FURTHER STUDIES ON PROTEUS HYDROPHILUS, THE ETIOLOGICAL AGENT IN "RED LEG" DISEASE OF FROGS

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INTRODUCTION AND HISTORICAL REVIEW

The frog has long been used as an experimental subject in various fields of research, yet rarely have reports been published concerning diseases to which it is naturally susceptible.

The first investigations on septicemic diseases of frogs were apparently those of Ernst (1890), who reported the isolation of a bacterium from the blood of infected frogs.

A year later Sanarelli (1891) described an organism isolated from frog's blood. He named it *Bacillus hydrophilus fuscus*. He did not believe it to be identical with that reported by Ernst. Sanarelli found that *Bacillus hydrophilus fuscus* was pathogenic for such cold-blooded animals as the frog, toad, salamander, lizard, sunfish and the fresh water eel. He also observed that injection of this organism into warm-blooded animals (guinea pig, rabbit, dog, cat, mouse, chicken and pigeon) resulted in death within a relatively short time. He described the growth of the bacillus on plain agar, glycerol agar, gelatin, blood serum and potato, and suggested that its natural habitat is water, since he found it to be present in two of twenty-six water supplies examined by him.

In 1893 Trambusti isolated what proved to be the same species from diseased frogs in his laboratory. His investigations of *Bacillus hydrophilus* were primarily concerned with the isolation of certain toxic metabolic products produced by it. He concluded from his experiments that the toxic products were of two kinds, one soluble and the other insoluble in alcohol. He made an attempt to determine the physiological action of both of these agents, and also of pure cultures of the organism, upon experimental animals.

An epizootic disease among frogs was described in 1893 by Roger. He found that the viscera and blood of these amphibia contained a small bacillus in pure culture. The same organism was easily obtained from the water of the aquarium which housed the infected frogs. From these observations this worker concluded that the organism was the same as that which Sanarelli had observed in a similar epizootic. A brief description was given by Roger of the gross pathological findings, in which he emphasized the appearance of general hemorrhagic areas in all species of artificially infected animals.

The first adequate description of the morphological, cultural, biochemical and

[1] The material published in this paper was taken from the thesis presented by the junior author in partial fulfillment of the requirements for the Master of Science degree at the University of Connecticut. For brevity, considerable detail has been omitted. For further detail, readers are referred to the thesis in the University of Connecticut library.

pathogenic properties of *Bacillus hydrophilus* was published by Russell in 1898, and his findings confirmed much of the work of Sanarelli and Trambusti. He made an extensive study of the gross pathological and histopathological findings of diseased frog tissues. His investigations on the metabolic products derived from the microorganisms showed that they elaborated two potent toxins, as was previously reported by Trambusti. One of these toxins resembled digitalis in its action, and the other veratrin. Russell concluded that no definite statement could be made concerning the route of infection in frogs. He thought, however, that infectious material was brought to the laboratory with the frogs and that infection became established through superficial skin lesions.

The common name of this frog disease in this country and in Canada is "red-leg." This designation first appeared in an article by Emerson and Norris in 1905. They claimed that the name is ideally suited, since it attracts attention to the most outstanding pathognomonic lesion common to diseased frogs, namely petechial haemorrhages on the surface of the abdomen and legs, varying from a light red to a deep scarlet color. Although these investigators observed some variation from the morphology as described by previous workers, they identified *Bacillus hydrophilus fuscus* of Sanarelli as the essential etiological agent. In the course of their studies they made several original observations both upon the pathological state of frogs and upon the products of the bacterial cultures. A series of carefully controlled experiments demonstrated that, while temperatures slightly above freezing have no harmful effect upon frogs, such temperatures completely control all manifestations of the disease in inoculated or naturally diseased frogs. They also found that the severity of the infection was definitely correlated with the destruction of the erythrocytes of the diseased frogs. This they believed was due to the action of the microorganism directly. Emerson and Norris concluded that the disease had a wide distribution throughout North American and Europe.

The substitution of the name *Proteus* for *Bacillus* originated with Weldin and Levine (1923). Bergey (1939) recognized the etiological entity of "red-leg" as *Proteus hydrophilus*. Throughout the remainder of this investigation, therefore, the species will be referred to as *Proteus hydrophilus*.

GROSS PATHOLOGY OF "RED-LEG" DISEASE IN FROGS

The information presented here on the gross pathology of "red-leg" disease in frogs was secured by observing over a hundred spontaneously infected animals and an additional thirty which were artificially infected with pure cultures of *Proteus hydrophilus*.

