SURVEY OF HEMOLYSIN PRODUCTION AMONG SPECIES OF PSEUDOMONADS

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Although *Pseudomonas aeruginosa* has been known to produce hemolysis on blood agar plates, attempts to obtain hemolysin in broth culture filtrates of this organism have been generally unsuccessful. Bullock and Hunter (1900) noted that filtrates of 3 to 4 week old broth cultures of this organism lysed suspensions of erythrocytes of several animal species, including man. This finding was confirmed by Landsteiner and Raubilschek (1908) and Fukuhara (1909). All of these authors agreed that the hemolysin could be demonstrated only in very old nutrient broth cultures and that filtrates from young, actively growing broth cultures were always devoid of hemolytic activity. The failure of *P. aeruginosa* to produce a soluble hemolysin in young broth cultures has been confirmed by this author.

In 1936, Birch-Hirschfeld described a cellophane plate technique for the production of staphylococcal hemolysin. This technique was extensively employed by William and Harper (1947), Marks and Vaughan (1950) and Liu (1954) in their studies of the *delta* hemolysin of staphylococci.

Recently this cellophane plate technique was applied to the production of the soluble hemolysin by *P. aeruginosa* with considerable success. Since several workers (Salvin and Lewis, 1946; Christie, 1948) have reported that the hemolysis by *P. aeruginosa* on blood agar could be used to differentiate this species from other pseudomonads, the hemolysin production with the cellophane plate technique was also applied to examine the hemolytic activities of several other pseudomonads which resemble *P. aeruginosa*. The present communication describes the findings of this study.

MATERIALS AND EXPERIMENTAL METHODS

Strains of pseudomonads used. The strains of *P. aeruginosa* used in this study were all isolated from cases of human infections as described previously (Liu, 1952). Strain P-A-1 which was used most extensively for hemolysin production was isolated from a case of otitis media. This strain was a good pyocyanin producer and showed a typical biochemical pattern. Ten more strains of *P. aeruginosa* were also examined with essentially the same type of hemolysin production.

Dr. W. C. Haynes (Northern Regional Research Laboratory) kindly supplied *Pseudomonas aureofaciens* (B-1543P), *Pseudomonas chlororaphis* (B-560), *Pseudomonas pseudomallei* (B-1110), *Pseudomonas caviae* (B-966), *Pseudomonas reptilivora* (B-963), *Pseudomonas syncyanea* (B-786), *Pseudomonas marginalis* (B-982) and *Phytomonas polycolor* (B-853). From Dr. P. W. Wetmore (Division of Veterinary Medicine, Walter Reed Army Institute of Research) were obtained 1 strain of *Pseudomonas fluorescens*, 1 strain of *Pseudomonas fragi*, 5 strains of *Pseudomonas ovalis*, and 6 strains of *Pseudomonas stutzeri*. One strain of *Pseudomonas viscosa* was obtained from Dr. G. Knaysi (Laboratory of Bacteriology, New York State College of Agriculture). Several strains of *Malleomyces pseudomallei* were obtained from Mr. L. S. White (Communicable Disease Center, Chamblee, Georgia).

P. aureofaciens and *P. chlororaphis* are both phenazine pigment producers and, therefore, were considered (Kluvyer, 1956) to be taxonomically closely related to *P. aeruginosa*. *P. syncyanea* and *P. marginalis* are both pyocyanin producers isolated from sources other than animals and they were considered (Haynes, 1951) to be identical with *P. aeruginosa*. *P. polycolor* is a plant pathogen which was considered by Elrod and Braun (1942) to be identical with *P. aeruginosa*. *P. caviae* (Scherago, 1936) and *P. reptilivora* (Caldwell and Ryerson, 1940) resemble *P. aeruginosa* in being animal pathogens. *P. pseudomallei* (*Malleomyces pseudomallei*) has recently been shown to be closely related to the pseudomonads, and its inclusion in the family Pseudomonadaceae was suggested (Wetmore and Gochenour, 1956). Strain B-1110 which was used most extensively in this study as the representative of this species

was originally obtained by Dr. Haynes from the National Collection of Type Culture in England and was labeled *Pfeifferella pseudomallei*. It is, therefore, an authentic strain of the species. The other strains of *P. pseudomallei* obtained from the Communicable Disease Center behaved essentially the same as the strain B-1110.

