was set to music in the early eighteenth century, but I fear I cannot sing it to you:

"The night is come like to the day,
Depart not thou great God away.
Let not my sinnes, blacke as the night,
Eclipse the lustre of thy light.
Keepe still in my Horizon, for to me,
The Sunne makes not the day, but thee.
Thou whose nature cannot sleepe,
On my temples sentry keep;
Guard me 'gainst those watchfull foes,
Whose eyes are open while mine close.
Let no dreames my head infest,
But such as Jacob's temples blest.
While I rest, my soule advance,
Make my sleepe a holy trance:
That I may, my rest being wrought,
Awake into some holy thought.

And with as active vigour runne
My course, as doth the nimble Sunne.
Sleepe is a death, O make me try,
By sleeping what it is to die.
And as gently lay my head
Upon my Grave, as now my bed.
How ere I rest, great God let me
Awake againe at last with thee.
And thus assur'd, behold I lie
Securely, whether to wake or die.
These are my drowsie dayes, in vaine
Now I do wake to sleepe againe.
O come that houre, when I shall never
Sleepe againe, but wake for ever.

"This is the dormitive I take to bedward; I need no other Laudanum than this to make me sleepe; after which I close mine eyes in security, content to take my leave of the Sunne, and sleepe unto the Resurrection."

Mechanisms of Renal Excretion of Urobilinogen

E. BOURKE,*† M.B., B.CH., B.A.O., M.R.C.P.ED.; M. D. MILNE,* M.D., F.R.C.P.; G. S. STOKES,*‡ M.D.

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Urobilinogen and its oxidized derivative, urobilin, have been regarded as compounds of great medical interest and importance for almost a century since their discovery by Jaffé (1868, 1869). Modern knowledge has been admirably reviewed by Watson (1963). Urobilinogen is in fact a mixture in varying proportion of three closely related chemical compounds, d-, i-, and l-urobilinogens. These are entirely derived from bacterial reduction of bilirubin in the intestinal tract. Broad spectrum antibiotics reduce the output of urobilinogen virtually to zero (Sborov *et al.*, 1951; Watson *et al.*, 1954), and none is formed by germ-free rats (Gustafsson and Lanke, 1960). The faecal output of urobilinogen in normal adults is 40-280 mg./day, and the urinary excretion 0-4 mg./day (Watson, 1937). Therefore only about 1% of the total amount formed appears in the urine. Nevertheless, estimation of urinary urobilinogen has long been used in the assessment of cases of hepatic insufficiency and of haemolytic disorders. The tacit assumption has always been made that urinary excretion parallels the plasma concentration of urobilinogen. This paper shows that this view is incorrect; urobilinogen is excreted by a combination of glomerular filtration, proximal tubular secretion, and pH-dependent back-diffusion in the distal tubules. Changes in urinary pH have a profound effect on urobilinogen excretion and clearance, these being increased in alkaline and reduced in highly acid urine. Gross changes in urinary output may therefore occur without any significant alteration of plasma urobilinogen content.

Methods

Urobilinogen was estimated in two-hourly urine collections from four normal adult subjects, three patients with haemolytic anaemias, and two patients with hepatocellular jaundice. Variation in urinary pH was produced by ingestion of

ammonium chloride, 0.1 g./kg. body weight, or of sodium bicarbonate at the same dosage. The normal diurnal rhythm of urinary pH was sufficient to obtain specimens in the intermediate range of pH 5.5-7. Urinary pH was measured by glass electrode immediately after collection, and specimens were stored for short periods at -4°C. in the dark before analysis. In clearance studies a blood sample was obtained by venepuncture at the mid-point of each urinary collection period and serum separated by centrifugation.

To estimate plasma-protein binding of urobilinogen an impure specimen of urobilinogen was prepared from the faeces of a case of hereditary spherocytosis by the method of Watson *et al.* (1953). A concentrated alcoholic solution was added to normal plasma to produce a urobilinogen concentration of 100 µg./100 ml. Protein-free specimens were obtained either by ultrafiltration or by dialysis through collodion membranes. If normal plasma was used the amount of unbound urobilinogen was too low to permit accurate analysis by the available methods.

To estimate the pK_a of urobilinogen partition coefficients between water and petroleum ether of boiling range 40°-60° C. were determined at a pH range from 3.5-7 by means of the buffer systems acetic acid:sodium acetate in the range 3.5-5.5, and monobasic sodium phosphate:dibasic sodium phosphate in the range 5.5-7.

