

# Enzymatic Degradation of Urinary Indoxyl Sulfate by *Providencia stuartii* and *Klebsiella pneumoniae* Causes the Purple Urine Bag Syndrome

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The etiology of the purple urine bag syndrome (PUBS), in which the urinary catheter bag of some elderly patients develops intense purple coloration, was studied. The purple was found to be a mixture of indirubin dissolved in the plastic and indigo on its surface. Six patients with PUBS were studied, and *Providencia stuartii* was isolated from the urine of five and *Klebsiella pneumoniae* was isolated from the urine of one. These strains produced indigo in 7.9 mM indoxyl sulfate-containing agar. One hundred and fifty isolates of 41 species of bacteria were tested for their ability to produce indigo on agar containing indoxyl sulfate, but only 17 of 27 strains of *P. stuartii*, a single strain of *Klebsiella pneumoniae*, and *Enterobacter agglomerans* were positive. All of the indigo-producing bacteria had an indoxyl phosphatase with a pI of 6.4. This enzyme also possessed indoxyl sulfatase activity and was not present in strains that were unable to produce indigo from indoxyl sulfate. We conclude that PUBS results from the decomposition of urinary indoxyl sulfate to indigo and indirubin by bacteria (notably *P. stuartii*). As elderly catheterized patients often have high urinary indoxyl sulfate levels and colonization of their urinary tract with *P. stuartii*, the condition is most commonly seen in them.

The purple urine bag syndrome (PUBS) is an uncommon condition in which the urinary catheter bag of a patient turns purple over a period of hours or days following urinary catheterization (1, 11, 13, 15). It is most commonly found in chronically catheterized women, although it has been reported for a surgical patient in the postoperative period (K. Weiner, personal communication) and in urinary ileal diversion bags (5). The chemical identity of the color is unknown, but a suggestion has been made that it is a mixture of indigo and indirubin, although indirubin was not specifically identified (14). The occurrence of indigo crystals in urine is well established and has been presumed to be of nonbacterial origin (10). We have located six cases of PUBS and identified the chemical nature of the color, the bacteria responsible, and the method of color production. In addition, we studied 150 isolates of 41 bacterial strains to survey their ability to produce indigo and indirubin and hence PUBS.

## MATERIALS AND METHODS

**Assessment of IS excretion by PUBS patients.** Urine samples from six PUBS patients and five clinically matched controls were collected in ice for 24 h, and indoxyl sulfate (IS) levels were measured by the method of Curzon and Walsh (2).

**Bacterial strains and patients.** Bacteria capable of producing indigo from IS were isolated by plating 100  $\mu$ l of urine onto ISA (blood agar base [Oxoid Ltd., Basingstoke, England] enriched with 7.9 mM IS [Sigma Chemical Co., Ltd., Poole, England]). Production of indigo was indicated by a deep blue color developing in the bacterial colony after 48 h of incubation at 37°C. Urine samples from six patients with PUBS and five controls matched for age, sex, and diagnosis were examined by this method. All bacteria isolated were identified by the API 20E system (API Systems S.A., Montalieu-Vervieu, France) and, in the case of isolates of

*Providencia* spp., tests for the fermentation of arabinol, trehalose, and adonitol (1% sugar peptone water) were included. To examine the distribution of the ability to produce blue colonies on ISA, 150 clinical isolates of 41 species of bacteria were examined. All isolates were identified by standard laboratory methods. *Escherichia coli* NCTC 10401 was used as a negative control strain when examining bacteria for the production of indigo.

**Identification of purple color.** Thin-layer chromatography was performed with the chloroform extract from the plastic of the catheter bags against known standard compounds of indigo and indirubin (Colour Chemistry Dept., Leeds University, England) (17). Absorption spectra of the separated colors were compared with those of indigo and indirubin (Unicam 1800 spectrophotometer; Pye Unicam Ltd., Cambridge, England).

**Purple production by bacteria.** Color production was tested by adding 50  $\mu$ l of an 18-h nutrient broth (Oxoid Ltd.) culture of the bacterium to be tested to 10 ml of 1% peptone water (Oxoid Ltd.) enriched with either 7.9 mM IS or 10% PUBS urine that was collected in ice, kept at 4°C, and filter sterilized [0.45  $\mu$ m pore size; Millipore (U.K.) Ltd., Harrow, England]. A piece of sterile polythene (5 by 0.5 cm) cut from a Simpla catheter bag (Simpla Ltd., Cardiff, Wales) was used to indicate color production. The polythene was examined daily for 3 weeks for purple color. After this time the cultures were subcultured for purity.

