THE UREASES OF PROTEUS STRAINS IN RELATION TO VIRULENCE FOR THE URINARY TRACT

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PLATES XXX-XXXII

THE ENZYME urease is thought to play a major role as a virulence factor in urinary-tract infections with *Proteus* (Braude and Siemieniowski, 1960) although it may not be the only factor (Eudy, Burrous and Sigler, 1971). Urease degrades urea with release of ammonia which may cause damage and death to the renal epithelium, inactivation of complement, and conditions favouring the development of renal stones. Many of these effects can be prevented by the administration of urease inhibitors (Griffith and Musher, 1973; Griffith, Musher and Campbell, 1973; Aronson, Medalia and Griffel, 1974; Musher *et al.*, 1975).

Phillips (1955) found wide variation in the pathogenicity of different strains of *Proteus* for the mouse kidney *in vivo*, and Senior (1979) has shown that particular proticine-production/sensitivity (P/S) types of *P. mirabilis* have a special affinity for the urinary tract whereas other P/S types and other *Proteus* species are seldom incriminated in serious urinary-tract infections. The aim of this study was to determine whether or not these differences in pathogenicity among strains of *Proteus* were associated with differences in the types of ureases produced by these strains.

MATERIALS AND METHODS

Bacterial strains. Strains of *Proteus* species were isolated from specimens of urine and faeces sent for routine bacteriological examination. They were maintained as pure cultures on nutrient-agar slopes at 4°C. In addition, two strains of *P. mirabilis* from boys with urinary-tract infection (Dr R. Maskell), five strains of *P. rettgeri* NCTC nos. 7475, 7476, 7477 and 7481, and strain GRR3 (Dr D. Jones) and 17 strains of *P. rettgeri* of different O serotypes (Dr J. Penner) were provided by colleagues. The species of each strain was determined or confirmed from the results of reactions in glucose, lactose, mannitol and sucrose peptone-water sugars, urea broth and tryptone water when tested for indole formation. *P. mirabilis* or *P. vulgaris* strains were typed by the method previously described (Senior 1977).

Growth of strains and preparation of sonic extracts. Preliminary investigations indicated that urease production by each strain was induced by urea. Cultures of strains actively producing urease were prepared therefore by 100-fold dilution of overnight broth cultures (Oxoid CM67) into one of two media; these were (1) sterile 2.5% (w/v) nutrient broth (Oxoid CM67) in 0.1M sodium phosphate buffer pH 7.5 supplemented with urea solution (40% w/v), sterilised by

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filtration, to a final concentration of 1% (w/v) or (2) pooled urine. This latter medium was prepared by adding 1M NaOH to pooled urine until the pH reached 8.3. After standing overnight at 4°C, the resulting alkali-precipitable material was removed by centrifugation. The pH of the clear supernatant urine was adjusted to 6.0 by the addition of 1M HCl before sterilisation by filtration. Phenolphthalein solution 1% (w/v) in propan-2-ol, was added to both media to a final concentration of 0.005% (w/v).

The cultures were incubated with shaking at 37°C until alkalinity developed through urease production and degradation of urea; this stage was indicated by the phenolphthalein indicator starting to change from yellow to pink. At this point, the cells were harvested by centrifugation at 4°C. The cell pellets were washed twice in PEM buffer (1mM sodium phosphate, 1mM disodium EDTA and 5mM 2-mercaptoethanol pH 7.0) and resuspended in PEM buffer containing glycerol 20% (w/v) and a trace of bromophenol blue dye. The cell suspensions were disrupted by sonication for 1 min. in an ice-water bath. The cell debris was removed by centrifugation at 3500 *g* for 5 min and the supernate was stored at -20°C.