Chemical Methods

Urinary urobilinogen output: the colorimetric method of Watson (1936) was used. Plasma and urinary urobilinogen in clearance studies: the fluorimetric method of Lozzio and Royer (1962) was modified to correct for the blank fluorescence of the alcoholic zinc acetate used in the method.

Urobilinogen methods are not completely satisfactory because of the difficulty of obtaining standards of certain purity. In both the colorimetric and the fluorimetric methods artificial standards by means of alkaline phenolphthalein and acriflavine

* Department of Medicine, Westminster Medical School, London.

† Travelling Fellowship, University College, Dublin; Grant from Wellcome Trust.

‡ Overseas Fellowship, National Heart Foundation of Australia.

respectively are usually recommended. If these are employed the investigator relies on the purity of the standard used by the originator of the method, and errors from variations between different photoelectric colorimeters and fluorimeters are inevitable. These inherent difficulties do not affect clearance determinations, where ratios between plasma and urinary concentrations are determined, but greatly influence absolute values in both plasma and urine. Preparation of pure urobilinogen is virtually impossible, and therefore a urobilin standard was used in the present study.

Chromatography of urobilinogen: three 15-minute collections of urine were obtained from an adult case of hereditary spherocytosis prior to splenectomy, the first without drugs at pH 6.2, the second after ammonium chloride ingestion at pH 4.8, and the third after sodium bicarbonate ingestion at pH 7.8. The urobilinogen in the three samples was oxidized to urobilin, which was then purified by the method of Watson *et al.* (1953). Chromatography was carried out by the method of Royer *et al.* (1964), methanol:butanol:ammonia, 1:3:2, being used as solvent. The developed chromatogram was sprayed with concentrated alcoholic zinc acetate and the fluorescent spots were photographed in ultra-violet light.

Creatinine: Jaffé's alkaline picrate method with Fuller's-earth absorption for serum analysis was used.

Plasma and urinary urate: the method of Henry *et al.* (1957) was employed.

Results

Variation of Urobilinogen Output with Urinary pH

In all subjects investigated there was a significant positive correlation between urinary urobilinogen output and urinary pH. The output, however, varied considerably in different subjects, presumably because of differences in plasma concentration. A representative series of results from a single normal subject is given in Fig. 1, where the log. of the output in $\mu\text{g.}$

FIG. 1.—Urobilinogen output in a normal subject related to urinary pH. There is a positive linear correlation between log. urobilinogen output and pH. The calculated regression line is given.

urobilinogen/min. is graphed against urinary pH. There was usually a better correlation between the logarithm of the excretion rate and pH than between the excretion rate itself and pH. This agrees with previous results in the case of ammonium output (Clarke *et al.*, 1955), and salicylate excretion (Macpherson *et al.*, 1955). The calculated regression lines relating log. urobilinogen excretion to urinary pH in three

normal subjects and two cases of hereditary spherocytosis are given in Fig. 2. In each individual there is a similar relationship between the two variables, but the absolute value given by the height of the intercept of the regression line and the ordinate varies considerably between individuals. The Table gives a statistical analysis of the results in the five subjects, and of the clearance data shown in Fig. 6. The correlation coefficients between log. urobilinogen excretion and urinary pH vary from 0.53 to 0.83, all values being highly significant statistically. The closest correlation was obtained, as expected, from the clearance data, as in these results variation of urobilinogen output from change of plasma concentration is avoided.

FIG. 2.—Calculated regression lines relating log. urobilinogen output and urinary pH in three normal subjects and two cases of hereditary spherocytosis.

Statistical Analysis of Relationship of Log. Urobilinogen Output and Log. Urobilinogen Clearance to Urinary pH

(a) Urobilinogen Output

Subject	n. No. of Observations	r. Correlation Coefficient of Log. Urobilinogen Output to Urinary pH	b. Gradient of Regression Line	P. Significance of Correlation Coefficient
Normal 1 ..	63	0.70	0.43	< 0.001
" 2 ..	52	0.58	0.25	< 0.001
" 3 ..	76	0.53	0.24	< 0.001
Hereditary spherocytosis:				
Adult ..	29	0.83	0.64	< 0.001
Child ..	7	0.75	0.56	< 0.02

(b) Urobilinogen Clearance

Subject	n	r. Correlation Coefficient of Log. Urobilinogen Clearance to Urinary pH	b	P
Grouped data of four patients, shown in Fig. 6	28	0.93	0.25	< 0.001

The gradient of the regression line varies in different subjects from 0.24 to 0.64. This gradient gives the rate of change of urobilinogen output with variation in urinary pH. The results agree with the theoretical aspects of pH-dependent excretion (Milne *et al.*, 1958), as this gradient in the case of an organic acid must lie between zero and unity.