**Enzyme preparation.** The six bacterial isolates from the PUBS patients capable of producing indigo on ISA were further examined for enzymes capable of producing indigo from indoxyl phosphate (IP), which was found to yield indigo more rapidly than IS but was a substrate for the same enzyme (see next section). Overnight 100-ml brain-heart infusion (Oxoid Ltd.) broth cultures of the organisms were washed twice with 100 ml of phosphate-buffered saline, pelleted by centrifugation at 800  $\times$  g, and suspended in 2.5 ml of 0.2 M phosphate-buffered saline, pH 7.0. The cells

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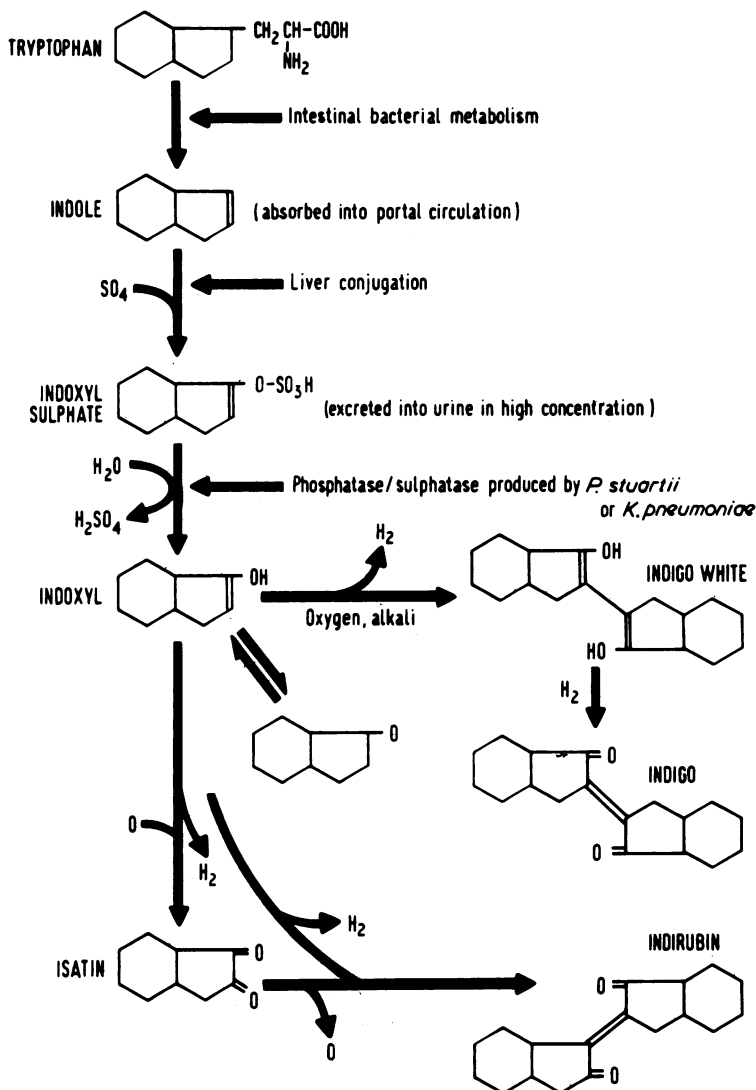


FIG. 1. Metabolites of tryptophan involved in PUBS.

were then sonicated for three 30-s periods. The supernatant was assayed for protein by the method of Lowry et al. (7) and stored at  $-20^\circ\text{C}$ .

**Assay of indoxyl phosphatase activity by indigo production from IP.** The production of indigo by enzyme preparations at  $37^\circ\text{C}$  in a 10-ml solution of 1.9 mM IP was measured by extracting it with 5 ml of chloroform (with shaking for 1 min). Indigo dissolved in the chloroform was quantified against standard preparations with a Cecil CE292 spectrophotometer (Cecil Instruments, Cambridge, England) by detection of  $A_{464}$ .

**Assay of indoxyl phosphatase and sulfatase activity by the detection of fluorescent intermediates.** Indoxyl produced by the breakdown of IS and IP spontaneously forms into the fluorescent indigo white (IW), which, in acid conditions, produces indigo (indigo blue) or indirubin (indigo red) (Fig. 1) (3). At a low pH and in the presence of ascorbic acid, the intermediates indoxyl and IW accumulate and may be measured in a fluorometric spectrophotometer (3). Relative rates of production of fluorescent intermediates were measured as fluorescence on a Baird SM200 fluorimeter with filters OY13 and OB10. As it was found that the rate of change of

