SEPARATION OF PORPHYRINS ON SEPHADEX DEXTRAN GELS

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Separation and characterization of the porphyrins by paper chromatography was first introduced by NICHOLAS AND RIMINGTON[1] and was followed by a systematic study of the behaviour of their methyl esters upon solid adsorption columns[2]. Several modifications of both techniques have since been described; see FALK[3,4]. A disadvantage of both methods is that they are applicable to only relatively small quantities and are thus not well suited for preparative purposes; for the column separation of unesterified porphyrins the only practicable method is that employing cellulose powder and the lutidine–water system as described by ERIKSEN[5].

During the course of a study, shortly to be published, of naturally occurring porphyrin–peptide complexes, the need arose for a method of separating and purifying them without esterification. Ion exchange columns had only limited applicability and we have therefore studied the behaviour of porphyrins upon Sephadex dextran gels. With many materials these gels function as molecular sieves, separation taking place according to molecular size, but in the case of aromatic and highly conjugated planar molecules adsorption phenomena play a prominent role[6]. This proved to be true of the porphyrins but by suitable choice of gel, buffer and particularly of buffer concentration, useful separation techniques have been evolved. With the aid of an automatic fraction collector, mixtures may be resolved on a preparative scale with the minimum of attention, once the sample has been placed on the column.

MATERIALS AND METHODS

Sephadex dextran gels were obtained from Pharmacia Ltd., London, W.13. Chromatographic columns were constructed from 8 mm internal diameter glass tubing. A plug of glass wool surmounted by some glass beads (height 2 cm) was introduced to support the gel which when packed formed a column 60 cm high. The void volumes of such columns, measured with Sephadex blue, were about 18 ml. Buffer was fed into the top of the tube from a reservoir placed on a shelf above it and through a hypodermic needle piercing a rubber stopper. Outflow from the bottom of the tube was controlled by a screw clip placed on polythene tubing of narrow bore. Operation was at room temperature. The fraction collector was a Shandon model CA-100 holding 200 tubes. In most experiments it was set to collect 2 ml samples gravimetrically. The receivers were 10 cm × 1 cm test tubes. Uroporphyrin I, coproporphyrin III, protoporphyrin IX, deuteroporphyrin IX and haematoporphyrin IX dihydrochloride were carefully purified materials from the laboratory stock; porphyrin c was a gift from

Dr. S. SANO, Kyoto. For application to the column porphyrins were dissolved in a minimal quantity (0.5–1 ml) of 0.2 *M* sodium borate buffer pH 8.6. To each 2 ml fraction was added 2 ml of 3 *N* HCl and the mixture further diluted, if necessary, by 1.5 *N* HCl and optical absorption at the Soret peak was measured on a Unicam SP 500 spectrophotometer using cells of 1 cm light path.

EXPERIMENTAL AND RESULTS

Since the molecular weights of the porphyrins range from about 500 to 800, it seemed possible that they might be separable on a Sephadex G-10 column. It was immediately evident, however, that factors other than molecular size played a dominant role. Even G-25 and G-100 gels adsorbed the porhyrins strongly in dilute acid or alkaline media or in barbital and phosphate buffers. On the assumption that there might be hydrogen bonding between the porphyrin carboxyl groups and the hydroxyls of the dextran, a change was made to borate buffers. Marked improvement followed and after some experimentation a sodium borate buffer of pH 8.6 containing 0.5 m*M* EDTA and 2–3 drops/l of phenol was selected as most suitable and a column of Sephadex G-25.

