SCIENCE

VOL. 74 FRIDAY, AUGUST 7, 1931 No. 1910

SCIENCE: A Weekly Journal devoted to the Advancement of Science, edited by J. MCKEEN CATTELL and published every Friday by

THE SCIENCE PRESS

New York City: Grand Central Terminal

Lancaster, Pa. Garrison, N. Y.

Annual Subscription, $6.00 Single Copies, 15 Cts.

SCIENCE is the official organ of the American Association for the Advancement of Science. Information regarding membership in the Association may be secured from the office of the permanent secretary, in the Smithsonian Institution Building, Washington, D. C.

OBSERVATIONS UPON THE FILTERABILITY OF BACTERIA, INCLUDING A FILTERABLE ORGANISM OBTAINED FROM CASES OF INFLUENZA[1]

STUDIES IN BACTERIAL METABOLISM, CI

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THERE is a heterogenous group of formidable diseases of man and animals, including some of the most rapidly spreading infections, whose inciting agents have thus far eluded cultivation upon artificial mediums. There can be little doubt that the incitants of this group are living entities and their cultivation outside the body should, judging from past experience in bacteriology and preventive medicine, be helpful, not only in solving important problems of their life history, but also in approaching the solution of prophylactic and curative measures. A few of these "viruses" are said to have been kept alive for periods of time in presence of large amounts of blood, or pieces of tissue from recently killed animals, but by common consent, this limited, restricted development is not regarded as equivalent to cultivation in the usual bacteriological sense.

[1] The James A. Patten Lecture in Bacteriology, Northwestern University Medical School, Chicago, Illinois, July 22, 1931.

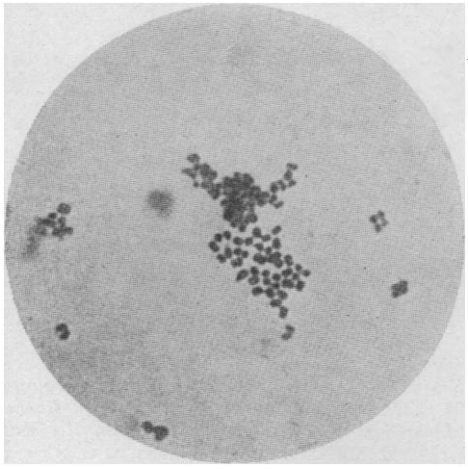

FIG. I. Diplococcus from a case of influenza, showing variation in size and intensity of staining.

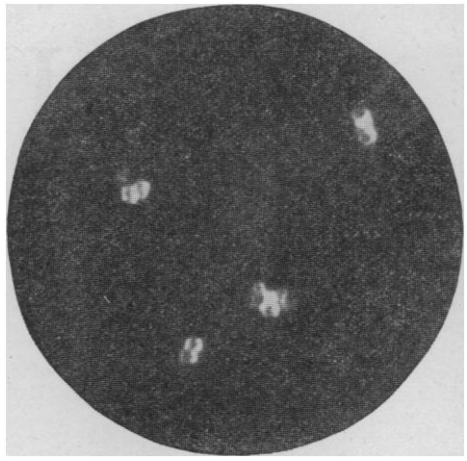

FIG. II. Diplococcus from a culture of influenza, 48 hour culture in K medium. Dark field illumination, showing granules.

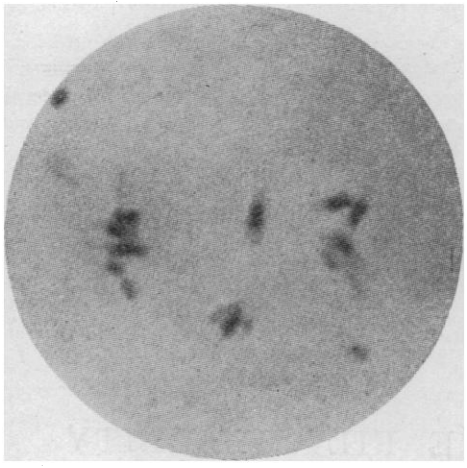

FIG. III. B. typhosus, 24 hours' growth in K medium, showing granules, and faintly staining residuum of organism.

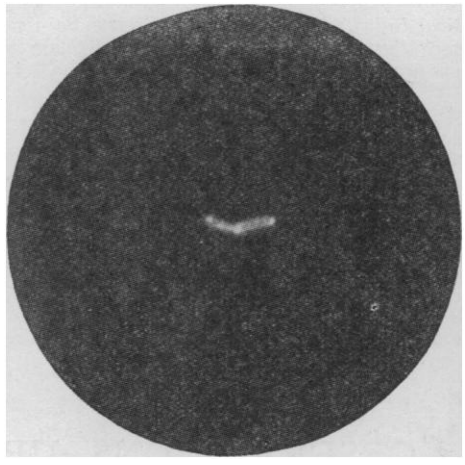

FIG. IV. B. typhosus, 48 hour culture in K medium. Dark field illumination, showing granules.

One can not but be impressed, in attempting to analyze the uniform failure to induce growth of these "viruses" in artificial mediums, with the fact that artificial mediums at present available for their cultivation depart radically from the physical nature and chemical composition of their natural habitats. Also, and this is significant, the more closely mediums approximating the nature and composition of living tissues are prepared, the more nearly has there been indication of at least some multiplication of these "viruses" outside the animal body. Comparing the artificial medium with the natural environment of these "viruses," one is struck by two outstanding differences, omitting for the present the question of inorganic constituents. The artificial medium contains protein degradation products, peptones and meat extractives, and little or no unaltered, or nearly unaltered, protein. The tissues of the body, on the contrary, contain unaltered protein, and little or no peptone or other protein degradation products, and there is clearly a difference as well as a distinction between limited life in fresh tissue or blood, on the one hand, and on the other hand true passage with almost limitless capacity for multiplication through a series of artificial cultivations. By comparison, current knowledge of the nature of cultivable microbes, their morphology, physiology and chemistry, contrasts strikingly with the paucity of information concerning the so-called "virus" diseases, of which but little is known, except objectively. And yet in this virus group are smallpox, vaccinia, measles, rabies, that extraordinarily contagious group com-

prising influenza, common cold and its clinical variants, poliomyelitis, encephalitis and several others.

