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KININASE PRODUCTION BY SOME MICROBES

BY

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Bradykinin can elicit the four cardinal signs of inflammation (Elliot, Horton & Lewis, 1960), and plasma kinins have been mentioned as chemical mediators in the inflammatory response. It would be of interest therefore to know if, and how, various microbes can influence the level of free kinins in an infected area. Amundsen & Rugstad (1965) were unable to detect kinin-forming enzymes in three types of microbes investigated. Kinin-inactivating enzymes, kininases, could, however, be demonstrated after ultrasonic treatment of these microbes. In the cultures of one of the microbes tested, *Pseudomonas aeruginosa*, kininase activity was found also in the medium. The amount of free kinin present in any one place and situation is determined not only by the available amounts of kinin-forming enzymes and of substrates for these enzymes, but also by the local concentration of kinin-inactivating enzymes.

Kinin-inactivating enzymes, kininases, are widely distributed in the mammalian organism and their presence has been demonstrated in plasma, in lymph (Trautschold & Rüdel, 1963) and in various tissues (Amundsen & Nustad, 1965). Such activity has also been demonstrated in other types of biological material such as yeast (Werle, Kehl & Koebke, 1950), in mushroom, where a protease has been shown to inactivate kallidin (Werle & Berek, 1950), and in microbial cultures (Amundsen & Rugstad, 1965). This paper analyses further the kininase activity developed in microbial cultures. Special emphasis has been laid on the analysis of an extracellular kininase which occurs in the medium of cultures from *Pseudomonas aeruginosa*. Subsequent papers will describe work on the purification of this enzyme.

METHODS

Bradykinin activity

This was tested on the isolated rat uterus preparation. Virgin rats, weighing 150–200 g were given 30 μg stilboestrol intraperitoneally 20 hr before being killed. The isolated uterus was suspended in a 10-ml. organ bath containing de Jalon solution at 30° C. The contact time was 1 min, and the intervals between tests were 5 min. The weight load on the uterus was adjusted so that a good distinction was obtained between contractions caused by the addition of 12.5, 25 and 50 ng of bradykinin.

Kininase activity

This was estimated by incubating at 37° C 500 ng of synthetic bradykinin (Sandoz) in 0.1 ml. saline with 0.9 ml. of the fluid to be tested. Aliquots of 0.1 ml. of the mixture were taken out for testing on the rat uterus every 5 min. The first test was made 1 min after the incubation started.

When the amount of bradykinin in the test sample taken after 11 min of incubation was judged to be 12.5 ng or less, whereas the test sample taken out after 6 min had contained more than 12.5 ng bradykinin, the kininase activity present in that incubation mixture was defined as 1 u. kininase. One such unit of kininase will thus under these conditions destroy 75% or more of the 500 ng of bradykinin in 11 min.

Media

Distilled, deionized water was used throughout and all reagents were of pro analysi quality.

The following media were used:

Medium A. This was a coli minimal medium similar to that used by Davis & Mingioli (1950). It contained /l. of water:

	(g)
K_2HPO_4	7
KH_2PO_4	2
Na_3–citrate · $5H_2O$	0.5
$MgSO_4$ · $7H_2O$	0.1
$(NH_4)_2$ SO_4	1.0
Glucose	1.0

Medium B. This contained /l. of water:

	(g)
K_2HPO_4	11.0
KH_2PO_4	1.0
Na_3–citrate · $5H_2O$	1.0
$MgSO_4$ · $7H_2O$	0.1
$(NH_4)_2$ SO_4	2.0
Glucose	5.0

Medium C. This had the same content of salts and glucose as medium B. The dialysable fraction of yeast extract in water was, however, used instead of pure water. Ten grams of yeast extract (Difco Laboratories, Detroit, Michigan, U.S.A.) were dissolved in 60 ml. of water. This mixture was dialysed in a Visking dialysis tube (Visking dialysis tubing 18/32: Visking Department, Union Carbide International Co., New York, U.S.A.) against 1 l. distilled, deionized water for 2 hr. The diffusate was then used in medium C instead of pure water.

Medium D. This was glucose-broth, containing 5 g glucose, 5 g peptone and 5 g meat extract (Difco) /l. of water.