Technique of hemolysin production. The medium used in most of these experiments was tryptone-glucose-agar (Difco) because it consistently gave a higher yield of hemolysin than any other type of agar. However, many other types of nutrient agar which were examined supported hemolysin production although the hemolytic titers were lower than that obtained with tryptone-glucose-agar. Enough glucose was added to make the final concentration 1 per cent. The addition of glucose greatly enhanced the production of hemolysin by most of the pseudomonads used in this study. An ordinary wrapping cellophane was cut to fit the bottom of the petri dish and then was sterilized under steam pressure. After the tryptone-glucose-agar was poured and solidified, the cellophane was placed on it. The wrinkles of cellophane were carefully stretched out with sterile forceps after the sheet had been moistened.

About two drops of a broth culture of the organisms were spread over the cellophane with a sterile cotton swab, and the plates were incubated according to the optimal growth temperatures of each strain. Hemolysin production usually reached its peak in 36–48 hr with *P. aeruginosa* and most of the other pseudomonads. At the end of the incubation periods, the growth on the cellophane plates was washed off with 3 ml of saline and centrifuged at 4000 rpm for 20 min to remove bacterial cells. The hemolysin of *P. aeruginosa* was removed to a considerable extent by the bacterial filter (particularly the Seitz) and, therefore, the supernatant fluids obtained by centrifugations were used directly as the hemolysins without filtration. When the hemolysins were to be stored for future use, 200 μg of streptomycin (or 50 μg of polymixin) were added to the preparation to prevent the further growth of the few viable cells remaining in the supernatant fluids which were not removed by centrifugation.

Separation of the hemolysin from pyocyanin. Although the bulk of pyocyanin produced by some of the pseudomonads diffused back to the agar through the cellophane sheet, a considerable amount of this pigment still remained in the hemolytic preparation. The separation of pyocyanin from the hemolysin was accomplished easily by the use of chloroform and a separatory funnel. About 0.4 ml of chloroform was added to every ml of the hemolytic preparation and shaken vigorously for a few minutes. The mixture was then placed in a separatory funnel and allowed to stand overnight at 4 C. The chloroform layer containing pyocyanin settled down to the bottom, leaving the hemolysin in the supernatant fluid. The pH of the hemolysin was adjusted to 7.2 before titration. The treatment with chloroform was necessary only for the preparations obtained with the pyocyanogenic group of pseudomonads. Other pseudomonads yielded colorless to slightly brown supernatant fluids and they were used directly in the titration of hemolytic titers.

Titration of the hemolysins. The hemolytic activities of the products were titrated by twofold dilution in 0.5 ml of saline. Five-tenths ml of a 1 per cent suspension of human erythrocytes was added to the tubes containing the dilutions of the hemolysins, and the final reading of the hemolysis was made after 2 hr incubation in a 37 C water bath. The reciprocal of the highest dilutions showing complete hemolysis was taken as the hemolytic units of the preparation.

Titration of the antihemolytic activities of various sera. When the antihemolytic activities of normal human and animal sera and fractions of normal human sera were titrated, the sera were diluted twofold in 0.5 ml and the hemolysins were diluted to contain one unit in 0.4 ml and were added in this amount to each tube of the serum dilutions. The mixtures were incubated at 37 C for 30 min and then 0.1 ml of a 5 per cent suspension of human erythrocytes was added to each tube. The reading of hemolysis was made at the end of another 2 hr incubation at 37 C.

Fractions of normal human sera. Fractions of normal human sera, II-III, IV and V, were obtained from E. R. Squibb & Son, New York.

Reading of hemolysis on plates. The technique for examining the hemolysis produced by pseudomonads is by no means standardized. Various kinds of peptones were used by different workers with different types of animal blood in several concentrations. This lack of standardization of technique may contribute to the present state of confusion and apparently has been responsible for the discrepancies in the results reported from

various laboratories. It was found in this study that (1) tryptone (Difco) gave the highest yield of hemolysin by pseudomonads among many brands of peptones employed, (2) the addition of simple carbon compounds, such as glucose, greatly enhanced the hemolysin production, (3) hemolysin production reached maximum in about 48 hr on cellophane agar, (4) the hemolysin of *P. aeruginosa* was inhibited by normal animal sera including that of man and (5) human erythrocytes appeared to be most sensitive to the action of this hemolysin among the types of animal erythrocytes commonly employed in bacteriological laboratories. Theoretically, therefore, the most sensitive technique for determining the hemolysis of pseudomonads would be incorporation of washed human erythrocytes, instead of whole blood, in tryptone-glucose-agar containing 1 per cent glucose and reading the hemolysis at the end of 48-hr incubation periods at optimal growth temperature. However, the addition of a high concentration of glucose into the medium resulted in the production of a moderate amount of acid which turned the whole plate brown after growth of the organisms. The reading of hemolysis on this type of medium, therefore, is not practical. Furthermore, the erythrocytes did not appear very stable in the tryptone-glucose-agar even after adjustment to isotonicity of the medium with NaCl and, therefore, another medium was substituted for this purpose. Brain-heart-infusion agar (Difco) appeared suitable for this purpose. Human erythrocytes were washed 3 times with saline and restored to their original concentration in the blood sample. Five per cent of this suspension was incorporated in brain-heart-infusion agar. The hemolysis of pseudomonads was more readily observed on this type of plate than on an ordinary blood agar plate containing whole blood of laboratory animals such as guinea pig, rabbit or sheep. Even with this type of medium, the detection of hemolysin production was not as readily accomplished as with the cellophane plate technique because glucose could not be incorporated into the medium and the titer of the hemolysin was low.