A theoretical method of approach to the problem of cultivation of these refractory "viruses," therefore, would appear to be the preparation of a sterilizable medium containing unaltered, or nearly unaltered, protein, and without peptone or significant amounts of other nitrogenous constituents, to confine discussion for the present to these most significant substances.

Such a medium has been prepared.[2] In brief, the essential ingredient is small intestine of man, swine, dog or rabbit, whichever is available, thoroughly extracted with alcohol to remove water and alcohol soluble extractives, followed by extraction with benzol to remove excess of lipoidal substances. This residue, dried, can be kept indefinitely. The addition of Tyrode solution, or even normal saline to this powdered extracted substance, makes a rather turbid medium which can be autoclaved without apparent harm. The autoclaved medium (referred to hereafter for brevity as the K medium) possesses most unusual qualities. These may best be expressed by citation of a series of experiments with it.

Fresh, aseptically drawn blood from seven cases diagnosed as mild influenza, four from cases in Passavant Hospital, three from cases in Evanston Hospital, was added to K medium, previously heated, then rapidly cooled to expel air, in the proportions of 10 cc of blood to 90 cc of medium. Incubation was practiced for several days at 30 degrees C. Three cultures showed increasing cloudiness with the progress of incubation; four did not. From the three cloudy cultures, an organism was isolated. The four remaining bloods continued sterile for nine weeks. Then they were discarded. Attention is drawn in passing to the difficulty in establishing a definite clinical diagnosis of influenza in these milder, sporadic cases, which occur in inter-epidemic periods. It is not without significance, therefore, that the three positive cases discussed here were found in two entirely separate institutions, and the diagnoses were made absolutely independently by physicians living in Chicago and Evanston, respectively.

Inasmuch as there has been, very properly, much just scepticism about the cultivation of filterable organisms in general, the details, including dates, of the two positive cultures from the Passavant cases are reported here at considerable length.

Isolation of Filterable Organisms from Cases of Influenza

Eight rabbits were put in stock under observation January 2, 1931. No spontaneous illness developed during the course of the experiments that followed.

[2] The details of preparation will appear in another communication.

A. February 3, 1931. 10 cc of blood from cases No. 3 and No. 4 (Passavant), respectively, were added to 90 cc of K medium.[3] Incubation was practiced for 10 days at 30 degrees, during which time distinct cloudiness developed. (Cultures No. 3A and No. 4A.)

B. February 13, 1931. Seven loopfuls of culture from No. 3A and No. 4A, respectively, were added to 10 cc of the K medium. They were incubated at 30 degrees for 10 days. The mediums became progressively clouded. (Cultures No. 3B and No. 4B.)

C. February 23, 1931. Seven loopfuls of culture from No. 3B and No. 4B, respectively, were added to 10 cc of the K medium. Incubated at 30 degrees for 10 days. The mediums again became clouded. (Cultures No. 3C and No. 4C.)

D. March 5, 1931. Seven loopfuls of culture from No. 3C and No. 4C, respectively, were added to 10 cc of the K medium. Incubated 5 days at 30 degrees. Considerable cloudiness developed. (Cultures No. 3D and No. 4D.)

E. March 10, 1931. One half cc of cultures No. 3D and No. 4D, respectively, was injected into the ear vein of rabbits No. 3 and No. 4. At the same time, as control, 1 cc of the medium from each of three negative flasks of K medium (from cases No. 1, No. 2 and No. 5), was injected into each of three rabbits. These control rabbits remained normal.

Ea. March 11, 1931. Rabbits No. 3 and No. 4 sneezed paroxysmally, and frequently during this day and for the next four following days, with apparently unabated violence. Rectal temperatures for March 11 (24 hours after inoculation) and for March 18 (one week later), were as follows:

	Temperature March 11	Temperature March 18
No. 1 (control rabbit)	102.2	102.0
No. 2 (control rabbit)	102.1	102.0
No. 3 ("flu" rabbit)	103.4	102.8
No. 4 ("flu" rabbit)	104.0	102.5
No. 5 (control rabbit)	102.4	102.2

By the sixth day, the acuteness of the sneezing had abated. Rabbit No. 4 made an uneventful but rather slow recovery; rabbit No. 3 still sneezes paroxysmally (June 23) and appears to be slowly losing ground.

F. March 12, 1931. 12 cc of blood was drawn from rabbits No. 3 and No. 4, respectively; it was distributed as follows:

1 cc to 10 cc of K medium = cultures No. 3Fa and No. 4Fa.
1 cc to 10 cc of dextrose broth = cultures No. 3Fb and No. 4Fb.
10 cc to ice box for 24 hours = blood, No. 3Fc and No. 4Fc.

Cultures No. 3Fa and No. 4Fa, after 10 days' growth at 30 degrees were plated on agar containing both dried intestine and proteose peptone.[4] Three days' anaerobic incubation followed by four days' aerobic incubation at

[3] K medium heated, and cooled rapidly each time to remove air.

[4] Proteose peptone, extracted thoroughly with absolute ethyl alcohol; meat infusion peptone agar is equally satisfactory, as was found out later.