Bacterial cultures

These were kindly identified at Kaptein Wilhelmsen og Frues Bakteriologiske Institutt. The microbes used were *Escherichia coli* (*E. coli*), *Providencia* (*Prov.*) (=*Proteus inconstans*) and *Pseudomonas aeruginosa* (*Ps. aer.*). The *Providencia* microbe was reputed to be a *Proteus vulgaris*, and it has been designated as such in some earlier work (Amundsen & Rugstad, 1965)

Culturing of microbes

Unless otherwise stated the different microbes were allowed to grow under constant shaking at 37° C in 500-ml. Ehrlenmeyer flasks containing 100 ml. medium each.

Investigation of bacterial growth and of kininase activity in bacteria and in media

At intervals samples were taken out from the different cultures. Care was taken to avoid contamination. The number of bacteria was estimated by measuring the optical density at 680 mμ in a Beckman Photometer (Model B). If the optical density of a sample were higher than 0.80, the sample was diluted with sterile medium so that it gave an optical density between 0.30 and 0.80 before the final reading was made. Five millilitre samples of the different cultures were centrifuged at 10,000 × g for 15 min. The supernatant and the sediment were investigated separately for

kininase activity. Portions of sediment were examined for kininase activity after ultrasonic treatment, and the activity then revealed has been designated "Kininase activity in microbes." The portions of sediment which were exposed to ultrasonic treatment were obtained from 5 ml. samples from the cultures. The sediments were first washed three times in 0.9% saline, and then suspended in 5 ml. of a 0.02 M Na-phosphate buffer of pH 7.5. The ultrasonic treatment was then carried out with an MSE ultrasonic disintegrator at 20,000 c/s. This treatment was applied for five periods of 1 min each and under cooling in order to avoid increase in temperature. This amount of ultrasonic treatment resulted in maximal kininase activity from the microbes.

The supernatants from the centrifuged portions of microbial cultures were investigated before and after adjustment of the pH to 7.5. If the kininase activity in the supernatant equalled more than 1 u./ml. dilution with 0.02 M Na-phosphate buffer of pH 7.5 was carried out before further tests on kininase activity were performed.

RESULTS

Amundsen & Rugstad (1965) found that *Ps. aer.*, but not *E. coli* or *Prov.* (then, as explained in Methods, thought to be *Proteus vulgaris*), gave marked kininase activity in the medium after 18 hr of growth. This could have been due to autolysis of *Ps. aer.* occurring at an earlier stage than for the other microbes. In the present investigation all three microbes were grown in medium C until marked autolysis had occurred in all three cultures. Bacterial growth, pH and kininase activity in the medium and in the microbes were examined at intervals. The results from a typical set of experiments are seen in Fig. 1.

Sediments were prepared from all the three types of microbial cultures during the growth period and from samples with about the same optical density. When these sediments were exposed to ultrasonic treatment approximately equal amounts of kininase activity were revealed. The same amount of kininase was thus apparently present in all three types of microbes.

The supernatant medium from *Ps. aer.* cultures contained marked kininase activity. No kininase activity was detectable, however, in the supernatant medium of cultures of *E. coli* or *Prov.*, not even when marked autolysis of the microbes had occurred. The considerable amount of kininase which was bound to these microbes thus did not appear in the medium during autolysis. When the microbes were grown in glucose broth the results were similar.

The pH values in cultures of *E. coli* and *Prov.* in glucose broth and medium C were considerably lower than in the cultures of *Ps. aer.* (Fig. 1). Low pH in the culture might have inactivated possible kininase present or interfered with its production. In some experiments 3 g $CaCo_3$/l. medium was therefore added to medium C in order to preserve a higher pH. In such a medium the pH of an *E. coli* culture changed during the growth from 8.2 to 7.8, the pH of a *Prov.* culture from 8.2 to 7.9, whereas the *Ps. aer.* cultures had a pH of 8.2 throughout the growing period. The three different microbes were cultured in this medium until marked autolysis had occurred. Kininase activity was again not detectable in the supernatant medium from cultures of *E. coli* and *Prov.*, whereas the kininase activity in the supernatant medium from cultures of *Ps. aer.* was about the same as when this microbe was grown in ordinary medium C.

