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Estimation of urinary 5-hydroxytryptamine-*O*-glucuronide, a metabolite of endogenous 5-hydroxytryptamine in sheep

A. L. BARTLET AND FRANCES M. GILBERT

Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, Edinburgh

Summary

1. 5-Hydroxytryptamine-*O*-glucuronide has been extracted from sheep urine and characterized by its chemical reactions and R_F values on paper chromatograms.

2. 5-Hydroxytryptamine-*O*-glucuronide in urine was separated from an inhibitor of β-glucuronidase using an anion exchange resin, and then estimated by measurement of the 5-hydroxytryptamine (5-HT) liberated on incubation with β-glucuronidase.

3. About 20% of the 5-hydroxyindoles found in sheep urine was 5-HT conjugated with glucuronic acid ($1{\cdot}18 \pm 0{\cdot}08$ mg 5-HT/24 h, thirty experiments).

4. Sheep urine also contained free 5-HT ($0{\cdot}35 \pm 0{\cdot}04$ mg/24 h, thirty experiments) and 5-hydroxyindolylacetic acid ($4{\cdot}43 \pm 0{\cdot}33$ mg/24 h, thirty experiments).

5. 5-Hydroxytryptamine-*O*-sulphate was not found in sheep urine.

Introduction

Conjugation with glucuronic acid has not been considered a principal route for the metabolism of endogenous 5-hydroxytryptamine (5-HT), but since hepatic UDP-glucuronyl transferase activity is quite high in sheep (Ellis & Newcomer, 1964) the excretion of conjugates of 5-HT has been investigated in this animal. Considerable amounts of 5-HT-*O*-glucuronide were found in sheep urine but it had to be separated from an inhibitor of β-glucuronidase before it could be estimated quantitatively.

Methods

Animals

Five black faced wethers kept on a diet of oats (150 g/day), unrestricted hay and water were used. The sheep were given the anthelmintic drug thiabendazole (2–3 g) not less than a week before use, and liver fluke and nematode eggs were always absent from samples of faeces.

Urine collection

Wethers fitted with urine collectors were put in metabolism cages (Warwick, 1969) and the urine collected in plastic bottles cooled by ice. The urine was adjusted to

pH 4·0 with HCl, filtered through Whatman No. 1 paper and a sterile Carlson-Ford bacteriological filter (grade HP/EK) and stored frozen.

Chemicals

The chemicals were of the analytical or microanalytical reagent grade except for *n*-butyl acetate (B.D.H. Laboratory Reagent). 5-Hydroxyindolylacetic acid diethylammonium salt (B.D.H.) , 5-hydroxytryptamine creatinine sulphate (B.D.H.), potassium D-glucarate (Sigma) and D-glucuronic acid (B.D.H.) were used, but amounts of 5-hydroxyindolylacetic acid (5-HIAA) and 5-HT have been given in terms of the acid and base, respectively.

Enzyme preparations

Two enzyme preparations made from *Patella vulgata* were used. One contained standardized β-glucuronidase activity and unstandardized aryl sulphatase activity and the other contained standardized aryl sulphatase activity and β-glucuronidase inactivated by acidification. A soluble powder made from *E. coli* containing only β-glucuronidase was also used. The preparation containing limpet β-glucuronidase and aryl sulphatase activities was used except where one of the other preparations has been specified. The enzyme standardizations were those of the manufacturer, the Sigma Chemical Company.

Ion exchange resins

Dowex-1 (X8, 100–200 mesh) (chloride form) was stirred four times with 1M NaOH (three vols.) for 30 min and then collected in a Buchner funnel. The Dowex-1 (hydroxide form) was washed with distilled water until the pH of the effluent fell to about 8 before conversion to the acetate form by stirring four times with 1M CH_3COOH (three vols.) for 30 minutes. The Dowex-1 (acetate form) was rinsed with distilled water until the pH of the effluent was about 4·5, and then stored at 5° C under 1M sodium acetate adjusted to pH 4·5 with concentrated CH_3COOH.

