four blood-serum slants, each prepared with about $\frac{1}{20}$ c.c. of milk of this date, did not reveal the presence of any colonies of bacillus diphtheriæ.

10th. No diphtheria bacilli could be detected in the milk of this date, though three samples from different teats were examined upon three sets of glycerin-agar plates.

In the course of these examinations all bacilli that might, by any possibility, have been mistaken for the bacillus diphtheriæ were isolated in pure culture and their biological peculiarities studied. In no case were any of them identical in their cultural peculiarities with the bacillus of Löffler. Seven different species that were isolated were inoculated subcutaneously into guinea-pigs. None of them possessed any pathogenic properties for these animals.

Of these seven species, three were at times a little like the bacillus diphtheriæ in their morphology; that is to say, irregular, curved, clubbed, and segmented forms that stained irregularly, could be seen upon microscopic examination. In some of their cultural peculiarities they had also certain points suggestive of Löffler's bacillus, but when all of their biological properties, and particularly their pathogenic powers were tested, it was easy to see that they bore no relation whatever to this organism.

The bacteriological studies of the milk of Cow No. 2 were made upon six different days prior to inoculation and upon nine days succeeding. The object in making these studies before the animal was subjected to the experiment, was to determine if there were any organism or organisms present in the milk that might be confounded with the bacillus diphtheriæ. Two or three organisms having irregularities in their morphological and staining peculiarities were detected,

but their behavior under different methods of cultivation readily demonstrated that they were not to be mistaken for the Klebs-Löffler bacillus.

The studies upon the milk, which, as stated, covered nine days succeeding the inoculation, were negative in so far as finding the bacilli that had been injected into the tissues of the animal is concerned. As in the studies made before inoculation, there appeared, occasionally, bacilli which in morphology alone were a little suggestive of the bacillus diphtheriæ, but which could easily be differentiated from it by culture tests.

Another interesting point in connection with these analyses is the unexpectedly small number of organisms of any kind that appeared in the milk. The teats were not disinfected or washed, but the samples of milk, collected by myself, were taken only after I had been milking for a short time. They were, as usual, collected in sterilized test-tubes.

The details of these analyses are as follows:

Examination of the Milk before the Cow was Inoculated.

March 29, 1893. Three blood-serum tubes were each smeared with three loopfuls of the milk. After forty-eight hours at 37° C., two of the tubes were still sterile, the third contained a few colonies of cocci.

31*st*. Two blood-serum tubes were each smeared with three loopfuls and one with five loopfuls of milk. Of the tubes inoculated with three loopfuls of milk, one remained sterile; on the other there developed one colony of a coccus and one of a short bacillus, while on the remaining tube there appeared a single, small, dry colony of a minute bacillus.

In morphology alone this latter organism might, *at times*, pass for the bacillus diphtheriæ. But in this respect it varies, and by culture methods is easily seen to be of no relation to that organism.

April 1. Three blood-serum slants each inoculated with $\frac{3}{10}$ c.c. of the milk. After forty eight hours at 37° C., there appeared on one of the tubes a group of lemon-yellow colonies; on another an orange colony of cocci and three or four colonies of a straight bacillus; while the third tube remained sterile.

3*d*. Three blood serum slants each inoculated with $\frac{3}{10}$ c.c. of the milk. After forty-eight hours at 37° C. one tube was sterile, another contained a single yellow or orange-colored colony, and the third had a diffuse white growth at the bottom consisting of a coccus.

5*th*. Four glycerin-agar plates made with 0.5 c.c., 0.4 c.c., 0.25 c.c., and 0.2 c.c. of the milk. After seventy-two hours there had developed three colonies, one colony, one colony, and four colonies on the plates in the order named. None of these organisms was in any way suspicious.

6*th*. Four Petri plates of glycerin-agar were made as follows: With 0.5 c.c., 0.5 c.c., 0.25 c.c., 0.25 c.c. of the milk. There developed respectively four, twenty-one, thirteen, and three colonies. They consist of small yellow colonies of cocci, colonies of large cocci, and a few of irregularly staining bacilli—the latter easily differentiated from bacillus diphtheriæ.

Bacteriological Examination of the Milk after Inoculation.