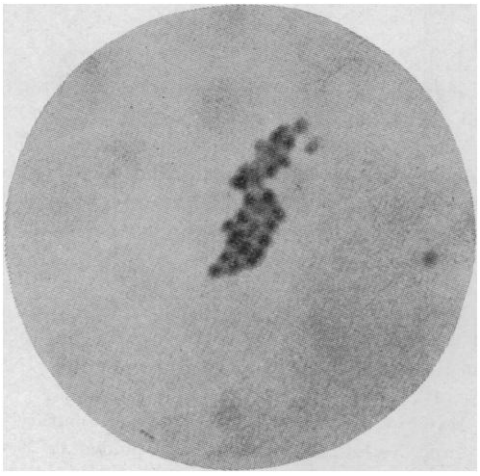

FIG. V. Staphylococcus from Staphylococcus bacteriophage. First culture on agar after K medium. Irregularities in size and staining are noticeable.

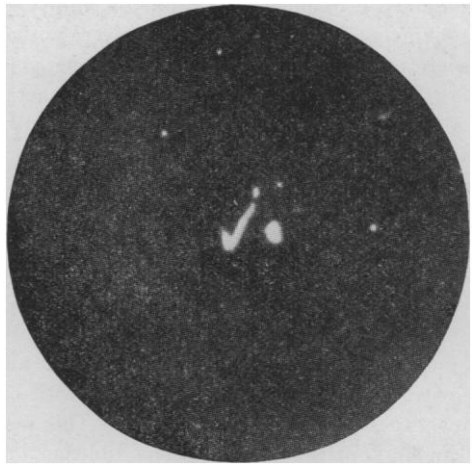

FIG. VI. Leptospira icteroides, second transfer in K medium. Beginning of granulation. Dark field illumination.

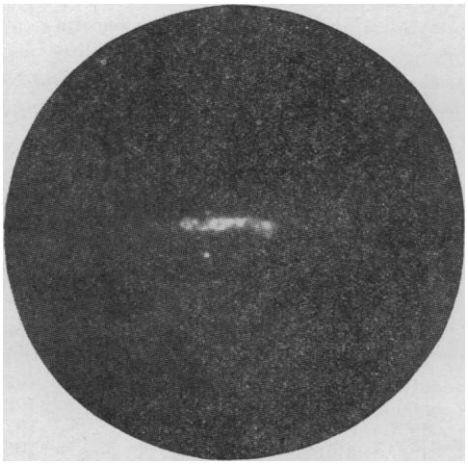

FIG. VII. Leptospira icteroides. Two weeks in K medium. Granulation complete. Organism filterable. Dark field illumination.

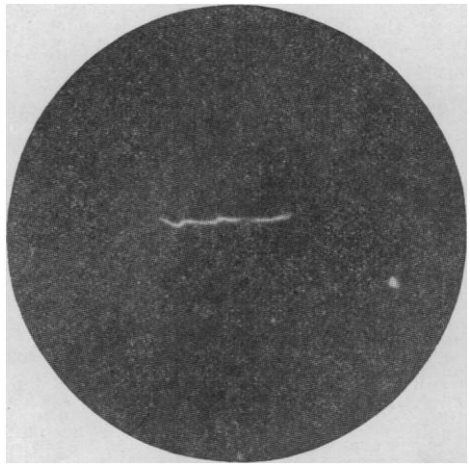

FIG. VIII. Leptospira icteroides. Growth in peptone-blood serum agar after filtration through Berkefeld N filter. Dark field illumination.

30 degrees, was productive of many small, almost invisible dew drop colonies. After transfer to blood agar (sheep), they grew as moist, fairly luxuriant white streaks. The organisms, which are members of the coccal group, appear to be somewhat pleomorphic, particularly in the first growth upon solid mediums. They tend to occur in pairs, they ferment the ordinary sugars, they are non-proteolytic, and stain with ordinary anilin dyes. They have little, if any, digestive action upon sheep hemoglobin.

Cultures No. 3Fb and No. 4Fb (in dextrose broth) remained absolutely sterile for several weeks. The coccus in the filterable state does not appear to grow in ordinary mediums, even when enriched with blood.

G. March 13, 1931. Serums from bloods No. 3Fc and No. 4Fc were filtered through Berkefeld N filters, after dilution to seven times their respective volumes with sterile physiological saline solution. One fourth cc of each serum Berkefeld filtrate was added to 10 cc of the K medium, and after incubation for 10 days at 30 degrees, was plated on the intestine-peptone agar. Colonies identical with those from cultures No. 3Fa and No. 4Fa were obtained after anaerobic incubation (3 days) followed by aerobic incubation (4 days).

Also, 5 cc of each of cultures No. 3D and No. 4D (the remainder after rabbit injections), diluted with three times their volume of sterile saline, were filtered through Berkefeld N filters. The filtrates, treated in precisely the same manner as filtrates No. 3Fc and No. 4Fc, yielded colonies which were morphologically and culturally like those had from No. 3Fa, No. 4Fa, No. 3Fc and No. 4Fc. Also, similar organisms were isolated, but more slowly, from blood No. 6 (Evanston Hospital case).

A brief recapitulation of these observations will indicate the several unusual features they present.

1. The seven influenza cases were quite similar clinically. Blood cultures made from four were sterile, even after prolonged incubation in the K medium. Three blood cultures yielded apparently identical organisms. This would seem to indicate that these organisms were not adventitious. The possibility of contamination at the time of taking the bloods is recognized, but from the nature of the organisms isolated, it is deemed rather remote, especially in light of the fact that the patients showing positive blood cultures were in two different hospitals. The four negative bloods also, from two separate hospitals, are an additional control on technique.

2. The mediums used for cultivation were autoclaved at fifteen pounds steam pressure for twenty minutes. Therefore, they were initially sterile.

3. The rabbits, both those showing symptoms (No. 3 and No. 4) and those remaining well (No. 1, No. 2, No. 5, No. 7 and No. 8), were under observation a full month before inoculation, and several weeks after inoculation. No symptoms of snuffles or other disease appeared among the control animals during this time. Three uninoculated rabbits (No. 5, No. 7 and No. 8) of the control series were kept in cages adjacent to rabbits No. 3 and No. 4, which, it will be recalled, sneezed vigorously for several days. They remained well. Apparently the infection induced in these rabbits was not very contagious for other rabbits. Blood from case No. 6, which developed turbidity in K medium much more slowly than bloods No. 3 and No. 4, was not injected into a rabbit.