RESULTS

The reading of hemolysis on plate. As mentioned previously, the reading of hemolysis of pseudomonads was enhanced by the use of washed human erythrocytes instead of whole blood of laboratory animals, but this technique was not as sensitive as the demonstration of hemolysin production on the cellophane plate. Many species of pseudomonads which produced low titers of hemolysin on the cellophane plate appeared nonhemolytic on the washed erythrocyte plate with only a slight clearing of the medium around the colonies. Figure 1 shows *P. pseudomallei* strain (B-1110) grown for 48 hr at 37 C on a blood agar plate containing 5 per cent whole guinea pig blood. Well isolated colonies appear entirely nonhemolytic in spite of the fact that they are much larger than those which were placed in a crowded environment and are hemolytic. The exact mechanism of this hemolysin production is unknown, but it seems to indicate that the mutual inhibition by similar organisms in a dense population alters their metabolic pattern to such an extent that they produce hemolysin, whereas it is not produced by the organism growing in a relatively sparse

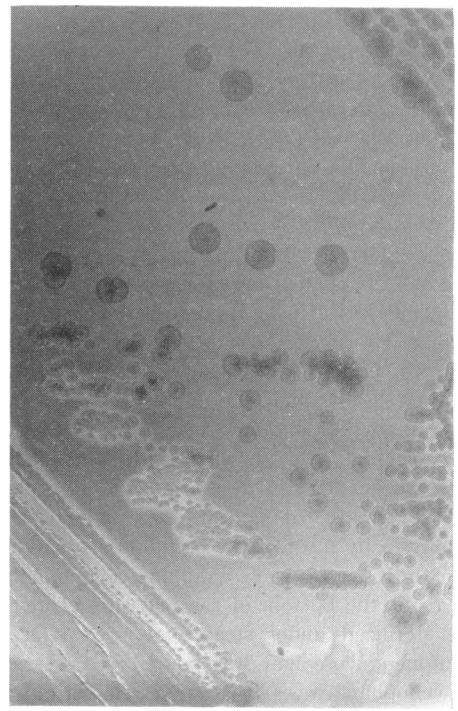

Figure 1. Hemolysis of *Pseudomonas pseudomallei* (B-1110) grown for 48 hr at 37 C on a plate containing 5 per cent whole guinea pig blood. The hemolysis is observed only where colonies are crowded and small. The larger, well-isolated colonies appear entirely nonhemolytic. × 1.5.

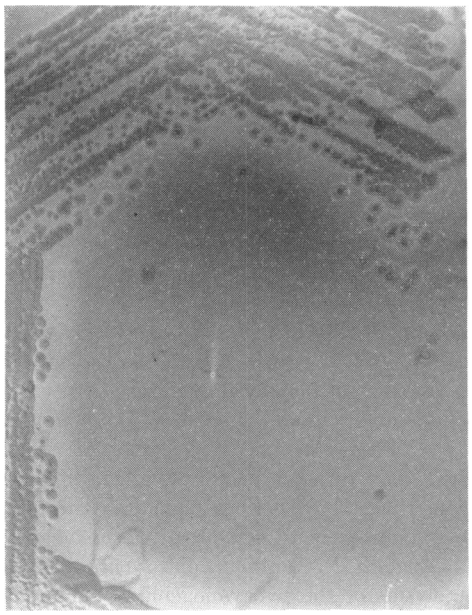

Figure 2. Hemolysis of *Pseudomonas pseudomallei* (B-1110) grown for 48 hr at 37 C on a plate containing 5 per cent washed human erythrocytes. The zone of hemolysis produced by the dense growth of the organism on the lower left side of the plate is much larger than that on the plate shown in figure 1, but the well-isolated colonies still appear nonhemolytic. × 1.5.

population. Figure 2 shows the same strain of *P. pseudomallei* grown in the same condition on a plate containing 5 per cent washed human erythrocytes. The zone of hemolysis produced by the dense population of the colonies at the left side of the plate is much wider than those in figure 1 but isolated colonies still appear nonhemolytic. This hemolysis can be increased by the addition of 1 per cent glucose to the basic medium but, as mentioned previously, the inclusion of glucose will result in acid production which turns the whole plate brown and the reading of the hemolysis on such media is not practical.