Assay of urease. The urease activity of the sonic extracts was determined by measuring, by the modified Berthelot reaction, the amount of ammonia formed from a standard excess amount of urea: 10- μ l portions of extract were added to 240 μ l of UPEM substrate (30mM urea, 20mM sodium phosphate buffer pH 7.5, 1mM disodium EDTA, 10mM 2-mercaptoethanol) and the mixture incubated for 5 min. at room temperature. The reaction was stopped by the addition of phenol nitroprusside solution 0.5 ml, alkaline hypochlorite 0.5 ml and distilled water 2.5 ml. After 5 min. at room temperature, the intensity of the developed blue colour was measured at 570 nm and compared with that given by standard solutions of ammonium chloride. One unit of urease was defined as the amount of extract required to liberate 1 μ mol of ammonia in 5 min. at room temperature in the above conditions.

Analysis of urease by polyacrylamide-gel electrophoresis. Sonic extracts containing urease were analysed by high-pH discontinuous polyacrylamide-gel electrophoresis. One unit of urease was applied to each slot of a slab gel comprising a resolving gel of acrylamide 6% (w/v) and N,N'-methylene-bis-acrylamide 0.3% (w/v) in 0.375M Tris HCl pH 8.9, 1mM disodium EDTA and 5mM 2-mercaptoethanol and a stacking gel of acrylamide 3% (w/v), N,N'-methylene-bis-acrylamide 0.15% (w/v) in 0.0625M Tris HCl pH 6.7, and overlaid with electrode buffer (0.38M glycine in 0.005M Tris). Electrophoresis was usually done at 75 V for 16 h at 4°C. The gels were then gently washed at 4°C in several changes of an aqueous solution of disodium EDTA 0.1% (w/v) and cresol-red indicator 0.02% (v/v) until the gel just became yellow, indicating neutral or slightly acid conditions. Gels were developed by discarding the washing solution and immersing them in a small volume of urea 1.5% (w/v) in water. Because urea is the specific substrate for urease, active urease isoenzymes were deduced to exist in areas where cherry-red bands appeared against the yellow background by the local production of alkali as a result of enzymic degradation of urea.

The developed gels were recorded by immediate photography with a Nikon F2 camera fitted with a Nikon XI dark-green filter. The film, Ilford Pan F, was developed in ID 11 developer for 8 min. at 20°C. To detect traces of urease isoenzymes, it was necessary to prolong the period of immersion in urea. These conditions, however, led to a broadening of the major bands and this eventually masked the location of minor bands. To prevent this and yet permit detection of minor bands, when major bands had been located, the gels were cut. The portion of the gel containing the major bands was removed from the urea solution and stored at 4°C in the EDTA-cresol red washing solution. When the minor bands could be clearly seen, the portions of the gel were reassembled and photographed together.

RESULTS

Initial experiments showed that the electrophoretic pattern of urease isoenzymes of strains grown in phosphate-buffered broth containing urea were indistinguishable from those given by the same strains when grown in treated urine. Thus the differences in enzyme patterns observed, within a species and

between the different *Proteus* species, cannot be attributed to variation in cultural conditions.

P. morgani urease

The ureases of 12 strains of *P. morgani*, isolated from the urine or faeces of 12 healthy male and female patients, ranging in age from 4 months to 86 years, were examined. Electrophoresis failed to reveal any differences between the urease isoenzymes of these strains. Each strain produced three isoenzymes (fig. 1a); the fastest moving isoenzyme, also called the main band or α urease, (Blattler, Contaxis and Reithel, 1967) was extremely active and rapidly appeared during the development of the gel. It was followed by β and γ ureases equidistant behind α . In some instances it seemed that the α urease might be a double band and this was possibly also true for the β and γ isoenzymes. All three isoenzymes seemed to be of high molecular weight because they did not move far into the gel. Further analysis of these isoenzymes in 5% acrylamide gels run overnight at 140 V gave better separation (fig. 1b).