4. The filters, Berkefeld N, failed to pass dextrose broth cultures of B. typhosus or the coccus above mentioned. They passed the filterable stage of the coccus readily, however.

RESULTS

From two bloods, taken from patients presenting clinical symptoms reminiscent of influenza, organisms were isolated which induced paroxysms of violent sneezing in rabbits. From the rabbit inocula, and from the blood of these rabbits, taken on the second day of infection, a filter passing "virus" was cultivated in the K medium. From the K medium, after incubation and development, cocci were isolated. These cocci, in their unfilterable state, could readily be retransformed to the filterable state upon K medium, refiltered, and recovered again, after a period of development in K medium, followed by growth on agar, as unfilterable cocci. It should be emphasized that the filterable state is readily induced by inoculation of the cocci in the special medium. The converse process, transformation of the filterable to the non-filterable state, is a relatively slow procedure. It can be done, however, by inoculation of K medium growth upon intestine-proteose-peptone agar, possibly better upon blood agar and usually, except for very fastidious microbes, as will appear later, upon plain agar. Colonies appear upon the solid mediums after a few days as tiny, clear, dew drop growths, which upon retransfer become heavier and more luxuriant. The coccus, therefore, can exist in a filterable state, in which condition it grows in the K medium, but does not appear to grow visibly in peptone mediums; and a non-filterable state, in which growth upon ordinary peptone-containing mediums is readily obtained. The rabbit infections were induced while the coccus was apparently in the filterable state. Attempts to induce infection with the non-filterable (coccus) state were unsuccessful. Experiments are under way to determine the nature and extent of changes in the virulence and infectivity of this microbe.

The relation of this coccus to the etiology of influenza naturally presents itself at this time. While the evidence, so far as it has been revealed, certainly is suggestive that more than accidental parallelism exists between the filterable state of this coccus through the rabbit syndrome to the human infection, proof is, of course, wanting. It can not be denied that possibly a filter-passing virus was simultaneously present. This might conceivably have caused the rabbit infection, and also perhaps explain the non-contagiousness by droplet infection from rabbit to rabbit. If so, it must be admitted that such an hypothetical "virus" either developed well in the K medium or else it was present in great abundance in the original blood cultures, which gave positive growths in K medium; also, if such were the case, it was also absent in the negative bloods, unless, perchance, a symbiotic action between the hypothetical "virus" and the coccus herein described was taking place.

It should be pointed out also that the dilution factor from the original blood (1:10) through cultures A–D inclusive in the K medium, would reduce the original constituents of the positive bloods many hundreds of times in the actual inocula of the rabbits. The possibility of the persistence of such a "virus" is freely admitted, nevertheless.

However, the most far-reaching phenomenon brought to light in this investigation is the obvious existence of the coccus isolated from these three cases of "flu" in two states, a filterable, and a non-filterable. Insofar as these experiments indicate, furthermore, the distinction between the two states does not depend primarily upon the somewhat academic question of porosity of filters, but upon actual, consistent differences in nutritive environment. The filterable form does not appear to develop into visible forms in plain

or dextrose broth (although occasionally they may develop upon agar under certain conditions), but it may be readily cultivated in the K medium. On the other hand, the non-filterable form, the coccus, grows readily in ordinary mediums. It changes rather readily to the filterable form, however, in K medium.

Having shown that this coccus exists in a filterable and a non-filterable state, it is not unreasonable to suspect that other bacteria might exhibit this same phenomenon of dual existence if exposed to the same cultural conditions. Such has proved to be the case.

The belief that bacteria may have a filterable state is a very old one.[5] In some instances, for example in the pus from cold abscesses, proof seems to have been procured. It is well known that formed tubercle bacilli are not found in such material, although Much granules,[6] which Fontes[7] showed could be passed unharmed through Berkefeld filters, have long been known. Not a few references to the successful passage of B. coli, B. dysenteriae (Shiga type), and B. typhosus through stone filters have also appeared from time to time in the literature, but these observations have not been very generally accepted, apparently because the experiments have not been repeatable in seriatim. On the other hand, there is a group of infections, incited by transmissible agents, capable of inducing pathological states in man, animals and plants, to which the term "filterable viruses" has been applied. The inciting agents have not, in most instances, been cultivated in artificial mediums, and little is known of the properties of these agents aside from their contagiousness, and the pathology of the lesions they incite. This embraces a large and important, but very heterogeneous group.[8] Within it are some of the most formidable infections of man. Some of the major problems in epidemiology, immunology and prevention and therapy are inextricably tied into this baffling group, whose members, resisting cultivation in ordinary mediums, have eluded science thus far.

To make this part of the investigation brief, it may be stated that the first organism examined for a filterable state was a culture of B. typhosus, which had been in stock in the laboratory for several years. It was put into the K medium, cultivated for 48 hours at 30 degrees, and after dilution with twice the volume of sterile physiological salt solution, was filtered through a new Berkefeld N filter. The clear Berkefeld filtrate was added to mediums in amounts as follows: 2 cc and 0.25 cc, respectively, to 10 cc of dextrose broth; 2 cc, 1 cc, 0.5 cc and 1 drop, respectively, to 10 cc of K medium.

Incubation of these filtrates was practiced for 48 hours in the K medium. The dextrose broth cultures remained sterile for three weeks. The 48-hour K cultures also failed to give cultures upon intestine-proteose peptone agar plates, but after 72 hours' incubation, agar cultures made from them and kept in the incubator at 30 degrees for several days, yielded numerous colonies of B. typhosus. These were characteristic morphologically and in staining reaction. Transfers of these colonies, introduced into either plain or glucose broth, agglutinated readily with specific typhoid serum.