Hemolysin production on cellophane agar. Cellophane plates were made with tryptone-glucose-agar containing 1 per cent glucose, and 3 plates were inoculated with each strain of pseudomonads mentioned above. The plates were incubated at the optimal growth temperatures of each organism. One plate each was washed off after 24, 48 and 72 hr of incubation and the hemolytic titers determined. The results are shown in the table 1. As will be seen in table 1, *P. marginalis*, *P. syncyanea* and *P. polycolor*, which were considered (Haynes, 1951) to be identical with *P. aeruginosa*, produced hemolysin with titers comparable to that produced by *P. aeruginosa*. This finding seems to provide additional evidence to support the contention that these species are indeed

TABLE 1

Hemolysin production on cellophane plates (tryptone-glucose-agar) by various species of pseudomonads

Organism	Optimal Growth Temp	Phenazine Pigments	Pathogenicity to Animals	Hemolytic Units at Times Indicated in Hr		
				24	48	72
	C					
Pseudomonas aeruginosa	37	+	+	128	256	128
Pseudomonas marginalis	37	+	+	256	256	128
Phytomonas polycolor	37	+	+	128	256	128
Pseudomonas syncyanea	37	+	+	256	256	128
Pseudomonas caviae	37	−	+	0	4*	4*
Pseudomonas pseudomallei	37	−	+	16	32	16
Pseudomonas reptilivora	37	−	+	0	4*	4*
Pseudomonas fragi	37	−	−	0	0	0
Pseudomonas ovalis	37	−	−	0	0	0
Pseudomonas stutzeri	37	−	−	0	0	0
Pseudomonas aureofaciens	28	+	−	16	64	64
Pseudomonas chlororaphis	28	+	−	32	256	128
Pseudomonas fluorescens	28	−	−	0	0	0
Pseudomonas viscosa	28	−	−	0	8	8

* Hemolysin production by these 2 species was very poor and irregular, sometimes none at all.

TABLE 2

Toxicities of the fresh and heated hemolysins of four pseudomonads as expressed by the mortality of mice injected intraperitoneally*

Organism	Type of Hemolysin	Hemolytic Units/Ml	Ml of Hemolysin Injected	Mortality of Mice at Times Indicated in Hr		
				24	48	72
Pseudomonas aeruginosa	Fresh	128	0.5	5/5		
			0.3	2/5	4/5	4/5
			0.1	0/5	2/5	3/5
	Heated	128	0.5	3/5	4/5	5/5
			0.3	2/5	3/5	3/5
			0.1	0/5	1/5	2/5
Pseudomonas pseudomallei	Fresh	32	0.5	5/5		
			0.3	3/5	4/5	5/5
			0.1	0/5	4/5	4/5
			0.05	0/5	2/5	4/5
			0.02	0/5	1/5	2/5
	Heated	16	0.5	0/5	0/5	0/5
			0.3	0/5	0/5	0/5
			0.1	0/5	0/5	0/5
Pseudomonas aureofaciens	Fresh	64	0.5	4/5	5/5	
			0.3	2/5	4/5	4/5
			0.1	0/5	1/5	1/5
	Heated	0	0.5	0/5	0/5	0/5
			0.3	0/5	0/5	0/5
			0.1	0/5	0/5	0/5
Pseudomonas chlororaphis	Fresh	256	0.5	4/5	5/5	
			0.3	4/5	4/5	5/5
			0.1	0/5	2/5	2/5
	Heated	0	0.5	0/5	0/5	0/5
			0.3	0/5	0/5	0/5
			0.1	0/5	0/5	0/5

* Heated for 15 min in a boiling water bath.

In a mortality data, the denominators indicate the number of mice used and the nominators indicate the number of mice that died at the time indicated.

identical with *P. aeruginosa*. It is interesting to note that *P. pseudomallei* which has often been described as nonhemolytic (Wetmore and Gochenour, 1956) did produce moderate amounts of hemolysin ranging from 16 to 32 units per ml. The hemolytic titers were much lower than that of *P. aeruginosa* and the hemolytic activity of this hemolysin was also inhibited by normal animal sera including that of man. This, apparently, is one reason why the colonies of this species on an ordinary blood agar plate appear nonhemolytic. *P. caviae* and *P. reptilivora*, both animal pathogens, produced very small amounts of hemolysin and the appearances of their hemolysins were very irregular, sometimes none at all.

