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A NEW METHOD OF ISOLATION OF NATURAL FLAVINS USING PHENOL-TYPE RESINS

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SUMMARY

It was shown that riboflavin (as lumiflavin and lumichrome) is sorbed by all resins with free phenolic groups, *e.g.* phenolsulphonic and phenolcarboxylic ion exchangers or a simple resin of resorcinol and formaldehyde. FMN and FAD, in aqueous solutions, passed through these columns without sorption, but can be retained in salt solutions, especially by a resorcinol resin. Flavins can be eluted from phenol resins by a mixture of water and acetone, propanol or dioxane and saturated aqueous solutions of urea with ethanol or cellosolve. The ability of phenol resins to form complexes with flavins can be used both for analytical and preparative purposes.

The ability of phenol and its derivatives to form complexes with flavins has been demonstrated[1–4] and recently accounted for as charge-transfer complexes between electron deficient flavins and electron rich phenols. We have shown that such or similar complexes are also formed in case of flavins and resins with free phenolic groups (containing phenol or its derivatives). This fact was found to be very useful for the isolation of flavins from highly contaminated and strongly diluted extracts, both for analytical and preparative purposes.

EXPERIMENTAL

Materials and apparatus

Riboflavin (Merck), chromatographically pure.

Riboflavin 5′-phosphate (Sigma), purified on phenolsulphonic resin K-28 before use (riboflavin is sorbed by the resin, FMN* passed through the column with water).

Flavin-adenine dinucleotide (Fluka) on chromatograms shows two additional greenish-yellow fluorescent spots of FMN and probably YAGI's "fourth flavin compound"[5]; used without further purification.

Lumiflavin and lumichrome, prepared from riboflavin by prolonged photolysis of riboflavin solutions in 0.1 *N* NaOH, extracted with chloroform and dried in vacuum. Both were separated on columns of Whatman cellulose powder: lumiflavin

* Abbreviations used: Rfl = riboflavin; FMN = riboflavin 5′-phosphate; FAD = flavin-adenine dinucleotide; Lfl = lumiflavin; Lch = lumichrome.

by elution with water saturated with isoamyl alcohol and lumichrome by means of methanol.

Ion exchangers. K-28*, phenolsulphonic acid type and K-16*, phenolcarboxylic acid type. Analogous results were obtained when commercially available phenolsulphonic exchangers such as ZeoKarb 215, Wofatit F, P and KS, Lewatit PN and KSN, Staionit FN and F-extra, resin MSF or phenolcarboxylic resins such as Lewatit CNS and ZeoKarb 216 were tested.

Resin R-15, resorcinol type, was prepared in the following way: 15 g of sodium hydroxide and 33 g of resorcinol were dissolved in 60 ml of water and placed in an ice bath. To the resorcinol solution 45 ml of 40 % aqueous formaldehyde was added (in small portions to prevent the temperature rising above 50°). A further 30 ml of water was next added to the reaction mixture which was then placed on a water bath at 50°. After 25–30 min the mixture begins to form long threads when a glass rod is drawn through it (the beginning of gelatinisation) and from this time must be kept still at 50° for 15 min. This resin was then pressed through a sieve (1 mm mesh diameter) under a stream of tap water. After an hour the resin was pressed through another sieve (0.5 mm mesh diameter) and then washed successively with water, 3 *N* HCl, water, 0.5 *N* NaOH, water, 3 *N* HCl, water, acetone–water mixture (1:1, v/v), 0.5 *N* NaOH, water, 3 *N* HCl and water. Washing with the acetone–water mixture and NaOH solutions must be carried out till the effluents became non-fluorescent and non-coloured respectively.

The fluorimeter used was constructed in the laboratory and has great sensitivity and selectivity[6].

Procedure

Aqueous solutions containing 1–2 μg of flavins (as riboflavin) in one milliliter were passed through columns of resin (resin bed 1.0–1.2 cm in diameter and 3.5–7 cm in height; flow rate 1 ml/2–3 min); 2.5 or 5 ml fractions were collected.

