Notes

Isolation and quantitative determination of pseudouridine in urine by anion-exchange chromatography*

A convenient method for the isolation of pseudouridine from urine was recently described by COHN *et al.*[1]. Urine was freed from cations and anions by successive passage through columns of strong-acid and weak-base ion-exchange resins. The neutral substances, including pseudouridine, which pass through both these columns were then applied in an alkaline borate solution to a strong-base ion-exchange resin and the uncharged or mono-ionic compounds including uracil, were eluted with a sodium borate-ammonium bicarbonate buffer. Pseudouridine which under these conditions exists as a doubly charged borate complex was subsequently eluted with ammonium bicarbonate. Removal of the buffer by evaporation and treatment with strong-acid resin furnished pseudouridine. However, this procedure in the hands of the checkers led to a product still contaminated with borate, the last traces of which had to be eliminated by paper chromatography.

This communication describes a simpler procedure which has been used extensively in our laboratories for preparative as well as analytical purposes. Pseudouridine is obtained from urine by the use of only two resin columns and a single, completely volatile buffer-system which avoids the danger of contamination with borate or other inorganic ions. Another advantageous feature of this method is the possibility of isolating components other than pseudouridine which are obtained in this fractionation, free of inorganic ions for purification and study (see below).

In a typical preparative experiment 3185 ml normal urine (24 h collection) was passed through a column (7.5 × 11 cm) of Dowex 50-X8 (H-form). The column was eluted with 4000 ml of water and the combined effluents were adjusted to pH 5–5.3 by stirring with an appropriate quantity of Dowex 3-X4 (free base) for 30–45 min. Approximately 400 g wet resin was required in this experiment. The mixture was then filtered and the resin washed with four 250 ml portions of water. The filtrate and washings were evaporated to dryness under reduced pressure and temperature. The residue was redissolved in 100 ml *N* ammonia and the solution applied to a column (3.2 × 32 cm) of Dowex 2-X8 (bicarbonate-form) which had been equilibrated previously with 400 ml *N* ammonia. The column was then eluted with 0.02 *M* triethylammonium bicarbonate buffer at pH 9.5. The absorption of the eluate at 253 mμ was monitored with a recording U.V. flow-photometer and collected in 15 ml fractions on a fraction collector at a flow rate of 3 ml/min. A large peak consisting mainly of N-methyl-2-pyridone-5-carboxamide emerged almost immediately. It was followed by three distinct peaks of varying size. In some urines screened in our laboratories peaks

* Contribution No. 3124 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, Calif.

No. 3 and 4 (see Fig. 1) were insignificant or absent. A preliminary examination of the fractions represented by these peaks has shown them to be a complex mixture of substances. The main components, however, appear to be peptides attached to a moiety with strong absorption in the 260 mμ region. A more detailed description of these interesting, as yet unidentified, metabolites will be presented in a separate report.

Pseudouridine began to emerge when approximately 5000 ml buffer had passed through the column and was collected in the next 2500 ml. Uracil (peak 6 in Fig. 1),

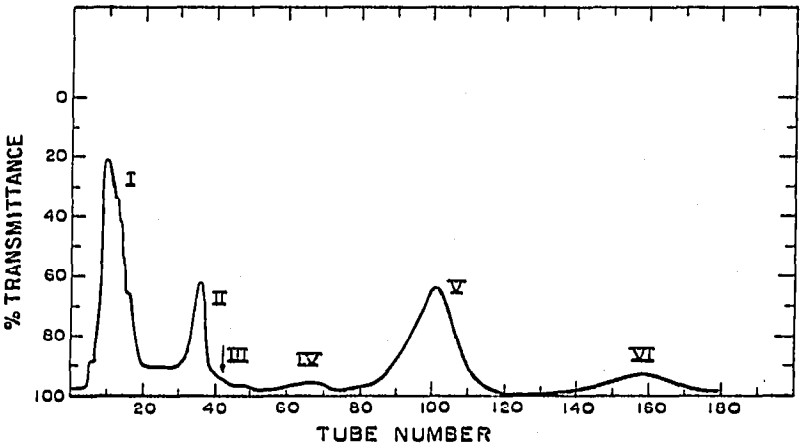

Fig. 1. Chromatographic analysis of pseudouridine in normal urine (8 mg creatinine/analysis) on a 0.8 × 20 cm column of Dowex 2-X8 (bicarbonate form). Elution with 0.025 *M* triethylammonium bicarbonate, pH 9.5. Flow rate: 1 ml/min, 4 ml/tube.

