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Studies on Urinary Pigments

I. Preparation of Crude Pigment from Human Urine

SVEND ERIK HANSEN

Department of Biochemistry A, University of Copenhagen, 30 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark

A method for preparing a crude pigment from human urine is described. Acidified urine is applied to a column of activated charcoal and celite, on which the pigments are adsorbed. They are eluted with pyridine, and from this solution a dry material is prepared by evaporation, dissolution in methanol and precipitation with ether. The yield is about 1 g/l of urine. The crude pigment is characterized by spectra in UV and IR, by its solubility properties, its strongly acid character and its content of glucuronic acid and certain amino acids. Darkening of pigment by treatment with acid and by storage is mentioned. The hydrolytic and chromatographic procedure for amino acid analysis is described. The findings are briefly discussed.

Very little is known up till now concerning the nature of the yellow, brown, and red pigments which may be prepared from human urine. Several trivial names have been assigned to them, the most generally accepted being urochrome, but these names will not be used in the following.

The composition of the pigments have been a matter of dispute. They have been claimed to be break-down products of hemoglobin [1] or fuscine pigments.[2] Amino acids, indoxyl groups, and glucuronic acid have been reported as constituents.[3,4]

The purpose of the present investigation is to establish a convenient procedure for preparation and fractionation of the substances and to obtain a chemical characterization of them. If possible, some light should be thrown on the biogenetic origin.

The method we have found to be most suitable for preparation makes use of adsorption of the pigments from acidified urine onto activated charcoal in a short column. After washing with water, the pigments are eluted with pyridine which is removed *in vacuo*. The residual material is dissolved in methanol, filtered, precipitated with ether and acetone and finally dried *in vacuo*, leaving a brown amorphous powder which has been found suitable for fractionation experiments and chemical investigation. The material prepared in this way

(in the following referred to as crude pigment) is readily soluble in methanol at acid pH, less at alkaline pH. In ether and most other organic solvents except the lower alcohols, the pigment is practically insoluble. The aqueous solution is strongly acid. By partition between butanol and 1 M HCl a partition quotient of about 2 in favour of butanol is seen. Partition between aqueous alkali and butanol gives practically all the pigment in the aqueous phase. The partition quotient is determined as the ratio of the absorbance of the two phases at 400 nm. The absorption spectra in the visible-ultraviolet range are of little informative value. They show a gradually increasing adsorption towards the shorter wavelengths. The only characteristic feature is a triplet of peaks in the region about 250—265 nm.

In the infrared region a spectrum is obtained which is characteristic of a rather high-molecular substance. Bands are seen which are caused by carboxylic groups. Besides that, bands from OH-groups and probably NH-groups are seen among several lines which are partly fused.

Heating the pigment with 0.1 M HCl in methanol for 1 h results in intensification of the colour, which at the same time is changed to a more reddish-brown. Heating with 0.1 M KOH in methanol results in less intensification of the colour.

Heating of freshly voided urine to 100°C after adjusting pH to 1—2 and 10—11, respectively, gives analogous results. The results from such a set of experiments are summarized in Table 1.

Table 1.

	Optical density at 400 nm		
	at start of experiment	after 60 min	Remarks
Pigment refluxed in 0.1 M HCl in MeOH	0.270	0.610	Optical density still increases after 60 min
Pigment refluxed in 0.1 M KOH in MeOH	0.300	0.470	Optical density still increases after 60 min
Urine pH 1—2	0.100	0.410	Optical density decreases after 60 min. Pigment precipitates
Urine pH 1—2 under N_2	0.120	0.300	Optical density still increases after 60 min. Pigment precipitates
Urine pH 10—11	0.180	0.270	
Urine pH 10—11 under N_2	0.200	0.240	

The experiments with urine show that the intensification of the colour occurs whether access to the atmosphere is allowed or the system is kept anaerobic by flushing with oxygen-free nitrogen, but in the latter case the reaction is slower. During heating of the acidified urine a dark brown precipitate is formed which removes much of the pigment from solution.

The intensification of colour by such treatment is accompanied by loss of part of the glucuronic acid content and presumably also of amino acids. To a smaller extent some darkening of the pigment may also occur during storage.

Treatment with 6 M HCl at 100°C overnight in a sealed tube liberates amino acids from the pigment preparation. These can be separated and identified by 2-way paper chromatography according to the method of Dent.[5] The following are always seen: glycine, alanine, valine, leucine, tyrosine, glutamic acid, and aspartic acid. Moreover small amounts of proline, hydroxyproline, and probably β-alanine are rather often seen.

During acid hydrolysis a fairly large amount of dark-brown humin-like substances are also formed.

The crude pigment is practically negative to ninhydrin when subjected to chromatography without prior hydrolysis. It is also negative to the biuret reaction.

By means of the Dische method [6] glucuronic acid is established as a component in amounts corresponding to 7—9 % of dry weight of the crude pigment.

Neither phosphorus nor sulfur containing amino acids are present in the pigment.

EXPERIMENTAL

Preparation of crude pigment. 10 l of mixed urines are collected without any addition. HCl is added till strongly acid reaction (pH below 2) and the urine is filtered through celite on a Büchner funnel. It is then divided into two portions of 5 l, each of which is passed with suction through a column of 25 g of activated charcoal (Norite A) thoroughly mixed with 100 g of celite. To prevent disturbance or washing out of the charcoal a layer of pure celite is placed above as well as below the celite charcoal mixture.

Each column is washed with 2—3 l of water. Both filtrate and washings should be clear and colourless.