Amberlite CG-50 (100–200 mesh ; hydrogen form) was stirred three times with 1M HCl (three vols.) for 30 min and rinsed with distilled water until the washings were free from chloride. To convert to the ammonium form the resin was stirred three times with 3M NH_4OH (three vols.) for 30 minutes. The ammonium form of the resin was washed first with distilled water until the pH of the effluent fell to about 9 and then with 0·2 M ammonium acetate adjusted to pH 7·5 with 2 M NH_4OH and was then stored at 5° C under the ammonium acetate buffer.

Extraction and paper chromatography of 5-HT-O-glucuronide

Urine (2 ml) diluted with water (3 ml) and adjusted to pH 7·0 was filtered through Whatman No. 1 paper, loaded on a column (1·5 cm × 3·0 cm) of Amberlite CG-50 (hydrogen form) and washed through with water (4 × 5 ml). This column removed free 5-HT from the sample. The effluent from the column was adjusted to pH 4·5, loaded on a column (1·5 cm × 3·0 cm) of Dowex-1 (acetate form) and washed through with water (4 × 5 ml). This removed strong acid substances from the urine but allowed the conjugated 5-HT to pass through. The effluent from the column was adjusted to pH 7·0 and concentrated to a volume of 5–10 ml in a rotary drier under reduced pressure and at an external temperature of 40° C. The concentrate

was loaded on a column (1·5 cm × 3·0 cm) of Dowex-1 (hydroxide form) which was washed with water (2 × 10 ml) before eluting 5-HT-*O*-glucuronide with 2 M acetic acid (20 ml). The rate of flow through the columns was kept at 0·1–0·2 ml/min with a small screw clip on fine rubber tubing attached to the base of the columns.

The eluates from four columns of Dowex-1 (hydroxide form) were pooled and acetic acid removed by six washings with equal volumes of *n*-butyl acetate. The residual aqueous extract was evaporated to dryness under reduced pressure and at an external temperature of 40° C. The residue was dissolved in water (0·3 ml) and streaked across a sheet of Whatman No. 1 paper (chromatographic grade) so that the applied solution formed a band which was about 45 cm long and not more than 1 cm wide. The chromatogram was developed at room temperature in the ascending direction with butan-1-ol/glacial acetic acid/water (12:3:5, by volume) for 20 hours. After drying in air a vertical strip (width 1 cm) was cut from the edge of the chromatogram for the location of 5-HT-*O*-glucuronide. The strip was sprayed with a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in 1M HCl (Ehrlich's reagent) and heated at 80–85° C for 3–5 min when 5-HT-*O*-glucuronide formed a blue spot, R_F 0·20. A horizontal strip (width 2 cm) from the remainder of the chromatogram, corresponding to the 5-HT-*O*-glucuronide, was cut into small pieces and shaken with distilled water (25 ml) in a glass stoppered tube. The eluate was rechromatographed in four solvent systems to determine the R_F values and colour reactions of 5-HT-*O*-glucuronide. In two experiments the eluate was adjusted to pH 4·0 and incubated with β-glucuronidase (500 Fishman units) for 72 h at 37° C before rechromatography.

The eluate was concentrated to a volume of 0·05–0·10 ml under reduced pressure and at an external temperature of 40° C and applied to a sheet of Whatman No. 1 paper so that the applied solution formed a circle of diameter not more than 3 cm. 5-HT, 5-HIAA and glucuronic acid (10–20 μg in each instance) were similarly applied to the origin of the paper. Chromatograms were developed at room temperature in the ascending direction with the solvents listed in Table 1. The chromatograms were dried in air before application of Ehrlich's reagent for indoles (as above), ninhydrin acetic acid reagent for tryptamines (Jepson & Stevens, 1963), nitrosonaphthol reagent for phenolic substances (Udenfriend, Weissbach & Clark, 1955) or naphthoresorcinol reagent for uronic acids (Harris & MacWilliams, 1954).

Removal of the urinary inhibitor of β-glucuronidase

Urine (2 ml) mixed with water (3 ml) and adjusted to pH 4·0 was filtered through Whatman No. 1 paper and run through a column (1·5 cm × 3·0 cm) of Dowex-1 (acetate form). After washing the column with water (4 × 5 ml) the combined effluent contained 5-HT-*O*-glucuronide separated from a urinary inhibitor of β-glucuronidase. The rate of flow through the columns was kept at 0·1–0·2 ml/minute.

