THE DIFFERENTIATION OF LIVING FROM DEAD BACTERIA BY STAINING REACTIONS

FREDERICK P. GAY AND ADA R. CLARK

Department of Bacteriology, Columbia University, New York

Received for publication May 11, 1933

Apparently the first efforts to differentiate living from dead bacteria by staining reactions were made in connection with Metchnikoff's theory of phagocytosis which was combated in its early phases by German authorities on the ground that phagocytes were only scavengers which, although capable of ingesting dead bacteria, were not endowed with the ability to include and destroy living microörganisms. Metchnikoff himself showed as early as 1887 that anthrax bacilli when ingested by phagocytes gradually acquired, coincident with their disintegration, the property of staining with Bismarck brown (Vesuvin) whereas living bacteria outside the cells and in the initial stages within the cells failed to take this dyestuff.

In 1895 both Mesnil and Bordet showed that the natural affinity for methylene blue of living bacteria, outside the phagocytes and, in their initial stage of ingestion, inside the phagocytes, was replaced by an avidity for alcoholic eosin under the influence of phagocytosis. Plato found that bacteria which failed to stain with neutral red outside phagocytes take this dyestuff when ingested. This latter reaction is not, however, a criterion of death but is due rather to the acid digestive vacuole that surrounds all ingested particles within a leucocyte.

All these studies although interesting from the standpoint of the mechanism of phagocytosis give little information as to differences which may exist between living and dead bacteria outside phagocytic cells. In fact, Bordet noted specifically that the destruction of bacteria by means of an immune serum did not lead to change in staining reaction as tested by the eosin-methylene-blue combination.

Congo red, in the experience of Henrici, stains dead but not living bacteria and Seiffert has found that gentian violet in the presence of serum or deutero-albumose does likewise. In this latter case the protein apparently prevents the entry of the stain into bacteria except when they are rendered more permeable by death. Methylene blue has also been used with indecisive results (Fulmer and Buchanan; Bickert) in differentiating living from dead bacteria. This dye has also been employed in combination with neutral red (Ruzicka).

Of all stains employed in differentiating living from dead bacteria the most reliable although perhaps the least well known is the one proposed by Proca in 1909 and subsequently advantageously modified by Kayser. This procedure in the hands of the latter investigator involves a somewhat prolonged staining with Loeffler's methylene blue, followed by rapid exposure to dilute Ziehl-Neelsen's carbol fuchsin.

RELIABILITY OF THE PROCA-KAYSER STAIN AS INDICATIVE OF DEATH OF BACTERIA, YEASTS AND SPORES

The Kayser modification of the Proca stain consists in separating the two elements combined in the original stain and applying first, for from three to five minutes, Loeffler's alkaline methylene blue to a specimen that has been fixed in the ordinary manner by drying and flaming, followed by the application to the drained and rapidly washed specimen of a dilute (1:10) solution of Ziehl-Neelsen's carbol fuchsin for five to ten seconds only, followed by rapid washing in water. The time relations of this process are extremely important, although certain substitutes in the way of dyes to be used and the order of their application may be made within certain limits, as will be later discussed. It should be specifically pointed out that living bacteria dried and fixed in this fashion are still viable and serve as controls to bacteria that have been killed by the various methods to be enumerated (Ficker).

We have studied a considerable range of both Gram-negative and Gram-positive bacteria, including Streptococcus, *Bacillus anthracis*, *Diplococcus pneumoniae*, *Coryn. diphtheriae*, Staphylococcus, the avian tubercle bacillus and yeast (*Saccharomyces cere-*

visiae) among the Gram-positive forms, and *Vibrio cholerae*, *Ebert. typhi*, *Esch. coli*, *Klebs. pneumoniae* and *Bacillus proteus* among the Gram-negative forms.

The nucleated red blood cells of the pigeon, chicken and incidentally some other nucleated cells, such as those of the alligator, have been employed to demonstrate certain apparent relationships to bacteria, as will be later related.

