LECITHINASE PRODUCTION BY GRAM-NEGATIVE BACTERIA

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Received for publication December 5, 1960

Lecithinase production by microorganisms has been investigated in an attempt to use it in the classification of bacteria, and to associate formation with toxicity and virulence. The first record of this reaction was made when Nagler (1939) and Seifert (1939) observed, independently, the formation of an opalescence in human serum by the α toxin of *Clostridium perfringens*. It was soon found that human serum could be replaced with egg yolk (Macfarlane, Oakley, and Anderson, 1941), and the nature of the reaction was identified as the lecithinase activity which resulted in the liberation of phosphorus and choline (Macfarlane and Knight, 1941), with precipitation of fat which was the source of the opalescence. Other members of the genus *Clostridium* have since been shown to produce this enzyme. *C. oedematiens* (Crook, 1942; Hayward, 1943), *C. bifermentans*, *C. sordellii* (Hayward, 1943), and *C. haemolyticum* (Jasmin, 1947) are the well known lecithinase producers in the genus *Clostridium*. Some members of the genus *Bacillus*, such as *B. cereus*, *B. mycoides*, and *B. anthracis* (McGaughey and Chu, 1948; Colmer, 1948), and several different types of acid-fast bacilli (Toda and Urabe, 1936) were also reported to produce lecithinase.

Most of the organisms which produced lecithinase were gram-positive, and only a few gram-negative bacteria were reported to have this activity. Lecithinase production by *Vibrio comma* and El Tor vibrio has been reported by Ruata and Caneva (1901), Kraaij and Wolff (1923), and Felsenfeld (1944). The lecithinase activities of the members of the genus *Serratia* (Monsour and Colmer, 1952) and of the pseudomonads (Klinge, 1957) have been reported recently. In view of the paucity of the literature on the lecithinase activity of gram-negative bacteria, a study was undertaken to see the extent of lecithinase activity by this group of organisms. The present communication describes these findings.

MATERIALS AND METHODS

Strains of gram-negative bacteria used. Twenty strains of *Pseudomonas aeruginosa* isolated from human sources were used. Strain P-A-1, the representative of this species, was isolated from a case of otitis and was a good pyocyanin producer. W. C. Haynes furnished *Pseudomonas pseudomallei* (B-1110), *Pseudomonas reptilivora* (B-963), *Pseudomonas caviae* (B-966), *Pseudomonas aureofaciens* (B-1543P), and *Pseudomonas chlororaphis* (B-560). Several strains of *Pseudomonas fragi* and *Pseudomonas stutzeri* were obtained from P. W. Wetmore and one strain of *Pseudomonas viscosa* was obtained from G. Knaysi. M. E. Rhodes supplied 11 strains of *Pseudomonas fluorescens* and several other strains of this species were obtained from K. Klinge. S. F. Snieszko kindly supplied 6 strains of *Aeromonas liquefaciens* (including some strains of *A. hydrophila* and *A. punctata* which were considered by him to be identical with *A. liquefaciens*) and 2 strains of *Aeromonas salmonicida*.

W. H. Burkholder supplied *Xanthomonas begonia* (XB-10), *Xanthomonas campestris* (XC-10), *Xanthomonas vesicatoria* (XV-25), and *Xanthomonas phaseoli* (XP-34). Several strains of *Vibrio comma*, El Tor vibrio, and a water vibrio were obtained from W. Burrows and W. B. Cherry. P. R. Edwards furnished *Salmonella typhimurium*, *Salmonella typhosa*, *Salmonella hirschfeldii*, *Salmonella paratyphi*, *Salmonella Schottmuelleri*, *Salmonella anatum*, *Salmonella newport*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella flexneri*, and *Shigella sonnei*. L. S. McClung supplied *Serratia marcescens*, *Serratia indica*, *Serratia plymuthica*, *Serratia kiliensis*, and *Serratia marinorubra*. W. B. Cherry kindly supplied *Pasteurella multocida* (4866), *Pasteurella haemolytica* (4960), *Pasteurella pestis*, and *Pasteurella pseudotuberculosis*.