Estimation of 5-HT-O-glucuronide

The effluent from two columns of Dowex-1 (acetate form) loaded with urine (as above) was combined, made up to a volume of 50 ml and adjusted to pH 4·0. The mixture was divided equally into two glass stoppered tubes, one tube being incubated with β-glucuronidase (*Patella vulgata*) (500 Fishman units) for 72 h at 37° C with constant agitation and the other being stored frozen. The 5-HT contents of

the two tubes were estimated, the difference being the 5-HT liberated from the 5-HT-*O*-glucuronide in 2 ml urine.

Extraction and estimation of 5-HT

The method is a modification of that described by Oates (1961). Urine (2 ml) diluted with distilled water (3 ml) and adjusted to pH 7·5 was filtered through Whatman No. 1 paper and loaded on a column (0·5 cm × 5·0 cm) of Amberlite CG-50 (ammonium form). The column was washed first with 0·02 M ammonium acetate (15 ml) adjusted to pH 7·5 with 0·5 M NH_4OH and then with 0·05 M H_2SO_4 (3 ml), before eluting 5-HT with 0·5 M H_2SO_4 (5 ml). The rate of flow through the columns was kept at 0·1–0·2 ml/minute. Concentrated HCl containing ascorbic acid, 0·5 mg/ml, was added to the eluate to make it 3 M with respect to HCl, and 5-HT estimated in a Farrand spectrofluorimeter by measuring the peak at 295 nm in the activation scan with fluorescence recorded at 535 nm.

Estimation of p-*nitrocatechol*

In some experiments aryl sulphatase (*Patella vulgata*) was added to incubates adjusted to pH 5·6 and otherwise similar to those described above. The activity of the enzyme in the incubates was checked by the addition of 2 nM *p*-nitrocatechol sulphate. After stopping the reaction by the addition of 1·5 M NaOH (eight vols.), liberated *p*-nitrocatechol was determined by measuring the intensity of the red colour which developed at a wavelength of 520 nm against a blank in which the substrate had been incubated in the absence of the enzyme before the addition of alkali (Roy, 1953).

Estimation of glucaric acid

A solution of glucaric acid was mixed with periodic acid and potassium ferricyanide as described by Ishidate, Matsui & Okada (1965) and E_{520} measured in a Unicam spectrophotometer (SP 500).

Extraction and estimation of 5-HIAA

The method has been described previously (Bartlet & Gilbert, 1970).

Estimation of creatinine

Urinary creatinine was determined by the sodium picrate method after extraction of the specimens with peroxide-free diethyl ether (Taussky, 1956).

Results

Bacterial contamination of urine

In preliminary experiments urinary 5-HT was sometimes increased when portions of urine were incubated at 37° C for 24 hours. This did not occur after bacteriological filtration of the urine specimens, and all specimens used in the present experiments were sterilized by filtration.

*Paper chromatography of 5-HT-*O*-glucuronide*

The R_F values in four solvent systems of the substance identified as 5-HT-*O*-glucuronide are given in Table 1. Ehrlich's reagent reacted with the glucuronide to form a blue product in the chromatograms, and after dipping papers in the

ninhydrin acetic acid reagent the glucuronide appeared as a greenish fluorescent spot in ultraviolet light. The nitrosonaphthol reagent did not form a colour with the glucuronide although 5-HT and 5-HIAA formed purple colours with this reagent. In papers treated with naphthoresorcinol reagent glucuronic acid appeared as a blue spot on a pale pink background, but 5-HT-*O*-glucuronide formed a colour which was so faint as to be hardly detectable. Extracts which had been incubated with β-glucuronidase did not contain 5-HT-*O*-glucuronide but free 5-HT and glucuronic acid were present and these were identified by their colour reactions and R_F values on paper chromatograms.

