ACTA CHEMICA SCANDINAVICA 23 (1969) 3466–3472

Studies on Urinary Pigments

II. Fractionation of the Pigments by Chromatography

SVEND ERIK HANSEN

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

A chromatographic fractionation procedure for pigments from human urine is described. The lipophilic Sephadex LH-20 is used as column material and the chromatogram is developed with pure methanol. By this method 4 brown, 1 blue, and 1 red fraction are obtained. Further purification of the most important fractions is achieved by rechromatography on similar but smaller columns. The fractions are characterized by spectra, nitrogen content, glucuronic acid content, and amino acid composition. The stability and the possibility of artefacts and interconversions between the fractions is discussed.

It has been reported in a previous paper [1] that it is possible to obtain a preparation of the pigments from human urine by adsorption on activated charcoal followed by extraction with pyridine.

The procedure which has been described in detail results in a preparation which seems to be rather pure. The content of free amino acids and other ninhydrin-positive material is negligible as judged from paper chromatography. Free carbohydrate cannot be detected by paper chromatography. Mucoids can also be excluded, as sialic acids are not present.

On the other hand it is quite clear that the crude preparation represents a mixture of substances even if these may be closely related chemically. The present paper describes attempts to fractionate the pigments.

To accomplish such a fractionation it must be taken into account that the pigments are highly polar substances with several ionisable groups. They show strong adsorption phenomena when brought into contact with practically any surface.

Investigation of several chromatographic systems has shown that the best results are obtained by means of the lipophilic crosslinked dextran gel filtration medium Sephadex LH-20 (Pharmacia Fine Chemicals AB, Sweden). Although there is some overlapping of the fractions, this difficulty may be overcome by rechromatography on smaller columns of the same kind.

The fractions obtained are characterized by their amino acid pattern after hydrolysis, their glucuronic acid content, and their spectra in the ultraviolet and infrared regions.

EXPERIMENTAL

Chromatography on gelfiltration columns. 500 mg of crude pigment is used for each experiment. To free the pigment from the last traces of amino acids and other impurities that might be present, the sample is dissolved in water, acidified to a pH below 2 and passed over a short column consisting of 2 g of activated charcoal mixed with 10 g of celite. The column is thoroughly washed with water and the pigment eluted with pyridine. The extract is evaporated to dryness *in vacuo* at about 35°C. During evaporation small portions of methanol are added to secure complete removal of pyridine. The dry material is dissolved in 2 ml of methanol, acidified to a pH of about 2 by the addition of a few drops of HCl and applied to the column. This has an internal diameter of about 35 mm and is filled to a height of 850 mm with Sephadex LH-20, swelled in methanol. Filling of the column requires about 200 g of the dry gel material.

The column is eluted with pure methanol on a fraction collector (LKB, Sweden). Fractions of 10 ml are taken and the absorbance at 400 nm of each fraction is plotted against elution volume by means of a Unicam SP 800 recording spectrophotometer.

According to the elution profile thus obtained the eluate is cut into 4 fractions as shown in Fig. 1.

Each fraction is evaporated *in vacuo* in pre-weighed flasks at about 35°C. They are washed with dry and peroxide free diethyl ether, and dried at oil pump vacuum.

Especially fraction No. 3 may give some trouble as it tends to remain sticky or oily after drying. To overcome this it is repeatedly dissolved in methanol and precipitated by means of acetone and diethyl ether.

In the dry state all the fractions are rather hygroscopic and they are usually dissolved in methanol to a concentration of 10 mg per ml. The solutions are used for the further investigations.

Rechromatography of fractions Nos. 2 and 3, the results of which are shown in Fig. 2, is performed in the same way as described above, but with smaller columns.

Between consecutive rechromatographies of one fraction this is not evaporated to dryness but only taken down to the volume which is necessary for application to the column again.

UV-spectra. These are recorded for each fraction in the range 200–450 nm by adding 5–25 μl of methanol solution (corresponding to 50–250 μg of pigment) to 3 ml of water in a 1 cm cuvette using water as a reference.

IR-spectra. The KBr pellet-technique is used. The methanolic solution (about 0.1 ml corresponding to 1 mg of pigment) is added to 300 mg of KBr. It is dried in a desiccator *in vacuo* over silica gel overnight before mixing and pressing the pellets. Spectra are recorded on a Beckman IR 12 infrared spectrophotometer.

Hydrolysis and paper chromatography of amino acids. 500 μl of methanolic solution (corresponding to 5 mg of pigment) is evaporated to dryness in a Pyrex tube. 125 μl of 6 M HCl are added and the tube is sealed *in vacuo* under nitrogen as already described in a previous paper. Two-dimensional paper chromatography is performed according to Dent.[2]

Glucuronic acid determination. Glucuronic acid is determined on 50 μl of methanolic solution (corresponding to 0.5 mg of pigment). After evaporation to dryness in a 10 ml Pyrex tube, which can be fitted with a ground glass stopper, 1 ml of water and 6 ml of concentrated H_2SO_4 are added and the determination is performed according to Dische.[3] Calculations are made as described previously.