The following stock cultures from our department were used: *Escherichia coli*, *Aerobacter*

aerogenes, *Serratia marcescens*, *Proteus vulgaris*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Neisseria catarrhalis*, and *Neisseria flava*.

Media. Trypticase soy agar and broth with the addition of 0.11% of calcium chloride and 5% egg yolk were used for the demonstration of lecithinase activity (Adams, Hendie, and Pappenheimer, 1947; Grogan and Artz, 1959). The final pH of all media was adjusted to 7.2. Blood agar base (Difco) plus 5% washed human erythrocytes was used to test hemolysis. Tryptone glucose extract agar (Difco) enriched with 1% glucose was used for toxin production by *P. aeruginosa*, whereas trypticase soy agar enriched with 4% glycerol and 0.5% dibasic sodium phosphate was used for toxin production by other pseudomonads.

Demonstration of lecithinase activity. Lecithinase activity was demonstrated by inoculation of the organism from a 24-hr agar slant to egg yolk agar; egg yolk broth was inoculated using 0.1 ml of a 24-hr broth culture. Incubation was at the optimal growth temperature and length of time required for reaction of each strain tested. Lecithinase positive colonies on egg yolk agar were clearly marked by an opaque zone extending from the edge of the colony and a curd usually appeared on the top of the egg yolk broth.

Determination of acid soluble phosphorus. Fiske and SubbaRow's (1925) method for the determination of acid soluble phosphorus with modifications suitable for this study was used. All pyrex glassware was rinsed with alcohol to remove any possible phosphorus residues that might have been left from phosphorus-containing detergents. All phosphorus determinations were performed in triplicate.

Determination of choline. Florence reagent was prepared by dissolving 2.4 g of potassium iodide and 3.82 g of iodine in 5 ml of deionized water. The inoculated and incubated egg yolk broth was centrifuged and the supernatant was used to determine the qualitative presence of choline. A drop of the Florence reagent was transferred to a glass slide and one drop of the supernatant to be tested was placed adjacent to it in such a way that the edges of the two drops came in contact. A cover glass was immediately placed over the mixture and examined microscopically for choline periodide crystals which were usually seen near the merging point of the two drops (Booth, 1935).

Production and titration of lecithinase. The cellophane plate technique as described previously (Liu, 1957) was used for the production of lecithinase. Cellophane plates were inoculated by spreading two drops of a broth culture of the desired organism over the whole plate with a sterile cotton swab. The plates were incubated at the optimal growth temperature of each species. At the end of the incubation period, which was usually 36 to 48 hr, 3 ml of sterile saline were used to wash off the growth. The washings from 12 cellophane plates of each desired organism were pooled and centrifuged for 1 hr at 2,000 $\times$ *g* to remove the bacterial cells. Thimerosal (Merthiolate, Lilly) was added to this supernatant fluid to make the final concentration 1:5,000 and the mixture was left overnight at room temperature (22 to 26 C). The sterility of the mixture was tested by the addition of 1 ml of the preparation to broth.

Lecithinase activity of the preparation was titrated by 2-fold dilution in 0.5 ml of saline; and 0.5 ml of the 5% suspension of egg yolk in saline was added to each of the tubes. The mixtures were incubated overnight in a 37 C water bath and read. The reciprocal of the highest dilution giving a complete reaction, detected by a ring of fat at the top of the mixture and clear underlying fluid, was taken as the titer of enzyme activity.

Production of antilecithinase serum. Albino rabbits were selected for the production of each antilecithinase serum. In the immunization of the rabbits with the lecithinase preparations, a mixture containing equal amounts of lecithinase and Freund's (1947) incomplete adjuvant (Arlacel and Bayol in the ratio of 1.5:8.5) was used. The rabbits were injected intramuscularly with 0.5 to 2 ml of the mixture. A series of 6 to 8 inoculations were given 1 week apart. If the material was not too toxic, lecithinase without adjuvant was administered intravenously in the last two injections. Trial bleedings were made on all rabbits 5 days after the last injection. When a titer sufficient for use was acquired a total bleeding from the heart was performed.