Effect of buffer strength

Initially, a buffer strength of 0.2 *M* was used but although uroporphyrin was rapidly eluted from the column, porphyrins with fewer carboxyl groups were more firmly retained and tended to spread; deuteroporphyrin was only eluted very slowly and occupied 100 tubes or more (Fig. 1). Porphyrin c (4–COOH groups) could be separated from coproporphyrin on this system but its rate of elution was also very slow. Decreasing the concentration of borate to 0.001 *M* accelerated elution to such an extent that coproporphyrin and deuteroporphyrin came off together in a single peak closely following uroporphyrin. The problem was therefore to find a satisfactory intermediate concentration. With 0.002 *M* buffer, coproporphyrin and deuteroporphyrin were separable with a slight overlap (Fig. 2) and separation was still better when using 0.005 *M* but the optimal concentration proved to be 0.01 *M*. This separated uroporphyrin, coproporphyrin and deuteroporphyrin very satisfactorily although the deuteroporphyrin band was sometimes slightly irregular (Fig. 3). The identity of the fractions was proved by measurement of the Soret maxima and by lutidine paper chromatography[7]. Recoveries were complete.

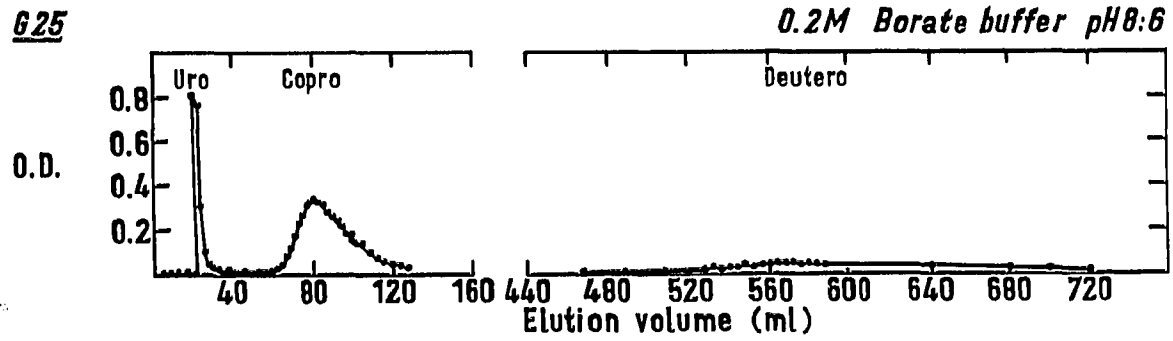
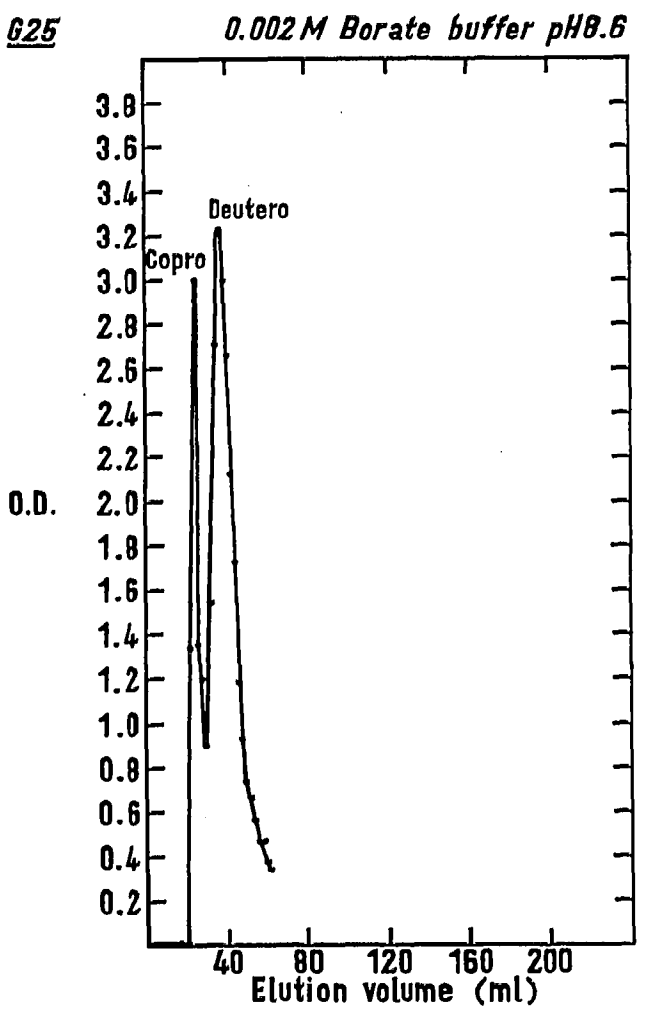