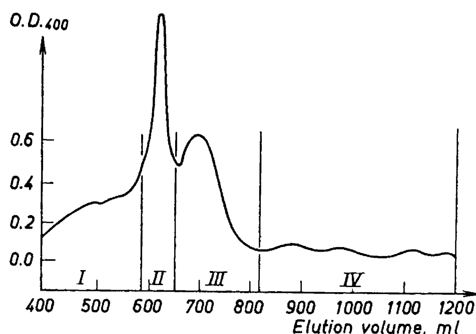

Fig. 1. Elution profile of LH-20 column. The data of the column and the procedure are as described in the experimental part.

RESULTS AND DISCUSSION

The typical profile from an LH-20-column, obtained as described, is shown in Fig. 1. The elution volumes are shown in Table 1, which summarizes the results from one fractionation. After rechromatography of the fractions

Table 1. Data obtained by examination of the fractions shown in Figs. 1 and 2. Figures (1) to (4) represent increasing intensity of the spots on the paper chromatogram.

	Fr. I	Fr. II	Fr. III	Fr. IV	Fr. II twice rechrom.	Fr. III twice rechrom.
Elution volume ml	400–570	580–630	640–790	800–1150		
Yield mg	25	40	125	100	30.5	14.5
Amino acids found in hydrolysate	Leu (2) Val (2) Ala (2) Gly (2) Glu (2) Asp (2) +traces	Leu (1) Val (1) Ala (1) Gly (2) Glu (2) Asp (2) +traces	Gly (2) Glu (2) Asp (2) +traces	Gly (4) Glu (3) Asp (2)	Gly (2) Glu (2) Asp (2) Leu (1) Ala (1) Val (1) +traces	Glu (3) Gly (2) Asp (2) Leu (1) Tyr (1) +traces
Glucuronic acid content % of dry wt.	4.2	6.4	11.6	15.5	8.2	11.8

II and III once and twice on a smaller column with the same system, the elution profiles shown in Fig. 2 are obtained. Judged by the shape of the profile the fractions are considered to be pure after the second rechromatography.

The results from determination of glucuronic acid and from amino acid chromatography of the hydrolyzed fractions show that the glucuronic acid

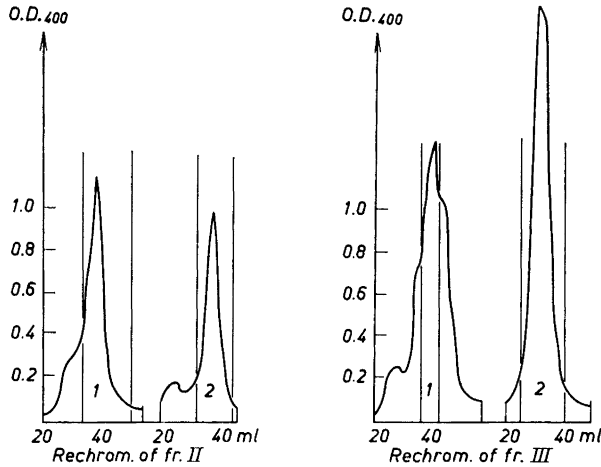

Fig. 2. Elution profile obtained by single and double rechromatography of fractions II and III from the column shown in Fig. 1. Only fractions between the vertical lines were used for the next chromatography or for further examination. The rest was discarded.

content increases with increasing fraction number and the number of amino acids which are found, decreases with increasing fraction number. On the other hand the amount of each of the three amino acids glycine, glutamic acid, and aspartic acid, which are found in all fractions, increases with increasing fraction number.

Table 2 shows the glucuronic acid content and nitrogen content from the rechromatographed fractions II and III from 4 fractionation experiments.

Much later than the fractions shown in the profile Fig. 1 and Table 1, two other fractions are eluted: a blue one with an elution volume of 1560—1640

Table 2. Glucuronic acid and nitrogen content in the twice rechromatographed fractions II and III from four consecutive fractionation experiments.

	II		III	
	Glucuronic acid, %	Nitrogen, %	Glucuronic acid, %	Nitrogen, %
1	8.6	5.9	6.2	4.0
2	8.2	5.5	11.8	4.7
3	7.4	6.1	7.6	3.2
4	4.1	5.7	7.2	2.4

ml and a red one with an elution volume of 1700—1850 ml. In the blue fraction glycine, glutamic acid, and aspartic acid are found, and in the red one rather small amounts of glycine and glutamic acid. In visible light they show absorption maxima of about 540 nm (red fraction) and 600 nm (blue fraction). In UV both show a maximum about 285—290 nm. The spectra are shown in Fig. 3. Both are rather unstable, becoming brown on standing. Evaporation to dryness also often results in transformation, partly or totally to brown

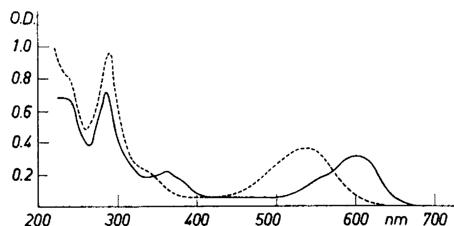

Fig. 3. Spectra of blue and red fraction (dotted line) from LH-20 column. The undiluted eluate is used directly for spectrometry. Path length 40 mm.

material. This invariably happens with the blue fraction, which also bleaches during a few days even if kept cold in the dark. Sample handling problems have prevented us till now from getting reliable IR-spectra of these — presumably very interesting — fractions.