The above results give excretion rates of the sum-total of the three separate urobilinogens—that is, the sum of d-, i-, and l-urobilinogen. Fig. 3 shows the chromatogram of urobilin prepared from 15-minute urinary collections from a patient with hereditary spherocytosis without drugs and at the extremes of

urinary acidity and alkalinity. The total amount of urobilin increases with urinary pH, being very high in alkaline urine. All three separate urobilins increase in urine with rise in pH.

FIG. 3.—Chromatogram of urobilin derived from the urobilinogen in three 15-minute urine collections from an adult case of hereditary spherocytosis. Left: acid urine after ammonium chloride. Centre: urine obtained without drugs. Right: alkaline urine after sodium bicarbonate. The letters refer to the positions on the chromatogram of i-, l-, and d-urobilins. Chromatogram developed by methanol-butanol-ammonia, 1:3:2, sprayed with alcoholic zinc acetate and photographed in ultra-violet light.

Diurnal Variation in Urinary and Serum Urobilinogen

Diurnal variation in urinary urobilinogen has been studied by many previous workers and therefore it was not reinvestigated in detail. In all except one subject the maximum urobilinogen output occurred at the time previously described—that is, between noon and 4 p.m.—the output during the night being virtually zero. In one normal subject the highest output occurred between 10 a.m. and noon. Fig. 4 gives a typical diurnal rhythm of output, together with the change produced by ingestion of sodium bicarbonate, 15 g. daily. There is a very high excretion rate during the first day of alkalinization of the urine, but this is not maintained on subsequent days, suggesting that high urinary excretion rates are sufficient to cause a reduction of plasma urobilinogen concentration. The output on the first day of alkalinization was 9 mg., which is 6% of the mean total faecal-plus-urinary output in this subject.

Figure 5 gives the variation in plasma urobilinogen in four normal subjects at two-hourly intervals between 9 a.m. and 7 p.m. There is considerable variation in concentration in all four individuals but the pattern of the change is inconstant. Normal plasma urobilinogen calculated from these results is 5.3 ± 3.2 $\mu\text{g./100 ml.}$

FIG. 4.—Diurnal variation in urinary urobilinogen in a normal subject with and without sodium bicarbonate ingestion.

FIG. 5.—Diurnal variation in concentration of plasma urobilinogen in four normal subjects.

Urobilinogen Clearance and Urinary pH

Fig. 6 gives urobilinogen clearances expressed as a fraction of the simultaneous endogenous creatinine clearance in relation to urinary pH. All the results were obtained from patients with hepatocellular jaundice or haemolytic anaemia, as the increased serum levels of urobilinogen in such patients greatly increases analytical accuracy. There is a significant correlation between the clearance ratio and urinary pH, the scatter being much less than in urobilinogen output studies (Fig. 1), because variation from change in urobilinogen concentration in plasma is eliminated in clearance data. The mean clearance ratio varies from 0.03 at urinary pH 5 to 0.22 at urinary pH 8. The correlation coefficients between the clearance ratio and pH are 0.89, and between log. clearance ratio and pH 0.93, both being statistically highly significant.

Plasma-protein Binding and the pK_a of Urobilinogen

The mean fraction of urobilinogen bound to plasma protein was 80%, with a range of 77–85%. Values for urobilin were even higher, averaging 95% protein-bound. There was no

significant difference in the results obtained from ultrafiltration and dialysis techniques. The pK_a of urobilinogen was estimated to be 5.45.

FIG. 6.—Urobilinogen clearance (expressed as a fraction of the simultaneous endogenous creatinine clearance) related to urinary pH in four patients with haemolytic anaemia or hepatocellular jaundice. There is a positive correlation between the two variables. The calculated regression line is given. Closed circles: hereditary spherocytosis. Open circles: sickle-cell anaemia. Triangles: cholangiolitic hepatitis. Squares: infective hepatitis.