April 10, 1893. Ten minutes after injecting 1 c.c. of a bouillon culture of virulent bacillus diphtheriæ into tissues of right shoulder, two blood-serum slants were each inoculated with $\frac{3}{10}$ c.c. of the milk. On one tube fourteen colonies developed, a portion of which were lemon-yellow and a few orange in color. On the second tube seven colonies similar to those on the first tube developed. Nothing to suggest bacillus diphtheriæ.

11*th*. Two samples of milk—one at beginning the other near the end of the milking.

Beginning sample. Two blood-serum slants inoculated with 0.2 c.c. of milk. On one tube there developed thirteen colonies; on the other, no colonies, but there were found a few lancet-shaped bacilli in the fluid at the bottom of the tube, after forty-eight hours at 37° C.

Sample at end of milking. Two blood-serum slants each inoculated with 0.2 c.c. of the milk. On one there developed eleven colonies; the second was still sterile after forty-eight hours at 37° C. No diphtheria bacilli appeared on the tubes from either sample.

12*th*. Four blood-serum slants each inoculated with 0.2 c.c of the milk. After forty-eight hours at 37° C., there developed five colonies, two colonies, and thirteen colonies on three tubes respectively, while the fourth remained sterile. No diphtheria bacilli.

13*th*. Four blood-serum slants each inoculated with 0.2 c.c. of the milk; two of them at beginning and two at end of milking. On the former two there developed two colonies and one colony respectively, and on the latter two, one colony and no colonies after forty-eight hours at 37° C. No diphtheria bacilli.

14*th*. Sample of milk at beginning and another at end of milking:

From former, each of three slants, two blood-serum and one glycerin-agar, were inoculated with 0.2 c.c. of the milk. Both blood-serum tubes remained sterile and one colony developed upon the glycerin-agar tube. The three slants, one blood-serum and two glycerin-agar, each inoculated with 0.2 c.c. of the milk from the end of the milking, resulted as follows: On the blood-serum tube one colony of a coccus, on one glycerin-agar slant twenty-four colonies of a white coccus, and on the other twenty-eight colonies of apparently the same organism. No diphtheria bacilli were found.

15*th*. Six glycerin-agar slants were each inoculated with 0.2 c.c. of the milk at beginning of milking. After forty-eight hours at 37° C., there developed two, three, seven, five, two, and three colonies respectively. None of them were bacillus diphtheriæ.

Six glycerin-agar slants were each inoculated with a similar amount of the milk from end of milking. There developed seventy, fifty-two, fifty-eight, fifty-six, forty-eight, and forty-eight colonies respectively, the majority of which are of a lemon-yellow color, a few are quite white, and two are salmon-color. There are no colonies that could in any way be mistaken for those of the bacillus diphtheriæ.

17*th*. Eight glycerin-agar slants inoculated with 0.2 c.c. each—four from sample at beginning, and four from sample at the end of milking. After forty-eight hours the former four showed no colonies; of the latter four, three remained sterile, and two colonies appeared on the remaining tube. Neither of them were bacillus diphtheriæ.

18*th*. A sample of milk at beginning of milking and one at end of milking, examined.

Eight glycerin-agar slants each inoculated with 0.2 c.c. of milk—four from first and four from second sample.

After forty-eight hours at 37° C., there developed on tubes from first sample, two, two, one, and one colony respectively. From second sample there developed twenty-three, seventeen, ten, and seven colonies respectively; all cocci. No diphtheria bacilli on any of the tubes.

20*th*. Two samples of milk examined:

Sample 1. Two blood-serum and two glycerin-agar slants each inoculated with 0.2 c.c. of the milk. There developed after forty-eight hours at 37° C., twelve, twenty-six, none, and no colonies respectively.

Sample 2. Two blood-serum and two glycerin-agar slants inoculated with 0.2 c.c. of the milk. Results after forty-eight hours at 37° C., twelve, none, one, and no colonies respectively. No diphtheria bacilli on tubes from either sample.

Though it is possible for a few scattered diphtheria bacilli to have been present in the milk and escaped efforts to detect them, I nevertheless think the above protocol speaks for a fair trial in this direction.