It appears, therefore, that B. typhosus, as well as the coccus from the influenza cases, can be rendered filterable. The filterable organisms fail to grow in ordinary broth mediums, but in the K medium, growth proceeds in due course, and eventually typical typhoid bacilli may be recovered in the manner indicated. The details of control of filters and pertinent associated phenomena will appear in another communication.

Other bacteria have been similarly made filterable, filtered and recovered. To date: Rosenow's poliomyelitis streptococcus, Dochez's scarlet fever streptococcus, B. paratyphosus alpha, Noguchi's Leptospira icteroides, as well as Staphylococcus aureus, B. typhosus, and the coccus from the "flu" cases, have thus been put through their paces. It should be pointed out here, as will be explained in another communication, that not infrequently the first tiny dew drop colonies appearing on agar plates from the up growth of the filterable organisms, are very inert, both chemically and culturally. One or more transfers in ordinary mediums may be required to elicit the usual luxuriance of growth and capacity for chemical activity.

Next, it appeared that certain much discussed, not well understood phenomena, bacteriophage and antivirus, which conceivably might have a similar explanation, should be investigated. Details must be left to other communications which will follow shortly, but it may be stated here that both Staphylococcus "phage" filtrates, and Besredka "Staphylococcus antivirus" have each yielded perfectly typical cultures of Staphylococcus aureus upon cultivation in the proper manner. A sample of rabies vaccine from a lot that apparently caused a case of human encephalitis, was inoculated into the K medium. After a period of acclimatization and growth, transfer to agar plates yielded a coccus that is under investigation. In light of what has been said, it is significant that growths were not had even upon prolonged incubation in enriched peptone-containing mediums. Positive blood

[5] An excellent summary by Klieneberger: "Bakterienpleomorphismus und Bakterienentwicklungsgänge," *Erg. d. Hyg., Bakt. Immunitätsforsch. u. Exp. Therapie,* Berlin, 11: 499–555, 1920, has the important details and literature.

[6] H. Much, *Beitr. z. Klin. d. Tuberk.,* 8: 85, 1907.

[7] A. Fontes, *Centralb. f. Bakteriol.,* Abt. I. Ref., 51: 244, 1912.

[8] See T. M. Rivers, "Filterable Viruses," *J. Bacteriology,* 14: 217–257, 1927, for excellent summary.

cultures have been obtained, quite readily, from a series of cases of common cold, of arthritis, of rheumatic fever, and rheumatoid endocarditis, measles (30 hours before the appearance of the rash), and German measles. These failed under parallel conditions to yield cultures upon any artificial mediums. In one case of endocarditis, with fever, and a rather sudden increase in symptoms, an hemolytic streptococcus was isolated both directly upon blood agar (sheep) and coincidently from the K medium. It is perhaps significant that the isolation with the K medium in this instance, where the blood agar culture was positive, was completed in 72 hours. Usually thus far, when cultures in peptone mediums are negative, final isolation of the organisms from the K medium requires from 10 days to 2 or even 3 weeks. Here again, the details must be presented in another place. Enough has been related, however, to focus attention upon the apparently fundamental and general character of the phenomenon which has been described, namely, that bacteria representative of many of the important divisions of this great group of organisms can, and apparently do, exist in two distinct states. One, readily filterable through Berkefeld N filters; the other, not filterable, exhibiting characteristic morphology and staining, and with appropriate organisms, immunological reactions, each distinctive after its kind. It is perhaps unnecessary to repeat and to reemphasize here, that the multitude of details surrounding each organism necessitates separate consideration of them individually. These will appear at the proper time.

Nothing has been said thus far about the morphology of bacteria in the filterable state. It is possible to see changes which appear to lead to the filterable state by inoculating, for example, B. typhosus, into the K medium. Better and clearer preparations can be had in a "clear" K medium which can be prepared from the original medium, as will be shown elsewhere. After 15 to 18 hours' incubation in this medium, many of the typhoid bacilli, seen under the dark field, lose their homogenicity and appear first as faintly discernible shadows, having the bacillary outline, without, however, their luminous substance. They are actively motile at this time. Several brilliantly luminous, but small granules, from two to four or more, appear within the shadowy outlined organisms. The addition of specific typhoid serum to such cultivations causes agglutination, but the time required is decidedly longer than that necessary to elicit agglutination in parallel peptone broth cultures, which contain only typical bacilli. Upon retransfer and reincubation in the K medium, the bacillary forms become much smaller, and eventually for the most part are lost. Methylene blue stains made at this stage frequently reveal a multitude of very small, faintly blue staining rod-shaped bodies, enclosing more deeply stained slightly reddish granules. These shadowy bluish haloes encircling the more deeply staining central parts are reminiscent of the Rickettsia bodies, found in lice. It should be stated here, that a culture of Proteus, X19, obtained from a case of typhus several years ago, presents a very similar appearance, when cultivated in the same manner.

Finally, the bacillary part of the organism disappears, and there remain merely the very small granules, appearing under the dark field as intensely bright, yellow oval bodies. Of course the dark field furnishes no index of the true size of these granules, and but little idea of their shape. In this state, the granular vestiges of the typhoid bacillus pass an N Berkefeld filter readily. It may be recovered in the bacillary state by appropriate recultivation, as explained previously. Staphylococci similarly become smaller, and colored with methylene blue appear as oval, faintly staining haloes surrounding a granule. Upon longer incubation the nebulous halo disappears, leaving granules, which under the dark field are quite similar to those of B. typhosus. Streptococci tend to retain their linear alignment through the nebulous state, but appear to lose it when the granules are finally left as vestiges of the original organisms. Usually a proportion of the bacilli or cocci remain unchanged during the earlier stages of the process, and cultures at this period can usually be had in ordinary mediums, presumably originating from these unaltered or partly unaltered forms. Filtration, however, appears to sift these out. They remain behind, and direct cultivation of the filtered granules into nonfilterable forms in ordinary plain or dextrose broth is almost always impossible. A few days' growth in the K medium, however, will almost always afford successful transit to the typical organisms. This inability of the filterable granules to develop in ordinarily favorable cultural mediums, such as plain or dextrose broth, seems to afford some evidence of the efficiency of the filters as restrainers of the normal bacillary or coccus forms of the organisms. It should be emphasized here that the mere fact that there is failure to elicit growth of the filterable forms of these bacteria in ordinary peptone-containing media is not proof they are not viable. A *small* amount of broth containing these granules, added to the K medium, will usually result in growth, even though a week or even two weeks elapses from the time of filtration to the date of recultivation. Much longer periods of survival of these granules are met with. Upon two occasions, perfectly typical strains of Staphylococcus aureus were cultivated from Staphylococcus phage which was 10 months old. Cultivation in nutrient