Titration of antilecithinase activity of antiserum. Neutralization tests were performed by making 2-fold dilutions of the serum in saline using

TABLE 1

Lecithinase activity among gram-negative bacteria

Family	Genus and Species	No. Positive/No. of Strains Tested*
Pseudomonadaceae	*Pseudomonas aeruginosa*	20/20
	Pseudomonas pseudomallei	3/4
	Pseudomonas reptilivora	1/1
	Pseudomonas caviae	1/1
	Pseudomonas aureofaciens	1/1
	Pseudomonas chlororaphis	1/1
	Pseudomonas fluorescens	13/16
	Pseudomonas fragi	0/1
	Pseudomonas stutzeri	0/1
	Pseudomonas viscosa	1/1
	Xanthomonas begoniae	1/1
	Xanthomonas campestris	0/1
	Xanthomonas vesicatoria	1/1
	Xanthomonas phaseoli	1/1
	Aeromonas liquefaciens	4/4
	Aeromonas punctata	1/2
	Aeromonas salmonicida	2/2
Spirillaceae	*Vibrio comma*	6/6
	Vibrio El Tor	2/2
	Vibrio sp. ? water vibrio	1/1
Enterobacteriaceae	*Escherichia coli*	0/2
	Aerobacter aerogenes	0/4
	Serratia sp.	14/16
	Proteus vulgaris	0/7
	Salmonella sp.	0/7
	Shigella sp.	0/7
Brucellaceae	*Pasteurella multocida*	0/1
	Pasteurella haemolytica	0/1
	Pasteurella pestis	0/1
	Pasteurella pseudotuberculosis	0/1
	Bordetella bronchiseptica	0/1
	Bordetella pertussis	0/1
	Brucella melitensis	0/1
	Brucella abortus	0/1
	Brucella suis	0/1
	Haemophilus influenzae	0/1
	Haemophilus parainfluenzae	0/1
Neisseriaceae	*Neisseria gonorrhoeae*	0/1
	Neisseria meningitidis	0/1
	Neisseria catarrhalis	0/2
	Neisseria flava	0/1

* Egg yolk lecithin was used to test for lecithinase production by gram-negative bacteria. Determinations of acid soluble phosphorus and of choline were made to verify the hydrolysis of egg yolk lecithin by all reactive lecithinase producing gram-negative organisms.

0.25-ml amounts. The same amount of lecithinase preparation, diluted to contain 4 units per ml, was added to each tube. These mixtures were incubated at 37 C for 1 hr, and 0.5 ml of 5% egg yolk in saline was added. The tubes were incubated again at 37 C overnight and read for inhibition of lecithinase activity. The reciprocal of the highest dilution showing complete inhibition of the lecithinase activity was taken as the titer of the antiserum.

RESULTS

Lecithinase activity of various organisms. Table 1 indicates combined results of testing with all three techniques, i.e., egg yolk agar, egg yolk broth, and testing the cell-free extract from the cellophane plate. Most lecithinase positive organisms will show activity with egg yolk agar and egg yolk broth with the exception of *P. aeruginosa* which shows no reaction on egg yolk agar and an atypical reaction on egg yolk broth. However, extracts from cellophane plates of *P. aeruginosa* were strongly positive. The family *Pseudomonadaceae* has the largest number of lecithinase producers. *P. pseudomallei* was very active in this respect. *P. reptilivora* and *P. caviae* produced weak lecithinase activity on egg yolk agar. Of the parasitic organisms only *P. pseudomallei* showed a rather typical precipitating zone around its colony in addition to displaying a definite clearing of the egg yolk media around the colony (Fig. 1, *top*). *P. reptilivora* and *P. caviae* showed a more flaky-like flocculation around their colonies in addition to a preceding zone of clearing of the egg yolk agar. *P. fragi* and *P. stutzeri* gave negative results.