The effectiveness of sorption was estimated on the basis of the resistance of the flavins to elution with pure water or aqueous buffer solutions. On the other hand, desorption was estimated as depending on the quantities of flavins eluted and the volumes of solutions used for this purpose.

The capacity of the resins in relation to riboflavin was established by passing solutions containing 15 μg of riboflavin/1 ml till the break-through point was reached.

Quantitative determinations were carried out based on the intensity of the yellow-green fluorescence of eluates. FAD was estimated according to CERLETTI's method[7] which depends on the increase of fluorescence after hydrolysis of FAD to FMN or Rfl. Qualitative analysis of flavins was accomplished by paper chromatography (paper Whatman No. 1, developing mixture: butanol–glacial acetic acid–water, 4:1:5; descending technique). Flavins were identified by the colour of fluorescence of the spots and their R_F values (Rfl = 0.30; FMN = 0.09; FAD = 0.03; Lfl = 0.42; Lch = 0.70).

As the flavins are highly photolabile, all operations have been carried out in a dark laboratory.

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* Ion exchangers K-28 and K-16 were prepared and kindly donated by Doc. Dr. H. WITKOWSKI of the Chair of General Chemistry of University A. M. in Poznań.

RESULTS AND DISCUSSION

Sorption of riboflavin, lumiflavin and lumichrome

All types of phenolic resins secured full, quantitative sorption of Rfl, Lfl and Lch from aqueous solutions, dilute acids (up to 0.5 *N*) and salt solutions (up to 2 *N*) over a pH range 1–8.

The capacity of resins as determined by the dynamic method (break-through point) was found to be practically unaffected by the resin form (H^+, Li^+, Na^+, K^+, NH_4^+) and closely related to the number of free phenolic —OH groups and was also probably affected by the presence of other functional groups (—COOH, —SO_3H) (see Table I and Fig. 1).

TABLE I

THE BREAK-THROUGH CAPACITY OF PHENOL TYPE RESINS* IN RELATION TO RIBOFLAVIN AND Na^+ (AQUEOUS SOLUTIONS)

Type of resin	*Rfl*	*Na^+*
K-28: phenolsulphonic acid type	3×10^{-3}	2.1
K-16: phenolcarboxylic acid type	8×10^{-3}	1.0
R-15: resorcinol type	23×10^{-3}	0.27

* In mequiv. per g of air-dried resin.

Riboflavin, as well as lumiflavin and lumichrome, adsorbed on phenolic resins could not be eluted with acids (up to 2.5 *N*) or neutral salt solutions (up to 3 *N*); higher concentrations gave traces of riboflavin in the effluents. Better elution was obtained with solutions of alkaline character such as NaOH, KOH, NH_4OH, Na_2CO_3, $Na_2B_4O_7$, but here again the riboflavin content in the effluents was low and the elution was not quantitative[8]. Because of serious lability of flavins in alkaline media (especially photolability) such solutions could not be used for elution purposes. The most effective eluants appeared to be aqueous solutions of certain organic compounds[8,9].

From a series of mixtures that were examined the best results were obtained when mixtures of water with acetone, propanol or *p*-dioxane and saturated aqueous solutions of urea with ethanol or cellosolve were used (see Fig. 2).