*Separation of 5-HT-*O*-glucuronide from an inhibitor of β-glucuronidase*

Urine was passed through a column of Dowex-1 (acetate form) to remove glucaric acid and its 1:4 lactone which inhibit β-glucuronidase (Levvy, 1952; Levvy, Hay &

TABLE 1. *R_F values of 5-hydroxytryptamine-*O*-glucuronide and related compounds*

	R_F in solvent system			
	1	2	3	4
Substance identified as				
5-Hydroxytryptamine-*O*-glucuronide	0·20	0·64	0·17	0·02
5-Hydroxytryptamine	0·51	0·33	0·61	0·58
Glucuronic acid	0·19	1·00	0·36	0·05
5-Hydroxyindolylacetic acid	0·78	0·49	0·55	0·19

The ascending method on Whatman No. 1 paper was used. Solvent systems were: (1), butan-1-ol/glacial acetic acid/water (12:3:5, by volume), 20h; (2), 20% (w/v) aqueous KCl, 4h; (3), methanol/butan-1-ol/benzene/water (2:1:1:1, by volume), 16h; (4), propan-2-ol/ammonia (S.G. 0·880)/water (20:1:2, by volume), 18 hours.

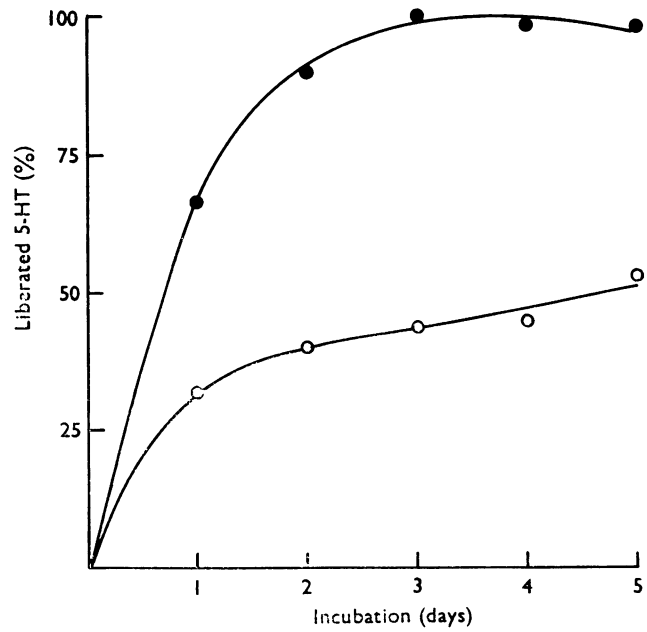

FIG. 1. Liberation of conjugated 5-HT by β-glucuronidase (500 Fishman units) in untreated urine (○——○) and urine passed through a column (1·5 cm × 3·0 cm) of Dowex-1 (acetate form) (●——●). Ordinate, liberated 5-HT (% conjugated 5-HT); abscissa, incubation at 37° C (days). Passage through Dowex-1 (acetate form) removed an inhibitor of β-glucuronidase from the urine.

Marsh, 1957). The colorimetric method for the estimation of glucaric acid (Ishidate *et al.*, 1965) could not be applied to sheep urine or to the effluent from the columns of Dowex-1 (acetate form) loaded with sheep urine as chromophore formation was inhibited in each instance. However, when glucaric acid (20 μg/ml, 5 ml) was loaded on a column of the resin and washed through with water (4 × 5 ml), glucaric acid was not present in the column effluent.

Urine and urine passed through Dowex-1 (acetate form) were incubated with β-glucuronidase (as described under **Methods**) and the liberated 5-HT estimated after incubation for 1–5 days. The results of one of six experiments are shown in Fig. 1. Sheep urine strongly inhibited β-glucuronidase and no more than 78% of the conjugated 5-HT was liberated even after incubation for 5 days. After passage through Dowex-1 (acetate form) sheep urine inhibited β-glucuronidase less strongly and incubation for 3 days with the enzyme seemed to be sufficient to break down the conjugate completely. Addition of further β-glucuronidase (500 Fishman units) to the incubates after incubation for 3 days did not liberate any more 5-HT, confirming that the 5-HT conjugate was completely broken down after incubation for 3 days with the enzyme.