The malady which resulted from either spontaneous or artificial infection of frogs with *P. hydrophilus* was characterized at the onset by a distinct sluggishness of the diseased animal. Within one or two days hemorrhagic areas more or less uniform in appearance were observed on the ventral surface of the body. An extensive edema of the abdomen and thighs occurred several hours before the frogs succumbed to the disease.

Post-mortem examinations consistently revealed the presence of considerable edematous fluid beneath the skin of the abdomen and thighs. Small hemorrhagic

areas were occasionally seen on the surface of the tongue accompanied by a slight amount of blood-tinged exudate in the mouth. Multiple petechial hemorrhages were always observed on the surface of the abdominal and thigh musculatures. In a few instances small ulcers penetrated from the abdominal cutaneous tissue to the rectus abdominal musculature. The peritoneal cavity of medium-sized infected frogs contained two or three milliliters of a hemolyzed bloody exudate in which were found large numbers of the infecting organisms.

The heart muscle was always pale and flaccid. In many of the infected frogs the lungs appeared highly congested, while in others no abnormal changes were noticed. A parasite, which is probably *Distonum cylindraeceum*, was obtained from the lungs of approximately one-fourth of the frogs examined.

The blood vessels on the surface of the stomach and intestine were intensely congested, while the organs themselves appeared greatly distended. An extremely viscid, bloody material exuded from both the stomach and the intestine when they were severed. The fluid of the gall bladder varied from a light yellow to a deep green color. It is of interest to note that *Proteus hydrophilus* was easily isolated in relatively large numbers from the yellow fluid, but rarely from the deep green fluid of the gall bladder. The liver appeared dark brown and mottled. The spleen seemed considerably enlarged and roughened due to the presence of abnormal uniform protusions of the spleenic capsule. No abnormal changes were observed in the kidneys.

Proteus hydrophilus was readily recovered from the various organs and the exudate and blood of infected frogs. Many of the data obtained were in agreement with the findings of Russell (1898) and Emerson and Norris (1905). The last two workers observed that a large number of infected frogs which presented only small areas of congestion and few vesicles recovered within a few weeks after the onset of the infection. In the present investigation recovery of frogs from "red-leg" disease was never observed after the clinical symptoms were once apparent.

In several instances *Proteus hydrophilus* was obtained in pure culture from the heart blood of dead frogs in which no clinical manifestations or gross pathological changes were observed. If we assume that the death of the amphibia was caused by invasion of *Proteus hydrophilus*, the absence of any apparent lesions may be explained on the basis of extreme susceptibility on the part of the host. Topley and Wilson (1936) state that when the host possesses no immunity to the etiological entity, it will succumb to a bacteremic infection in which local lesions are rarely observed.

METHOD OF ISOLATING PROTEUS HYDROPHILUS AND HISTORY OF CULTURES

Blood broth and blood agar having the following basic composition were the mediums used in isolating the organism:

	per cent
Bacto peptone	1.0
Savita (yeast concentrate)	1.0
NaCl	0.5
Distilled water	

Agar (1.8 per cent) was added to the above, in the preparation of a solid medium. The addition of three per cent by volume of sterile defibrinated blood completed the formula.

For primary isolation, a loopful of material (usually heart's blood) was transferred to a tube of blood broth. A second loopful was streaked over the surface of a blood agar plate. Usually, there was good colonial growth on the blood agar plate after from 24 to 48 hours' incubation. Occasionally, no colonies developed on the agar but there was growth in the blood broth. In such cases streak plates from these broth cultures yielded good growth of the organism. All broth and agar plate cultures for primary isolation were incubated at 25°C.

One hundred and twenty-one strains were isolated from 96 sources, most of these being infected frogs. The discrepancy between the total number of isolations and the sources can be explained by stating that in several instances two isolations were made from the same animal, one from the heart blood and one from the gall bladder. Eight strains were secured from dead frogs which showed no gross pathological evidence of "red-leg" disease. Ten isolations were made from aquarium water in which infected frogs were kept. Eighteen came from either the heart's blood or gall bladder of apparently healthy frogs. Three strains were obtained from the water of an aquarium containing frogs which appeared healthy. After assurance that the isolations were pure, they were grown and stored on blood agar slants.