P. rettgeri urease

The ureases of 26 strains of different serotypes of *P. rettgeri* were examined. Although some strains of different O serotype gave the same electrophoretic pattern, there was considerable diversity among other strains in their urease isoenzymes, and representatives of the different types found are shown in fig. 2a. The α urease isoenzymes of different strains electrophoresed over a range of positions. When comparisons were made between *Proteus* species into the mobility of their α urease, it was clear (figs. 2b and 2c) that, in general, those of *P. rettgeri* were the furthest moving, followed by those of *P. vulgaris*, *P. mirabilis* and *P. morgani*. After the development of the α -urease band, the next isoenzyme to appear was one that electrophoresed only a short distance into the gel, ω urease. Only some strains of *P. rettgeri* appeared to possess this isoenzyme, which banded in a position close to that of the α urease of *P. morgani* (fig. 2a). Further analysis (fig. 2c) showed that the ω urease banded in a position between the α and β ureases of *P. morgani*. Occasionally, the α and the ω urease bands of some strains each appeared to be double bands (fig. 2c slots 2, 4, 6). Prolonged development of the gels revealed minor isoenzymes (usually two) behind the α urease and there appeared to be differences between the strains in the relative proportions of the two components of these minor isoenzymes (fig. 2a).

P. vulgaris urease

The ureases of 12 strains of *P. vulgaris* belonging to nine different P/S types were examined. All appeared to give similar electrophoretic patterns but there was some variation between the strains in the mobility of the α urease (fig. 3).

One strain, *P. vulgaris* F35865 (fig. 3, slot 11) was unusual in possessing a very mobile α urease which moved considerably faster than that of the other strains of *P. vulgaris* and even faster than that of one *P. rettgeri* strain (fig. 2b, slots 3 and 4). There was no correlation between mobility of the α urease and the P/S type of a strain. Prolonged development of the gels revealed two minor isoenzymes behind the α isoenzyme (figs. 3 and 4c).

P. mirabilis urease

The ureases of 33 strains of *P. mirabilis* were examined; 16 strains were of those P/S types commonly found in faeces and in urine (Senior, 1979), i.e., P/S types P0/S4, P0/S7, P1/S0 and P2/S1, and included local and distant isolates from the faeces of healthy patients and the urine of patients with urinary-tract infections; 17 strains were of P/S types P3/S1,8, P3/S1,13 and P3/S1,8,13 which are known to have a special affinity for the urinary tract (Senior, 1979), 15 of them being isolated from different patients with severe urinary-tract infection and two from the stools of healthy patients. The α -urease isoenzymes of 30 of the strains were electrophoretically indistinguishable from each other but three strains, F51259 (P0/S4) and F35549 (P0/S7) both from faeces and 1427/77 (P0/S7) from urine, had an α urease that consistently ran faster than that of the other strains (fig. 4a). Other strains having P/S types the same as those possessing "fast" α urease were found to have "normal" α urease. The α -urease isoenzymes of *P. mirabilis* were very similar in mobility to those of many strains of *P. vulgaris* (fig. 4b). Prolonged development of the gels revealed two minor isoenzymes which appeared to be different from those of *P. vulgaris* in that the latter were evenly spaced behind the α urease whereas those of *P. mirabilis* appeared to be grouped together (fig. 4c).

Although minor differences were observed in the urease isoenzyme patterns of some strains within a species, the greatest differences were observed between strains representing the different *Proteus* species. The patterns of urease isoenzymes fell into four groups: (1) that given by strains of *P. morgani*, (2) that given by strains of *P. rettgeri* and (3) that given by strains of *P. vulgaris*, which was similar to (4) that given by strains of *P. mirabilis* (fig. 5).

DISCUSSION

Most of our understanding of urease has come from research on jack-bean urease, whereas little is known about the enzyme produced by strains of *Proteus* despite the fact the proteus urease is considered to be a major factor in the development of pyelonephritis (Braude, Shapiro and Siemienski, 1959). Guo and Liu (1965) made a limited study of the serological specificities of *Proteus* ureases and found those of *P. mirabilis*, *P. vulgaris* and *P. rettgeri* to be serologically indistinguishable from each other and quite different from that of *P. morgani*. The results presented here substantiate the view that *P. morgani* urease is distinct from that of other *Proteus* species and even though the ureases of *P. mirabilis*, *P. vulgaris* and *P. rettgeri* may be serologically indistinguish-

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FIG. 1.—The urease isoenzymes of strains of *P. morgani* after overnight electrophoresis on (a) 6% polyacrylamide gels at 75 V and (b) 5% polyacrylamide gels at 140 V.