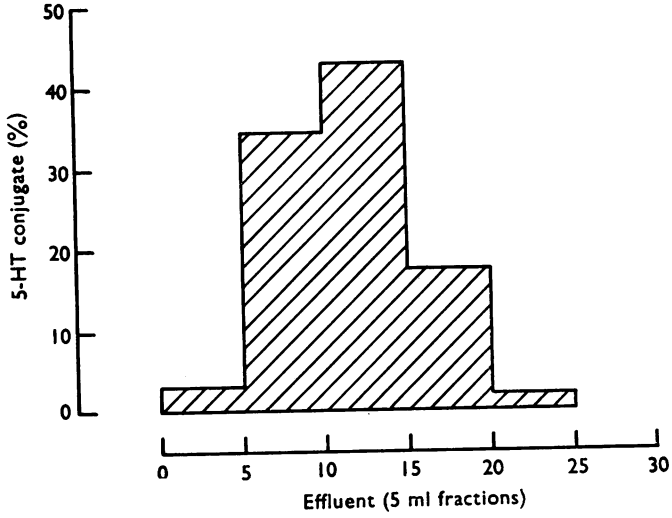

FIG. 2. Recovery of conjugated 5-HT in the effluent from a column (1·5 cm × 3·0 cm) of Dowex-1 (acetate form) loaded with sheep urine and then washed with water. Ordinate, 5-HT conjugate (%); abscissa, effluent (5 ml fractions). All the conjugated 5-HT was recovered in 25 ml effluent.

TABLE 2. *Liberation of free 5-HT on incubation with various enzyme preparations*

Enzyme	5-HT liberated (μg/ml urine)	*P* (when compared with untreated extract)
Limpet β-glucuronidase (500 Fishman units)	1·45 ± 0·35 (5)	< 0·02
Bacterial β-glucuronidase (500 Fishman units)	1·62 ± 0·38 (5)	< 0·02
Limpet aryl sulphatase (10 units)	0·02 ± 0·02 (5)	> 0·3

Urine specimens collected from five sheep were passed through columns (1·5 cm × 3·0 cm) of Dowex-1 (acetate form) before incubation with the enzyme preparations. The values are expressed as means ± S.E.M. with numbers of observations in parentheses.

Recovery of 5-HT-O-glucuronide from a column of Dowex-1 (acetate form)

The effluent from a column of Dowex-1 (acetate form) loaded with sheep urine and then washed with water was collected in 5 ml fractions and the 5-HT content of each fraction estimated before and after incubation with β-glucuronidase (three experiments). Most of the conjugated 5-HT was found in the second, third and fourth fractions of effluent (Fig. 2), so that in subsequent experiments 25 ml of effluent was collected from the columns.

In five further experiments the conjugated 5-HT was estimated after passing the urine through one or two columns of Dowex-1 (acetate form). The amount of conjugated 5-HT was not reduced after passage through a second column of the resin, confirming that it was all recovered when 25 ml of effluent was collected from the columns.

Ability of β-glucuronidase and aryl sulphatase to split the 5-HT conjugate

The effluent from columns of Dowex-1 (acetate form) loaded with sheep urine was incubated with β-glucuronidase and aryl sulphatase to see whether 5-HT was conjugated with glucuronic acid only. β-Glucuronidase (*E. coli*) (500 Fishman units) or β-glucuronidase (*Patella vulgata*) (500 Fishman units) or aryl sulphatase (*Patella vulgata*) (ten units) were added to incubates adjusted to pH 7·0, 4·0 or 5·6, respectively, and incubated as described under **Methods**. Liberated 5-HT was measured in the incubates (Table 2).

Incubation with aryl sulphatase did not liberate a significant amount of 5-HT although 2 mM *p*-nitrocatechol sulphate added to the incubates was broken down completely with the release of *p*-nitrocatechol. Incubation with the preparation of bacterial β-glucuronidase, which was without sulphatase activity, liberated as much 5-HT as did incubation with limpet β-glucuronidase, which contained aryl sulphatase activity.

5-Hydroxyindole content of sheep urine

The 24 h urine specimens contained 0·35 ± 0·04 mg 5-HT (mean ± S.E.M., thirty experiments), 1·18 ± 0·08 mg 5-HT-*O*-glucuronide (expressed as liberated 5-HT), 4·43 ± 0·33 mg 5-HIAA and 550 ± 37 mg creatinine. The volume, pH and creatinine and 5-hydroxyindole contents of the 24 h specimens collected from each sheep are given in Table 3, together with the body weights of the animals.