P. aureofaciens and *P. chlororaphis*, both phenazine pigment producers and found by Liu (1957) to be good producers of heat labile hemolysin, were active producers of lecithinase. Fig. 2 shows *P. chlororaphis* on egg yolk agar and blood agar. The zone of hemolysis is not as wide as the zone of precipitation on egg yolk agar and it is not certain whether the lecithinase is identical with the heat labile hemolysin. *P. aureofaciens* and *P. chlororaphis* gave a typical lecithinase reaction on egg yolk agar which features a dense, fine, cream-colored precipitate

with an even border, regardless of the size or morphology of the colony (Fig. 2, *right*). As the culture aged, a clearing of the egg yolk medium directly adjacent to the colony itself appeared. *P. chlororaphis* formed chlororaphine crystals after 5 to 6 days of incubation. These appeared as fine emerald green needles that sometimes radiated from the center. These crystals appeared near the surface of the egg yolk agar and egg yolk broth.

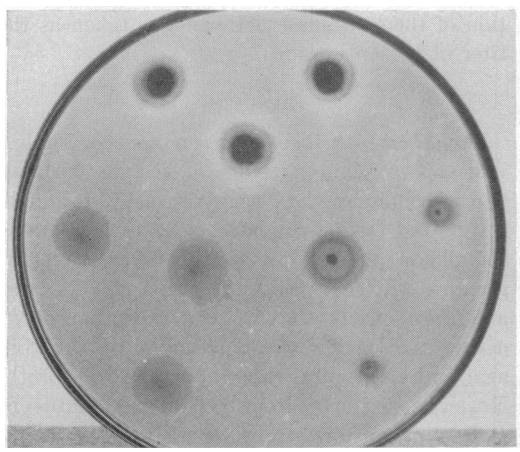

Fig. 1. Egg yolk agar plate. Reading *clockwise from top:* three colonies of *Pseudomonas pseudomallei* strain B-1110, showing a zone of opacity due to lecithinase surrounded by a zone of clearing of the media; three colonies of El Tor vibrio strain 34-D-23, showing a zone of opacity with a slight zone of clearing of the medium; three colonies of *Escherichia coli* showing a negative reaction.

Table 1 shows many strains of *P. fluorescens* to be good lecithinase producers, with the exception of three strains which were received from Dr. Rhodes. All the positive *P. fluorescens* strains gave typical lecithinase reactions on egg yolk agar with no preceding zone of clearing.

The only difference between *P. aureofaciens* and the lecithinase-producing *P. fluorescens* is the fact that *P. aureofaciens* produced a phenazine pigment (phenazine α-carboxylic acid). Therefore, if a strain of *P. aureofaciens* loses its ability to form this pigment it becomes indistinguishable from *P. fluorescens*. Many of the strains of *P. fluorescens* used in this study produced small amounts of orange pigment which appeared similar to the pigment of *P. aureofaciens*.

The genera *Xanthomonas* and *Aeromonas* were active producers of lecithinase but were much slower to show visible activity than the genus *Pseudomonas*. One species of *Xanthomonas* failed to show any lecithinase activity or hemolysis of human erythrocytes, whereas three members of this genus did. All members of the genus *Xanthomonas* tested developed a yellow nondiffusable carotenoid pigment. The lecithinase-active members of this genus showed a flaky fluocculation type of reaction around the colony which was uneven and preceded by a clearing zone of the egg yolk agar.