CELL MORPHOLOGY AND GROWTH STUDIES

Primary isolations of *Proteus hydrophilus* may possess as many morphological variations as the species has had names in the past. In smears made from the heart's blood of infected frogs and stained by Wright's method (Gradwohl, 1938) the organism presented a wide variety of shapes. Plump, short, encapsulated diplobacilli measuring approximately 2.0 microns in length and about 0.8 micron in width predominated. The ends of many cells were rounded, while others were truncate or pointed. Bipolar staining was usually observed in the cells having rounded ends. Clavate and elongated dumb-bell forms were present to a considerable extent in the smears. Filamentous variants were never observed. Encapsulated coccobacilli and lance-shaped varieties of the organism were fairly common. Narrow elongated granular forms were present in very small numbers. These observations were made on heart's blood from which *Proteus hydrophilus* was obtained in pure culture.

When grown on standard nutrient agar for a period of 18 hours at 30°C. and stained by Gram's method, *Proteus hydrophilus* appeared as a gram-negative straight rod with rounded ends and measured about 2.5 microns by 0.6 micron. Under these conditions the organism occurred either singly or in pairs. Irregular forms such as coccobacilli and clavate types were occasionally seen. Bi-polar staining was a very characteristic feature of newly isolated strains. The organism was non-sporing. It possessed a capsule and a single flagellum which was readily demonstrable by Liefson's method of staining (1930) (Figure 1). Bergey (1939) states that this organism has peritrichous flagella, but Russell

(1898), on the other hand, reported monotrichous flagellation. In the present study no non-motile strains of *Proteus hydrophilus* were observed following their growth in nutrient broth at 30°C. for 18 hours.

Two strains, P_2 and B-f-1, were selected as representative of the entire group of isolations and were subjected to further study. These two strains differed from each other only in their serological relationships. When grown on standard nutrient agar plates for 24 hours at 37°C., the translucent colonies which formed were approximately 2 mm. in diameter and were creamy white in color. They were slightly convex and circular in appearance, with an entire edge. The internal structure was finely granular and the surface smooth and moist. Growth was butyrous in consistency. The swarming phenomenon described by Cantu (1911) and Moltke (1927, 1929) as being characteristic for members of the

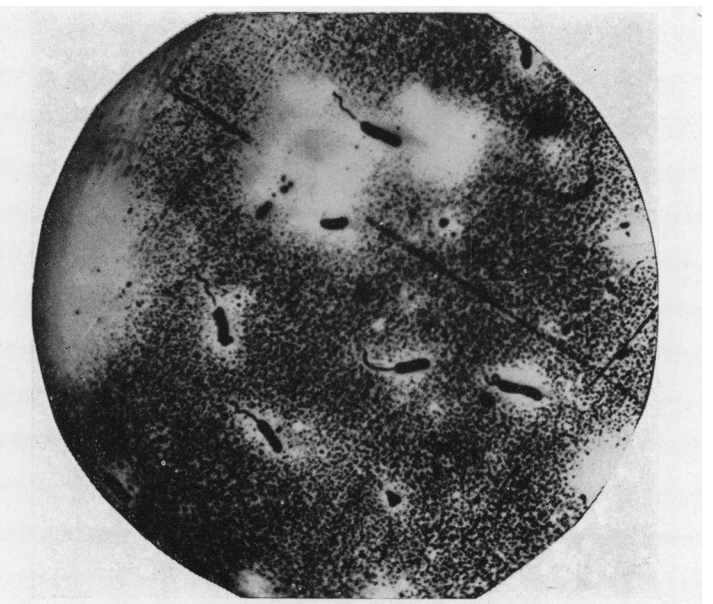

FIG. 1. FLAGELLA OF PROTEUS HYDROPHILUS

Proteus genus was never observed when *Proteus hydrophilus* was cultured on moist standard nutrient agar plates or on any other solid medium.

When grown on 3 per cent ox blood Savita agar plates which were incubated at 37°C. for 24 hours, the two strains produced circular colonies approximately 3 mm. in diameter, surrounded by a zone of beta type hemolysis which was about $1\frac{1}{2}$ mm. in diameter. The colonies were convex, finely granular, smooth, moist and glistening, with an entire edge. Although they seemed to be creamy white in color, a slight brownish tinge was observed in the peripheral portion. This effect may have been produced initially by the red color of the medium; in older cultures, however, it was observed to be a characteristic property of the colony. Distinct central and peripheral areas were seen in all colonies.