FIG. 2a.—The urease isoenzymes of *P. rettgeri* strains of different O serotype.

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FIG. 2b.—Comparison of the mobilities of a range of α urease isoenzymes isolated from strains of (left to right) 1–3 *P. rettgeri*, 4–6 *P. vulgaris*, 7–9 *P. mirabilis*.

FIG. 2c.—Comparison of the mobility of the ω urease isoenzyme from four strains of *P. rettgeri* with that of the urease isoenzymes of *P. morgani*

FIG. 3.—The urease isoenzymes of strains of *P. vulgaris* of different P/S type. The urease from strain F35865 was placed in the second slot from the right.

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FIG. 4.—(a) The α urease isoenzymes of *P. mirabilis* strains of different P/S type. (b) Comparison of the mobilities of the α urease isoenzymes of (left to right) 1-7 *P. mirabilis* and 8-13 *P. vulgaris* strains of different P/S type. (c) Comparison between the urease isoenzyme pattern of (left to right) 1-6 *P. mirabilis* and 7-12 *P. vulgaris* strains of different P/S type.

FIG. 5.—The differences in mobility of the urease isoenzymes among the different *Proteus* species. Left to right 1-3 *P. morgani*, 4-6 *P. vulgaris*, 7-9 *P. mirabilis* 10-12 *P. rettgeri*.

able, their isoenzyme patterns as determined by gel electrophoresis suggest that they are quite different from each other.

Jack-bean urease can be rapidly and reversibly dissociated into several catalytically active forms, dissociation being favoured by conditions of low ionic strength, high pH and high concentrations of reducing agents (Blattler *et al.*, 1967; Fishbein, Spears and Scurzi, 1969). Because of the standard ways in which all enzyme extracts were prepared, stored and run it is highly improbable that these explanations could account for the variation in urease isoenzyme patterns found within and between the *Proteus* species examined in this study. It is more probable that the different isoenzyme patterns of different *Proteus* species are produced by isoenzymes that have dissociated from genetically different enzymes.

A recent survey of the DNA relatedness between strains of *Proteus* species (Brenner *et al.*, 1978) has suggested that *P. morgani* strains form a single unique group of closely similar organisms which are quite different from other *Proteus* species, whereas *P. rettgeri* strains form another group with three different but related groups of strains within it. *P. mirabilis* strains form another distinct homogenous group which, while different from other groups, is partially related to the *P. vulgaris* group of strains which comprise two related groups of organisms. The results of urease isoenzyme patterns agree very closely with this classification scheme. The ureases of *P. morgani* were indistinguishable from each other but quite different from those of other *Proteus* species. The greatest diversity of isoenzymes was seen among *P. rettgeri* strains and, considering ω -urease isoenzyme alone, two distinct groups of strains could be defined—those that possess the isoenzyme and those that do not. There was less variation in the enzyme patterns of the different *P. vulgaris* strains, some of which appeared to be very similar to those of *P. mirabilis*, the latter forming, almost without exception, a single pattern of urease isoenzymes.

The absence of any correlation between P/S type and urease isoenzyme pattern among the large number of *P. mirabilis* strains examined suggests that the variation in pathogenicity for the urinary tract among strains with different P/S types is not related to the types of ureases that the strains form. It may be related, however, to the factors that control urease production in these strains and this problem is to be investigated.

SUMMARY

The ureases produced by a large number of strains of different *Proteus* species, some of which were known to have a special affinity for the urinary tract, were examined by polyacrylamide-gel electrophoresis. Each *Proteus* strain gave a pattern of urease isoenzymes that was characteristic and unique to its species although strains of *P. mirabilis* and *P. vulgaris* gave isoenzyme patterns that were closely similar. There was some minor variation in the patterns of urease isoenzymes even between strains of the same species. This was most noticeable among *P. rettgeri* strains and to a lesser extent among

P. vulgaris strains. No correlation was found between the types of ureases a strain produced and its pathogenicity for the urinary tract.

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