broth of the same composition as the phage solution itself, however, was always negative. It is of some importance to note that the coccus which was isolated from the blood of case No. 3 on February 3 was re-isolated as late as June 12. The flask containing the blood had been in the ice box for about three months. A few loopfuls were placed in K medium, and after two weeks at 30 degrees, colonies were obtained upon transfer to agar plates. Inoculations at the time into ordinary mediums were negative. Apparently the protein ingredients of the extracted intestine, which are lacking in the peptone medium in which these filterable forms exist, furnish the conditions essential for growth of the granules which seem to constitute the non-filterable state of the organism. Whether there is, or may be, multiplication of these filterable granules in phage, Besredka "antivirus" or in peptone solutions, can not be answered definitely at this time.

One rather unexpected phenomenon intruded itself during these filtration experiments, for which no explanation can at present be given. It seems to be, quite in opposition to what common sense would predicate. Filtration experiments were conducted in general in accordance with this routine: the growth in the K medium, after proper development, was diluted with twice its volume of sterile, physiological saline solution, and filtered with very little vacuum; usually less than four inches below normal atmospheric pressure as measured by a vacuum gauge. The water-clear filtrate (which always showed granules, both of media, and organisms under the dark field), was distributed as follows: to dextrose or plain broth 2 cc and 0.25 cc respectively in 10 cc of medium. To the K medium, 2 cc, 1 cc, 0.5 cc, 0.25 cc and 1 drop respectively to 10 cc. Almost always, but not invariably, the tubes receiving 0.25 cc and 1 drop of filtrate from the K medium showed more rapid and better growth than those corresponding cultures containing the larger amounts of inocula from K medium. In no case thus far has growth ever been detected in the ordinary broth cultures, although sub-cultures of a few loopfuls of broth to K medium usually afforded growth eventually. This strange phenomenon has been noted so often and so consistently, it seems to be more than a coincidence. On the other hand, it seems to violate the law of diminishing returns. As an oft-repeated happening, it is proffered for what it is worth.

It is pertinent to inject here a few observations drawn from the field of bacteriology, which may have significance in light of what has been related.

Several types of infection are known in which organisms, at first demonstrable, disappear, even though symptomatology persists. Thus, in syphilis, the Treponema are clearly demonstrable in mucus patches, and in the initial lesion by dark field illumination. The organisms are rarely found in the spinal fluid in later stages, although reports of the presence of "granular bodies" in material from the cerebrospinal axis are in the literature. The ovoid bodies found by Noguchi and Flexner, and cultivated by them from cases of poliomyelitis, especially in light of the filterable nature of the material from which they were isolated, is certainly equally suggestive that these bodies are more than mere débris of media, even though current texts appear to have abandoned belief in the etiological relationship of these ovoid bodies to disease. Thus far it has been impossible to obtain an authentic culture of these ovoid bodies for study. It is very important to recall that Rosenow has repeatedly injected his streptococcus, isolated from poliomyelitis cases, into animals with the elicitation of symptoms. In many instances, especially when the animals survive for a few days before examination, it has been found difficult to demonstrate the injected organisms by staining methods, or even by culture. Reference is made at this point to the filterability of his streptococcus, of which he very courteously sent cultures, described above. It is not at all impossible to believe that the Rosenow organisms injected in the non-filterable state gradually change to the filterable state in presence of the brain substance, as previously explained. If this be true, cultivations from such animals in a K medium (or if necessary with K medium modified by substituting brain for intestine) should be successful.

Granules from cold (tuberculous) abscesses, and the appearance of Rickettsia bodies, has already been commented upon, as has the Besredka Antivirus (staphylococcus) and Staphylococcus bacteriophage. Many lesions occur in the tissues of man and of animals, in which bacteria can not be demonstrated by staining methods, and from which bacteria may or may not be isolated. It is not improbable that in many instances the same type of phenomenon of filterable and non-filterable stages of bacteria is involved in these. A culture of Leptospira icteroides (Arias strain), obtained through the courtesy of Dr. Sawyer, likewise proved filterable, thus apparently confirming Noguchi's contention that his spiral organism had a filterable stage. Blood from cases of yellow fever has not been available, consequently the rather obvious attempt to cultivate a non-filterable organism from the filterable "virus" of yellow fever has of necessity been postponed.

Granted that many bacteria, and by implication, many "filterable viruses" do have a filterable, and a non-filterable stage, the question arises, what determines the one state or the other? While a final an-

swer can not be given at this time, sufficient evidence has been gathered to formulate at least one procedure which has been uniformly successful in causing non-filterable forms of representative types of bacteria to become filterable, and after filtration, to be forced to pass back again into the non-filterable state. The phenomenon indeed appears to be quite general among the types of bacteria.