The effect of death on the staining reactions of these bacteria has been extensively and thoroughly studied and on the supposition that the method of killing might have some effect on the result produced, the cultures have been killed in a number of different ways. Thus, the killing of bacteria by heat, by formalin, by phenol, by $HgCl_2$, by gentian violet and by saponin, has been carefully studied, each with several of the forms enumerated above. Destruction of bacteria by means of normal serum as exemplified in the destruction of *B. anthracis* by normal rabbit serum which is due to the thermostable beta-lysins of Pettersson, and also the specific destruction by immune serums of the cholera vibrio and of the typhoid bacillus have been investigated. In cholera the destruction by an immune serum is recognized to involve visible disintegration (Pfeiffer's phenomenon) whereas in the majority of other bacteria, the cell may remain apparently intact though dead. Another form of bacterial destruction is that produced by means of a specific bacteriophage and in this regard the staphylococcus, *Coryn. diphtheriae* and *Esch. coli* have been investigated. It is well recognized (Bronfenbrenner, Bayne-Jones and Sandholzer) that phage action involves a swelling and granular disintegration that is not unlike that present in Pfeiffer's phenomenon. Investigations in this laboratory (Stone and Hobby) indicate that destruction of the diphtheria bacillus takes place principally when the culture is in its coccoid stage.

Death produced by any of the methods that have been outlined results in a change of staining reaction in the Proca-Kayser method, that is to say, whereas living bacteria in controls stain blue they tend to take the red stain after death. This color shift varies in degree and clearness and various stages of change appear ranging from the original blue to purple to pink to red, depending

not only on the organism that is involved but on the surrounding medium in which it is suspended when subject to the lethal influence, and possibly on the rapidity of death. Some of these variable factors in the staining method will be specified, but it may here be categorically stated that there is a change produced in bacterial death which is invariably in the direction of a shift from blue to red, and which follows death by whichever of the methods employed. It is scarcely necessary to state that the death of the bacteria is in every instance authenticated by cultures which are sterile or which show a greatly decreased number of organisms.

Among the limitations of the method the following should be noted:

Early cultures of bacteria, for instance typhoid bacilli, grown for only five hours frequently show many pink organisms instead of uniformly blue forms.

Cultures of from forty-eight to seventy-two hours of certain organisms, as for example anthrax and proteus, show many pink forms.

Suspensions or cultures that have stood in the icebox for a considerable period of time, although many may be living, show many pink organisms.

We do not regard these exceptional pink forms as indicating any failure of accuracy in the method, but simply as indicating that the cultures concerned actually contain a number of dead organisms.

These remarks may be amplified by two examples which to us are particularly illustrative as to what these apparent exceptions mean. We have studied rapidly desiccated cultures of three organisms, the bacillus of anthrax, the pneumococcus and the typhoid bacillus. Previous studies have assured us that although such dried preparations are living when tested by subculture (Gay, Atkins, and Holden), the great majority of the bacteria, in fact from 91 to 93 per cent, have been destroyed, and we are not surprised to find that such preparations contain a majority of pink forms with an exceptional blue one. *Bacillus anthracis* from stock cultures, uncapsulated and without spores in the early

stages of its growth (four to six hours in saline suspension from agar) is invariably a blue-staining organism. When examined in agar cultures aged from twelve to eighteen hours, prespores are visible and unstained inside the rods which are stained purple or pink. These latter findings were interpreted to mean that the vegetative phase of the cell is dead as soon as the spore is formed, a conception which we believe is new but wholly consistent with the accepted explanation as to the mode of formation of anthrax spores from the chromatin granules present in the cytoplasm (Eurich and Hewlett; Bayne-Jones and Petrilli).