Three sheep were put in metabolism cages for 9 days and fed cabbage instead of hay on days 4, 5 and 6. The cabbage diet increased the volume of the 24 h urine

TABLE 3. *5-HT, 5-HT-O-glucuronide, 5-HIAA and creatinine contents of sheep urine*

Sheep	Body weight (kg)	Urine pH	Urine volume (ml/24 h)	Creatinine (mg/24 h)	5-HIAA (mg/24 h)	Conjugated 5-HT (mg/24 h)	Free 5-HT (mg/24 h)
1	28	8·3 ± 0·3	750 ± 90	648 ± 44	5·13 ± 0·58	1·22 ± 0·08	0·24 ± 0·03
2	19	8·5 ± 0·1	558 ± 60	483 ± 30	4·92 ± 0·99	0·80 ± 0·06	0·22 ± 0·02
3	33	8·6 ± 0·1	702 ± 48	803 ± 25	4·71 ± 0·73	1·41 ± 0·09	0·32 ± 0·02
4	24		362 ± 65	467 ± 94	3·46 ± 0·45	1·29 ± 0·27	0·64 ± 0·13
5	36		460 ± 75	349 ± 52	3·93 ± 0·84	1·19 ± 0·24	0·33 ± 0·06

Each value is the mean obtained from six urine specimens ± S.E.M.

specimens as much as 10-fold without affecting the amounts of 5-hydroxyindoles and creatinine excreted.

The pH of the urine specimens was usually about 8·5, but in two experiments it fell to 6·0, spontaneously. The fall in urinary pH was not accompanied by any marked change in 5-hydroxyindole excretion (Fig. 3).

Discussion

The urine specimens were sometimes contaminated with bacteria which may have produced β-glucuronidase as the 5-HT content of these specimens increased on incubation. Keeping the urine collections at 0° C followed by bacteriological filtration prevented bacterial activity. Urinary creatinine was estimated as a check on the completeness of the urine collections. The daily excretion of creatinine did not