Growth on agar slants occurred readily at 30°C. Following an incubation

period of 48 hours, the creamy white growth was abundant, slightly spreading and possessed an undulate edge. The surface was smooth, moist and raised. Old agar slant cultures occasionally became yellowish in color.

Standard nutrient broth was a very satisfactory medium for growth of the organism. Multiplication in this medium was very rapid during a 24-hour incubation period at either 30°C. or 37°C. A dense uniform turbidity was always seen in the cultures examined and a deposit was usually present which broke up completely when the tube was shaken. Occasionally surface pellicles were observed in young cultures. Old broth cultures possessed an offensive odor.

BIOCHEMICAL STUDIES

With only slight differences here and there, the various strains had the following biochemical properties.

Acid and gas were produced from glucose, fructose, galactose, maltose, sucrose, salicin and starch. Raffinose, sorbitol and xylose were not attacked, and action on lactose was, at best, weak and should be described as doubtful. Napiform liquefaction occurred in gelatin tubes. Indole was produced and H_2S also was formed. All strains were Voges-Proskauer positive and all failed to grow in Koser's citrate broth. Methylene-blue milk was reduced and a rennet curd without acid production was formed, followed by peptonization of this curd. Ox, sheep, rabbit, horse and fowl erythrocytes were hemolyzed. Urea was not attacked, in strong contrast to several strains of *Proteus vulgaris* which were urea-positive.

Moltke (1927) stated that two very important properties which distinguish the *Proteus* group from other gram-negative, gelatin-liquefying, rod-shaped bacteria are the production of H_2S and the decomposition of urea. Wolf (1918–19) isolated three members of the *Proteus* genus from three different infections in man and found that they hydrolyzed urea with the formation of ammonia. Approximately 43 per cent of the total nitrogen present in the given medium (urine or urine-containing medium) was transformed into ammonia. Taylor (1928) observed that urine contaminated with *Proteus* organisms possessed an offensive odor which eventually became ammoniacal. He concluded that sterile normal urine, when inoculated with *Proteus* strains from various sources (suppurative wounds, feces and urine), soon became ammoniacal and deposited phosphates. Minning and Ritter (1937) also reported that urea is hydrolyzed with ammonium production by members of the *Proteus* genus.

The comparative action on urea of *Proteus vulgaris* and *Proteus mirabilis* (which are probably one and the same organism) and *Proteus hydrophilus* was studied.

The first medium employed was that of Murray (1916), but none of our cultures would grow in it. Next, a medium of the following composition was used:

	per cent
NaCl	0.5
K_2HPO_4	0.2
$MgSO_4$	0.5
Glucose	1.0
Urea	1.0
Distilled water	

This medium was sterilized by filtration. No growth occurred in it, but when meat extract in a concentration of 0.05 per cent was added all of the organisms grew. With the exception of *Proteus hydrophilus*, all of the cultures produced ammonia. To determine whether meat extract served as a source of ammonia, it was substituted in the original formula for the urea. All species grew in it, but no ammonia was produced by any of them. It was concluded that the *Proteus* species other than *Proteus hydrophilus* do decompose urea.

A few strains and also filtrates from broth cultures of some strains produced a startling zonation effect (Figure 2). This occurred in blood agar made with sheep, fowl, rabbit, horse and ox blood, but the greatest degree of zonation was noted in agar to which ox erythrocytes had been added.