Thus far, emphasis has been laid upon the microbic side of this problem of bacterial filterability, and return to their non-filterable forms. Some important correlative information may be gleaned from a brief discussion of the conditions bacteria meet in the body itself, especially in relation to infection. Generally speaking, microbic infection of the body may be considered as taking place when the prospective invading microbe passes across the barriers which usually suffice to keep it out, and actually penetrates the protein fastness itself. A majority of bacteria, and probably a majority of "viruses" gain entrance through epithelia, principally those of the intestinal and respiratory tracts. A remarkable chemical difference between these two tracts should be emphasized here. The intestinal mucosa is almost constantly bathed in a medium rich in protein digestive products, which pass successively from highly complex peptones and albumoses to simple peptids and amino acids. The latter, so it appears, are normally absorbed through the villi of the intestine, and pass to the blood stream. Hence the mucosae of the intestinal tract are in a peptone environment, using the term "peptone" merely to indicate protein in various stages of digestion. The nutritive value of this medium is reflected in the luxuriance of the intestinal flora. The respiratory tract, and the oral cavity, on the other hand, especially the former, is by design a sterile tract. Absence of protein degradation products, except at those times when purulence is present, is the usual state of affairs. Whether mucus is of significance in this connection can not be answered in light of current information. Stated differently, the digestive tract is proteolytic, the respiratory tract is apparently aproteolytic. Bacteria that gain entrance to the digestive tract in the usual manner find abundance of protein derivatives for their nutrition, whereas bacteria that gain entrance to the respiratory tract do not normally find protein derivatives for their nutrition. Just what factors, singly and collectively, normally shield both these mucosae from bacterial invasion, are not well known, and for the moment they must be disregarded. The fact that deserves emphasis here is that bacteria within the intestinal canal are in an environment that should encourage their continued existence in the non-filterable state. Rather the contrary condition would appear to prevail in the respiratory tract. Perhaps in light of this, it is not without significance that many, if not most of the contagious so-called "filterable viruses" appear to enter, and to leave, the body through the respiratory path. Few, if any, are suspected of entering or leaving the body through the intestinal tract.

An interesting parallel is found in the observations presented above, namely, that the filterable state of bacteria can apparently be induced by cultivating them in the K medium, which by design is rich in substance approaching tissue protein in composition, and free from peptones and most alcohol soluble nitrogenous extractives. Bacteria that pass from the respiratory tube actually into the tissue of the lung, whether they are filterable or not as they enter the respiratory tract, become confronted with a protein-rich peptone-poor medium. From what has been stated, this is one condition which tends to induce and perpetuate the filterable state. Similarly, upon passing back from the lung tissue to the respiratory tube, the same condition prevails, unless there is purulence, which, as is well known, is associated with protein digestion. Therefore, filterable states of bacteria should theoretically be not uncommon in infections of the respiratory tract, in absence, of course, of pus formation. Perhaps it is unnecessary to reiterate that many, if not most of the "filterable viruses" are found in association with the respiratory, rather than the intestinal tract. Bacteria leaving the body through the intestinal tract are exposed to nutritive conditions conducive to the non-filterable, rather than the filterable state. Such appears to be the case in light of current information. This is not the whole story, however. The fact that bacteria may pass readily into the filterable state does not explain just why bacteria, even in this state, can induce infection. Colon bacilli and typhoid bacilli, as has been shown above, are readily filterable, when grown in presence of protein and absence of peptone, but colon bacilli do not, except relatively rarely, invade the body. The nature of the weapons with which certain kinds of bacteria force entrance through intact epithelia, which ordinarily suffice to keep microbes out, is still to be determined.

Not enough work has been done to date to be dogmatic, but sufficient experimental evidence has accumulated to show quite clearly that the bacteria studied can be made to pass rapidly and readily to the filterable state by cultivation in the K (protein) medium, and what is perhaps equally significant, the converse is true, because several successful cultivations of bacteria of various types have been made from the blood stream in cases of influenza, common cold, arthritis, rheumatic fever, measles, and other

pathological states, where bacteria have for a long time been confidently expected to exist, but from which cultivations in artificial mediums thus far have been rather regularly negative. Applying the same reasoning with experience gained with this K (protein) medium to infections in the body, it seems not unreasonable to believe that as soon as bacteria as they are known in culture (the non-filterable state) pass into the tissues through the barriers that ordinarily suffice to keep them from the tissues, they are in a protein environment, which is free, or nearly so, from peptones and similar products of protein disintegration. Under these conditions, bacteria may be expected to change from the non-filterable to the filterable state. In this state their migration from place to place might reasonably be facilitated, and what is equally significant, their existence in the granular or a filterable state would probably explain why it is that bacteria as such are so infrequently demonstrable by stain or by culture in the body tissues except where pus collects. Pus is rich in degraded protein. Possibly an interesting exception may be leprosy, where, as is well known, large numbers of stainable bacilli may be found in nodules devoid of evidence of local proteolysis. In arthritis, and many other long drawn out microbic processes, culture and stain alike have usually failed to furnish unequivocal evidence of the presence of microbes, yet in the few cases studied in this series, using a medium adapted to the growth of these filterable forms, bacteria have been grown, where in the past sterility has been the rule. It should be emphasized that even in these cases, referred to above, cultivations in the ordinary mediums, even enriched with blood, have usually been wholly in vain. The objection may well be raised that the successful cultivation of organisms from a very few such cases mentioned above is not necessarily an indication of etiological relationship. Bacteria might be present more or less normally in the blood stream. It may be remarked, parenthetically, that if such be the case, the fact is well worth further investigation. Normal tissues from animals are notoriously bacteria infested. In this connection also it may be pointed out that organisms have been cultivated from two breast carcinomas rich in lymphoid tissue, and one lymph gland adjacent to a rectal carcinoma. In these it would appear reasonable to suspect however that the regional lymph glands have merely strained out the bacteria that happened to be brought to them from time to time. Even tubercle bacilli have been found in lymph glands without evidence of infection.[9] However, bloods taken from nine normal persons have been most carefully examined as controls upon this point. They were all free from cultivable bacteria.