Fig. 1. Elution of uroporphyrin, coproporphyrin and deuteroporphyrin from Sephadex G-25 by 0.2 *M* borate buffer, pH 8.6.



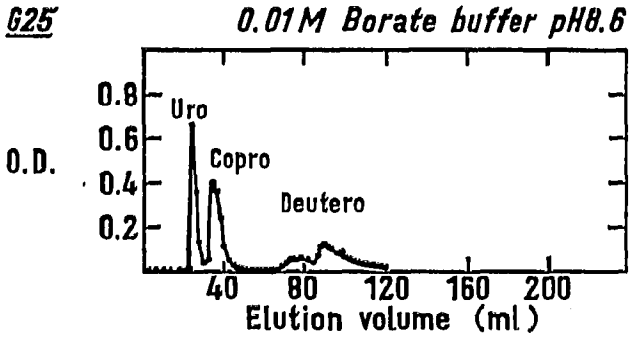


Fig. 3. Elution of uroporphyrin, coproporphyrin and deuteroporphyrin from Sephadex G-25 by 0.01 *M* borate buffer, pH 8.6.

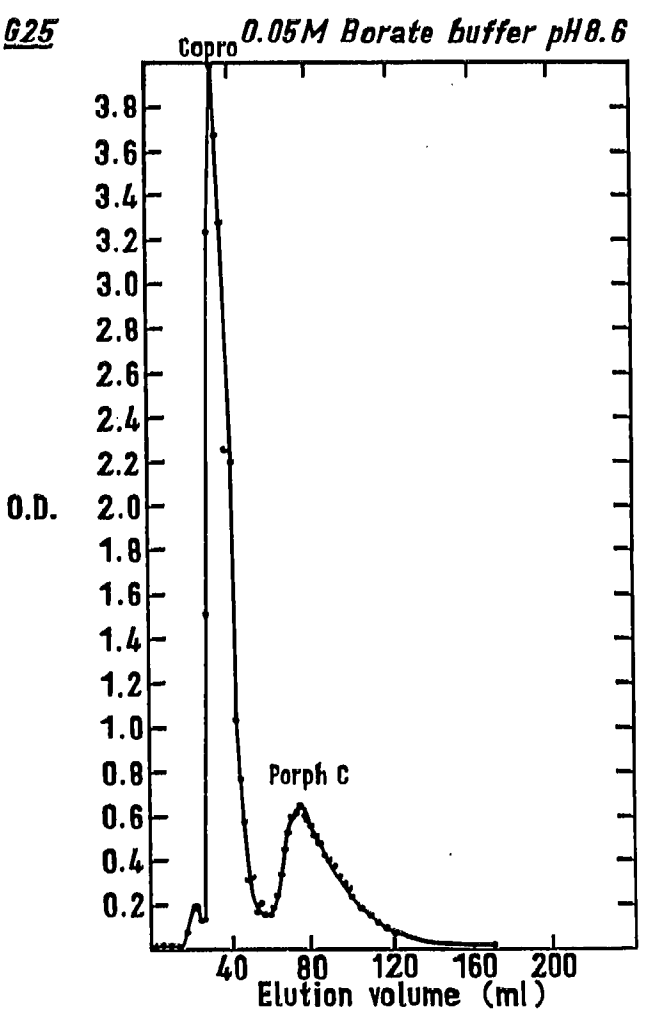
Fig. 2. Elution of coproporphyrin and deuteroporphyrin from Sephadex G-25 by 0.002 *M* borate buffer, pH 8.6.