fluorescence ( $\Delta F$ ) was constant for 4 h after the addition of up to  $100\ \mu\text{l}$  of crude enzyme (with phosphatase activity at  $0.11\ \text{mmol/s per g}$  and protein at  $48\ \mu\text{g/ml}$ ) to 10 ml of 1.0 mM IP, fluorescence was measured for up to 4 h only. The enzyme preparation ( $10\ \mu\text{l}$ ) was added to 10 ml of 7.9 mM IS or 1.9 mM IP in 0.05 M phosphate-citrate (PC) buffer (pH 5.2). The reaction mixture was incubated at  $37^\circ\text{C}$ , and the rate of production of IW was calculated from the relative increase in fluorescence compared with controls without enzyme preparation. The optimum pH for enzyme activity was found by comparing the rate of IW production at  $37^\circ\text{C}$  from pH 4.5 to 8.5 in 0.05 M Tris or PC buffer.

**IEF.** Isoelectric focusing (IEF) was performed by the agarose gel method of Vecoli et al. (16) with pH 3 to 10 ampholines (Sigma Pharmalytes; Sigma Chemical Co.). Indoxyl phosphatase activity in the gel was detected with the application of Whatman number 41 filter paper soaked in 1.9 mM IP in 0.9% NaCl to the surface of the gel by the appearance of blue bands of indigo blue after 3 h of incubation at  $37^\circ\text{C}$ . Sulfatase activity in the gel was found to be too low to produce blue bands after application of 7.9 mM IS directly to the gel on filter paper. The sulfatase activity of

bands with and without phosphatase activity was tested by cutting out fragments of the gel, mixing them with a Whirlmixer (Fisons Ltd., Loughborough, Leicestershire, England) for 5 min in 1 ml of 0.05 M (pH 5.2) PC buffer, and adding 100  $\mu$ l of this to 10 ml of 7.9 mM IS in 0.05 M PC buffer. The production of fluorescent intermediates was then measured by using a fluorimeter as described above.

**Distribution of indigo production among bacteria.** One hundred and fifty isolates of 41 bacterial species were tested with a multipoint inoculator (Dynatech Ltd., Billingshurst, England) to inoculate approximately  $10^4$  CFU onto ISA and blood agar base enriched with 1.9 mM IP. These cultures were incubated at 37°C and examined daily for 3 days for visible color production, which was graded as follows: strong (++), colony completely opaque with color; weak (+), usual color of the colony tinted with blue. Urea hydrolysis was tested on identification medium IDM 32 (Mast Ltd., Mast House, Bootle, England).

## RESULTS

**Bacteria isolated from PUBS urine.** Inoculation of six PUBS urine samples onto ISA revealed blue colonies from all six. Five of these isolates were identified as *Providencia stuartii*, of which four were urease positive, and one as *Klebsiella pneumoniae*. No similar colonies were isolated from control urine samples obtained from five persons without PUBS.

**Identification of the purple color.** The relative mobilities of color extracts from three purple catheter bags on thin-layer chromatography were identical to those of indirubin and indigo standards. This was confirmed by the fact that the absorption spectra of the separated colors were similar to those of the standards, with a peak at 600 nm (indigo) and one at 550 nm (indirubin).

**Purple production by bacteria.** Inoculation in pure culture of the five *P. stuartii* and one *K. pneumoniae* strains into 1% peptone water enriched with either 7.9 mM IS or 10% filtered PUBS urine produced purple discoloration of the plastic in the broth in 2 weeks at 37°C. This discoloration did not occur without inoculation, with control *E. coli* NCTC 10401, or without IS present.

**Indoxyl phosphatase and sulfatase activity by the detection of fluorescent intermediates.** Maximum indoxyl sulfatase and phosphatase activities (measured as rate of change of fluorescence,  $\Delta F$ ) of the enzyme preparations were both found to occur at pH 5.2.

**IEF and chromogenic detection of indoxyl sulfatase and phosphatase activity.** IEF showed no bands when developed with IS but a series of blue bands when 1.9 mM IP was used (Fig. 2). It was found that 5  $\mu$ l of enzyme preparation with a protein concentration of 20 mg/ml gave discrete bands with organisms not associated with PUBS, but the organisms from patients with PUBS, because of their greater phosphatase activity, required 0.5  $\mu$ l to avoid blurring of the focused IEF bands. All five *P. stuartii* and the *K. pneumoniae* isolates from PUBS urine showed a major band at pI 6.4. Three *P. stuartii* not able to produce blue colonies on ISA did not show this band on IEF. When the band with a pI of 6.4 was cut out of the gel and washed with 0.05% PC buffer at pH 5.2, the washings were found to have both indoxyl phosphatase and indoxyl sulfatase activity in the ratio of 56.5:1. Blue bands from *P. stuartii* not associated with PUBS and agarose gel fragments not associated with indigo production did not show any sulfatase activity with the same methods. Indoxyl phosphatase activity as measured by the