P. aureofaciens and *P. chlororaphis*, both phenazine pigment producers which do not grow at 37 C, also produced moderate amounts of hemolysin. It is of interest to note that *P. fluorescens*, which has often been confused with *P. aeruginosa* in the past, completely failed to produce any detectable hemolysin.

Qualitative differences of the hemolysins produced by various species of pseudomonads. During the titration of hemolysins produced by various pseudomonads it was noted that the hemolysins of *P. aureofaciens* and *P. chlororaphis* took a longer time to show hemolysis and also took a longer time to reach their maximum titers than the hemolysins of *P. aeruginosa*, *P. pseudomallei*, *P. syncyanea*, *P. marginalis* and *P. polycolor*. The hemolysins of the pyocyanogenic pseudomonads took only a few seconds to produce complete hemolysis in the first tube of the serial dilutions when incubated at 37 C, and the maximum titers were reached within 40–50 min of incubation. The hemolysins of *P. aureofaciens* and *P. chlororaphis* took about 20 min to show complete hemolysis in the first tube of the serial dilution and their maximum titers were reached only after 2 hr incubation at 37 C regardless of their final hemolytic titers. It was also found that the hemolysins of *P. aureofaciens* and *P. chlororaphis* were completely inactivated by heating in a boiling water bath for 15 min, while the hemolysins of the pyocyanogenic pseudomonads and that of *P. pseudomallei* were not destroyed by this treatment. The toxicities, as reflected by the mortality in mice injected intraperitoneally, of the hemolysins of *P. aeruginosa*, *P. pseudomallei*, *P. aureofaciens* and *P. chlororaphis*, before and after heating, are shown in table 2. The hemolysin of *P. pseudomallei* used in this experiment was filtered through a sintered glass filter after centrifugation because a few viable cells remaining in the supernatant fluids were enough to start infections and thus increase death rates of the animals after 48 hr. The hemolysins of other pseudomonads were obtained by mere centrifugation.

As will be seen in table 2, the hemolytic titer of the preparation of *P. aeruginosa* remained exactly the same after heating. The toxicity of

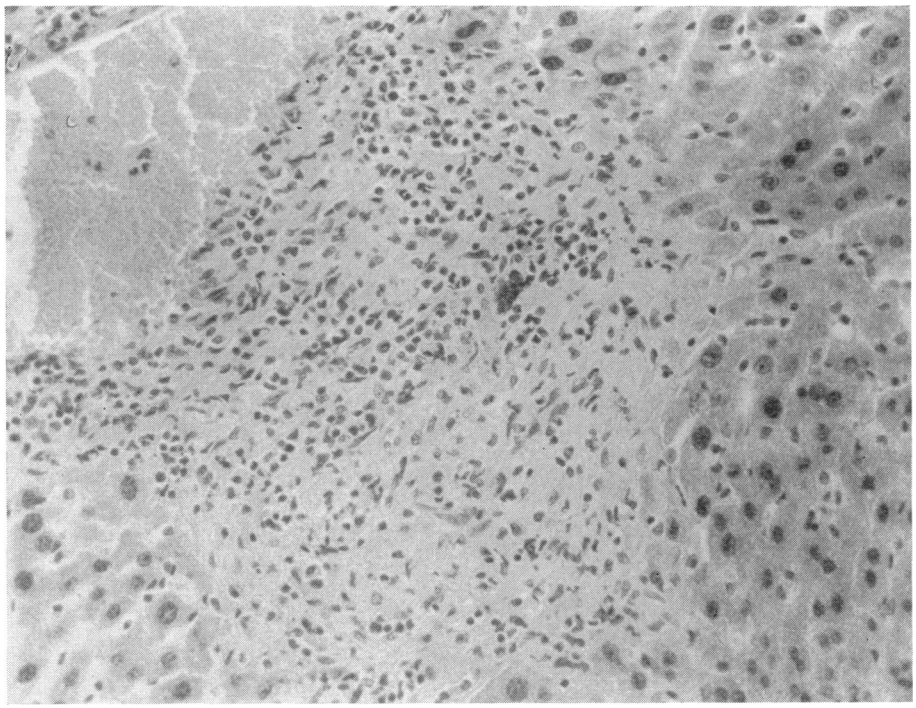

Figure 3. Infiltration of liver with inflammatory cells. The liver was taken from a mouse that died 18 hr after intraperitoneal injection of the hemolysin of *Pseudomonas aeruginosa*. The area of infiltration was always found connected with large vessels (upper left) indicating that the hemolysin was transported to the liver by the portal vein. Normal liver tissues are seen in the right side of the picture. × 250.