Saline suspensions from agar cultures give better results both with living and dead forms than do bacteria grown in broth cultures. Bacteria killed after suspension in distilled water in which they are originally blue in color may not show as definite a transformation to the red forms as when suspended in saline. The presence of serum in the suspension tends to prevent the transformation to red forms after death. The granules of the cholera vibrio in Pfeiffer's phenomenon are purplish rather than red, a partial transformation which may be due to the rapidity of death.

The staining of spores by the Proca-Kayser method presents a special and interesting problem in itself which yields results that differ from those obtained with vegetative bacterial cells. As Proca and Danila have already described, living anthrax spores (presumably extra-cellular) fail to take the stain, whereas dead spores stain blue. Our experience would modify their statement only in saying that living anthrax spores whether intracellular (prespores) or extracellular, fail to stain by the Proca-Kayser method, or at best show a faint bluish areola. This areola may be modified by a pinkish adherent rim of dead vegetative protoplasm. Blue-stained spores are rarely seen (dead?) under normal conditions. When, on the other hand, anthrax cultures are autoclaved, the spores stain a deep blue as contrasted with the red of dead vegetative forms.

In our experiments the spores of *B. subtilis* reacted somewhat differently from those of anthrax. In the first place, the spores are more ovoid in appearance than those of anthrax and in the presumably living condition, more spores are stained uniformly

blue. In autoclaved cultures the spores are stained blue although not so intensely as are those of *B. anthracis*.

In general, the spores of anaerobic bacteria, so far as can be judged from cultures of *Clos. tetani*, *Clos. sporogenes* and *Clos. histolyticum* are in agreement with the results obtained with the anthrax bacillus except as regards their intracellular position.

ON THE MECHANISM OF, AND VARIATIONS, IN THE PROCA-KAYSER STAINING REACTION

The study of staining methods has been of enormous significance not only in its relationship to bacteriological identification and taxonomic distinctions, but as bearing on the fundamental questions of bacterial structure and bacterial physiology. It is the Gram stain in particular which has served as an extensive theme of study for many investigators whose various conclusions we do not plan to mention here. Furthermore, the penetration of bacteria by stains bears an intimate relationship to the process of disinfection by dyes and to dye therapy. And, again, the question of vital staining both of bacteria and of animal cells is obviously dependent on the use of bacterial stains. The changes in protoplasm that typify a change from life to death have largely been based on a study of dye absorption, as for example in the experiments of Kite and of Lepeschkin.

We have considered the indications afforded by the Proca stain, and by other stains to be mentioned, as regards their mechanism and their significance in representing changes in bacterial protoplasm in death as contrasted with life. The first impression that one would gain from the original methylene blue-eosin contrast stain of Bordet is that the change in avidity for one or the other of the dyestuffs involved is due to a crude change in the reaction of the bacterial protoplasm, that is to say, whereas living bacteria have an acidic protoplasm that naturally unites with basic dyes, the reaction changes with death toward a basic reaction which makes them more eager for an acid dye. Although this might seem in agreement when eosin is used as a counter stain, it would not apply to the use of carbol fuchsin, which is a basic dye, in the Proca stain.

In general the pH of the suspension medium, so long as it is on the alkaline side of the iso-electric point of most proteins, is not a factor in this shift of staining reaction, although some degree of interference with the development of a typical red color occurs when bacteria are killed by heat in buffer solutions at lower pH levels (4.3; 5.3). Similarly, since both dyes in the stain mixture are basic, i.e., since the cation is the colored portion of the molecule in each case, we cannot look upon this differential staining as resulting from reversal of electric charge during the process of death.

The shift from blue to red in the Proca-Kayser staining cannot be fully explained until the chemical changes incident to death are more fully understood. Since the effect of an alteration in pH or of reversal of electric charge on the staining substrate does not seem to operate, we may regard the reaction as incident to the denaturation of nucleoproteins during death, whereby, because of alteration in the number or kind of reactive groupings of the denatured protein molecules, affinity for both the methylene blue cation and the fuchsin cation is reduced. Fuchsin can more readily replace methylene blue owing to its greater staining ability and the greater permeability of dead bacterial protoplasm.