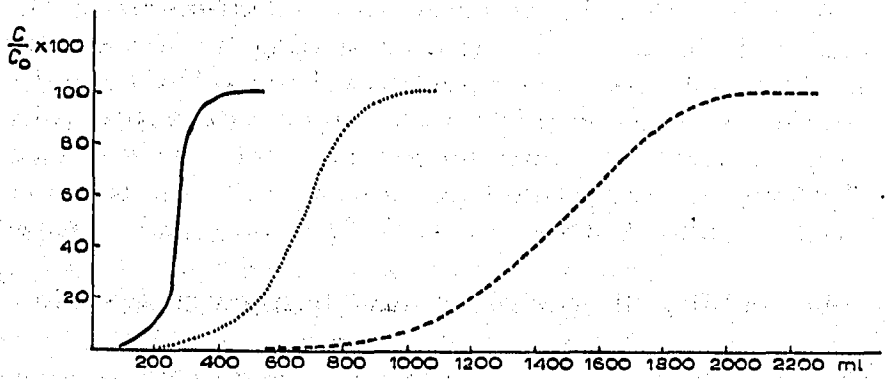

Fig. 1. The break-through curves (isoplanes) for aqueous solutions of riboflavin on phenol-formaldehyde resins: K-28, phenolsulphonic acid type (———); K-16, phenolcarboxylic acid type (···); R-15, resorcinol type (– – –).

Sorption of FMN and FAD

Flavin mononucleotide and flavin-adenine dinucleotide from aqueous solutions are only partially bound by resorcinol type resins and are not sorbed on sulphonic and carboxylic resins. Both nucleotides undergo sorption under conditions exerting an inhibitory effect on their phosphate groups and probably other fragments of the molecules. In the case of resins of the phenolsulphonic acid type sorption of FMN and FAD takes place from saturated solutions of salts (for instance KCl, KH_2PO_4) as well as from solutions of pH below 1. In the case of resins of the phenolcarboxylic acid type lower concentrations of salts and acids are necessary, in both cases however,

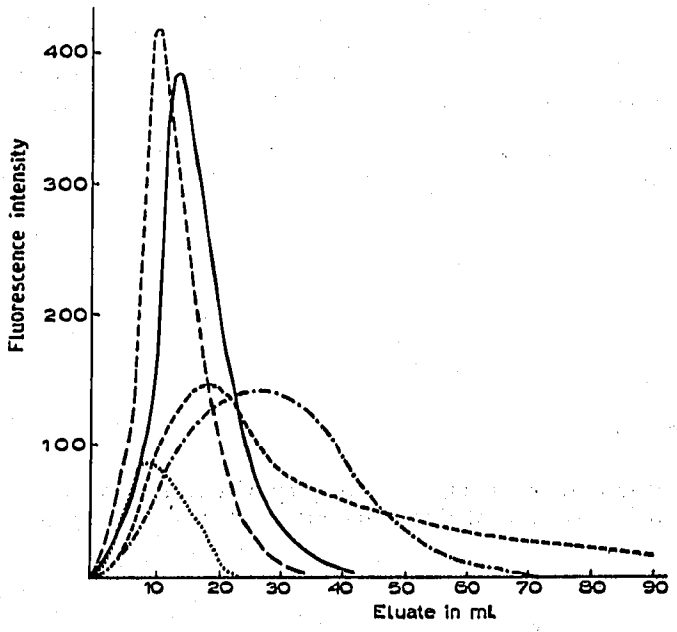

Fig. 2. Elution curves of riboflavin from K-28 resin for different solvents: acetone–water, *p*-dioxane–water, propanol–water (1:1) (– – –); cellosolve–water, ethanol–saturated aqueous solution of urea (1:1) (————); ethanol–water (1:1) (—·—·—·—); absolute ethanol (- - -); acetone (···).

significant hydrolysis of nucleotides occurs. Using resorcinol resins traces of FMN and FAD are retained from aqueous solutions; complete sorption is effected from 0.1–0.05 *N* solutions of well dissociated salts. But it was found that many salts, especially salts of K^+, Na^+, Cl^-, PO_4^{3-}, can cause in the course of sorption and/or elution slight destruction of the FMN to riboflavin. This was not observed when, for instance, $(NH_4)_2SO_4$ was used. Both nucleotides can be eluted from resorcinol resin by a 10% aqueous solution of acetone.

Separation of riboflavin from FMN and FAD

The separation of riboflavin from flavin nucleotides was successful with all types of phenolic resins using two-step elution. From phenolsulphonic and phenolcarboxylic type resins FMN and FAD can be eluted with water and pure riboflavin in the next step when an acetone–water (1:1, v/v) mixture is poured through the column. In the case of a resorcinol type resin the nucleotides can be eluted with a 5–10% aqueous solution of acetone and riboflavin with a 50% acetone–water mixture.