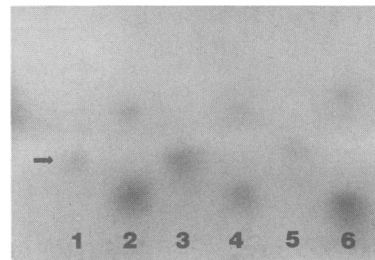


FIG. 2. IEF of indoxyl phosphatase enzymes from six strains of *P. stuartii*. Enzyme preparations used in lanes 1, 3, and 5 were from strains associated with PUBS. The indoxyl phosphatase unique to them (at pH 6.4, arrow) was so active that these preparations had to be diluted 10-fold with respect to strains not associated with PUBS (in lanes 2, 4, and 6).

production of indigo (constant up to 8 h) was 0.11 mmol/s per g for the *P. stuartii* isolate from one patient.

**Distribution of indigo production among bacteria.** Of the 19 strains that produced blue colonies within 48 h on ISA, 17 were *P. stuartii*, one was a strain of *Enterobacter agglomerans*, and one was a strain of *K. pneumoniae* (Table 1).

**Assessment of IS excretion by PUBS patients.** The concentration of indican ranged from 1.36 to 8.2 mg/liter in the samples from six patients with PUBS, compared with 0.16 to 1.66 mg/liter in those from the control patients. The range of daily urinary output of indican was from 99 to 225 mg/day for the six patients with PUBS compared with 3 to 136 mg/day for the control patients. The normal value for daily excretion of indican is less than 100 mg/day. No bacteria capable of producing indigo from IS were found in the urine of the control patients, whereas *P. stuartii* was isolated from the urine of five patients and *K. pneumoniae* from that of a sixth patient with PUBS. All of these bacteria were capable of producing indigo from IS.

## DISCUSSION

There has been considerable debate about the etiology of PUBS, some writers suggesting that indigo was the cause of the color and that it came from mammalian metabolism (1, 13, 15). Our careful investigation of the purple in plastic urinary bags shows that it is due to the presence of two pigments: indirubin, which is dissolved in the plastic, and indigo. As urinary IS is ultimately derived from the products of gut bacterial metabolism of tryptophan, the bacteria known to metabolize these substrates may well be involved in the conversion of IS to indigo and indirubin in the urine of patients with PUBS. Likely candidates are the members of the tribe *Proteeae*, which can deaminate aromatic amino acids such as tryptophan and phenylalanine, produce indole from tryptophan, degrade tyrosine, and oxidatively metabolize aromatic amino acids to produce melaninlike pigments (9). Hence, it was not surprising that *P. stuartii*, commonly found in the urine of catheterized patients and able to deaminate a variety of aromatic amino acids, was involved in IS catabolism (4, 8). Of 41 species tested, only 3 were able to produce indigo from IS: *P. stuartii*, *K. pneumoniae*, and *E. agglomerans*. Only *P. stuartii* could produce opaque blue colonies in 18 h on ISA. The finding that four of the five strains of *P. stuartii* isolated from PUBS patient urine samples produced urease is unusual, as this property is relatively rare (4). It is possible that genes for the production of indigo from IS reside on a plasmid, as urease production is often a plasmid-encoded trait. The ability to produce