Though the bacillus diphtheriæ was not found in the milk of either of the two cows experimented upon, I should not have been very much surprised had it appeared; for it has recently been demonstrated by Ghriskey and myself, working together, (1) that in exceptional instances diphtheria bacilli when deposited under the skin of smaller animals (guinea-pigs) may be found in the lymphatic apparatus of the omentum, and that when the injections are made into the testicles of these animals they were also found in masses in the omental lymph spaces of three out of four animals inoculated. In all these experiments there was strong evidence in favor of many, if not all of them, having been conveyed from the seat of inoculation, either

in the loose subcutaneous tissues or the tissues of the testicle, to the points in the omentum at which they were found, through the phagocytic activity of the wandering blood-corpuscles, the leucocytes.

Morphological Variations of the Bacillus Diphtheriæ.

One of the points upon which Klein lays particular emphasis is the presence in the degenerated muscle fibres, at the seat of inoculation in one of his cases, of long threads in tangled clumps. He refers to them in the following terms (*loc. cit.*, p. 173):

"Microscopic examination of the masses of bacilli in the necrotic portions of the tumor, as also of those implicating the muscle fibres, revealed the following remarkable fact, namely, that many bacilli had become threads, some of considerable length, and containing granules in their course, characterized by intermediate or terminal buds or swellings, spherical, oval, or flask-shaped. In a word, the organisms had taken on forms which are but exaggerations of what had been before observed in cultures of this bacillus. I draw particular attention to the bacilli cultivated from the milk of Cow No. 2, where some of the threads were seen to be possessed of terminal flask-shaped enlargements, reminding one vividly of the growing and germinating hyphæ of a mycelial fungus."

We have searched carefully the sections through the seat of inoculation of both of our cows, but have failed to find this remarkable appearance described by Klein. In the first cow clumps of rather long, irregularly staining and beaded bacilli were easily to be seen in the necrotic areas, while in the second cow the organisms were not arranged in clumps, were smaller, and were scattered, as stated, through masses of leucocytes lodged in the connective-tissue lymph spaces.

We have not felt called upon to consider the long, irregular forms seen in the tissue from the first cow as remarkable, for similar forms of this organism are practically constant when it is cultivated upon Löffler's blood-serum mixture. We have made a series of studies upon the variations in the morphology of this organism, and find that its form changes markedly under certain different conditions of environment. That is to say, its morphology is always more regular and smaller on glycerin agar-agar than on any of the other media used for its cultivation; while upon Löffler's blood serum the other extremes of development appear; here one sees (instead of the very

short, spindle, lancet, club-shaped, always segmented and regularly staining forms as seen upon glycerin-agar) long, irregularly staining threads, that are sometimes clubbed and sometimes pointed at their extremities. They are usually marked by areas that stain more intensely than do the rest of the rod, and at times they may be a little swollen at the centre. These differences are so conspicuous that microscopic preparations from cultures of the bacillus diphtheriæ upon glycerin agar-agar and blood serum when placed side by side, would hardly be considered as of the same organism, unless its peculiar behavior under these circumstances was already known.

We have endeavored to discover the cause of this variation, and for this purpose have studied the growth of the organism upon a number of different media, with very interesting but not entirely satisfactory results. On plain nutrient agar-agar (that is, nutrient agar-agar without glycerin); on solidified egg-albumen; on a medium that we are now using consisting of dried albumin, as found in commerce, dissolved in bouillon (about 10 grammes albumin to 100 c.c. of bouillon containing 1 per cent. of grape-sugar); in bouillon without glycerin and in bouillon to which a bit of hard-boiled egg has been added, the morphology of the organism is about intermediate in both size and outline between the forms seen upon glycerin agar-agar and Löffler's blood serum. There will appear about an equal number of short segmented and long irregularly staining forms, but in general the longest are rarely as long as the long forms seen on blood serum, and throughout they are not so conspicuous for the irregularity of their staining.

In cultures made upon two sets of nutrient agar-agar tubes differing only in the fact that one set contains glycerin to the extent of 6 per cent., while the others contain none, a noticeable difference in morphology can usually be made out; while the forms on the glycerin-agar cultures are throughout small, pretty regular in size, shape, and staining, those on the plain agar are larger, stain more regularly, vary more in shape, and when stained by Löffler's blue are not so uniformly marked by the pale transverse lines that give to them the appearance of being made up of numerous short segments.