this hemolysin was somewhat reduced by heating but it still remained quite toxic for mice. The hemolytic titer of the preparation of *P. pseudomallei* was generally reduced to half of its original value by heating. The toxicity of this preparation was much higher than would be expected from its hemolytic titer and the loss of its toxicity after heating was entirely out of proportion to the reduction in its hemolytic titer. It was obvious that the toxicity of the preparation of *P. pseudomallei* was due to some nonhemolytic, heat labile substances. The preparations of *P. aureofaciens* and *P. chlororaphis* completely lost both their hemolytic activities as well as toxicities after heating for 15 min in a boiling water bath.

Pathological pictures produced by the hemolytic preparations of pseudomonads in mice. After the hemolysin of *P. aeruginosa* was injected into the mice intraperitoneally, the animals showed restlessness and ruffled hair in about 5 min. Many of them showed labored abdominal respiration and they tended to crouch together in the corner of the cage. When the animals died, within 12 hr after the injection, no significant pathology of the internal organs was noted except a copious serous exudate in the abdominal cavities. When the animals survived 18 hr or longer, several pathological changes were noted. Macroscopically, the most significant change at autopsy was the change of the color of the liver which appeared light brown. Microscopically, small areas of focal necrosis comprising neutrophile nuclear debris and degenerating liver cells were seen throughout the liver tissues. Frequently, areas of infiltration with inflammatory cells (neutrophile and mononuclear) were seen along both periportal space and around central vein. An example of this infiltration is shown in figure 3. The next most frequent change observed macroscopically was hemorrhage of the kidney. Microscopically, the kidneys showed acute congestion and slight hydropic degeneration of tubular epithelium together with hemorrhages. Figure 4 shows the kidney of a mouse which died

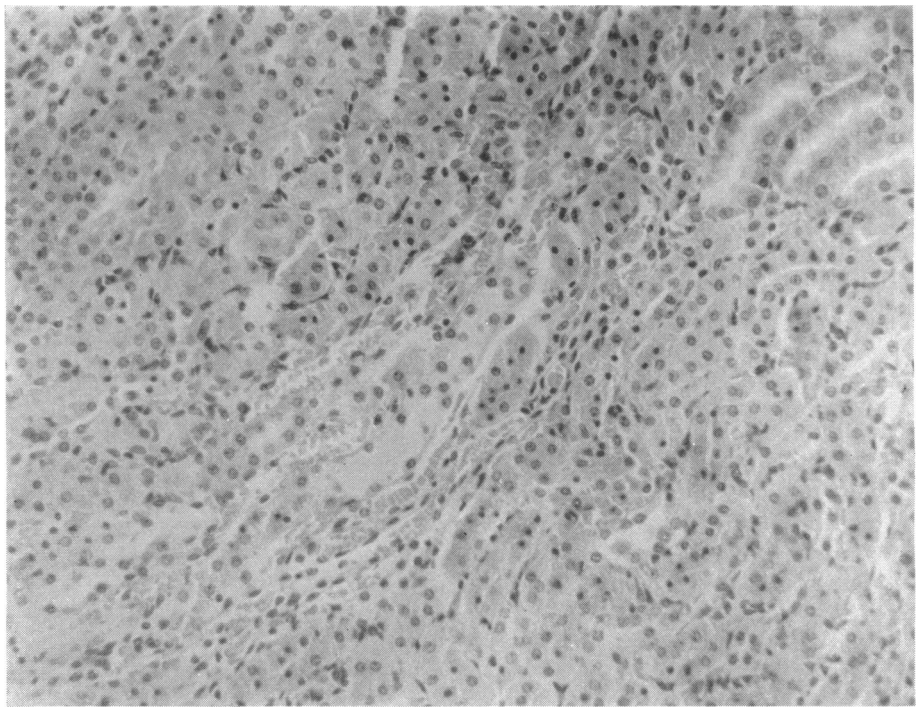

Figure 4. The kidney of a mouse that died 18 hr after the intraperitoneal injection of the hemolysin of *Pseudomonas aeruginosa*. Acute congestion, slight hydropic degeneration of tubular epithelium and hemorrhage are seen. × 250.