Effect of Probenecid on the Urobilinogen Clearance

The high clearance ratio of urobilinogen in highly alkaline urine (Fig. 6) makes it unlikely that the total amount excreted could have been entirely derived from glomerular filtration. Owing to the high proportion of plasma urobilinogen bound to plasma protein, only about 20% of the amount in plasma can pass into the glomerular filtrate. Most organic acids excreted by a pH-dependent mechanism are secreted by proximal tubular cells, and their clearance is competitively reduced by probenecid (Weiner and Mudge, 1964). Probenecid at a dosage of 0.5 g., q.i.d., was administered for two days in three patients

FIG. 7.—Urobilinogen clearance (expressed as a fraction of the simultaneous endogenous creatinine clearance) related to urinary pH in a patient with hepatocellular jaundice. Open circles: control day. Closed circles: clearance values obtained on the second day of probenecid ingestion.

with raised plasma and urinary urobilinogen concentrations; the clearances of urobilinogen and urate were estimated on a control day and on the second day of probenecid therapy. Urobilinogen clearance was significantly reduced by probenecid in one of the three patients (Fig. 7). This patient showed the greatest probenecid effect as estimated by increase of urate clearance: the urate clearance was raised from 10% to 50% of the coincident creatinine clearance, whereas in the other two patients urate clearance was increased only one and a half times and two and a half times respectively.

Discussion

The present results indicate that urobilinogen is about 80% bound to plasma protein. This is likely to represent a minimum value, because, owing to the virtual impossibility of obtaining absolutely pure urobilinogen, some competition for binding sites by impurities cannot be excluded. Therefore only a fraction of the total plasma content is filtered at the glomerulus. The amount filtered is too low to account for the relatively high urobilinogen clearance recorded in highly alkaline urine. This suggests that some urobilinogen must pass from the plasma into the lumina of the renal tubules by proximal tubular secretion, a view supported by the reduction of clearance in one patient after administration of probenecid, a competitive inhibitor of secretion of many organic acids. Protein-binding of a compound reduces the rate of diffusion into interstitial fluid but does not otherwise render the substance unavailable for proximal tubular secretion. As the concentration in the interstitial fluid is reduced by cellular uptake, the equilibrium between the bound and the free compound is altered with promotion of dissociation of the protein-bound fraction.

Urobilinogen is a weak divalent organic acid which is lipid-soluble in the undissociated form and water-soluble in the ionized form. The pK_a value of 5.45, determined by a solvent partition technique, is presumably the dissociation constant between the completely unionized fraction and the monovalent salt. A partition method is particularly relevant when the property of diffusion across lipid-containing cell membranes is concerned. Excretion by a pH-dependent mechanism is particularly likely to occur in the case of a weak acid of this order of pK_a (Milne *et al.*, 1958), and the clearance is likely to increase with urinary pH throughout the whole range of urinary acidity and alkalinity (Milne *et al.*, 1960). In these experiments the urobilinogen clearance expressed as a fraction of the endogenous creatinine clearance varied from an average minimum of 0.03 in extremely acid urine to an average maximum of 0.22 in highly alkaline urine (Fig. 6).

Comparison of the present results with those of other workers is difficult for three reasons. (1) Investigation of the mechanisms of urobilinogen excretion has previously been neglected, despite the wide use of estimation of urinary urobilinogen in clinical pathology. (2) Urinary pH has not previously been known to affect the clearance of this compound. (3) Urobilinogen is difficult to obtain pure as a satisfactory reference compound; this makes comparison of absolute values between laboratories difficult, although it does not affect clearance determinations; the use of artificial standards in both the colorimetric and the fluorimetric methods is an unsatisfactory substitute.

Royer and Solari (1941) reported that the urobilinogen: creatinine clearance ratio was 0.95–1.86 in man and 0.53–1.35 in the dog. These results are much higher than the present figures, but were obtained after injection of urobilin, which may have altered the clearance. Probably the proportion bound to plasma protein falls if plasma urobilinogen levels are artificially high. In a more recent paper (Lozzio *et al.*, 1963) normal plasma urobilinogen concentration is quoted as $15.1 \pm 10.1 \mu\text{g./100 ml.}$, which is considerably higher than the present estimate of $5.3 \pm 3.2 \mu\text{g./100 ml.}$ This may partly be due to the modification of the fluorimetric method of Lozzio and Royer (1962) by correction for the high reagent blank value of the method. Lozzio *et al.* (1963) do not quote clearance values but give the coincident 24-hour output of urobilinogen. Calculation of the clearance of a compound with considerable diurnal variation of plasma concentration is obviously inaccurate. Nevertheless values calculated from their data agree with the present results, averaging 4.2 ml./min. in normal subjects and 4.75 ml./min. in various pathological states associated with increase of plasma and urinary urobilinogen. The range varied from 0.5 to 31.8 ml./min., and in retrospect this was probably due to variation in urinary pH.

