The Urease Activity of Fluorescent *Pseudomonads*

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SUMMARY

Media frequently used for the detection of urease activity were found to be unsuitable for fluorescent pseudomonads since the presence of free ammonia was found to suppress the formation of urease in growing cultures. Incubation of cultures for 40 hr at 25° in a low concentration Casitone + yeast-extract + glucose medium, followed by the addition of urea to 0.2% (w/v), resulted in the development of an alkaline shift in the medium after further incubation for a few more hours at 37°. This method gave good results in the detection of urease activity.

INTRODUCTION

De Turk (1955) recorded the adaptive (inducible) formation of urease in washed suspensions of *Pseudomonas aeruginosa* harvested from nutrient broth. Nevertheless Klinge (1959, 1960) defined the genus *Pseudomonas* as 'urease negative', Brisou (1958) found no ureolytic strains among pseudomonads from various sources and Rhodes (1959) was unable to show with certainty the possession of urease by any of 169 fluorescent pseudomonads which she examined. However, Lysenko (1961), who used essentially the same medium as Rhodes (1959), described pseudomonad isolates which apparently possessed urease activity. These workers and others used media either originally devised for use with the Enterobacteriaceae or inorganic media containing urea with or without another organic carbon source. The present work was done to evaluate the various urea media described in the literature for urease detection, and to determine whether fluorescent pseudomonads do possess urease activity and, if so, under what cultural conditions the enzyme might best be demonstrated.

METHODS

Bacteria. Fifty isolates classified as belonging to the genus *Pseudomonas* from hen eggs, soil, river and ditch water, milk, and chicken droppings, were examined. All were Gram-negative, polarly flagellate rods which grew well at 25° but not at all at 42°. All oxidized glucose, were oxidase positive, penicillin resistant and produced pyoverdine (fluorescin) but not pyocyanine on the appropriate media proposed by King, Ward & Raney (1954). Nine distinct bio-groups had been recognized among 30 of the isolates on the basis of more than 70 biochemical features (Stewart, 1964); by using the criteria proposed by Klinge (1959, 1960), 24 of these 30 isolates were assignable to *P. putida* and the other 6 to *P. fluorescens*. Similarly 15 of the other 20 isolates were classified as *P. putida* and the remaining 5 as *P. fluorescens*. The organisms were maintained on nutrient agar slopes.

Basal modified urease (MU) medium. Casitone (Difco), 0.02 g.; yeast extract (Difco), 0.02 g.; glucose, 0.05 g.; NaCl, 0.3 g.; K_2HPO_4 , 0.02 g.; distilled water 98 ml.; mixed indicator solution, 2 ml. Adjusted to pH 7.4. Sterilized for 15 min. at 120°.

Glucose was chosen in preference to galactose, which was used by Rhodes (1959), because manometric studies showed that glucose was more rapidly oxidized by the isolates. The buffer salt (K_2HPO_4) was kept at a low concentration to allow a more rapid increase in pH value with base production. The mixed indicator system proposed by Singer (1950) was chosen since its green colour at pH 7.4 changes to blue at about pH 8.2 and to a distinct violet at about pH 9.0, thus allowing relative degrees of base production to be assessed. It consists of three indicator solutions prepared by dissolving 0.20 g. of bromothymol blue, cresol red and thymol blue in 6.4 ml., 10.6 ml. and 8.6 ml., respectively, of NaOH (0.05 N) and adding 100 ml. of distilled water to each solution; the three solutions are then mixed in the proportions 12.5:4:10 by vol.

Chemicals. Analar grade NH_4Cl , $(NH_4)_2SO_4$ and urea (Hopkin and Williams) and 40% (w/v) sterile urea solution (Oxoid) were used.

Delivery of 0.02 ml. amounts. Platinum-tipped dropping pipettes (Astell) were used.

Assay of urease activity in washed suspensions. To 0.40 ml. of a 0.25% (w/v) solution of urea (Hopkin and Williams) in veronal buffer (0.04 M, pH 7.2) was added 0.10 ml. of bacterial suspension containing the equivalent of 200 μ g. dry wt. bacteria. The ammonia released after incubation for 1 hr at 30° was estimated by the Berthelot reaction on 0.10 ml. of reactant mixture with UN Kit reagents (Hyland Laboratories, California). Endogenous and substrate ammonia were similarly determined on appropriate incubated mixtures. NH_3 -N values were calculated from a standard curve prepared from $(NH_4)_2SO_4$ solutions in ammonia-free distilled water.

Urease activity (qNH_3 -N) was expressed as μ g. NH_3 -N released under the assay conditions by equiv. 1 mg. dry wt. bacteria/hr at 30°.