Separation of coproporphyrin and porphyrin c

Attention was next turned to the separation of porphyrin c (2,4-dicysteinyldeuteroporphyrin IX) from coproporphyrin, both of which possess 4–COOH groups. With 0.2 *M* buffer, retention of porphyrin c was nearly as strong as that of the dicarboxylic porphyrins and its band spread out considerably. It would appear that the amino acid carboxyl groups are not comparable, in the effect they exert, to the carboxylic side chains of the porphyrin ring system. 0.01 *M* buffer gave fairly good resolution but the most satisfactory separation of coproporphyrin and porphyrin c was achieved with 0.05 *M* buffer concentration. A small impurity in the porphyrin c even came off immediately in front of the coproporphyrin peak (Fig. 4).

Separation of porphyrin c and haematoporphyrin IX

Particular attention has been paid to this separation since during the working up of natural materials structurally resembling porphyrin c, some fission of the thioether linkages is apt to occur giving rise to haematoporphyrin. Although this porphyrin is ether-soluble, in contradistinction to the c type porphyrin, it is also hydrophilic and a chromatographic separation would be very useful. The best result was again obtained by using a 0.05 *M* buffer, very sharp peaks of the two porphyrins



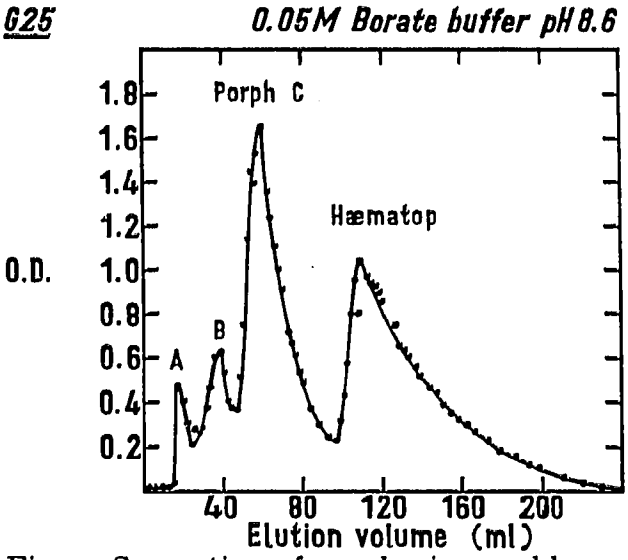


Fig. 5. Separation of porphyrin c and haematoporphyrin by elution from Sephadex G-25 with 0.05 *M* borate buffer pH 8.6. A and B are impurities in the materials.

Fig. 4. Separation of coproporphyrin and porphyrin c by elution from Sephadex G-25 with 0.05 *M* borate buffer, pH 8.6.

being obtained. As expected, porphyrin c with its 4 COOH groups preceded the dicarboxylic porphyrin (Fig. 5); minor impurities (A and B) were once more detectable.

Separation of dicarboxylic porphyrins

Although a sharp separation of the dicarboxylic porphyrins, deuteroporphyrin and haematoporphyrin, seemed unlikely in view of their closely related chemical structure and their behaviour in lutidine paper chromatography[7], the hydroxylic functions of haematoporphyrin should nevertheless influence to some extent its behaviour on dextran gels. Protoporphyrin which possesses two vinyl side chains should be considerably more firmly retained than the other two porphyrins. These expectations were realized in practice.