Diurnal variation in urobilinogen excretion was first reported over seventy years ago by Grimm (1893). Saillet (1897), using

the relatively insensitive methods then available, was unable to demonstrate any urobilinogen in urine excreted during the night. Salén (1924) confirmed that the highest output was between noon and 4 p.m. and warned against drawing any conclusions from arbitrary urine samples rather than 24-hour collections. Quodbach (1936) obtained similar results, and found that the rhythm was unaffected by taking the main meal at 2 a.m. or by feeding every two hours throughout the day by a gastric tube. He considered that the regular variations in urinary urobilinogen were due to an "endogenous liver rhythm." In addition, there is up to 300% variation in urinary urobilinogen from day to day (Royer, 1943).

The present results show that the diurnal variation in urinary urobilinogen is due to at least two factors: variation in renal clearance from diurnal changes in urinary pH, and alterations in plasma urobilinogen concentration. A high rate of excretion can occur only when there is both a high plasma concentration and a relatively high urinary pH. This combination usually occurs between noon and 4 p.m., when plasma levels are generally above 6 µg./100 ml. and the urine is at a maximum pH value in relation to diurnal change.

These results are of practical importance in the interpretation of urinary urobilinogen output. Determination of the 24-hour output is of little value unless the average urinary pH is known. Urobilinogen is much more stable in alkaline than in acid conditions, and storage for a whole day without alkalization is likely to give too low a result. Probably the best specimen for analysis would be a two-hour or four-hour specimen collected between noon and 4 p.m. The urinary pH should be determined immediately, and if there is any delay in analysis the specimen should be alkalized before storage. Normal values can be given only if corrections are made for urinary pH (Fig. 1).

Summary

Urobilinogen output and clearance are correlated with urinary pH, the values being higher in alkaline urines and lower in acid specimens. The ratio between the urobilinogen and endogenous creatinine clearance averages 0.03 at urine pH 5

and 0.22 at urine pH 8. Normal plasma urobilinogen is estimated as 5.3 ± 3.2 µg./100 ml. Urobilinogen is about 80% bound to plasma protein, and in one subject the urobilinogen clearance was significantly reduced by probenecid. Reasons are advanced for a triple mechanism of urobilinogen excretion by the kidney—that is, glomerular filtration, proximal tubular secretion, and distal tubular pH-dependent back-diffusion. Urobilinogen is shown to resemble other lipid-soluble organic acids in these respects and to obey the theoretical laws of excretion by pH-dependent diffusion.

Determination of urinary urobilinogen as an index of haemolytic states or of hepatic dysfunction is unreliable unless corrections are made for variation in urinary pH.

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Outbreaks of Conjunctivitis due to the Newcastle Disease Virus Among Workers in Chicken-broiler Factories

D. G. TROTT,* M.B., CH.B., D.P.H., D.I.H.; R. PILSWORTH,† M.D., DIP.BACT.

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Newcastle disease, a disease of world-wide distribution in fowls, was described originally by Doyle in 1927 as a viral pneumo-encephalitis. Burnet (1943) reported the first proved human infection as an acute follicular conjunctivitis without keratitis in a laboratory worker who became infected by the accidental introduction of infected material into the conjunctival sac. Subsequently eight cases of human infection in which the virus was isolated have been reported (Anderson, 1946; Freymann and Bang, 1949; Ingalls and Mahoney, 1949; Gustafson and Moses, 1951; Hunter, Keeney, and Sigel, 1951).

Only two cases reported by Ingalls and Mahoney (1949) occurred naturally—one in a broiler-plant operative and the

other in a veterinary student. Yatoom (1946) reported an outbreak of 17 cases of conjunctivitis among kitchen workers handling poultry at the Agricultural School at Mikyeh, Israel, where an outbreak had recently occurred in fowls; and Nelson *et al.* (1952) reported 40 cases of conjunctivitis occurring in a produce plant where poultry were eviscerated, processed, and canned.

This paper describes outbreaks of conjunctivitis at three different broiler factories owned by the same company occurring between December 1960 and December 1963. It is interesting to note that, though the Newcastle disease virus was suspected to be the causal agent from the very first outbreak in December 1960, repeated inquiry failed to discover fowl pest or signs of the disease at any of the farms supplying the

* H.M. Medical Inspector of Factories, Eastern and Southern Division.
 † Public Health Laboratory, Chelmsford.