In another type of experiment the microbes were grown in medium C containing some bradykinin. One hundred micrograms of bradykinin was added to 25 ml. of medium in

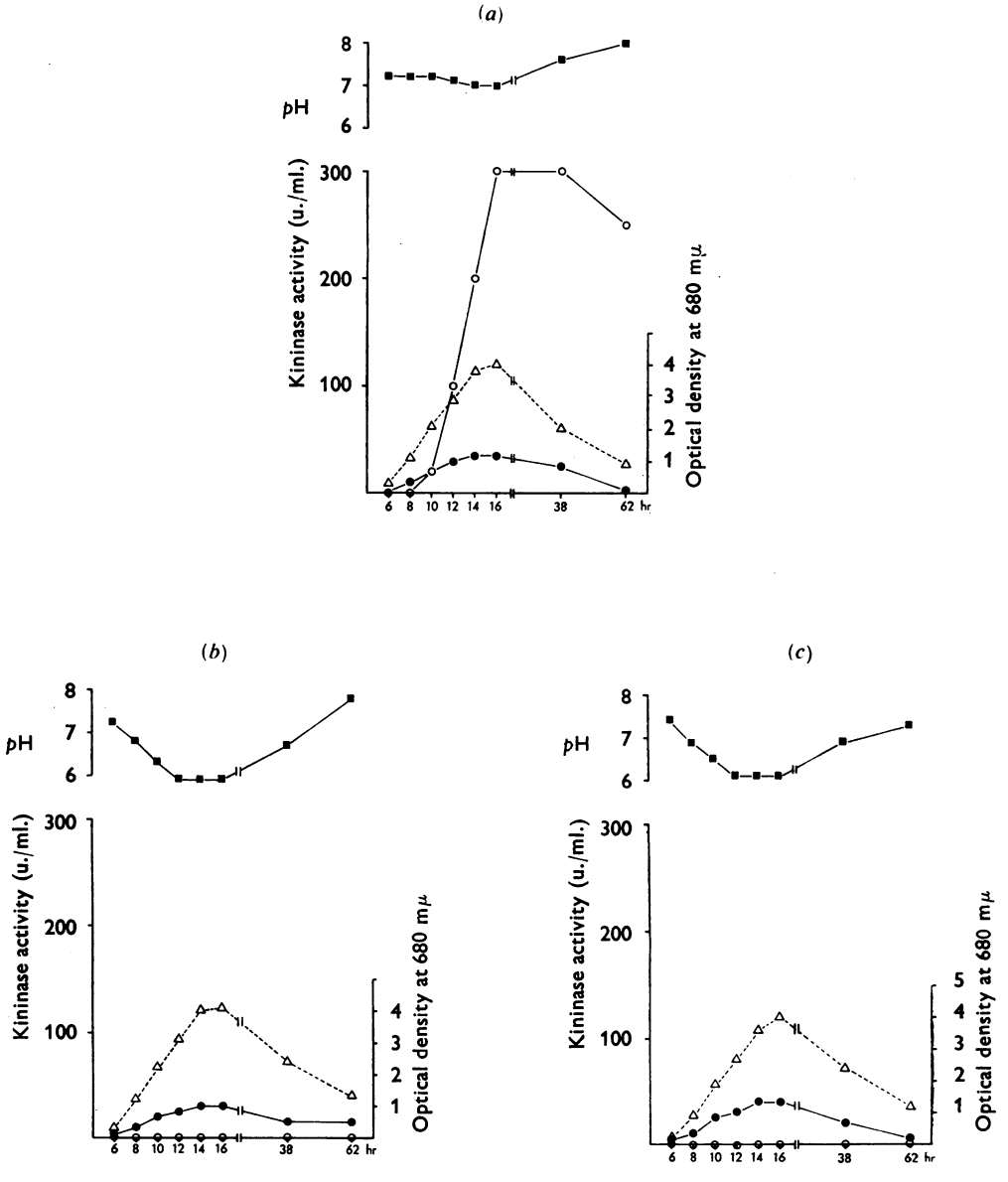

Fig. 1. Bacterial growth, pH and kininase activity in medium and in microbes in cultures of *Pseudomonas aeruginosa* (A), *Escherichia coli* (B) and *Providencia* (C). The different microbes were grown for 62 hr at 37° C in a shaking bath in 500-ml. Ehrlenmeyer flasks containing 100 ml./medium each. Samples were taken out at intervals for analysis of bacterial growth and kininase activity. Bacterial growth was estimated by measuring the optical density at 680 mμ. For the composition of medium C and for the methods used in testing kininase activity in medium and in microbes see Methods. △——△ optical density; ●——● kininase activity in microbes; ○——○ kininase activity in the medium.