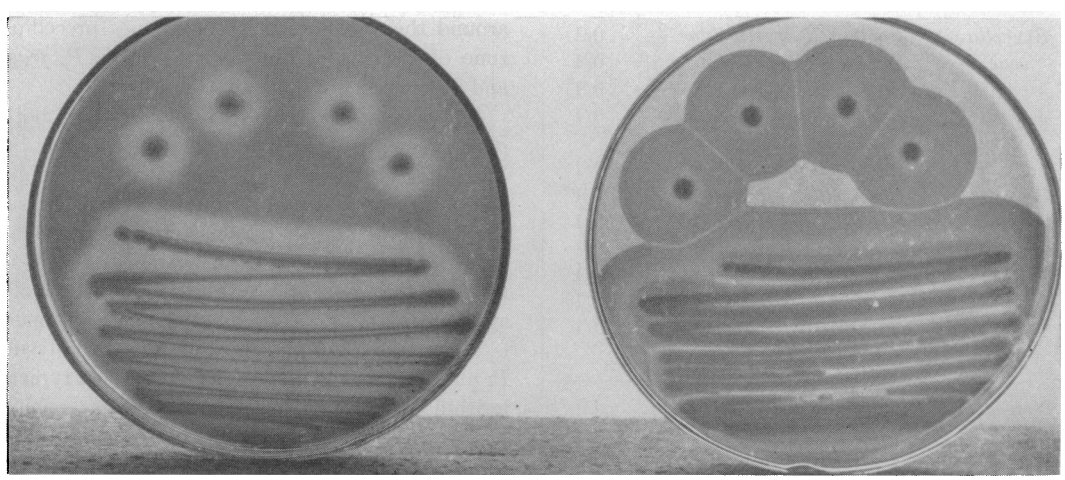

Fig. 2. Pseudomonas chlororaphis strain B-560 on human blood agar plate (*left*) and egg yolk plate (*right*). The zone of hemolysis is not as wide as the zone of precipitation on egg yolk agar. There is a clearing of the agar around the colonies of *P. chlororaphis* on egg yolk agar.

All the strains of *A. liquefaciens* produced lecithinase after 1 to 2 weeks. One strain of *A. punctata* (U-21) did not show hemolytic activity toward human erythrocytes nor did this organism exhibit a lecithinase reaction on egg yolk agar or egg yolk broth. However, another strain of *A. punctata* (U-23) hemolyzed human red blood cells and was an active lecithinase producer. *A. salmonicida* formed a soluble, brown melanin-like pigment. This organism was slow to produce lecithinase on egg yolk agar and egg yolk broth.

Findings of Felsenfeld (1944) were confirmed since two strains of *V. comma*, El Tor vibrio, and the water vibrio showed the production of lecithinase on egg yolk agar and egg yolk broth in 4 to 7 days. Fig. 1 shows the reaction of El Tor vibrio on an egg yolk agar plate. The zone of precipitation is not as even as the typical zone formed around colonies of the reactive *Pseudomonas* species as seen in Fig. 2 (*right*). The zones around all the vibrios tested were preceded by a zone of clearing of the media which might have been due to its proteolytic activity.

Only the genus *Serratia* of the *Enterobacteriaceae* family showed a precipitate on egg yolk agar in 1 to 4 days. Five strains of *S. marcescens* produced typical zones of precipitation on egg yolk agar, whereas two strains did not produce a zone. Two strains of *S. indica* were slow to produce a slight zone of precipitation on egg yolk agar and choline could not be demonstrated. Three strains of *S. plymuthica* and two strains of *S. marinorubra* produced marked zones of precipitation on the agar substrate, whereas two strains of *S. kiliensis* produced partial zones. All the reactive *Serratia* species tested showed zones of precipitation preceded by zones of clearing of the media. The members of the family *Brucellaceae* and of the genus *Neisseria* failed to demonstrate any lecithinase activity.