This observation, in connection with what has already been said, might lead to the suspicion that the small amount of glycerin present was instrumental in bringing about these conspicuous differences. To

determine if this is the case, Löffler's blood serum was prepared containing 6 per cent., 10 per cent., and 20 per cent. of glycerin, but cultures upon it were in no way identical with those found upon agar-agar containing 6 per cent. of glycerin. The morphology of the organisms that grew differed from that seen upon Löffler's serum without glycerin only in being a trifle shorter and more markedly beaded. The protoplasmic portions that took up the staining were often perfectly round and extended a little beyond the lateral margin of the rod in which they were located. Many of these beads were almost black in color; indeed, these cultures were conspicuous for the number of these blackish points, even after only twenty-four hours in the incubation. No growths appeared upon the tubes containing 20 per cent. of glycerin. The effect of pure glycerin was tested upon long forms that had already developed upon Löffler's serum for twenty-four hours, by scraping the growth from the surface and mixing it with pure glycerin, but after twenty-four hours of this treatment the morphology of the long, irregularly staining rods was not altered.

In Figs. 2 and 3 we have endeavored to depict the appearances of this organism as obtained from glycerin-agar and from Löffler's blood serum, and, as can be seen, the difference is marked.

Fig. 2 represents the appearance of the organism as obtained from a glycerin-agar culture twenty-four hours old, while Fig. 3 is the same organism from a blood-serum culture of the same age.

It is particularly interesting to note the sudden transition that occurs in the morphology of this organism when placed upon blood serum after it has been cultivated upon glycerin-agar through a number of generations, and *vice versa*. We have repeatedly isolated it upon glycerin-agar from single colonies directly from the throat of a diphtheritic patient or the seat of inoculation of an animal dead of the experimental form of the disease, and continued its cultivation upon this medium for six or eight generations, in all of which practically none but the short segmented varieties could be seen, but with the first generation that appeared, when now transferred to Löffler's blood serum, the tendency throughout was to the formation of the long, irregular threads diagrammatically represented in Fig. 3. The reverse is true when it is carried along for a time on serum and then transferred to glycerin-agar.

We have made these observations on five cultures from undoubted cases of primary diphtheria, upon cultures from two cases of membranous rhinitis, and upon one culture of the so-called pseudo-diphtheritic bacillus, and find no deviation in the behavior of any of them from what has been said.

It is impossible to say what it is that causes these differences, and with the evidence at our disposal it is, perhaps, premature to offer an hypothesis, but certainly it appears that the blood serum offers something that is particularly conducive to the growth of the outer envelope of the bacilli, and that this portion of the cell grows more rapidly than do its protoplasmic contents, causing in this way breaks in the continuity of the cell protoplasm as seen in stained preparations. (Figs. 3 and 4.) Whether this is true or not, there is apparently a difference in constitution between the portions of the cell, as cultivated upon blood serum, that take up staining, and that portion that is seen as either a colorless or only faintly stained sheath or tube. These differences can readily be brought out by micro-chemical means; for example, if one prepares a cover-slip from a fresh (twenty-four hours old) serum culture of the bacillus diphtheriæ, and stains it for a few minutes in saturated watery solution of Bismarck-brown, and subsequently by Gram's method, that portion of the cell that is usually colorless, or nearly so, is now seen to be brown, while the protoplasmic contents will be of an intense violet color. Fig. 4 represents a preparation made from a blood-serum culture by this method.

From what has been said it is easy to conceive that one accustomed to study this organism upon agar-agar or gelatin would hardly be prepared for the striking alteration that it undergoes in its appearance when under more favorable conditions of growth, and for this reason it is important to recognize the necessity of an acquaintance with the growth of the organism under the different conditions of environment.

Long, irregularly staining, clubbed, and curved forms similar to those that are constantly seen upon Löffler's blood serum, even after only twenty-four hours at the temperature of the incubator, are often referred to collectively as involution or degenerate forms of this organism; but from these studies I feel justified in questioning the accuracy of this view; at all events it is difficult to reconcile the

voluminous growth of this organism, as seen upon blood serum after twenty-four hours, with the opinion that the organisms of which it is composed are in a condition of degeneration.

SUMMARY.—The results of our experiments have differed from those of Klein in several essential points. We have failed to obtain cultures from the seat of inoculation of either of our cows at autopsy. In one case the animal died sixteen days after inoculation, and in the other she was killed on the twentieth day after the experiment was begun. In both cases microscopic examination of the tissues revealed the presence of the bacillus diphtheriæ in relatively large numbers.

In neither of the two cows operated upon by us did an eruption appear upon the udder or teats. Careful watch was kept, but at no time during the experiments could anything abnormal be detected upon this organ.