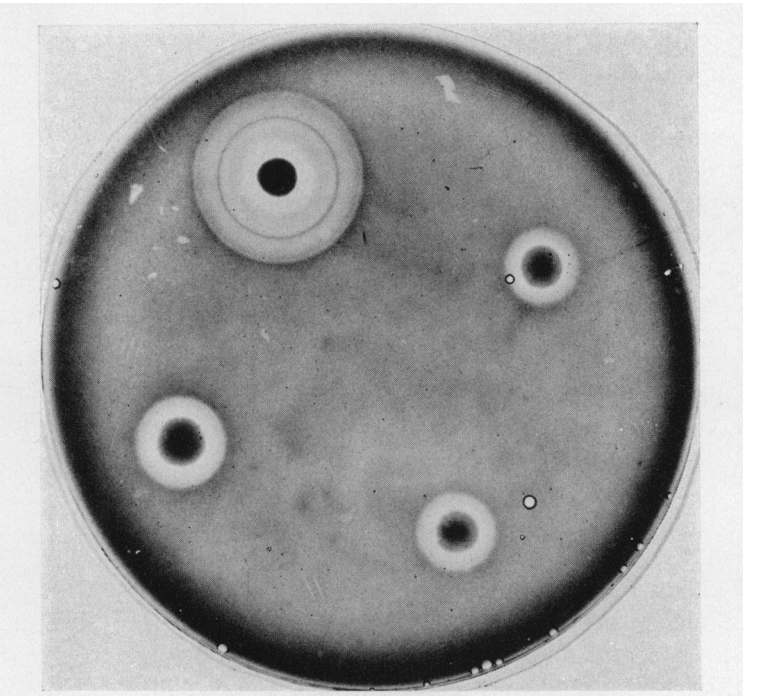

FIG. 2. ZONATION EFFECT OF PROTEUS HYDROPHILUS ON BLOOD AGAR

Brown (1919), Kortenhaus (1929), Rhodes (1938) and others have reported this zonation effect, called "Leisegang ring formation," in blood-agar cultures of streptococci, staphylococci and pneumococci. This phenomenon has been ascribed to a combination of chemical and physical factors.

SEROLOGICAL STUDIES

Antisera were prepared for several strains of *Proteus hydrophilus*. A study of these sera indicated that there were two distinct serological types of this organism in our collection, represented here by the P_2 and B-f-1 strains. It is interesting to note that of 24 normal rabbit sera examined, seventeen showed somatic agglutinins specific for B-f-1, while none reacted with P_2. It is known that normal

rabbits often harbor *Pasteurella cuniculicida* (*Bacillus leptisepticus*) in their nasal passages de Kruif (1922), Webster (1924). This organism is associated with a disease in rabbits called "snuffles." It contains Forssman's antigen (Zinsser *et al*, 1939). On this basis it is possible to explain the presence of so-called "normal" agglutinins found in the serum of rabbits which are specific for the B-f-1 strain of *Proteus hydrophilus*. Only rabbits showing no agglutinins for either strain were used in the preparation of specific *P. hydrophilus* antisera.

Using the above antisera, an attempt was made to determine the relationships of the different isolations to the above two strains. Both flagellar and somatic factors of the cell were considered in this study. It was found that, contrary to the observations of other workers, there was little difference between the flagellar and the somatic titers. The fact that this species is monotrichous may help to explain this finding.

The following conclusions are offered for this phase of the investigation:

1. Two distinct serological types of *Proteus hydrophilus* predominate.
2. Evidence points to the presence of other types of this organism not employed in this study.
3. More than one serological type of the pathogen may be isolated from the body of an infected frog.
4. Strains of *Proteus hydrophilus* vary in the degree of their antigenic relationship to the B-f-1 and P_2 serological types, on the basis of flagellar and somatic agglutination reactions.
5. Strains of the frog pathogen may possess common somatic, but dissimilar flagellar, antigenic components for the serological type B-f-1 strain.
6. The homologous flagellar titers of strains of B-f-1 and P_2 do not exceed the somatic titers of these organisms.

HABITAT

Bergey (1939) states that the habitat of *Proteus hydrophilus* is unknown. Sanarelli (1891) thought that the organism was a normal inhabitant of water, but he was able to isolate it from this source only twice in 26 attempts.

In the present research the waters of seven aquaria used to house apparently normal frogs were examined and *Proteus hydrophilus* was isolated from three of these sources. Proceeding on the hypothesis that in this instance, as in many other infectious diseases, the healthy carrier may be the source of the organism, several frogs from each of the above aquaria were autopsied. The internal organs appeared to be normal, and *Proteus hydrophilus* was isolated from the gall bladder of only three frogs. In these three cases, however, the fluid in the gall bladder was yellow in color, in contrast to the green-colored bile generally found in normal frogs. A fourth isolation was made from the intestine of another frog, but in no other instance was this organism recovered from animals which appeared healthy.

It was easy, as a rule, to secure *Proteus hydrophilus* from the waters of aquaria which harbored infected frogs. However, in a few instances frogs that were segregated in small individual aquaria did not eliminate the organism, even

though pure cultures were secured from the gall bladder of these animals at autopsy. This is in agreement with the findings of Amoss (1922), Topley (1926) and Knorr (1926) in their study of laboratory rodents which were experimentally infected with members of the *Salmonella* group. They concluded that these organisms may be eliminated continuously or intermittently in the feces or urine, being harbored in these instances in the spleen, liver, gall bladder and lymph nodes. Meyer, Neilson and Feusier (1921) claimed that in rodents surviving *Salmonella* infections the persistent carrier state resulted from a bacterial embolism in the gall bladder wall.