Attempts were undertaken to separate all flavins sorbed on the resins. On phenolsulphonic and phenolcarboxylic acid type resins we could not separate FMN

from FAD. Using a gradient elution technique in the system: 0.1 *N* KCl–water–acetone, only partial separation of FMN from FAD was obtained; this is due to the tendency of FAD to remain on the resin as long as FMN (see Fig. 3).

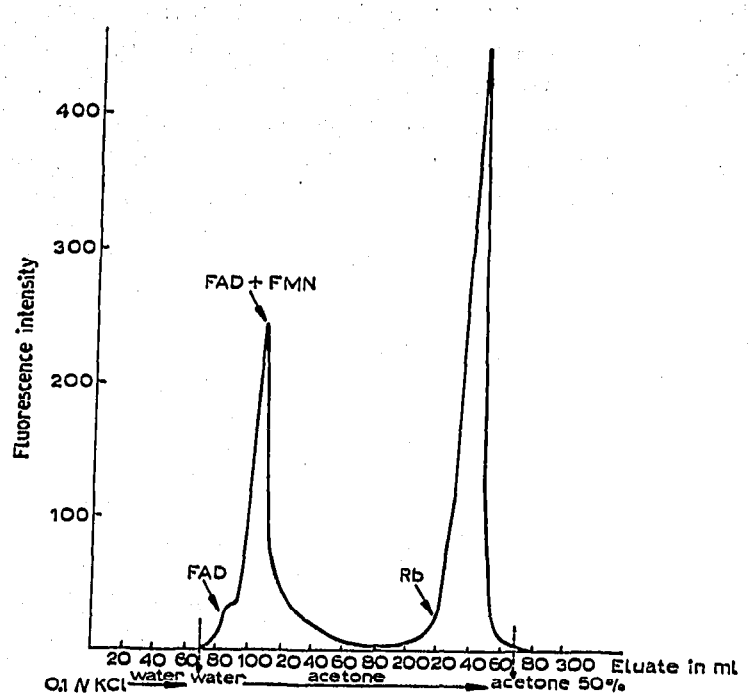

Fig. 3. Gradient elution on the R-15 resin, on which were sorbed equal quantities (25 μg) of riboflavin, flavin mononucleotide and flavin-adenine dinucleotide. Solvent system: aqueous 0.1 *N* KCl, water, acetone.

Use of phenol resins for estimation of flavins

The use of phenol resins allows the:

isolation of riboflavin from highly contaminated and strongly diluted extracts,

concentration of riboflavin solutions,

determination of individual forms of flavins occurring in natural products.

For the determination of flavins phenolsulphonic[9,10] as well as resorcinol type resins can be used.

For the determination of total flavins the crude extract obtained from samples of the analysed product (hydrolysis in 0.1 *N* H_2SO_4, neutralization by NH_4OH, enzymatic hydrolysis) was passed through the column of the resorcinol resin. The column was washed with 0.1 *N* $(NH_4)_2SO_4$ to remove the non-flavin compounds and then riboflavin and FMN were eluted with the 50 % acetone–water mixture (or the urea–water–ethanol mixture). Using the acetone–water mixture the fluorescence of the eluate was measured after acetone was removed under reduced pressure (acetone strongly enhances the fluorescence intensity of flavins[11]).

When Rfl, FMN and FAD were to be estimated, the crude extracts (obtained in 0.1 *N* $(NH_4)_2SO_4$ at 80°) were passed through the resorcinol resin. FMN and FAD were eluted with the 10 % acetone–water mixture and determined according to the method of CERLETTI[7]. Riboflavin was eluted from the column using the 50 % acetone–water mixture.

The efficacy of removal of the quenching substances and fluorescent non-flavin

compounds from extracts is of a high degree. The process of determination has been reduced, in this method, to few simple operations; the results obtained are accurate and of good reproducibility[10].

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