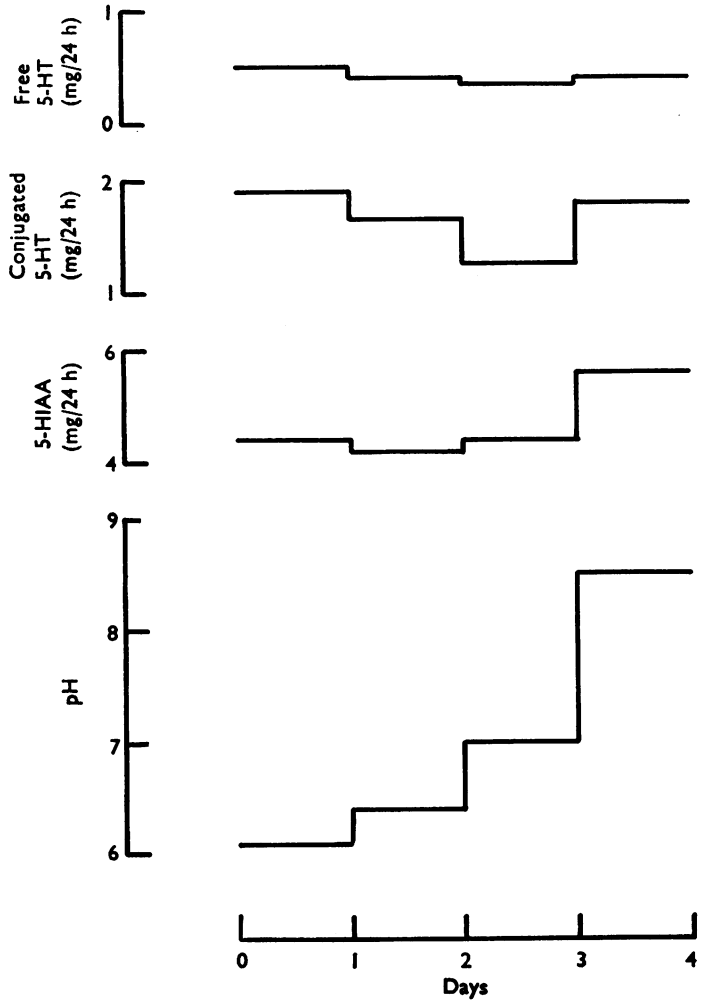

FIG. 3. Excretion of 5-hydroxyindoles in urines of different pH. Sheep, 33 kg. From above downwards: urinary 5-HT (mg/24 h), 5-HT-*O*-glucuronide (mg/24 h), 5-HIAA (mg/24 h) and pH. The spontaneous shift of pH was not associated with a change in 5-hydroxyindole excretion.

change significantly when the volume of the specimens varied greatly, as for example when the sheep were fed cabbage instead of hay.

On incubation with β-glucuronidase the conjugated 5-HT was broken down with the liberation of 5-HT and glucuronic acid. The positive reaction of the ninhydrin-acetic acid reagent with the glucuronated 5-HT on paper chromatograms indicated a tryptamine structure with little substitution in the side chain (Jepson & Stevens, 1953), and the negative reaction with nitrosonaphthol reagent showed that the phenolic group of the glucuronated 5-HT had been blocked by ether or ester formation. Thus the conjugate seemed to be 5-HT-*O*-glucuronide. It has been characterized by its R_F values in four solvent systems.

Sheep urine contained substances which strongly inhibited the breakdown of 5-HT-*O*-glucuronide by β-glucuronidase. The liberation of 5-HT from its glucuronide was probably inhibited competitively by other urinary glucuronides and by the 1:4 lactone of glucaric acid, a powerful inhibitor of β-glucuronidase (Levvy, 1952; Levvy *et al.*, 1957) excreted in the urine of sheep and other species (Marsh, 1963). 5-HT-*O*-Glucuronide was displaced from a column of Dowex-1 (hydroxide form) by acetic acid indicating that it had only weak acidic properties. The pK_a of most glucuronides, however, is less than that of acetic acid (Smith & Williams, 1966). This suggested that the weakly acidic 5-HT-*O*-glucuronide might be separated from strongly acidic inhibitors of β-glucuronidase by ion exchange chromatography. Glucaric acid was adsorbed by a column of Dowex-1 (acetate form) and, when urine was loaded on the resin, 5-HT-*O*-glucuronide was recovered in the column effluent without loss and with much of the β-glucuronidase inhibitor removed. This allowed 5-HT-*O*-glucuronide to be estimated by measurement of the 5-HT liberated by β-glucuronidase.

About 1·2 mg 5-HT-*O*-glucuronide was found in a 24 h urine specimen, corresponding to about 20% of the urinary metabolites of 5-HT in sheep. The glucuronidation of substantial amounts of 5-HT accords well with the report of a relatively high activity of hepatic UDP-glucuronyl transferase in sheep (Ellis & Newcomer, 1964), and does not seem to be the outcome of a deficiency of monoamine oxidase since a 24 h urine specimen contained about 4·4 mg 5-HIAA. The glucuronide of 5-HIAA was not found in sheep urine (Bartlet & Gilbert, 1970), however, indicating that little 5-HT-*O*-glucuronide was deaminated by monoamine oxidase *in vivo*.

5-HT-*O*-Sulphate may be eluted from an anion exchange resin with acetic acid (Davis, Huff & Brown, 1966). However, incubation with aryl sulphatase did not liberate 5-HT in the effluent from a column of Dowex-1 (acetate form) loaded with sheep urine, although *p*-nitrocatechol sulphate added to the incubates was broken down completely with the liberation of *p*-nitrocatechol; thus sheep urine does not seem to contain 5-HT-*O*-sulphate.

Free 5-HT (about 0·35 mg) was found in a 24 h urine specimen despite the pH of sheep urine being about 8·5. Changes of specimen pH had little effect on the excretion of 5-HT, 5-HT-*O*-glucuronide or 5-HIAA, however, indicating that the renal tubules do not reabsorb these substances in substantial amounts.

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