TABLE 1

BACTERIAL GROWTH AND KININASE PRODUCTION IN CULTURES OF *PSEUDOMONAS AERUGINOSA* IN VARIOUS MEDIA

For the composition of the different media, see Methods. The microbes were cultivated in 500-ml. Ehrlenmeyer flasks containing 100 ml. medium each. The different cultures were examined after 14 hr of incubation (first values given) and after 38 hr (values in parentheses). Bacterial growth was estimated by measuring optical density at 680 mμ. Kininase activity was measured as described in Methods

Medium	Optical density	pH	Kininase activity in medium (u./ml.)	
A	0·6 (0·6)	0·7 (7·3)	2	(2)
B	1·3 (1·2)	6·9 (7·3)	100	(100)
C	3·4 (2·3)	6·8 (7·4)	400	(400)
D	3·5 (2·5)	6·9 (7·8)	400	(400)

each of several 100-ml. Ehrlenmeyer flasks before they were autoclaved. Autoclavation for 15 min at 120° C did not destroy the bradykinin. The cultures were examined after 14 and 38 hr, and the results from one such set of experiments are seen in Table 1. The presence of bradykinin in the medium apparently did not influence the growth of the microbes or the content of kininase in microbes or media. After 14 hr, when the optical density of the cultures had nearly reached maximum, some of the bradykinin added was still present in the media of *E. coli* and *Prov.* This confirms that these bacteria do not liberate significant amounts of kininase into the medium, and bradykinin cannot have penetrated the bacteria to any great extent. If the bradykinin in the medium had had access to the inactivating enzyme within the microbes or *vice versa*, the amount of kinin added would have been inactivated in a few minutes.

The production of kininase by *Ps. aer.* in different media has also been investigated. The purpose of this investigation was twofold. Frstly, would *Ps. aer.* produce kininase in all media which gave growth to the organism ? Secondly, it was important to find a medium which was suitable for work on purification of the kininase. Such a medium ought to give a high, preferably a maximal, amount of kininase, and it should ideally be without proteins. The results of the investigations with the different media are seen in Table 2.

TABLE 2

CULTURING OF *PSEUDOMONAS AERUGINOSA*, *E. COLI* AND *PROVIDENCIA* IN MEDIUM C WITH SYNTHETIC BRADYKININ 4 μg/ml. ADDED

The same microbes were cultured in medium C without bradykinin as a control. For the composition of medium C, see Methods. The microbes were cultured at 37° C in a shaking bath in 100-ml. Ehrlenmeyer flasks containing 25 ml. medium each. Bacterial growth was estimated by measuring the optical density at 680 mμ. The different cultures were examined after 14 hr of incubation (first values given), and after 38 hr (values in parentheses). Bradykinin and kininase activity were measured as described in Methods

	Pseud. aer. Bradykinin		*E. coli* Bradykinin		*Providencia* Bradykinin	
	With	Without	With	Without	With	Without
Optical density	3·3 (1·4)	3·1 (1·3)	3·8 (2·5)	3·2 (2·6)	3·4 (1·9)	3·8 (2·2)
Bradykinin in the medium (in % of amount added)	0 (0)	—	50 (20)	—	90 (0)	—
ininase activity supernatant (u./ml.)	200 (300)	200 (300)	0 (0)	0 (0)	0 (0)	0 (0)
Kininase activity n microbes (u./ml.)	30 (5)	20 (5)	30 (20)	30 (20)	30 (20)	30 (20)

A coli minimal medium (medium A) gave rather scarce microbial growth and also a small kininase production. Addition of different salts, of glucose, and amino acids to this medium was tried. Increased amounts of glucose and of various salts to the concentration of medium B increased the bacterial growth as well as the kininase production considerably. Further increase in content of glucose, $(NH)_2SO_4$ or citrate, or the The purpose of this investigation was twofold. Firstly, would *Ps. aer.* produce kininase production.