Data on the demonstration of phosphate and choline. Each milliliter of filtrate used in the phosphorus determination was equivalent to 0.01 ml of egg yolk. *P. aeruginosa* strain 20 showed the liberation of 16.1 mg of phosphate per 100 ml in 10 days and choline was demonstrated in 4 days. *P. aeruginosa* strain P-A-7 did not produce choline in a 5% egg yolk broth culture. However, the demonstration of choline was possible by mixing equal amounts of 5% egg yolk saline and its lecithinase produced by the cellophane plate technique. Therefore, the failure to demonstrate choline may be due to the utilization of choline by the organism. *P. pseudomallei* (B-1110) and *P. caviae* (B-966) freed phosphate equal to 17.3 and 8.3 mg per 100 ml, respectively, in 10 days, whereas *P. reptilivora* (B-963) liberated 11.2 mg per 100 ml in 16 days. Choline was demonstrated in the two former organisms in 4 to 5 days, whereas the latter did not produce choline.

P. aureofaciens and *P. chlororaphis* liberated 16.4 and 18.0 mg acid soluble phosphorus per 100 ml, respectively, in 8 days. Choline crystals were present on the 6th day of incubation in the case of *P. aureofaciens*, whereas *P. chlororaphis* did not show choline until the 14th day. *P. fluorescens* strain 28/4 liberated 16.1 mg of acid soluble phosphorus per 100 ml in 10 days, whereas the demonstration of choline was possible in 3 to 5 days of incubation. *P. viscosa* freed 20.9 mg of acid soluble phosphorus per 100 ml in 12 days, and choline was demonstrated in 8 days.

The Florence test was positive in all the lecithinase producers of the genera *Xanthomonas* and *Aeromonas*, thus proving that choline was liberated. Acid soluble phosphorus values after 22 days of incubation were 12.4 mg for *X. phaseoli* (XP-34) and 11.6 mg for *A. punctata* (U-23), per 100 ml.

V. cholerae (Hikojima 18) and El Tor vibrio (34-D-23) were strong producers of acid soluble phosphorus, liberating 24.0 and 16.8 mg per 100 ml, respectively, and choline crystals were demonstrated in 8 days. *S. marcescens* (16a) and *S. plymuthica* (146) liberated 16.8 mg of acid soluble phosphorus per 100 ml in 4 days. The former organism showed choline periodide crystals in 1 day, whereas the latter showed the presence of choline in 2 days. *S. marinorubra* (60) liberated 18.4 mg of acid soluble phosphorus per 100 ml in 4 days and choline was demonstrated in 1 day. *S. indica* (11) and *S. kiliensis* (113) did not produce choline. The former organism liberated only 10.0 mg of phosphate, whereas the latter liberated 9.5 mg of phosphate, per 100 ml.

Cross neutralization of antilecithinase of closely related organisms. Normal rabbit serum did not neutralize the lecithinase of *P. aeruginosa*, *P. pseudomallei*, and *P. aureofaciens* but did

TABLE 2

Cross neutralization of lecithinase activity of some Pseudomonas species by antilecithinase serum

Lecithinase	Antilecithinase Serum				
	P. aeruginosa	*P. pseudomallei*	*P. aureofaciens*	*P. chlororaphis*	Normal rabbit
P. aeruginosa	256*	0†	0	0	0
P. pseudomallei	0	8	0	0	0
P. aureofaciens	0	0	128	64	0
P. chlororaphis	8	16	512	1,024	64
P. fluorescens	16	64	128	512	128

* Number indicates the reciprocal of the highest dilution showing complete neutralization of lecithinase activity.

† Indicates no inhibition of lecithinase activity at the lowest dilution (1:8).

neutralize the lecithinase of *P. chlororaphis* and *P. fluorescens*. The significance of this is not known but it may indicate latent infections of *P. chlororaphis* in the rabbits. Table 2 shows the results of cross neutralization of the lecithinase of *Pseudomonas* species by antilecithinase sera. The lecithinases of *P. aeruginosa* and *P. pseudomallei* were specific, whereas *P. aureofaciens*, *P. chlororaphis*, and *P. fluorescens* showed cross neutralization in their lecithinases. It has been reported in a previous communication (Liu, 1961) that extensive cross reactions of the extracellular antigens of *P. aureofaciens*, *P. chlororaphis*, and many strains of the hemolytic, lecithinase positive *P. fluorescens* exists. Cross neutralization of these three species indicate that they are closely related. It is felt at present that these lecithinase-producing *P. fluorescens* strains are probably strains of *P. aureofaciens* or *P. chlororaphis* which have lost their ability to form phenazine pigments.

