# SEPARATION AND IDENTIFICATION OF PTERIDINES BY PAPER CHROMATOGRAPHY*

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During our attempts to elucidate the pathways of enzymic oxidation of pteridines, it became necessary to separate mixtures that contain isomers as well as pteridines in different stages of hydroxylation[1]. Paper-chromatographic analysis has been successfully applied to pterines, extracted from biological sources[2–6]. Systematic investigations regarding the suitability of various solvent mixtures for the separation of pterines have been carried out by TSCHESCHE AND KORTE[7,8]. Since all naturally occurring pterines have an amino group at position 2, the solvents selected by previous investigators were of special value for the identification of *basic* pteridines. ALBERT *et al.*[9–12] used $R_F$ values extensively for the characterisation of their synthetic pteridines, including hydroxy derivatives devoid of amino groups. Some of the figures obtained by these authors are included in Table II. However, the methods adopted were not chosen for the purpose of analysing mixtures of closely related compounds, such as occur in the course of enzymic oxidation. Especially when there are no side-chains attached to the heterocyclic nucleus and the only difference consists in the number or position of hydroxyl groups, the problem of quantitative separation becomes much more intricate.

In the present study, we have tried to develop new methods for the chromatographic separation and analysis of pteridines, with two main objectives in mind: (a) The procedure should concentrate the compounds tested in well-defined spots. This point is of great importance, in view of the fact that in many solvents certain pteridines tend to streak, while other derivatives may give more than a single spot. The former phenomenon, the tendency to trail, may be ascribed to the presence at each point of equilibrated mixtures of molecular and ionised forms[13]. The latter observation, *viz.* the appearance of more than one circumscribed spot, may be explained by the formation of hydrates (by addition of water to a reactive double bond) that are in equilibrium with the non-hydrated structure. Naturally, if the two members of such a pair are rapidly interconvertible, it would not be easy to understand how they could separate during chromatography. Therefore one is led to assume that during chromatography, conditions are unfavourable for equilibration so that the

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mixture of hydrated and non-hydrated molecules may behave as though two independent entities were present. Finally, there are indications for keto–enol tautomerism in the pteridine series (SCHOU[14]). If this should prove to be the case, it could lead to the appearance of 2 spots, if the tautomers do not equilibrate easily during development of the chromatogram. (b) The method should be of wide applicability, *i.e.* it should be useful for the separation of a large number of pteridines when present together.

## MATERIALS AND METHODS

### *Substances*

The pteridines used in the present investigation were synthesised by ALBERT *et al.*[9–12] and were obtained through their courtesy. The purity of these compounds was checked by ultraviolet spectrophotometry and by paper chromatography. Only for 2,6,7-trihydroxypteridine has the absorption spectrum not been reported previously. An aqueous solution of this derivative apparently consists of a mixture of two different structures[12]; during chromatography it separated into two well-defined spots. However, the synthetic material is undoubtedly pure, because it proved identical in every respect with the substance resulting from enzymic oxidation of 6,7-dihydroxypteridine[1]. At pH 8, 2,6,7-trihydroxypteridine from either source exhibited maxima at 353 and 232 m$\mu$. Although this spectrum is probably not characteristic for a single structure, it is very useful for identification purposes*.

### *Determination of dissociation constants*

The p$K$ values of the compounds used were determined spectrophotometrically, by observing the changes of $\lambda_{max}$ as a function of pH[15]. The results are unequivocal in all cases in which a steady function is obtained. However, with some 6-substituted pteridines, notably 6-Pt, 2,6- and 6,7-Pt, the number and the position of the absorption maxima change suddenly in a certain pH range, so that an exact evaluation of the p$K$ values by this procedure proved impossible. In these cases, the figures of ALBERT *et al.*, who applied potentiometric methods, were used. However, it should be recalled that the p$K$ values of most derivatives, containing a 6-hydroxyl group, are ambiguous; they depend on the direction of the titration. Inspection of Table I reveals certain discrepancies between our own measurements and those of the Australian investigators. These differences can be ascribed to the different procedures used. In the spectrophotometric method, the substance is dissolved in a given buffer, where it may equilibrate between all possible structures, and then its $\lambda_{max}$ is measured. In the potentiometric procedure, the pH of a given solution is changed continuously and there may not always be sufficient time for equilibration to take place. Table I demonstrates that most pteridines are very weak bases; they are converted into