The addition of meat extract or of yeast extract gave, however, better microbial growth and an increased kininase production. Meat extract and yeast extract were equally effective in this respect. The dialysable part of yeast extract was found to be almost as effective as the undialysed extract. Glucose broth was the best medium for bacterial growth and kininase production in the present experiments. Medium C, which contains the diffusate of yeast extract (see Methods), gave almost as good bacterial growth and kininase production as glucose broth. Medium C (see Methods) was therefore chosen for further work on purification of the kininase.

The relation between bacterial growth and kininase production by *Ps. aer.* was studied in greater detail. One litre of medium C was autoclaved in a 3-l. flask. The medium was stirred with a magnetic stirrer and vigorously aerated with sterile air. At zero time 100 ml. of a 6-hr culture of *Ps. aer.* in the same medium was inoculated in the 3-l. flask. Every second hour the culture was examined for bacterial growth, pH and amount of kininase. Some kininase activity could be detected in ultrasonically treated microbes before such activity appeared in the surrounding medium (Fig. 2). The amount of enzyme in the medium was maximal when the optical density of the culture was maximal

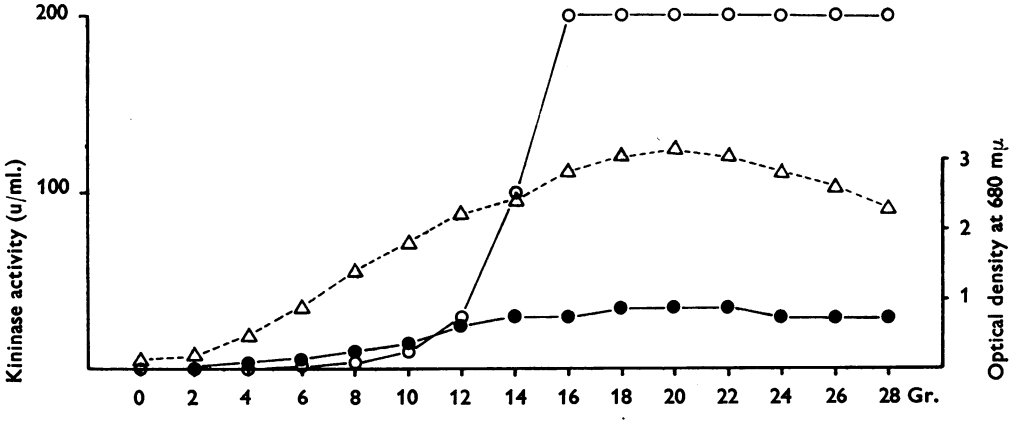

Fig. 2. Bacterial growth and kininase activity in the microbes and in the medium in a culture of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* was cultured in a 3-l. flask containing 1 l. of medium C. The medium was stirred with a magnetic stirrer and vigorously aerated. At zero time 100 ml. of a 6-hr culture of *Ps. aer.* in the same medium was inoculated in the 3-l. flask. Samples were taken out at 2-hr intervals for analyses of bacterial growth and kininase activity. Bacterial growth was estimated by measuring the optical density at 680 mμ. For composition of medium C and the methods used in testing kininase activity in microbes and in medium, see Methods. △——△ optical density; ●——● kininase activity in microbes; ○——○ kininase activity in the medium.

(in some experiments a short time before). Kininase activity of the medium then remained nearly constant for the following day or two.

The stability of the kininase in the medium was also tested in another experiment. A portion from a *Ps. aer.* culture was centrifuged 2 hr after the growth curve had reached its top, and the supernatant was kept for a further period at 37° C. After 48 hr no bacterial growth had occurred in this supernatant (as judged by measurements of the optical density), and its kininase activity had remained almost constant. The kininase activity remained constant in *Ps. aer.* cultures from the point where the maximal optical density had been reached (Fig. 2), and this indicates that the enzyme is produced during the growth phase only.

The relationship between enzyme production and growth of microbes was examined further in another experiment. Here a 30-ml. sample was taken out from a *Ps. aer.* culture (in medium C), which showed rapid growth. When this sample was taken out the optical density of the culture was 2.2, the kininase activity in the supernatant 50 u./ml., whereas the kininase activity bound to the microbes equalled 20 u./ml. of microbial culture. Two hours after removing the sample optical density was 3.9 in the culture itself, which had been allowed to grow further. The kininase activity in the medium was now 150 u./ml.