Dry weights of pseudomonad suspensions. These were determined by nephelometry and the use of a calibration curve.

RESULTS

Behaviour of the pseudomonad isolates in various urea-containing media

The following media were prepared: A (Christensen, 1946); B (Brisou, 1958); C (Stuart, van Stratum & Rustigian, 1945); D (Ferguson & Hook, 1943); E (Rhodes, 1959); MU medium (present paper). Their compositions are given in Table 1. The various media were inoculated, with a straight wire, from nutrient agar cultures of the 50 isolates and incubated at 25°. Daily for the first 14 days and then every few days for a total of 42 days, the tubes were examined for growth and change in pH value. The results (Table 2) clearly showed that media B, C and D were unsuitable for the growth of all but a few of the isolates. Most of the isolates grew in medium E although only 3 of 45 of these gave an alkaline reaction. All the isolates grew well in medium A to produce in most cases (45/50) a slow alkaline reaction. Forty of these 45 isolates, however, gave a similar reaction in this medium even when the urea was omitted, a phenomenon earlier reported by Rhodes (1959), and one which renders medium A useless in testing such organisms for urease activity.

Table 1. *Urea-containing media*

Composition of the five urea-containing media examined: A (Christensen, 1946); B (Brisou, 1958); C (Stuart *et al.* 1945); D (Ferguson & Hook, 1943); E (Rhodes, 1959); MU (present paper)

Constituent	Percentage (w/v) composition in medium:					MU
	A	B	C	D	E	
Peptone (Oxoid L37)	0.1	—	—	—	—	0.02 (Casitone)
NH ₄ H ₂ PO ₄	—	—	—	—	0.1	—
Yeast extract (Difco)	—	—	0.01	—	—	0.02
Glucose	0.1	—	—	—	—	0.05
Galactose	—	—	—	—	0.1	—
Ethanol	—	—	—	1.0	—	—
KH ₂ PO ₄	0.2	0.1	0.91	0.1	—	—
K ₂ HPO ₄	—	0.1	—	0.1	—	0.02
Na ₂ HPO ₄	—	—	0.95	—	—	—
NaCl	0.5	—	—	0.5	—	0.3
KCl	—	—	—	—	0.02	—
MgSO ₄ ·7H ₂ O	—	—	—	—	0.02	—
Agar	2.0	—	—	—	—	—
Urea (Oxoid)	2.0	2.0	2.0	2.0	2.0	[0.2]*
Indicator	PR	BTB	PR	PR	PR	S
Initial pH	6.8	7.0	6.8	7.0	7.0	7.4

PR = Phenol red; BTB = Bromothymol blue; S = Singer (1950).

* Added after culture incubated at 25° for 44 hr.

Table 2. *Behaviour of the 50 pseudomonad isolates in the urea-containing media*

Showing the proportion of isolates able to grow and to produce an alkaline reaction in media: A (Christensen, 1946); B (Brisou, 1958); C (Stuart *et al.* 1945); D (Ferguson & Hook, 1943); E (Rhodes, 1959)

Medium	Proportion able to grow	Proportion producing an alkaline reaction	Time for an alkaline reaction to develop (weeks)
A	50/50	45/50	3-4
B	8/50	8/8	3-4
C	5/50	0/5	—
D	7/50	7/7	3-4
E	45/50	3/45	2-3

Behaviour of the pseudomonad isolates in MU medium containing various concentrations of urea

Urea (Oxoid) was added aseptically to batches of sterile basal MU medium (pH 7.4) to final concentrations (% w/v) of: 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0. These media were dispensed in 2 ml. amounts into test tubes (100 mm. × 10 mm.) and inoculated with 0.02 ml. of dilute suspensions of organisms in $\frac{1}{4}$ -strength Ringer's solution prepared from nutrient agar cultures of 6 of the isolates (3 classified as *Pseudomonas putida*, 3 as *P. fluorescens*). These cultures were incubated for 14 days at 25° with daily examination for growth and change in pH value.

The 6 isolates grew well in all seven media; the results with one of them (S. 60) typical of the 6, are shown in Table 3. It appeared that urea concentrations greater

than 0.2% in the MU medium delayed the change to a more alkaline pH value. All 50 isolates were then tested by growth in the 0.2% urea MU medium; they all produced a blue colour within 7 days and a violet one within 12 days. In the MU medium without urea, all the isolates caused a slight but permanent acid change (yellow-green, pH 6.5) after incubation for a day or two.

Table 3. *Reactions of pseudomonad strain S. 60 in the MU media*

The time (in days) for the pH value to increase to 8.2 (indicator blue) and to 9.0 (indicator violet) in the MU media containing (% w/v): 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 urea, inoculated with strain S. 60 and incubated at 25°.