As bearing on this question and also as to other stains that might be employed, table 1, showing possibilities which give as good or nearly as good results as the original alkaline methylene blue followed by Ziehl-Neelsen's carbol fuchsin, is offered. Many other dyestuffs have been tested both as primary and as secondary stains with negative results. Those dyes which are included in parentheses give partial but not clear cut results in a shift from the blue after death. Carbol fuchsin, aqueous basic fuchsin and safranin are all equally good as contrast stains after methylene blue. Apparently the only dye which is as good as alkaline methylene blue is saturated aqueous methylene blue. Toluidine blue and thionin, although they give fairly good results when used as a primary stain, are distinctly inferior to the two varieties of methylene blue.

The stains as applied in the Proca-Kayser method do not of course indicate that dead bacteria will not stain with methylene

blue. As a matter of fact, we find that dead bacteria stain *more faintly* with methylene blue than do living bacteria. This should not be confused with a somewhat generally accepted fact (Eisenberg, Nicolle, Seiffert) that dyes *penetrate* dead bacteria better than they do living ones; nor again with the fact that certain dyes are reduced by living as contrasted with dead cells.

And, on the other hand, carbol fuchsin will stain living bacteria as well as dead ones. Its selective property of replacing methylene blue in dead, as contrasted with living bacteria, is apparently due to the fact that the dead cells are less deeply stained by the primary stain than are living ones. In fact, carbol fuchsin will

TABLE 1

Possible modifications of Proca's stain

PRIMARY STAIN	SECONDARY STAIN
Loeffler's methylene blue	Carbol fuchsin
Loeffler's methylene blue	Aqueous basic fuchsin
Loeffler's methylene blue	Safranin
Loeffler's methylene blue	(Bismarck brown)
Loeffler's methylene blue	(Alcoholic eosin)
Saturated aqueous methylene blue	Carbol fuchsin
(Toluidine blue)	Carbol fuchsin
(Thionin)	Carbol fuchsin

replace methylene blue even in living bacteria if applied for from fifteen to thirty seconds instead of five to ten. This technical difference is one of the points involved in correct application of the method itself.

The order of staining cannot be reversed, that is to say, if carbol fuchsin is applied first to dead bacteria, followed by methylene blue, they stain pale blue instead of pink or red. In other words, the carbol fuchsin replaces the methylene blue in dead bacteria apparently because dead protoplasm has failed to retain the original dye as well as when alive and because the fuchsin is recognizedly a much more active and rapid dye stuff for bacteria.

NEUTRAL RED AS A DIFFERENTIAL STAIN FOR LIVING AND DEAD BACTERIA AND FOR ANIMAL CELLS

We have already referred to the work by Plato which showed that bacteria that have been ingested by phagocytes are stained red whereas they are unstained outside the cell. Metchnikoff, although he mentions this reaction as indicative of the destructive effect of the process of phagocytosis on living bacteria emphasized that it indicates simply the formation of an acid digestive vacuole about the organism. In other words, it does not mean that the ingested bacteria are dead and the stain is not in any degree analogous to the Proca stain. A dilution of neutral red in saline solution of 1:8000 is almost invariable in its reaction on bacteria, in accordance with whether they are inside or outside phagocytes. The cells remaining in the surrounding fluid whether they be living or dead usually fail to take any stain or at most show a faint brownish, yellow color. Nor does it make any difference whether such bacteria are treated with normal or immune serum. There is one exception, in that anthrax bacilli outside of cells stain a faint pink in about 5 per cent of their numbers which probably indicates that they are becoming acid, which may or may not mean that they are about to undergo degeneration. On the other hand, bacteria of various kinds that have been taken up by phagocytes, whether living or dead, although at first unstained, rapidly take on a distinct brownish-red coloration provided that the cells in which they are ingested are still alive, as is indicated by the fact that they do not take a diffuse pinkish coloration with the red but may show fine granules (polymorphonuclear cells) or segregation vacuoles (clasmatocytes). We have studied this process both in the animal body and in the test tube and with exudates obtained from rabbits and from guinea pigs, and with exudates containing large proportions of polymorphonuclear or of mononuclear cells, with the results that have already been briefly summarized.