The portal of entry necessary for *Proteus hydrophilus* to become established as an infectious organism in the frog appeared to be the abraded skin. Frogs experimentally scratched to break the skin and placed in water containing the organisms developed the typical disease very quickly. Attempts to produce the infection by feeding were unsuccessful.

TAXONOMY

Although the organism causing "red-legs" has been named differently by past investigators as *Bacillus ranicida* (Ernst, 1890; Weldin, 1926–7), *Bacillus hydrophilus fuscus* (Sanarelli, 1891), *Bacterium hydrophilus fuscus* (Chester, 1897), *Bacillus hydrophilus* (Chester, 1901) and *Bacterium ranicida* (Lehman and Neumann, 1931), it is now known as *Proteus hydrophilus*.

Bergey's Manual (1939) recognizes the *Proteus* genus on the following basis:

> Highly pleomorphic rods. Filamentous and curved rods common as well as involution forms. Gram negative. Generally actively motile, possessing peritrichous flagella. Characteristically produce amoeboid colonies, etc., on moist media and decompose proteins. Ferment dextrose and generally sucrose, but not lactose. Do not usually yield a positive Voges-Proskauer test. Urea decomposed.

Topley and Wilson (1936) agree with the above description of this genus and add that mannitol is fermented.

Members of the *Proteus* genus cause coagulation and digestion of casein in milk, and indole may or may not be formed, according to the Medical Research Council (1929). Fifty-three strains of the *Proteus* group were examined by Taylor (1928) who found only three which produced indole. Moltke (1927) reported only 36 strains out of 194 examined as giving the reaction.

As previously stated (Moltke, 1927; Wolf, 1918–9), urea decomposition and the production of hydrogen sulfide are claimed as important characteristics, distinguishing species of the *Proteus* genus from other gram-negative, gelatin-liquefying bacteria.

Proteus hydrophilus, according to the present investigation, has the following properties which are characteristic of the genus: it is gram-negative, actively motile, gives rise to involution forms (present only in the animal body), ferments glucose and sucrose, and produces hydrogen sulfide. Its variable properties include indole and acetyl-methyl-carbinol production and gelatin liquefaction. The absence of the "swarming" phenomenon, urea decomposition,

peritrichous flagella, filamentous and curved rod formation makes it differ from characteristics usually considered significant for the *Proteus* genus.

TOXIN STUDIES

Proteus hydrophilus and bacteria-free culture filtrates of this organism are strongly hemolytic.

An attempt was made to determine whether a hemotoxin was the lethal agent in mice dying from infection with *Proteus hydrophilus*. Erythrocyte counts were made on a series of eight adult mice. These mice were then inoculated with a lethal dose of *Proteus hydrophilus*. They were kept under close observation, and immediately after death the erythrocyte count was repeated. No deviation was found from the original average of 8,000,000 per cu. mm. which would seem to indicate that the mice did not die because of erythrocyte destruction. This parallels the observations of Zinsser, Enders and Fothergill (1939) regarding streptolysin and its relationship to streptococcal infections. They reported that hemolysin has little significance in the lethal effect of streptococci which are strongly hemolytic *in vitro*.

A substance which was definitely toxic for mice, rabbits and guinea pigs was demonstrable in bacteria-free filtrates of *Proteus hydrophilus* broth cultures which were from six to thirty days old. Mice were more resistant to intraperitoneal than to intravenous injection of this material. Heating such a filtrate to the boiling point for one hour reduced the toxicity for mice to about 10 per cent of the original. This substance was quite stable when kept at 10°C. in the dark. Rabbits were given a series of sub-lethal intravenous injections of this filtrate, but the resulting antisera had no protective action for mice subsequently injected with the above filtrate. In contrast, mice immunized with *Proteus hydrophilus* bacterins were not killed by intravenous injection of this toxic substance. These experiments seem to indicate that the toxic material in old broth cultures of *Proteus hydrophilus* is endotoxic in nature.