A mixture of deuteroporphyrin, haematoporphyrin and freshly prepared protoporphyrin were placed on a G-25 column in 0.05 *M* sodium borate buffer pH 8.6 and developed with the latter. A small red fluorescent impurity left the column rapidly (this is usually seen with protoporphyrin preparations) and was followed by a large unsymmetrical band appearing between 116 ml and 360 ml of eluate. After 400 ml of eluate had passed, the fraction collector was stopped and all porphyrin remaining on

the column allowed to collect in a single overnight fraction. Spectrophotometric analysis and paper chromatography of the esterified porphyrins by the method of CHU, GREEN AND CHU[8] showed that haematoporphyrin was eluted first but that there was some overlapping with deuteroporphyrin. The large band from 116–360 ml did, in fact, exhibit two peaks with different Soret band absorption. The final overnight fraction consisted of dicarboxylic porphyrin with Soret maximum in 5 % HCl of 407–8 mμ and was therefore protoporphyrin. There is no doubt that repeated filtration on Sephadex of the first, composite band, would effectively separate the haematoporphyrin and deuteroporphyrin which it contained.

DISCUSSION

Separation techniques employing Sephadex dextran gels and an automatic fraction collecting device have the advantages of requiring minimum attention, having high flow rates, and of being easily reproducible. They may be used on a preparative scale, the quantity of material handled being virtually limited only by the size of the column and the volume of solvent necessary to dissolve the applied mixture. Recoveries of material, moreover, are complete or very nearly so. In the case of proteins, separation generally occurs according to molecule size, the process being one of molecular sieving, but with aromatic substances and highly conjugated planar molecules, adsorption effects become increasingly evident and may predominate. The effects of pH and of buffer concentration then become important[9].

In the present study, physical interaction between porphyrins and the dextran gel were found to be very marked. Only by the use of a borate buffer, pH 8.6, containing some EDTA to remove traces of metals, and careful selection of the appropriate buffer strength could good resolution be achieved without serious tailing. Recoveries were then quantitative. Sephadex G-25 proved to be the most satisfactory gel and, in general, rate of elution increased with increasing number of carboxyl groups in the molecule. Thus uroporphyrin suffers little or no retention whether the buffer concentration be 0.2 *M* or 0.001 *M*. In the weaker buffer, coproporphyrin and deuteroporphyrin also move fairly rapidly and are eluted together but as the buffer concentration is increased their retention on the column is also increased and their bands are broadened. The interval between them becomes greater. For general purposes a 0.01 *M* borate buffer was found optimal in that it gave clear separation in well defined bands.

The behaviour of porphyrin c is particularly interesting. It has 4 carboxyl groups, 2 being present in the cysteinyl groups and 2 in the propionic acid side chains at positions 6 and 7 of the porphyrin ring. On lutidine paper chromatography, porphyrin c runs level with the tetracarboxylic coproporphyrin. On Sephadex G-25, however, it is somewhat more firmly retained than coproporphyrin but less firmly than dicarboxylic porphyrins. It would appear, therefore, that the carboxylic functions of the cysteinyl groups exert a weakened de-adsorptive effect due, no doubt, to the adjacent α-amino groups. For good separation from coproporphyrin, a 0.05 *M* was found preferable to a 0.01 *M* buffer. The fact that haematoporphyrin and porphyrin c may be readily separated under these same conditions is exceedingly important since the thioether linkage of this latter porphyrin easily suffers fission during chemical manipulations with resulting formation of some haematoporphyrin.

The methods described in this paper have already been applied successfully to the separation of complex mixtures of porphyrins derived from natural sources. Obviously, however, different problems may demand differing treatment and it is one of the virtues of the Sephadex gel system, as revealed by this study, that one can vary the behaviour of porphyrins in it in a predictable manner. It may be useful, at times, to carry out a preliminary separation at one buffer concentration and then to re-run some fractions in a weaker or stronger buffer.

The use of Sephadex LH 20 for the separation of porphyrin esters or of the free porphyrins was examined with various solvent systems but it was found to be distinctly inferior to the G-25 gel.

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SUMMARY

Porphyrins can be separated on columns of Sephadex G-25 gel by sodium borate buffer, pH 8.6. Alteration in buffer molarity influences separation in a predictable manner; for general purposes 0.01 *M* is optimal but for special separations appropriate concentrations should be used. Recovery is quantitative and the technique is suitable for preparative purposes.

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