It will be recalled that both Mesnil and Bordet used a compound staining method consisting of methylene blue followed by alcoholic eosin to indicate the destruction of bacteria within

phagocytes, assuming that the dead microörganisms alone stain with eosin. In our experience the Proca stain is distinctly better but with neither of these staining methods have we obtained uniformly demonstrative results of the intraphagocytic death of bacteria. Red organisms can, to be sure, be found after varying intervals among the bacteria that have been phagocyted but they are difficult of demonstration as bearing on the universally accepted purpose of phagocytosis. We do not mean these remarks to indicate any invalidity in the generally accepted hypothesis that phagocytes do actually destroy living bacteria but simply that an initial change in staining reaction as preceding disappearance of bacteria within phagocytes is hard to demonstrate.

The yeast cell offers an interesting intermediary organism between bacteria and animal cells as regards staining by neutral red. Yeast cells take up neutral red in small amounts in their vacuoles. When yeast cells have been killed or when they die as a result of ingesting too great a concentration of neutral red, they become diffusely stained. This must mean that dead yeast cells, in contrast to dead bacteria, become definitely acid in reaction.

The effect of neutral red on nucleated red blood-cells

The reaction of bacteria within phagocytes to neutral red suggests interesting analogies with the staining of the nucleus of nucleated red blood-cells by this same dyestuff. We have made certain observations with alligator cells and have particularly studied pigeon and chicken red cells. The reaction of nucleated red blood cells to neutral red raises acutely the question of the existence of a true "vital" stain, whether for bacteria or for animal cells, in contradistinction to a staining which simply represents a stage in a process of injury. Although the experiments of Churchman would indicate that certain Gram-negative bacteria are alive and motile, after staining with gentian violet, and similar results are claimed for animal cells in several articles reviewed by Möllendorff, and in the experiments of Churchman and Russell with paramecium, the question of injury must still remain a debatable one. For example, we find under conditions specified below that dilutions of neutral red give a perinuclear dot stain in

chicken or pigeon cells in dilutions as great as 1:2,000,000, and stain the whole nucleus uniformly when used in concentration of 1:4000 to 1:8000. Neutral red hemolyzes and agglutinates these same cells in dilutions of from 1:400 to 1:1000, depending on the specimen of dye and the particular cells in question.

Although stated thus categorically the nuclei of red blood cells are not always stained by neutral red as indicated for reasons that are by no means clear. Thus, when saline washed cells are suspended in mixtures of isotonic (0.85 per cent) saline and serum, or in broth (pH 7.4) they do not stain; when washed and suspended in saline they do stain. Since we know that unbuffered saline soon becomes acid on standing ($\pm$ pH 6) it was natural to assume that staining depended on the acidity of the medium. But when phosphate-buffered isotonic saline solutions ranging in pH from 4.3 to 8.5 were employed, the nuclei stained in all although more markedly in the more acid range. It is apparent by staining fixed preparations that the cells in the acid range of these buffered isotonic solutions are injured without presence of neutral red, but the reaction alone does not account for staining by neutral red in saline buffered at pH 7.4. They apparently are still injured when neutral red sufficient to stain the nuclei is added. It seems probable that the living red blood cell, particularly when suspended in the most favorable menstrum (broth or serum) reduces a certain amount of neutral red that may penetrate the cell. When the cell is injured by acid the dye penetrates more readily. At all events, staining of the nucleus by neutral red would seem to be a reaction of injury rather than a true vital staining. Nucleated cells when killed and fixed with formalin do not stain with neutral red.

These and many other staining experiments were originally begun in an attempt to find some staining reaction which might be indicative of the sensitization process produced by immune serum but in this respect they have been completely negative.