18 hr after the injection of the hemolysin of *P. aeruginosa*. Hemorrhage of lung and spleen was also frequently observed in these mice.

The hemolysins of *P. aureofaciens* and *P. chlororaphis* failed to produce lesions comparable to those produced by the hemolysin of *P. aeruginosa* in spite of the fact that in high concentrations they were able to kill mice within 18 hr. The liver of the animals which died between 24 and 48 hr after the intraperitoneal injections of these hemolysins appeared gray instead of the light brown color which was observed in animals killed by the hemolysin of *P. aeruginosa*. Microscopically, the liver showed acute congestion, neutrophile infiltration and occasional focal necrosis. Hemorrhage of the lung, kidney and adrenals was seldom observed.

Neutralization of the hemolysin of P. aeruginosa by normal animal sera and fractions of normal human sera. The hemolytic extracts obtained by Bullock and Hunter (1900), Landsteiner and Raubilschek (1908) and Fukuhara (1909) from old broth cultures of *P. aeruginosa* were all described as heat resistant. The hemolysin produced by this organism on cellophane plates was also heat resistant. The hemolytic extracts obtained by Fukuhara (1909) were neutralized by normal human sera and its fractions. It was, therefore, decided to examine the effects of normal human sera and its fractions on the hemolysin of *P. aeruginosa* produced on cellophane plates. The results are shown in table 3. More than 20 samples of normal human sera were tested and they all neutralized the hemolysin of *P. aeruginosa* at 1 to 128 dilutions. Fractions of normal human sera were also examined and they all showed some neutralizing effects to this hemolysin. Fraction V (albumin) showed the highest antihemolytic activity. Since the antihemolytic titer of fraction V was about the same as that of a normal serum, it can be stated that practically all of the antihemolytic activity of a normal human serum is contained in its albumin fraction. Sera of normal laboratory animals, such as guinea pig, rabbit, sheep, and

TABLE 3

Inhibition of the hemolysin of Pseudomonas aeruginosa by normal human serum and its fractions

Fractions	Dilutions							
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Whole serum	0	0	0	0	0	0	3	4
II-III	0	0	0	3	4	4	4	4
IV	0	0	3	4	4	4	4	4
V (Albumin)	0	0	0	0	0	0	3	4

Each fraction was diluted so that the starting dilutions contained approximately the same concentration of each component as they exist in normal human sera. Fractions II-III, IV and V contained 17, 10 and 52 mg/ml respectively.

Fraction I (fibrinogen) was not tested because it does not exist in normal sera.

The numerals in the data indicate the degree of hemolysis. '4' = complete hemolysis. '0' = complete absence of hemolysis. '3' = degree of partial hemolysis.

monkey also neutralized this hemolysin in dilutions from 32 to 128.

DISCUSSION

It is obvious from this study that hemolysin production among pseudomonads is quite common and it certainly cannot be used for the identification of *P. aeruginosa*. The characteristic of hemolysin production was found in both animal pathogens and nonpathogens. It appeared that phenazine pigment producers tended to produce high titers of hemolysin regardless of their optimal growth temperatures or pathogenicity to animals. However, no parallelism was noted between the quantities of the phenazine pigment produced by a strain and the amount of hemolysin obtained on the cellophane plate. Some strong pyocyanin producers yielded relatively small quantities of hemolysin and the strain of *P. aeruginosa* which produced the highest titer of hemolysin was a poor producer of pyocyanin.

The differences in heat resistance and toxicity of the hemolysins of various pseudomonads for mice indicate that there are some qualitative differences among these hemolysins. The hemolysin of *P. pseudomallei* was similar to that of *P. aeruginosa* in that the appearance of hemolysis was fast and the hemolytic activity was not destroyed by heating in a boiling water bath. The hemolysins of *P. aureofaciens* and *P. chlororaphis* acted very slowly on erythrocytes causing delayed hemolysis, and their toxicities as well as hemolytic activities were completely lost by heating. For lack of convenient designation, the fast-acting, heat-resistant hemolysin of *P. aeruginosa* and *P. pseudomallei* will be designated tentatively as type A in the subsequent discussion. The slow-acting, heat-labile hemolysins of *P. aureofaciens* and *P. chlororaphis* will be called type B. They will be called type A and type B instead of A and B, respectively, because it is still uncertain whether they are completely identical.

The type A hemolysin was much more toxic to mice than the type B as can be seen by the mortality of these animals after intraperitoneal injections. It is interesting to note that type A hemolysin was produced only by two human pathogens, *P. aeruginosa* and *P. pseudomallei*. It also should be mentioned in this connection that the hemolysins of two other animal pathogens, *P. caviae* and *P. reptilivora*, were also heat resistant although their titers were very low and sometimes they were not detected at all in a one to four dilution. The hemolysin produced by other nonpathogenic pseudomonads generally appeared heat-labile.