IMMUNOLOGICAL STUDIES

Kulp, Lackman and Borden (1940) were able to immunize mice and leopard frogs against *Proteus hydrophilus* infection. They employed as immunizing antigen heat-killed suspensions of *Proteus hydrophilus;* also living and heat-killed suspensions of *Proteus mirabilis*. Successful immunization was secured in from 60 to 100 per cent of the animals tested. They were unable to determine any antigenic relationship between the immunizing strains of *Proteus vulgaris* and *Proteus mirabilis*, on the one hand, and the pathogen, *Proteus hydrophilus*, on the other.

This work was repeated and extended in the present investigation. Immunization experiments were conducted with the two serological types of *Proteus hydrophilus*, B-f-1 and P_2. It was shown that a bacterin of one of these types protects frogs against both strains. Antibodies could not be demonstrated in the blood of immunized frogs except in one instance where agglutinins were found in the blood of a large bull-frog which received repeated injections

of bacterin. In the experiments with mice the bacterin was prepared by alternate freezing and thawing of the suspensions until they were sterile. Cross-immunization was secured in mice with B-f-1 and P_2 bacterins prepared in this manner. Blood from mice immunized against one strain did not show the presence of either somatic or flagellar agglutinins specific for the other strain. Antiserum from mice immunized against P_2 bacterin did not confer passive immunity against B-f-1 and *vice-versa*, nor did antisera prepared in rabbits against one of the above types protect mice passively against the other strain of this organism.

An attempt was made to explain the mechanism of immunity in mice. A definite increase in leucocytes was noted in these animals, but since the study of the bactericidal power of the blood was made on the whole blood, the immunity may not be entirely leucocytic. Borden (1936) found that blood from mice which had been immunized with living cultures of *Proteus vulgaris* and *Proteus mirabilis* was bactericidal for *Proteus hydrophilus in vitro*. It is quite possible that both humoral and cellular responses may be involved. This phase of the problem will be studied further.

SUMMARY AND CONCLUSIONS

"Red-leg" disease, an epizootic of aquarium frogs, is a form of septicemia that is caused by an organism which is a member of, or so closely related to, the *Proteus* group as to merit the name *Proteus hydrophilus*.

Proteus hydrophilus is a gram-negative monotrichous rod; it is a more or less pleomorphic, non-sporulating, strongly fermentative, gelatin-liquefying organism. This species produces acetyl-methyl-carbinol, is indole-positive, grows readily on the ordinary nutrient mediums, but does not produce the "swarming" type of growth generally attributed to the *Proteus* genus. It develops a potent lysin for the erythrocytes of several animal species. Certain strains produce a zonation effect on blood agar mediums. A toxic substance has been demonstrated in culture filtrates, which appears to be endotoxic in nature.

There are at least two definite serological types in these species. When employed antigenically as bacterins, each brings about immunity in experimental animals against both types, whereas passive immunization has been demonstrated only against the homologous type.

Frogs which appear normal seem to act as carriers, the infective agent in all probability being carried in the gall bladder.

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REFERENCES

AMOSS, H. L. 1922 Experimental epidemiology. II. Effect of the addition of healthy mice to a population suffering from mouse typhoid. J. Exptl. Med., **36**, 45–69.

BERGEY, D. H., ET AL. 1939 Manual of Determinative Bacteriology. The Williams & Wilkins Company, Baltimore, Md.

BORDEN, A. G. 1936 The effect of a non-specific vaccine on the immunity of mice to pneumococcus type I and *Proteus hydrophilus*. A thesis in the Library of the University of Connecticut.

BROWN, J. H. 1919 Monograph of the Rockefeller Institute for Medical Research, No. 9.

CANTU, C. 1911 Le Bacillus Proteus sa distribution la nature. Ann. inst. Pasteur, **25**, 852–864.

CHESTER, F. D. 1897 A preliminary arrangement of the species of the genus *Bacterium*. Contributions to determinative bacteriology. Part I. Ninth Ann. Rept. Del. College Agr. Expt. Sta., 92.

CHESTER, F. D. 1901 Manual of Determinative Bacteriology. 235.

EMERSON, H., AND NORRIS, C. 1905 "Red-Leg"—an infectious disease of frogs. J. Exptl. Med., **7**, 32–58.

ERNST, P. 1890 Die Frühjahrsseuche der Frösche und ihre Abhängigkeit von Temperature-einflüssen. Beitr. path. Anat., **8**, 203–220.

GRADWOHL, R. B. H. 1938 Clinical Laboratory Methods and Diagnosis. The C. V. Mosby Company, St. Louis, Mo. (2d ed.) 328 and 785.