We are interested here in comparing this, and other staining methods applied to nucleated red blood cells, either in their natural condition or after hemolysis by various methods, as compared with their effect on dead bacteria to which these red blood

cells are to some extent analogous. For this purpose red blood cells have been hemolyzed by a specific immune serum, or rather a specific immune serum heated to 56° and reactivated by guinea pig alexin, or else laked by the addition of ether, or water, or saponin. Cells destroyed by any one of these methods present with minor variations the same appearance, that is to say, they lose their hemoglobin and the cytoplasm disappears more or less completely. It is characteristic of all methods of hemolysis, however, that the nuclei remain relatively intact for longer periods of time although they tend to swell and become otherwise distorted. When lysed nucleated blood cells of the pigeon produced by the various methods enumerated are stained with neutral red the swollen nuclear material surrounded by fragments of cytoplasm fails to take the dye in the characteristic fashion. In other words, it has lost its power of natural staining with neutral red, being described in our protocols as "tinted" instead of reddish. In other words, the remnants of killed (laked) blood cells change in their reaction to neutral red whereas bacteria when killed by the methods we have enumerated do not; the latter are still unstained outside the cell and are stained by neutral red almost as well as are living bacteria within the phagocyte.

When hemolyzed nucleated pigeon cells are stained by either the Proca or the Wright stain, they show on the other hand a complete analogy to the change in staining affinity demonstrated by bacteria. Whereas the original intact cells of the pigeon show a blue nucleus with either Wright or Proca stain, and a coppery red cytoplasm with Wright stain and a yellowish protoplasm with Proca stain, when hemolyzed the nuclear remnants change from blue to pink as do bacteria.

Red blood cells are known not only to be phagocyted but to be destroyed in phagocytes. It is known that macrophages exercise a selective action in this regard as contrasted with polymorphonuclear cells which are concerned primarily in disposing of bacteria. It has perhaps been taken for granted that the destruction of red blood cells within phagocytes is essentially a process of hemolysis but a careful study of the matter by these staining methods in which we are interested indicates certain distinctive differences

from extracellular hemolysis which are of interest. When examined by the Proca or Wright stain, nucleated pigeon cells in macrophages of the rabbit or guinea pig gradually disintegrate through a shrinking of the cytoplasm and swelling of the nucleus which latter part of the cell becomes a brownish pink in color in contradistinction to the normal blue. When examined by the neutral red stain it is seen that the cell cytoplasm, which is somewhat brownish yellow in color within the phagocyte, slowly shrinks. The nucleus, at first unstained, takes the neutral red as it does in saline suspension and gradually swells but continues reddish in color as contrasted with what happens to it outside the cell where it finally fails to stain.

SUMMARY AND CONCLUSIONS

The Proca-Kayser differential stain, applied to living as contrasted with dead bacteria, has been extensively studied and its accuracy confirmed. Provided certain specified conditions as to order and duration of application of each of the stains involved (methylene blue then carbol fuchsin) are observed, the stain clearly indicates whether the bacteria were alive (blue stained) or dead (purple to red) when placed on the slide on which they are subsequently dried and fixed by heat, in the usual fashion. The apparent exceptions, in failure of a shift in staining on the part of individual organisms, are found to be simply confirmatory of the accuracy of the method.

Death as produced by physical or chemical agents, by bacteriophage, or by serum results in each instance in a similar shift in staining.

Gram-positive and Gram-negative bacteria, acid-fast bacteria and yeast all show a similar shift in staining reaction. The distorted but relatively resistant nucleus of nucleated red blood cells hemolyzed in different ways shows a corresponding shift from blue to red when stained by the Proca method.

Living bacterial spores, whether intracellular or extracellular, take the faintest if any stain by the Proca method; when dead they stain blue.

Certain substitutions in either the primary or the secondary dye

stuff employed in the Proca-Kayser method are possible as explained in the text.