* For the sake of brevity, the pteridines will be designated as follows: 4-hydroxypteridine = 4-Pt; 2,4-dihydroxypteridine = 2,4-Pt; 4,6,7-trihydroxypteridine = 4,6,7-Pt, etc. All monohydroxy derivatives are classified as group I, all dihydroxy derivatives as group II and all trihydroxypteridines as group III.

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## TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF PTERIDINES

For examination of fluorescence or of staining properties, 10–20 $\gamma$ of a pteridine were chromatographed with solvent 6, with the exception of pteridine and 2-Pt. For these two substances solvent 2 was used instead (see Table II). After developing for 12 h, the paper was air-dried and then sprayed with a 0.1% solution of copper acetate in 95% ethanol. After drying, a second spray was applied with 0.5% diphenylcarbazide in 95% ethanol. When solvent 2 was used for development, the paper became impregnated with ammonium chloride and therefore stained red-brown with the above reagents. The spots of pteridine and 2-Pt, which were first detected by observation of their fluorescence, could not be recognised against the background, even when 50 $\gamma$ of material were used.

| *Substance* | *pK* | | | | | | *Fluorescence** | *Color of complex with Cu++ and diphenylcarbazide* |
|---|---|---|---|---|---|---|---|---|
| | ALBERT *et al.*[9–12] | | | *Our determinations* | | | | |
| | *Cation* | *Mono-anion* | *Bis-anion* | *Cation* | *Mono-anion* | *Bis-anion* | | |
| Pteridine | 4.1 | 12.2 | | 3.5 | 12.5** | | Violet | ? |
| *Group I* | | | | | | | | |
| 2-Pt | < 2.0 | 11.3 | | ~ — 2.0 | > 12.0 | | Greenish | ? |
| 4-Pt | < 1.5 | 7.9 | | ~ 1.0 | 7.9 | | Blue | Orange |
| 6-Pt | 3.7 | 6.7 | | | | | Black-violet | (weakly orange) |
| 7-Pt | — 2.0<br>+ 1.2 | 6.4 | | 0.5 | 5.9 | | Blue-violet | Orange |
| *Group II* | | | | | | | | |
| 2,4-Pt | < 1.0 | 7.9 | | ~ — 2.0 | 7.2 | 12.5 | Greenish | Yellow-orange |
| 2,7-Pt | | 5.8 | 10.1 | | 5.3 | (11.5?) | Sky blue | Red-orange |
| 4,7-Pt | | 6.1 | 9.6 | ~ — 2.0 | ~ 5.5 | > 9 | Blue-Violet | Red-orange |
| 2,6-Pt | | 6.7 | 11.6 | 2.0 | 9.5*** | | Green | None |
| 4,6-Pt | | 6.1 | 9.7 | 0.7 | 6.6 | 9.7 | Blue | (Very faint) |
| 6,7-Pt | < 2.7 | 6.9 | 10.0 | | | | Violet | None |
| *Group III* | | | | | | | | |
| 2,4,7-Pt | | 3.6 | | | (3.0?) | 9.8 | Blue-violet | Dark red-violet |
| 2,4,6-Pt | | 5.7 | 9.4 | — 2.0 | 5.2 | 9.6 | Blue-green**** | (Very faint) |
| 4,6,7-Pt | | | | — 2.0 | 6.7 | 9.5 | Blue | (Very faint) |
| 2,6,7-Pt | | 3.5 | 6.7 | 0.5 | 6.6 | 9.1 | Upper spot: black-violet | None |
| | | | | | | | Lower spot: sky blue**** | None |
| 2,4,6,7-Pt | | | | | ~ 3.5 | ~ 9.5 | Blue | None |

* It should be noted that the color of the spots under ultraviolet irradiation depends not only on the concentration of the substances, but also on the pH of the solvent used for chromatography. The colors reported are observed when the solvent contained ammonia.