The red fraction can be purified by thin layer chromatography on silica gel with light petroleum (b.p. 70—100°C):ether:acetic acid, 35:15:1, as the solvent but this has only been done on minute amounts that have not allowed physical or chemical investigations yet.

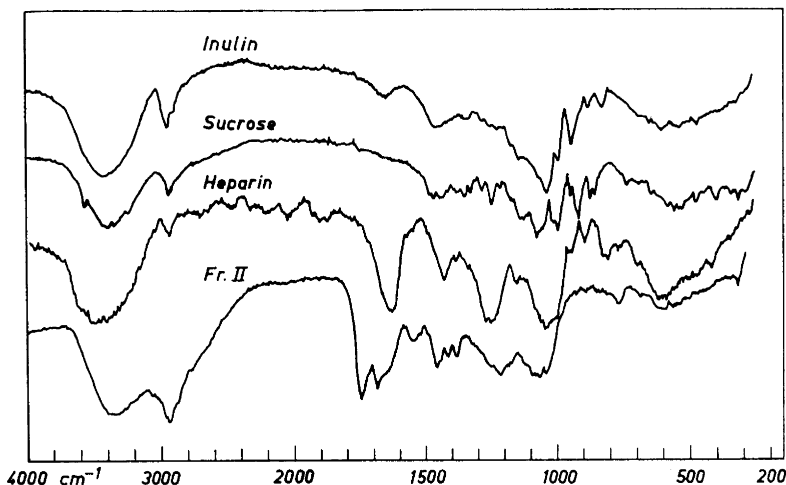

Fig. 4. Comparison of IR-spectra from twice rechromatographed fraction II with the spectra of heparin, inulin, and sucrose.

The most constant and reproducible of the fractions described is fraction No. II which always contains the 6 amino acids: glycine, glutamic acid, aspartic acid, leucine, valine, and alanine. Its glucuronic acid content after rechromatography shows a mean value of 7.1 % and the nitrogen content which is determined by the Natelson modification of the Kjeldahl procedure [4] shows a mean value of 5.8 %.

The infrared spectra suggest that a large part of the molecule is of carbohydrate nature. This view is supported by comparing the IR-spectrum with those of heparin, inulin (chosen as high molecular and rather complex substances), and sucrose. The spectra are shown in Fig. 4.

The ultraviolet spectrum of this fraction is not very informative; absorbance is gradually increasing towards shorter wavelengths and a rather weak triplet of peaks is seen in the range of 252—265 nm.

In fraction III the same triplet plays a dominating role in the spectrum (Fig. 5). It could be taken as evidence for aromatic groupings. This is supported by the IR-spectra. Fraction III is of less interest than fraction II as its composition is not so reproducible. Glucuronic acid content may vary from 6.2 to 11.8 % of dry weight from one preparation to another. The nitrogen content is about 4 % by weight. As mentioned above this fraction is somewhat difficult to prepare in a dry and convenient state as it tends to remain oily and sticky. IR-spectra taken by the KBr-technique also very often show a rather strong depression of the transmission at the higher frequencies. For further investigations we would therefore choose fraction II.

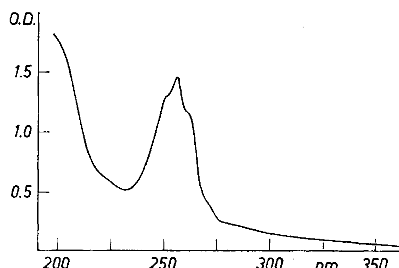

Fig. 5. Ultraviolet spectrum of twice rechromatographed fraction III.

It is a possibility that all the fractions are in reality artefacts formed from one and the same substance. It may be mentioned that after heating the crude pigment with dilute HCl in methanol, the red fraction is enhanced and a greater number of brown fractions are seen. Heating of the apparently homogeneous fraction II with dilute HCl in methanol and rechromatography on the same LH-20-column on which it was prepared shows two or more fractions, which are probably similar to the original fractions.

It should be noticed also that the gel filtration procedure described offers no possibility for determination of the molecular weights of the fractions as even the fastest moving fractions exhibit a K_D which exceeds 1. This must be due to strong absorption to the matrix. The same problem arises in dialysis experiments.

Acknowledgement. The work was subsided by grants from *Statens Almindelige Videnskabsfond.* Technical assistance by Mrs. Bente Grane Madsen is gratefully acknowledged.

REFERENCES

1. Hansen, S. E. *Acta Chem. Scand.* **23** (1969) 3461.
2. Dent, C. E. *Biochem. J.* **43** (1948) 169.
3. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
4. Natelson, S. *Microtechniques of clinical chemistry for the routine laboratory*, Thomas, New York 1957, p. 272.

Received March 24, 1969.