identified by its λ_{max} and 260/280 mμ ratios at pH 2 and 12, was not eluted until about 9500 ml buffer had passed through the column. The eluate containing the pseudouridine was evaporated to dryness under reduced pressure and temperature and the residue was distilled 3 to 4 times with 15–20 ml of water in order to remove the last traces of buffer. The crude pseudouridine thus obtained was dried in a desiccator over phosphorous pentoxide for 12 h and extracted with two 2 ml portions of boiling 95 % ethanol. The extracts were filtered and allowed to cool. The first extract which had an amber color yielded 13.6 mg pseudouridine with a diffuse m.p. at about 190°. The second, colorless extract gave 8.7 mg product with m.p. 213–215°. These two crops when pooled and recrystallized from 3 ml methanol furnished 14.4 mg crystalline pseudouridine, m.p. 221–222°; λ_{max} 262 mμ; ε_{max} 7980; A 290/260:0.066 at pH 2, and λ_{max} 287 mμ; ε_{max} 7960; A 290/260:2.26 at pH 12. The product migrated as a single component in isopropanol–acetic acid–water (60:30:10), R_F 0.29; *n*-butanol-water (86:14), R_F 0.11; 3-methyl-1-butyn-3-ol–water (75:25), R_F 0.32; 3-methyl-1-pentyn-3-ol saturated with *N* ammonia, R_F 0.12 and acetonitrile–water–formic acid (70:25:5), R_F 0.36. The solid which remained after the first two extractions with 95 % ethanol was dissolved in 5.2 ml boiling methanol and filtered. On cooling 20.25 mg crystalline pseudouridine was obtained, m.p. 220-221°; λ_{max} 262 mμ; ε_{max} 7580; A 290/260:0.069 at pH 2, and λ_{max} 287 mμ; ε_{max} 7530; A 290/260 2.20 at pH 12. The product was chromatographically homogeneous in the above solvent systems. The mother liquors yielded a further 8 mg pseudouridine with m.p. 219–222°. Total yield of crystalline pseudouridine: 42.4 mg. Slight modifications

of this isolation procedure may be necessary to take into account variation in the level of urinary pseudouridine in different individuals and samples.

In the analytical version of this procedure a quantity of urine corresponding to 10 mg creatinine was passed through a Dowex 50 column (0.8 × 10 cm) followed by 450 ml of water. The effluents were neutralized with Dowex 3 resin to pH 5 and filtered. The resin was washed with four 25 ml portions of water and the pooled filtrates were evaporated to dryness. The residue was redissolved in 10 ml *N* ammonia and 8 ml of the solution was chromatographed on a column (0.8 × 20 cm) of Dowex 2 (bicarbonate-form). Triethylammonium bicarbonate (0.025 *M*) of pH 9.5 was used for elution and fractions of 4 ml were collected at a flow rate of 1 ml/min. Fig. 1 illustrates the excellent separation obtained with this system. The tubes containing pseudouridine were pooled and evaporated to dryness. The residue was redissolved in 3 ml of water and a portion representing approximately 1.4–3 optical density units was further purified by paper chromatography (duplicate spots) on Whatman No. 1 paper with methylbutynol–water (75:25) as a solvent. This eliminates U.V. absorption originating from impurities in the buffer system and resin. Pseudouridine was located on the paper under U.V. light, the absorbing spots were cut out and eluted with 3 ml glycine buffer (pH 2) each and the nucleoside determined spectrophotometrically by its absorption at 262 mμ. For calculations ε_{max} 7980 was used. A blank was prepared by identical treatment of a similar area of the chromatogram free of pseudouridine. The reproducibility of this assay in 6 duplicate runs was ± 5%. In three experiments in which authentic pseudouridine was added to urine 85%, 89% and 90% were recovered by this method. Approximately two thirds of the loss occurred during the paperchromatography step and about one third during the other operations of the procedure.

N-Methyl-2-pyridone-5-carboxamide (peak I) was determined quantitatively by evaporation of the eluate to dryness, passage of the redissolved residue through a composite column consisting of successive sections (0.8 × 5 cm each) of Dowex 50 (H-form) and Dowex 20 (OH-form) and spectrophotometric assay of the effluent at 258 mμ[2].

Acknowledgement

This work was supported by a grant from the National Institutes of Health, Public Health Service.

Gates and Crellin Laboratories of Chemistry,
California Institute of Technology,
Pasadena, Calif. (U.S.A.)

HEINRICH RINDERKNECHT
VICTORIA MA

[1] W. E. COHN, V. KURKOW AND W. CHAMBERS, *Biochem. Prep.*, 10 (1963) 135.
[2] J. M. PRICE, *J. Biol. Chem.*, 211 (1954) 117.

Received May 25th, 1964