** The absorption maximum of pteridine remains constant at 298 m$\mu$ between pH 4 and 12. Above pH 12, it jumps to 320 m$\mu$, indicating ionization due to hydration. This change has been reported previously by LISTER, RAMAGE AND COATES[20] and ALBERT, BROWN AND WOOD[21].

*** The p$K$ of 9.5 for 2,6-Pt is a pseudo constant, characteristic for the equilibrium mixture of 2,6-Pt and its hydrate. The true ionization constants of ALBERT *et al.* belong to a single species only, because they are determined before equilibration can take place.

cations at high acid concentrations. The hydroxy derivatives form anions, the p$K$ decreasing in general with the number of hydroxyl groups present. However, a limit is set by formation of bis-anions— an observation analogous to previous experience in the purine series[15].

*Detection of spots*

The ability of pteridines to form metal chelates has been studied by ALBERT[16]. Taking advantage of this property, we have tried to stain the chromatograms by spraying the paper first with a solution of a metal acetate and then with a staining agent, specific for the cation used. Among the more stable metal complexes, the $Zn^{++}$ and the $Cu^{++}$ chelates were the most promising. The former, after spraying with an alcoholic solution of diphenylcarbazone, gave intensely red spots. However, since the background was also stained red, only quantities above 50 $\gamma$ could be recognised unequivocally. Copper acetate and diphenylcarbazide, on the other hand, produced pink to red spots on a bluish-gray background; spots representing 10 $\gamma$ could be recognised at once and became still more distinct after 24 hours when the background had faded. This proves that the complex pteridine–$Cu^{++}$–diphenylcarbazide is much more stable than the complex formed in the absence of pteridines. The results of the staining experiments with copper ion are also included in Table I. This detection method is, however, of limited value, since only certain derivatives give a positive reaction. Pteridines containing a 6-hydroxyl group produce only a weak color or may not stain at all. Pteridine and 2-Pt could not be tested, since they concentrate in well-defined spots only in solvents containing ammonium chloride. Chloride ions were, however, found to destroy these complexes. In contrast to the staining procedure, fluorescence in ultraviolet light is of general applicability and was therefore used in all experiments. For this purpose a Mineralight ultraviolet lamp, which emits radiation of about 255 m$\mu$ was used.

*Chromatography*

All experiments were carried out by the descending method, using Whatman paper No. 1. The paper was cleaned by immersion in 0.05 $M$ borax (pH about 9) for one hour and then in 10% acetic acid for the same period. Development extended usually over 12 hours, with the solvent front 35–40 cm from the starting line. In those cases, where the low rate of migration required developing periods of 100 hours (group III in solvent 6 or 8, see Table II) so that the solvent was dripping off the edge of the paper, $R_F$ values were determined by simultaneously running 2,4,7-Pt as reference substance.

## RESULTS

*Separation of pteridines with various solvent mixtures*

The first solvent tested was 3% ammonium chloride (= solvent 1), which has been introduced for pterine studies by TSCHESCHE AND KORTE[7]. As shown in Table II,