The 30-ml. sample taken out was centrifuged for 15 min at 10,000 g and the sediment washed three times in 0.02 Na-phosphate buffer of pH 7.5, and finally suspended in 30 ml. of the phosphate buffer. This suspension had an optical density of 1.9. When a part of it was centrifuged at 10,000 × g for 15 min, the supernatant showed traces of kininase activity only. Kininase activity bound to the microbes equalled 20 u./ml. The remaining part of the 30-ml. portion was kept at 37° C for 24 hr. Its optical density had then declined to 1.7, the supernatant after centrifugation had a kininase activity of 1 u./ml. only, whereas the kininase activity bound to the microbes was still 20 u./ml. These findings indicate that kininase is produced into the medium by growing bacteria. Bacteria stored under conditions where they are unable to grow apparently do not produce kininase.

DISCUSSION

The ability of microbes to interfere with formation and inactivation of plasma kinins may be related to their pathogenicity. For the three microbes *E. coli*, *Ps. aer.* and β-haemolytic streptococci, Amundsen & Rugstad (1965) were unable to detect kinin-forming ability. These and other microbes could still, however, cause formation of kinins by activating the hosts own plasma kinin-forming enzymes. The inactive precursor of a plasma kinin-forming enzyme in plasma may thus, for example, be activated by acidification (Lewis, 1960; Elliott, 1963). Lowering of pH will, at the same time, delay inactivation of formed kinins by plasma kininase (Edery & Lewis, 1962). It is well known that inflammatory exudates may have a pH below 7.

On the basis of the findings presented by Amundsen & Rugstad (1965), and in the present paper the participation of microbes in kinin inactivation seems to deserve special attention. All three microbes examined in this paper, *E. coli*, *Ps. aer.* and *Prov.* have approximately the same amount of kininase activity bound to the bacterial bodies. The significance of this activity is uncertain. It does not apparently interfere with bradykinin in the surrounding medium.

Pseudomonas aeruginosa, but not the other microbes investigated here, produces large amounts of extracellular kininase. Such a kininase should favour kinin elimination in the surroundings of the microbe, and it might be that the ability of *Ps. aer.* to produce such kininase is related to the types of infection caused by this microbe.

The production of this extracellular kininase by *Ps. aer.* into a medium containing very little protein is very abundant. A portion of such kininase-containing medium with its high content of the enzyme and with the relatively low content of other proteins represents a convenient starting preparation for purification of the enzyme. Work on such purification of the kininase is now being carried out in our laboratory, the intention being to study the mode of action of the purified enzyme preparation.

Two identified, well-known enzymes, are known to inactivate bradykinin, namely chymotrypsin (Werle *et al.*, 1950; Boissonnas, Guttman & Jaquenod, 1960) and carboxy peptidase B (Erdös, 1962). Both these enzymes inactivate bradykinin by initially splitting off the C-terminal arginine. It would be of interest to see if the enzyme produced by *Ps. aer.* splits bradykinin in the same manner. Preliminary work with purified preparations seems to indicate that this is not the case.

In a purified form this enzyme could be useful in studies on the possible roles of plasma kinins—that is, during infections. This enzyme could also be useful in the investigation of the biosynthesis and the mechanisms for excretion of extracellular enzymes. With the methods described here, which represent a very sensitive system, such an enzyme can be detected and its production followed. For the analyses of the substrate, bradykinin is needed in amounts of a few ng only. The study of this kininase and its production could therefore possibly be useful where our knowledge of extracellular enzymes is concerned.

SUMMARY

1. Cultures of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*Ps. aer.*) and *Providencia* (*Prov.*) have been investigated for content of kininase activity. Such activity was revealed in suspensions of washed microbes of all three types after ultrasonic treatment.

2. *Pseudomonas aeruginosa* also produced large amounts of kininase into the surrounding medium. Kininase activity was not detected in the medium from cultures of *E. coli* and *Prov.*, not even when marked autolysis of the microbes had occurred.

3. The extracellular kininase from *Ps. aer.* is apparently being produced into the medium during the phase of bacterial growth.

4. Production by *Ps. aer.* of the extracellular kininase was studied in cultures grown in various media. The kininase was produced into all media which gave growth to the microbe, including a protein-free medium. The kininase production was most marked, however, in media which allowed good growth of the microbe.

5. The addition to the medium of 4 μg/ml. of synthetic bradykinin did not apparently influence the growth or the kininase content in cultures of the three microbes.

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