The problem of immunity, in light of these observations, takes on a new aspect. On the one hand, the beneficial effects reported during the use of phage and Besredka Antivirus for therapeutic purposes would seem to be related, not to enzymes or toxins, but rather to the presence of viable, filterable stages of bacteria, although, of course, enzymic and toxic effects are not disproven by any means. Experience with vaccine virus and rabies vaccine, on the other hand, shows very clearly the protective efficacy of living, but (thus far) uncultivable "viruses," whose virulence has been purposefully profoundly altered by growth in other animals for sufficient time to change the host infectiveness. Suggestions of the mechanism of alterations in host infectibility seem to be foreshadowed in some of the filterable forms of bacteria studied in this series. It has repeatedly been noted, for instance, that filterable typhoid bacilli, cultivated serially in K medium prepared from hog intestine, grow scantily and slowly for several transfers in corresponding K mediums prepared in the same manner from dog or rabbit intestine. Later, when culture acclimatization has taken place, it is rather difficult to pass the cultures in the filterable state back to hog intestine medium again. A more rapid procedure of adaptation is to start with the typical, non-filterable typhoid bacillus, and inoculate directly from ordinary mediums to hog, dog, or rabbit intestine mediums as desired. This is perhaps a cultural analogue to the well-known passage of human smallpox virus through a series of heifers to the vaccine virus state, or the passage of dog rabies through rabbits to the virus fixé state. However, it should also be noted that some bacteria which grow fairly readily in a hog intestine K medium grow badly, or even not at all in dog intestine, and vice versa.

The relation of the filterable forms of bacteria to phagocytosis, and to serologic immunity, have thus far not been studied at all. A large amount of work will have to be done to cover the multitude of problems that immediately arise from this dual stage of microbic existence.

A word of caution must be interjected. It is absolutely necessary to be assured of sterile mediums. A double autoclaving has been found satisfactory for the K medium, but this can not be practiced with egg white. It coagulates. Egg white, filtered through Berkefeld W filters (after dilution with sterile physiological saline solution) is rarely sterile. Bacterial growth can usually be obtained from such egg white filtrates, although otherwise, fresh egg white filtered and diluted with Tyrode solution is a rather good

[9] See Trudeau and Krause: *J. Med. Research*, 22: 277, 1910.

medium for the elicitation of filterable forms of bacteria. This is again evidence, it would seem, of the existence of filterable, viable forms of bacteria in purely protein solutions. And not only must mediums for the cultivation of the filterable states of bacteria be sterile; material from the human or animal body, and even cultures of bacteria themselves, in light of what has been stated, must be thought of as having possibly two or more distinct organisms living side by side. It has long been known, for example, that the virus of foot and mouth disease can be carried along with vaccine virus for considerable periods, and there is therefore the possibility that blood cultures may harbor more than one organism. In the foot and mouth disease-vaccine association, and in the filterable virus group generally, the protein which is usually associated, either from the body or in the artificially enriched medium, might suffice to keep the viruses in the filterable state, in which instance their presence would be readily overlooked. The details of recognition and separation of such complexes will naturally vary with individual cases.

Bacteria also may be similarly associated: there is some evidence already indicating that a filterable state of one microbe may be actually carried along, unrecognized, with a stock culture of another microbe, especially if transfers are made upon plain, nutritive meat infusion agar. One form may remain hidden, and entirely unsuspected until cultivation is practiced for a period of time in K medium. Reestablishment of the non-filterable form may reveal two organisms, where only one was presumed to be present.

Finally, a word about mediums. It is very obvious that the K medium, even with its variants as suggested above, is by no means the best that may be concocted. The same tissue from different animals affords, in specific instances, some differential advantages for specific cultivation of one or another microbe; therefore different mediums should be available. Reasoning from analogy, with some experimental evidence also, it would seem to be consistent to utilize homologous tissue from homologous species for difficult isolations. Also, within limits which vary with the type of microbe, there is advantage in training them to develop in alien host tissue protein for purposes of immunization and experimental infection.

SUMMARY AND CONCLUSIONS

1. The isolation of a filter-passing diplococcus from the blood of certain cases of influenza by means of a special cultural medium is described. The experimental effects of this organism, while in the filterable state, upon rabbits, is discussed.

2. A procedure is formulated for inducing at will both a filterable and a non-filterable state in bacteria. Mention is made of a series of experiments in which both the filterable and the non-filterable state has thus been induced in a series of well-known bacteria comprising a variety of types.

3. It is postulated that a majority, if not all, known bacteria can and do exist in a filterable and in a non-filterable state.

4. A preliminary report of the isolation of microbes in the blood, not only of cases of influenza, but also from common cold, rheumatic fever, arthritis, from Staphylococcus bacteriophage and Besredka's Staphylococcus Antivirus is presented in evidence of the ubiquity of the procedure.

5. An explanation of the chemical basis for the existence of bacteria, both in the filterable and non-filterable states, in the animal and human body, and in culture, is proffered.

6. The relation of this chemical concept to microbic infection, and the state of microbes in the body during infection is discussed.

In conclusion, it is a privilege as well as a pleasure to inscribe here my appreciation for the courteous cooperation of Dean Irving S. Cutter, Doctors Charles A. Elliott, Paul Starr, James G. Carr, Walter Nadler, Howard Alt and Herbert Barker, of the department of medicine, and to Northwestern University for the generous facilities and unrestricted opportunity for research which have contributed immeasurably to this investigation.

ARREST OF GEOLOGIC, ARCHEOLOGIC AND PALEONTOLOGIC WORK IN CENTRAL ASIA

By President HENRY FAIRFIELD OSBORN
AMERICAN MUSEUM OF NATURAL HISTORY

A PEIPING society originally known as the Cultural Society, but now more definitely organized as the Commission for the Preservation of Antiquities, on June 3, 1931, addressed the following letter to Dr. Roy Chapman Andrews in reply to an application of May 5, 1931, for the continuation of the American Museum explorations and researches in Mongolia:

Sir:

We beg leave to acknowledge the receipt day before yesterday of your note stating that members of your