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TABLE II

$R_F$ VALUES OF PTERIDINES IN VARIOUS SOLVENTS

| Substance | Solvent No. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Pteridine | streaks | 0.87 | diffuse | (0.75) diffuse | diffuse | (0.44) | 0.84 | diffuse | 0.25 |
| *Group I* | | | | | | | | | |
| 2-Pt | 0.37 | 0.89 | 0.66 | (0.83) diffuse | diffuse | 0.47 | 0.84 | 0.23 | 0.8 |
| 4-Pt | 0.6 | 0.88 | 0.44 | 0.67 | 0.48 | 0.5 | 0.87 | 0.46 | 0.5 |
| 6-Pt | 0.57 | 0.81 | 0.66 | 0.78 | 0.63 | 0.53 | 0.83 | 0.49 | 0.3–0.7 (streaks) |
| 7-Pt | 0.58 | 0.83 | 0.49 | 0.68 | 0.64 | 0.57 | 0.87 | 0.49 | 0.75 |
| *Group II* | | | | | | | | | |
| 2,4-Pt | 0.56<br>0.36 | 0.86 | 0.37 | 0.64 | 0.54 | 0.5 | 0.80 | 0.22 | 0.5 |
| 2,7-Pt | 0.48 | 0.71 | 0.32 | 0.51 | 0.35 | 0.43 | 0.77 | 0.13 | |
| 4,7-Pt | 0.52 | 0.79 | 0.24 | 0.53 | 0.35 | 0.21 | 0.86 | 0.24 | 0.3 |
| 2,6-Pt | streaks | streaks | diffuse | streaks | diffuse | 0.12 | 0.75* | 0.11 | 2 spots |
| 4,6-Pt | 0.52<br>0.4<br>0.19 | 0.75 | 0.26 | diffuse | diffuse | 0.17 | 0.82 | 0.15 | 0.5 |
| 6,7-Pt | 0.48 | 0.68 | 0.25 | diffuse | 0.45 | 0.22 | 0.79 | 0.13 | 0.35 |
| *Group III* | | | | | | | | | |
| 2,4,7-Pt | 0.45 | 0.63 | 0.3 | 0.4 | 0.2 | 0.32 | 0.77 | 0.13 | |
| 2,4,6-Pt | 0.5 | 0.7 | 0.21 | 0.5 | 0.43 | 0.12 | 0.76 | 0.07 | |
| 4,6,7-Pt | 0.47 | 0.61 | 0.17 | diffuse | 0.13 | 0.07 | 0.75 | 0.035 | 0.05 |
| 2,6,7-Pt | 0.69<br>0.47 | 0.96<br>0.62 | 0.1** | diffuse | 0.17** | 0.17<br>0.035 | 0.85<br>0.7 | 0.03** | |
| 2,4,6,7-Pt | 0.34 (diffuse) | 0.5 | 0.12 | diffuse | 0.06 | 0.06 | 0.67 | 0.025 | 0.05 |

* Most of the material accumulated in a "head" with green fluorescence. A long tail with faint, blue fluorescence was also observed.

** A second spot was not detected.

Solvent 1: 3% $NH_4Cl$ in water.
Solvent 2: 3% $NH_4Cl$ + 5% $NH_3$ in water.
Solvent 3: 65 ml isopropanol + 25 ml DMF + 10 ml water.
Solvent 4: 65 ml isopropanol + 25 ml DMF + 10 ml glacial acetic acid.
Solvent 5: 65 ml isopropanol + 22.5 ml DMF + 2.5 ml 90% formic acid + 10 ml water.
Solvent 6: 65 ml isopropanol + 25 ml DMF + 10 ml 25% ammonia.
Solvent 7: 50 ml 95% ethanol + 50 ml 5% ammonia.
Solvent 8: 80 ml 95% ethanol + 20 ml 12.5% ammonia.
Solvent 9: 67 ml *n*-butanol + 33 ml of 5 *N* acetic acid (ALBERT *et al.*[9]).

column 2, all members of group I, with the sole exception of 2-Pt, have about the same $R_F$. Within group II, the $R_F$ values of all isomers are again close to each other. However, 2,4-Pt gave two spots and 4,6-Pt even three. The $R_F$ of 2,6-Pt could not be measured, because the compound streaks over the whole chromatogram. In group III likewise little variation is observed. All its members develop well-defined, single spots, with the exception of 2,6,7-Pt, which gives two spots in accordance with the experience of ALBERT, LISTER AND PEDERSEN[12]. An important feature is the sharp separation of group III as a whole from 2,4,6,7-Pt, one of the end-products of enzymic oxidation[1]. To summarise: with solvent 1 there is little differentiation between individual compounds or groups of isomers.