Now the pigments are eluted with pure pyridine (BDH lab. reagent). The colourless forerun is discarded and the dark-brown pigment solutions from the two columns are mixed. The total volume is 1—2 l.

The extinction of the eluate at 400 nm is measured. In 9 consecutive experiments the value $E_{400} \times$ ml eluate/ml urine has shown a mean of 0.539 with a standard deviation of 0.027.

The solution is evaporated *in vacuo* at 30—40°C; methanol is added and the evaporation is repeated. This is done a couple of times to remove the last traces of pyridine.

The residue is dissolved in 1 l of methanol. The material which precipitates is discarded and the solution evaporated to a syrupy consistency. 20 ml of acetone is now added followed by 1 l of diethyl ether in portions. When precipitation is considered complete the supernatant is discarded. The residue is dissolved in as little methanol as possible and precipitation is repeated with smaller volumes of ether and acetone. At last the precipitate is washed with pure diethyl ether and dried *in vacuo* to a voluminous and brittle dark brown mass. This is powdered and stored at −20°C in 0.5 g portions carefully protected from air and moisture as the material is very hygroscopic. The yield is 10—13 g from 10 l of urine.

Spectral examination. The UV-visible spectra are recorded on a Unicam SP 800 recording spectrophotometer. The sample is a solution containing 50—250 μg in 3 ml of water.

IR-spectra are recorded using the KBr-disc technique. Our first spectra were run on a Perkin-Elmer 221 spectrophotometer. Now a Beckman IR 12 is used.

Hydrolysis and paper chromatography. Hydrolysis experiments are performed by placing 5 mg of pigment and 125 μl of 6 M HCl (BDH microanalytical grade) in a Pyrex tube. The tube is placed in a CO_2-acetone cooling mixture until the content is completely frozen whereafter nitrogen is blown in to displace the air.

It is then evacuated and sealed by means of a blowpipe. Hydrolysis is performed for 16 h at 110°C. 25 μl of the content are placed on a 20 × 20 cm sheet of Whatman No. 4 filter paper which is subjected to two-way chromatography in phenol:water (first) and collidine:lutidine:water (second) according to the method of Dent.[5]

Phosphate is determined according to Brun [9] after wet ashing.

Glucuronic acid is determined by the Dische method.[6] In each determination 0.5 mg of pigment is used. For calculation the Morton-Stubbs procedure [7] is used: the spectrum of the reaction mixture is recorded from 600 to 450 nm and the two end points of the curve are connected by a straight line. The vertical distance from this line to the spectral curve at 525 nm is used as extinction value for the sample in question.

Standards containing 5 μg of glucuronic acid are used for calibration. The use of this procedure is necessitated by the fact that the red colour due to glucuronic acid is overlayered by a brown colour which gives a variable and non-horizontal baseline.

Treatment of pigment and urine with acid and alkali. A: Pigment. Crude pigment (100 mg) was dissolved in methanol (40 ml). Conc. hydrochloric acid (0.4 ml) was added to make the solution 0.1 M with respect to HCl.

The mixture was refluxed and samples of 1 ml were drawn before and just after acidification and after 5, 10, 20, 30, 45, 60, 90, and 120 min of heating. The samples were diluted 1:10 with methanol and the spectra recorded from 700 to 315 nm. In the case of treatment with alkali the hydrochloric acid was substituted by KOH (200 mg solid substance) and the diluted samples were acidified with a drop hydrochloric acid just before recording the spectra.

B: Urine. Freshly voided urine without any additions was filtered and stored in the refrigerator overnight. A sample (50 ml) was adjusted to a pH of 1—2 by means of hydrochloric acid. It was heated with reflux on a boiling water bath. Samples were drawn at the same intervals as above and treated in the same way. Dilution before recording the spectra was 1:10. The experiment with alkali treatment was performed similarly, but the pH was in this case adjusted to 10—11 before refluxing. Samples were only diluted 1:5 before the spectra were recorded.

Both acid and alkali treatment were performed twice, first without any special precautions and thereafter under anaerobic conditions by flushing the system by pyrogallol-washed nitrogen during the whole refluxing period.

DISCUSSION

The amount of urinary pigments is rather surprising: about 1 g per day excreted in the urine. Gross contamination can probably be ruled out as repetition of the purification procedure followed by a chromatographic fractionation which will be dealt with in the following paper, will give a recovery of about 80 % by weight, even though the procedure implies several steps that might cause loss of material.

If this is accepted, the origin of the pigment must be sought among the precursors or metabolites of substances that have a high metabolic turnover rate. In view of the content of glucuronic acid and amino acids the glycoproteins may be considered possible precursors, even if this gives no immediate explanation of the colour.

Another possibility is the porphyrin metabolism. If that represents the origin of the pigments pyrrole carboxylic acids should arise from oxidation with alkaline potassium permanganate. Such oxidations have been performed according to the method of Nicolaus,[8] but the yield of oxidation products which after paper chromatography and treatment with diazobenzenesulphonic acid give rise to strongly coloured spots was always low. In view of the rather low specificity of the colour reaction we would therefore not take this as a definite proof for the presence of pyrrole ring systems.

It should be mentioned in this connection, that tryptophan has never been found in alkaline hydrolysates of the pigments even if hydrolysis is performed in a teflon tube which is supposed to give the maximal protection of that amino acid.

It should be noted that no basic amino acid have ever been found in the pigment but only neutral and acid ones. This together with the rather high content of glucuronic acid can explain the strongly acid character of the pigments.

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