DISCUSSION

The results of this survey indicate that lecithinase production is by no means limited to gram-positive bacteria. Many gram-negative bacilli belonging to the genera of *Pseudomonas*, *Xanthomonas*, *Aeromonas*, *Vibrio*, and *Serratia* are active producers of this enzyme.

It was interesting to note that, in the gram-positive group, most of the lecithinase producers are anaerobes, whereas in the gram-negative group, most of the lecithinase producers are aerobes.

The absence of a positive reaction on egg yolk agar with *P. aeruginosa* may be due to a lipase acting on the precipitate formed by its lecithinase activity. Choline and phosphorus were split from egg yolk lecithin in spite of the failure of this organism to produce a typical egg yolk reaction.

The lecithinase produced by animal pathogens such as *P. aeruginosa*, *P. pseudomallei*, *P. reptilivora*, and *P. caviae* may play an important role in the pathogenesis of these organisms. However, it should be noted that this enzyme was also produced by other species such as *P. fluorescens*, which are not pathogenic for warm blooded animals. The failure of this organism to produce disease in warm blooded animals may be due to its inability to grow at 37 C. *P. chlororaphis*, which appears to be closely related to *P. fluorescens*, will grow at 37 C and is known to be pathogenic to some warm blooded animals such as mice and guinea pigs (Breed, Murray, and Smith, 1957).

The lecithinase positive gram-negative organisms studied showed variations in the time required for a curd formation, in the production of acid soluble phosphorus, and in the demonstration of choline. The reasons for this are unknown. However, these observations do indicate that the hydrolysis of egg lecithin into phosphorus and choline takes place in stages rather than simultaneously. The active lecithinase producers in this study were all found to be hemolytic.

Cross neutralization of the antilecithinases of closely related organisms appears to exist. The results of this study show that the lecithinases of *P. aureofaciens*, *P. chlororaphis*, and *P. fluorescens* are immunologically related, but are not related to the lecithinase of *P. aeruginosa*, *P. pseudomallei*, *P. reptilivora*, or *P. caviae*.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the following staff members of the Veterans Administration Hospital, Louisville, Kentucky, for the use of their facilities: Edward L. Foote, Jerre L. Noland, Bruce E. Rawlings, Mildred F. Edinger. Wendell Gillett is responsible for the photography.

The work was supported in part by a senior research fellowship (SF-259) given to P. V. Liu by the U. S. Public Health Service.

SUMMARY

A survey of lecithinase production among gram-negative bacteria was made using egg yolk agar and egg yolk broth.

The families *Pseudomonadaceae* and *Spirillaceae* contained the most active lecithinase producers. *Pseudomonas* and *Vibrio* species were the most active, whereas the *Xanthomonas* and *Aeromonas* species showed less activity.

Pseudomonas aeruginosa, *Pseudomonas pseudomallei*, *Pseudomonas reptilivora*, *Pseudomonas caviae*, *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, and *Pseudomonas viscosa* were active lecithinase producers, whereas *Pseudomonas fragi* and *Pseudomonas stutzeri* gave negative results.

Lecithinases of *P. aeruginosa*, *P. pseudomallei*, *P. reptilivora*, and *P. caviae* were immunologically distinct and species specific. Antilecithinase sera of *P. aureofaciens* and *P. chlororaphis* not only neutralized their own lecithinase but cross neutralized. Their antilecithinase sera also neutralized the lecithinase of many strains of *P. fluorescens*.

In the family *Enterobacteriaceae*, *Serratia* was the only genus producing lecithinase. The members of the family *Brucellaceae* and of the genus *Neisseria* failed to produce lecithinase.

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