We have met with little success with the methylene blue-eosin contrast stain employed by Mesnil and by Bordet to show similar changes in bacteria within phagocytes. The Proca stain, although better, fails to show convincingly that phagocytes actually change live to dead bacterial protoplasm.

The precise nature of the change that bacterial protoplasm undergoes in death still eludes us. It is not simply a crude change in reaction of the bacterial protoplasm or reversal of electric charge in the staining substrate. Apparently changes in permeability of the protoplasm and in the reactive groupings of the protein are involved.

Neutral red fails for the most part to stain bacteria until they become ingested by phagocytes. The intracellular stain is not however indicative of bacterial death but rather of the acidity of the digestive vacuoles. The nuclei of chicken or pigeon red blood corpuscles also take neutral red in great dilution. For reasons that are given this staining reaction is interpreted as indicative of injury to the cell rather than as a true vital staining.

REFERENCES

Bayne-Jones, S., and Petrilli, A. 1933 Jour. Bact., **25**, 261.

Bayne-Jones, S., and Sandholzer, L. A. 1933 Jour. Exp. Med., **57**, 279.

Bickert, F. W. 1930 Centralbl. f. Bakt., Orig., **117**, 548.

Bordet, J. 1920 Traite de l'Immunite. Paris, Masson et Cie, 190.

Bordet, J., and Gay, F. P. 1909 Studies in Immunity. Wiley and Sons, New York, 33.

Bronfenbrenner, J. 1928 "Bacteriophage," Newer Knowledge of Bacteriology. Jordan and Falk, University of Chicago Press, 525.

Churchman, J. W. 1912 Jour. Exp. Med., **16**, 221.

Churchman, J. W., and Russell, D. G. 1913–14 Proc. Soc. Exp. Biol. and Med., **11**, 120.

Eisenberg, P. 1913 Centralbl. f. Bakt., Orig., **71**, 420.

Eurich, F. W., and Hewlett, R. T. 1930 British System of Bacteriology, National Research Council, H. M. Stationery Office, London, **5**, 439.

Ficker, N. 1929 Handb. der path. Mikroorgan. Kolle, Kraus und Uhlenhuth, third edition. Gustav Fischer, Jena, **9**, 793.

Fulmer, E. I., and Buchanan, R. E. 1923–4. Jour. Gen. Physiol., **6**, 77.

Gay, F. P., Atkins, K. N., and Holden, M. 1931 Jour. Bact., **22**, 295.

Henrici, A. T. 1923 Proc. Soc. Exp. Biol. and Med., **20**, 293.

Kayser, H. 1912 Centralbl. f. Bakt., Orig., **62**, 174.

Kite, G. L. 1915 Amer. Jour. Physiol., **37**, 282.
Lepeschkin, W. W. 1931 Quart. Rev. Biol., **6**, 167.
Lepeschkin, W. W. 1932 Amer. Jour. Bot., **19**, 568.
Mesnil, M. A. 1895 Ann. de l'Inst. Pasteur, **9**, 301.
Metchnikoff, E. 1887 Ann. de l'inst. Pasteur, **1**, 321.
Metchnikoff, E. 1901 L'Immunité. Paris, Masson et Cie, pp. 87, 176, 191, 208.
Möllendorff, W. 1920 Ergeben. der. Physiol., **18**, 141.
Nicolle, M. 1903 Bull. de l'inst. Pasteur, **1**, 137.
Plato, J. 1900 Arch. f. Mic. Anat., **56**, 868.
Proca, G. 1909 Compt. rend. Soc. de Biol., **67**, 148.
Proca, G. and Danila, P. 1909 Compt. rend. Soc. de Biol., **67**, 307.
Ruzicka, V. 1905 Pflüger's Archiv. f. Physiol., 107, 497.
Seiffert, W. 1922 Centralbl. f. Bakt., Orig. **88**, 151.
Stone, F. M., and Hobby, G. Unpublished communication.