Percentage (w/v) urea in medium	Days for alkaline reaction to develop	
	pH 8.2 (blue)	pH 9.0 (violet)
0	n. al.*	n. al.
0.05	3	5
0.1	3	5
0.2	3	5
0.5	4	6
1.0	5	10
2.0	5	10

* n. al. = no alkaline reaction developed within 14 days.

Table 4. *Urease activities (qNH₃-N) of pseudomonad strain S. 1 incubated in MU media with and without urea*

Age of culture (days)	qNH ₃ -N of bacteria harvested from:		
	MU medium	MU medium + 0.2% urea	MU medium + 2% urea
1	5.1	0.4	0.3
2	25.3	1.2	0.5
4	32.0	4.5	0.9
6	84.1	4.4	1.2

The development and activity of pseudomonad urease in MU media

Modified basal MU medium with 0.4% (w/v) K₂HPO₄ and without indicator was prepared. Batches containing: no urea, 0.2% (w/v) urea (Oxoid) and 2% (w/v) urea (Oxoid), respectively, were dispensed in 100 ml. amounts in round bottles of 230 ml. capacity. A dilute suspension of one of the pseudomonad isolates (S. 1) was inoculated (1 ml.) to each of four bottles of the three MU media (0, 0.2, 2% urea). After incubation for 1, 2, 4 and 6 days at 25°, the crop of isolate S. 1 was harvested by centrifugation from, on each occasion, one bottle of each of the three media. The various lots of deposited organisms were washed twice in distilled water, each re-suspended to 1 ml. in veronal buffer (0.04 M, pH 7.2) and held at -20° for urease assay. The urease activities (qNH₃-N) of the suspensions are shown in Table 4. The bacteria grown in the absence of urea showed significant urease activity which increased greatly with the time of incubation of the cultures. Very little urease was formed in the bacteria grown in the 0.2% urea medium and even less in the 2%

urea medium. It was concluded that urea added to MU medium exerted a considerable inhibitory effect on the formation of urease.

The effect of urea and of ammonia on the development of urease

Organisms of pseudomonad strain S. 1 were harvested after growth for 6 days in MU media containing (w/v): (1) 0.0038 % NH_4Cl (equivalent to 10 μg . $\text{NH}_3\text{-N/ml.}$); (2) 2 % Oxoid urea; (3) 2 % Hopkin and Williams urea (urea added as a Seitz-filtered solution). Urease activities of the organisms and the supernatant $\text{NH}_3\text{-N}$ concentrations of the culture fluids before and after incubation were measured. It can be seen from Table 5 that little or no ammonia disappeared from any of the media during incubation. The results indicated that the low concentration of glucose (0.05 %, w/v) in the medium was insufficient to allow the assimilation of any more nitrogen than was already provided in organic forms in the peptone and yeast extract and that the presence of very low concentrations of unassimilable $\text{NH}_3\text{-N}$ was sufficient to suppress urease formation by the growing bacteria.

Table 5. *Ammonia-N assimilation by, and urease activity of pseudomonad strain S. 1 grown in MU media with or without the addition of NH_4Cl or urea*

Urease activity ($\text{qNH}_3\text{-N}$) of organism S. 1 harvested from, and the $\text{NH}_3\text{-N}$ concentrations in the culture supernatant fluids of, MU medium with and without urea.

Medium	$\text{NH}_3\text{-N}$ ($\mu\text{g./ml.}$)		$\text{qNH}_3\text{-N}$
	At inoculation	After 6 days at 25°	
MU (no addition)	1.0	0.9	104
MU + 0.0038 % NH_4Cl	10.1	9.7	< 1
MU + 2 % Oxoid urea	38.0	36.5	< 1
MU + 2 % Hopkin and Williams urea	3.0	14.0	< 1

Technique adopted for demonstrating urease activity in fluorescent pseudomonads

On the basis of the above results, the following procedure was used to examine urease production by the 50 pseudomonad isolates. The isolates were inoculated by a straight wire from nutrient agar cultures into 2 ml. of basal MU medium (in 100 mm. $\times$ 10 mm. tubes). After incubation for 40 hr at 25°, good growth was apparent in all the tubes and the pH values had decreased to about 6.5. To each tube was then added aseptically 0.02 ml. of 20 % (w/v) urea solution, the tubes were shaken and re-incubated at 37°. Alkalinity developed within a few hours in all tubes; in no case did the incubation period for a pH value of 9.0 (violet) to be reached exceed 6 hr.

In addition to these 50 isolates, all of 25 isolates of *Pseudomonas aeruginosa* from clinical specimens and milk were found by this technique to hydrolyse urea actively (with these organisms the initial incubation for growth was at 37°).

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