TABLE 1. Ability to produce indigo from IS and IP among 41 species of bacteria

Organism	No. of strains	Blue colony formation <sup>a</sup> on agar containing:				Urea hydrolysis <sup>b</sup>
		IS		IP		
		18 h	48 h	18 h	48 h	
<i>Acinetobacter calcoaceticus</i> subsp. <i>anitratus</i>	2	-	-	-	-	±
<i>Alcaligenes denitrificans</i>	1	-	-	-	-	-
<i>Citrobacter diversus</i>	1	-	-	++	++	±
<i>Citrobacter freundii</i>	2	-	-	+	++	+
<i>Enterobacter agglomerans</i>	1	-	-	-	+	-
	1	+	+	++	++	-
<i>Enterobacter cloacae</i>	1	-	-	++	++	-
<i>Escherichia coli</i>	6	-	-	+	+	-
<i>Hafnia alvei</i>	1	-	-	++	++	-
<i>Klebsiella pneumoniae</i>	1	-	-	-	++	±
	2	-	-	++	++	+
	1	+	+	++	++	+
<i>Klebsiella oxytoca</i>	3	-	-	++	+	+
<i>Morganella morganii</i>	11	-	-	++	++	+
<i>Proteus mirabilis</i>	9	-	-	++	++	+
<i>Proteus penneri</i>	5	-	-	++	++	+
<i>Proteus vulgaris</i>	5	-	-	++	++	+
<i>Proteus vulgaris</i> biogroup 2	5	-	-	++	++	+
<i>Providencia alcalifaciens</i>	12	-	-	++	++	-
<i>Providencia rettgeri</i>	11	-	-	++	++	+
<i>Providencia rustigiani</i>	7	-	-	++	++	-
<i>Providencia stuartii</i>	9	-	-	++	++	-
	6	-	+	++	++	-
	6	-	++	++	++	-
	1	+	++	++	++	-
	3	++	++	++	++	+
	1	-	+	++	++	±
	1	-	-	++	++	±
<i>Pseudomonas maltophilia</i>	2	-	-	++	++	-
<i>Pseudomonas acidovorans</i>	1	-	-	-	-	-
<i>Pseudomonas putida</i>	1	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	5	-	-	-	+	±
<i>Pseudomonas fluorescens</i>	1	-	-	-	+	-
<i>Salmonella typhimurium</i>	1	-	-	-	+	-
<i>Serratia marcescens</i>	1	-	-	-	-	-
<i>Shigella sonnei</i>	1	-	-	+	+	-
<i>Yersinia enterocolitica</i>	1	-	-	++	++	-
<i>Bacillus</i> sp.	1	-	-	-	-	-
<i>Erysipelothrix rhusiopathiae</i>	1	-	-	-	-	+
<i>Listeria monocytogenes</i>	1	-	-	-	-	-
<i>Staphylococcus aureus</i>	5	-	-	-	+	-
<i>Staphylococcus epidermidis</i>	1	-	-	-	-	-
<i>Streptococcus agalactiae</i>	1	-	-	-	++	-
<i>Streptococcus bovis</i>	1	-	-	-	-	-
<i>Streptococcus capitis</i>	1	-	-	-	-	-
<i>Streptococcus faecalis</i>	1	-	-	-	+	-
<i>Streptococcus pyogenes</i>	1	-	-	+	+	-
<i>Streptococcus sanguis</i>	1	-	-	-	-	-
<i>Staphylococcus aureus</i>	5	-	-	-	+	-
<i>Staphylococcus epidermidis</i>	1	-	-	-	-	-

<sup>a</sup> Symbols: -, no indigo production; +, indigo production resulting in a blue-tinged colony; ++, indigo production resulting in opaque deep blue colony.

<sup>b</sup> Symbols: ±, alkaline discoloration of the medium in the zone of the colony only; +, discoloration in a halo around the colony.

ammonia from urea is not related to the production of indigo, as some 13 urease-negative strains were able to produce indigo. However, when strains of *P. stuartii* from other sources were tested for indigo production, it was clear that strong urease producers gave opaque blue colonies faster than other strains.

Bacterial strains that produced blue colonies on ISA were isolated from six patients with PUBS and none of five controls. These organisms, when inoculated into sterile urine (from a PUBS patient) or into broth containing 7.9 mM IS, produced a purple color in the plastic indicator strip. Thus, bacterial metabolism of IS is responsible for the production of indigo and indirubin in PUBS patients.

All the bacteria found that had indoxyl sulfatase activity also had indoxyl phosphatase activity. The strongly positive indoxyl phosphatase band on IEF unique to IS-splitting organisms at pI 6.4 was also found to have indoxyl sulfatase activity, and the optimum pH for indoxyl sulfatase and indoxyl phosphatase activity was found to be the same, 5.1, suggesting that it is the same enzyme hydrolyzing the two substrates.

Our finding of an increased urinary concentration and daily excretion of IS in patients with PUBS was in contrast to the report of Stott et al. (15). However, they did not collect the samples tested in ice, and when this was not done we found that 30% of the IS was destroyed (unpublished data), presumably by bacterial metabolism, before testing took place. There have been conflicting reports of the excretion of IS in elderly patients, some finding elevated levels and some the same levels as in younger patients (6, 12). The cause for the high IS excretion in our PUBS patients was not clear, but presumably intestinal bacterial overgrowth, metabolism of dietary tryptophan, and absorption of those products were responsible. Although color will develop with normal levels of IS excretion, the presence in most of our patients of higher than normal levels presumably led to intense color development in the catheter bag.

We conclude that PUBS results from the action by a bacterial enzyme (a phosphatase with minor sulfatase activity, pI 6.4) on the IS in the urine of elderly catheterized patients with high urinary IS levels. The enzyme is found in many strains of *P. stuartii* and rarely in other bacteria.

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