KNORR, M. 1926 Akute Gastroenteritis und Typhöser Paratyphus. Zentr. Bakt. Parasitenk., **99**, 25–46.

KORTENHAUS, F. 1929 Zonenzyklohämolyse auf Blutagar bei Staphylokokken sawie bei Viridans—Streptokokken und Pneumokokken. Zentr. Bakt. Parasitenk., **113**, 499–503.

DEKRUIF, P. H. 1922 Mutation of the bacillus of rabbit septicemia. J. Exptl. Med., **35**, 561–574.

KULP, W. L., LACKMAN, D., AND BORDEN, A. G. 1940 Non-specific immunization with members of the *Proteus* group. Unpublished data. Department of Bacteriology, University of Connecticut.

LEHMAN, K. B., AND NEUMANN, R. G. 1931 Determinative Bacteriology. Edited by R. S. Breed. G. E. Stechert and Company (Alfred Hafner) New York, 474.

LEIFSON, E. 1930 A method of staining bacterial flagella and capsules together with a study of the origin of flagella. J. Bact., **20**, 203–211.

Medical Research Council. 1929 A system of bacteriology in relation to medicine. His Majesty's Stationery Office, London, **4**, 308.

MEYER, K. F., NEILSON, N. M., AND FUSIER, M. L. 1921 Experimental typhoid-paratyphoid carriers. IV. A comparative study of the infections produced by intravenous injections of typhoid, paratyphoid A and B bacilli in normal and immunized rabbits. J. Infectious Diseases, **28**, 408–455.

MINNING, W., AND RITTER, G. 1937 Zur Kenntnis der Biologie und Systematik von Proteus und Kloakenbakterien (Kloacinae Haupt). Zentr. Bakt. Parasitenk., **140**, 27–35.

MOLTKE, O. 1927 Contributions to the characterization and systematic classification of *Bacillus proteus vulgaris* (Hauser). Monograph. Levin and Munksgaard, Copenhagen.

MOLTKE, O. 1929 Untersuchungen über das Phänomen des Schwarmens der Proteus-Bazillen. Zentr. Bakt. Parasitenk., **111**, 399–403.

MURRAY, I. J. 1916 The oxygen requirements of biological soil processes. J. Bact., **1**, 597–614.

RHODES, G. B. 1938 Satellite hemolytic zones in blood agar staphylococcus cultures. J. Infectious Diseases, **62**, 124–128.

ROGER, M. 1893 Une epizootic observée ches des grenouilles. Compt. rend. soc. biol., **5**, 709–711.

RUSSELL, F. H. 1898 An epidemic septicemic disease among frogs due to the *Bacillus hydrophilus fuscus*. J. Am. Med. Assoc., **30**, 1442–1449.

SANARELLI, G. 1891 Ueber einen neuen Mikro-organismus des Wassers, welcher für Thiere mit veränderlicher und constanter Temperatur pathogen ist. Zentr. Bakt. Parasitenk., **9**, 193–199.

TAYLOR, J. F. 1928 *B. proteus* infections. J. Path. Bact., **31**, 897–915.

TOPLEY, W. W. C. 1926 The third Milroy lecture on experimental epidemiology. Lancet **1**, 645–651.

TOPLEY, W. W. C., AND WILSON, G. S. 1936 The Principles of Bacteriology and Immunity. William Wood & Company, Baltimore, Md. 2d ed.

TRAMBUSTI, A. 1893 Ueber die physiologische Wirkung der Stoffwechselprodukt des Hydrophilus Fuscus. Beitr. path. Anat., **14**, 317–336.

WEBSTER, L. T. 1924 The epidemiology of a rabbit respiratory infection. V. Experimental snuffles. J. Exptl. Med., **40**, 117–127.

WELDIN, J. C. 1926–27 The colon-typhoid group of bacteria and related forms. Relationships and classification. Iowa State Coll. J. Sci., **1**, 151.

WELDIN, J. C., AND LEVINE, M. 1923 An artificial key to the species and varieties of the colon-typhoid or intestinal group of bacilli. Abstracts Bact., **7**, 13.

WOLF, C. G. L. 1918–19 Contributions to the biochemistry of pathogenic anaerobes. VII. The biochemistry of *Bacillus proteus*. J. Path. Bact., **22**, 289–307.

ZINSSER, H., ENDERS, J. F., AND FOTHERGILL, L. D. 1939 Immunity Principles and Applications in Medicine and Public Health. The Macmillan Company, New York, 164.