The possibility still exists that *P. aeruginosa* and *P. pseudomallei* also produce the type B hemolysin. Since the activity of this hemolysin is slow, it will be masked by the type A hemolysin unless the titer of the former is much higher than that of the latter. The reduction in the titer of the hemolysin of *P. pseudomallei* and the similar reduction in the toxicities of the hemolysin of *P. aeruginosa* without reduction in the hemolytic titer after heating may well be due to the destruction of the heat labile hemolysin contained in the preparations.

It has been well known that the cultures of *P. aeruginosa* contain several toxic substances. As early as 1899, Emmerich and Loew drew attention to the fact that old broth cultures of *P. aeruginosa* were highly bactericidal to many microorganisms. They also noted that culture filtrates of this organism lysed the cell suspensions of *Vibrio cholerae* and *Bacillus anthracis*. They ascribed this effect to the action of an enzyme called

"pyocyanase." The enzyme nature of this bactericidal, toxic substance was contradicted by Klimoff (1901) and was finally disproved by Schoental (1941). In a study of the antibacterial activities of *P. aeruginosa*, Schoental (1941) proved that all of the toxic, antibacterial activities of old broth cultures of this organism were due to three chloroform-soluble and dialyzable substances, i. e., pyocyanine, α-oxyphenazine, and a colorless, oily, bacteriolytic substance. The hemolysins described in this study did not pass through the cellophane sheet and their hemolytic activities were not affected by the chloroform treatment which removed the pyocyanine and related substances. It is, therefore, obvious that these hemolysins were distinct from those bactericidal substances described by Schoental (1941). The type A hemolysin, however, is very similar, if not identical, with the hemolytic extracts obtained by early workers (Bullock and Hunter, 1900; Landsteiner and Raubilschek, 1908; and Fukuhara, 1909) in that it was heat resistant and was neutralized by normal animal sera and fractions of normal human sera.

Most of the toxicity of the preparation of *P. pseudomallei*, however, appeared to be due to some nonhemolytic, heat labile substance. Its toxicity was quite out of proportion as would be expected by the hemolytic titer and, after heating, the loss of toxicity was entirely disproportional to the reduction in the hemolytic titer. The mode of action of the nonhemolytic, heat-labile toxin of *P. pseudomallei* also appeared entirely different from that of the hemolysin of *P. aeruginosa* because it was able to kill mice within 5–6 hr of injection while the hemolysin of the latter was never observed to do so in this short period of time. A study of this heat labile, toxic factor of *P. pseudomallei* is being conducted at the present time and the results will be reported later.

It should also be mentioned that the hemolysins described in this study and the three chloroform-extractable substances reported by Schoental (1941) are probably not all of the toxic substances that can be produced by *P. aeruginosa*. When cellophane plates were made with a medium which was inadequate for hemolysin production (nutrient agar (Difco), for example) the nonhemolytic preparation obtained after the growth of *P. aeruginosa* was also toxic, although the toxicity was not as high as that of the hemolytic preparations.

ADDENDUM

After this manuscript was sent to the Journal for publication, an article by Nigg *et al.* (1955) came to my attention. These workers demonstrated that a heat labile toxin as well as a heat resistant type could be obtained from 8–12 day old cultures of *P. pseudomallei* using a beef extract broth containing 4 per cent glycerin.

The heat labile toxin produced by these workers caused a hemorrhagic lesion on the skin of guinea pig within 5–10 min of injection. The same observation was made with the heat labile toxin produced on the cellophane plate.

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SUMMARY

A survey of hemolysin production among species of pseudomonads was made, using the cellophane plate technique. Several pseudomonads, both animal pathogens and nonpathogens, were found to produce hemolysin and it appears probable that hemolysin production cannot be used to differentiate species among pseudomonads. It was noted, however, that phenazine pigment producers, regardless of their pathogenicity to animals, tend to produce high titers of hemolysin.

There appear to be some qualitative differences in hemolysins produced by various pseudomonads. The hemolysins produced by *Pseudomonas aeruginosa* and *Pseudomonas pseudomallei* were heat resistant and act more rapidly on erythrocytes than the hemolysins produced by *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis* which were heat labile and produced hemolysis only after prolonged incubation.

P. pseudomallei produced a heat labile, nonhemolytic substance which was responsible for most of the mouse toxicity.

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