We have subjected the milk from both cows to careful and prolonged study, and have failed in both instances to detect the bacillus diphtheriæ in it.

The pathological appearances in the internal organs noted by Klein were not looked for in Cow No. 1, for the reason given; but in Cow No. 2 there was no trace of disease of any character in either the lungs, pleuræ, or serous surfaces of the heart.

From the description of the seat of inoculation in the cows experimented upon by Klein, we cannot say whether the pathological lesions found by us were similar to those obtained by him or not. In neither cow have we found at the seat of inoculation bacilli sufficiently peculiar in their morphology to justify us in likening them to the germinating hyphæ of mycelial fungi; but we did find in Cow No. 1 bacilli the morphology of which was strikingly like the long irregular forms of this organism that predominate when it is grown upon Löffler's blood-serum mixture. In Cow No. 2 the majority of the bacilli were short and irregular in outline, presenting nothing peculiar in their appearance.

NOTE.—Before closing, I wish to express my indebtedness to Dr. Simon Flexner, Associate in Pathology, Johns Hopkins University and Hospital, for his kindly assistance in my studies of the pathological lesions found in these experiments; and to Professor Pearson

and Dr. Turnbull, of the Department of Veterinary Medicine of the University of Pennsylvania, for their valuable aid during the time that the cows were under observation in this institution.

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3. *Welch and Flexner:* The Histological Changes in Experimental Diphtheria. Johns Hopkins Hospital Bulletin, August, 1891, No. 15.

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DESCRIPTION OF PLATES.

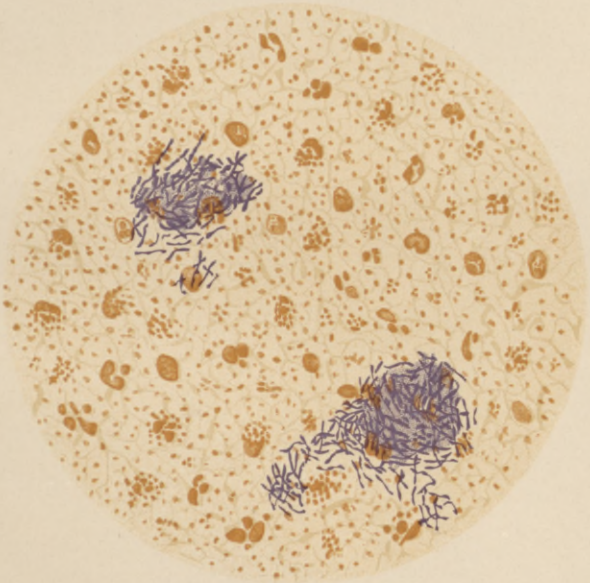
FIG. 1.—Area of necrosis at seat of inoculation of Cow No. 1, showing the condition of fragmentation of the cell nuclei and clumps of irregularly staining bacillus diphtheriæ. Stained with Bismarck-brown and Gram's method. Leitz oil immersion $\frac{1}{12}$, ocular 4, tube 160 mm.

FIG. 2.—Bacillus diphtheriæ, second generation on glycerin agar-agar, after twenty-four hours at 37° C. Stained with Löffler's blue. × about 1500 diam.

FIG. 3.—Bacillus diphtheriæ after twenty-four hours at 37° C. on Löffler's blood-serum mixture. Stained with Löffler's blue. × about 1500 diam.

FIG. 4.—Bacillus diphtheriæ after twenty-four hours at 37° C. on Löffler's blood-serum mixture. Stained with Bismarck-brown and Gram's method. × about 1500 diam.

FIG. 1.



A. C. ABBOTT, *del.*

Fig. 2.

Fig. 3.

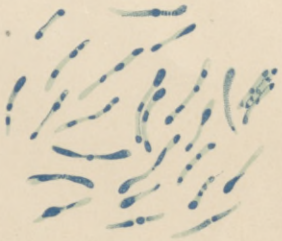
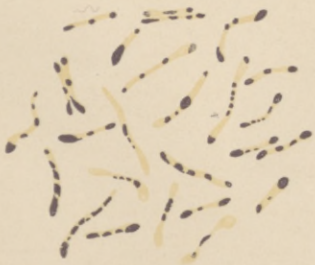


Fig. 4.



A. C. Abbott, *del.*