The pH of solvent 1 was usually between 5 and 6. It is evident from the p$K$ values in Table I that in this pH range most pteridines exist as neutral molecules or as mixtures of the latter with anions. It appeared therefore possible that separation might improve, if all substances were present as anions only. The effect of addition of 5% ammonia to solvent 1 was therefore studied (solvent 2, pH 10.5; column 3 in Table II). Curiously enough, all $R_F$ values increased considerably. Otherwise, the results with solvent 2 were similar to those with solvent I. In group I, it is remarkable that 2-Pt now behaves like its isomers. In group II, 4,6-Pt is concentrated into a single spot, whereas 2,4-Pt still gives two spots. Like in solvent 1, 2,6-Pt spreads over a considerable length of the paper. The titration curve of 4,6-Pt shows a hysteresis loop, indicating the formation of a hydrate[11]; however, in alkaline media equilibration is very rapid. Therefore, in solvent 2 this derivative does not give more than a single spot. In contrast, 2,6-Pt at alkaline pH represents a mixture of two neutral molecules and two anions[12] and the slow transition between the different forms makes the formation of a well-defined spot impossible. The behaviour of 2-Pt is very interesting. This derivative is distinguished from its isomers by its exceptionally high p$K$ value (see Table I). In solvent 1, where it is present exclusively as the neutral molecule, it migrates *slower* than its isomers, which are in equilibrium with their anionic forms. Usually, the opposite behavior is observed in paper chromatography, ions travel at lower speed than the neutral molecules. However, the reverse relationship holds in general for solvent 1 and 2, since the mobile phase contains only water and possesses a higher ionic strength than the stationary phase. As compared to solvent 1, solvent 2 is somewhat more suitable for group separation and could indeed be used for certain analytical problems arising from enzymic oxidation reactions[1].

In the special case of 6-Pt, ALBERT, BROWN AND CHEESEMAN[10] could eliminate streaking by the use of dimethylformamide (DMF) as solvent. In the light of our experience with solvent 1 and 2, it was decided to test DMF in three different forms: (a) in combination with neutral solvents only; (b) together with organic acids, and (c) with ammonia.

The combination of 25% DMF with 65% isopropanol and 10% water (= solvent 3) was selected from about 10 different mixtures for a more thorough study. The results in Table II, column 4, show that only pteridine and 2,6-Pt give diffuse spots. Group I appears to be divided into two pairs of substances, the members of each

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pair having identical $R_F$ values: 2- and 6-Pt migrate considerably faster than the pair 4- and 7-Pt. In group II and III, the $R_F$ values overlap, and similarly no sharp separation is found between group III and 2,4,6,7-Pt. A most remarkable feature is the appearance of only a single spot for 2,6,7-Pt, a phenomenon observed also with solvent 5. Summarising, it can be said that solvent 3 does not provide the desired solution of the present problem and is suitable only for special purposes (*e.g.* for the separation of a mixture of 2- and 4-Pt or of 2,4- and 4,7-Pt).

In solvent 4, DMF (25%) and isopropanol (65%) were combined with 10% glacial acetic acid instead of water. The results in Table II, column 5, demonstrate the inferiority of this mixture. Many compounds produced diffuse spots and 2,6-Pt showed streaking. ALBERT *et al.*[10] used DMF as its azeotrope with formic acid (6%). We examined therefore solvent 5, which contains 2.5% formic acid and 10% water (see Table II, column 6). Although superior to solvent 4, it was still unsatisfactory, because four derivatives gave diffuse spots. However, some new interesting properties came to light: In group I, 4-Pt could easily be separated from 7-Pt, analytically a very valuable feature. In group II, 6,7-Pt can be differentiated from 2,4-Pt on the one hand and from the pair 2,7- and 4,7-Pt on the other. In group III, 2,4,6-Pt differs from its isomers by its high $R_F$ value. This group as a whole is also well separated from tetrahydroxypteridine.

More encouraging results were obtained by the addition of 2.5% ammonia (solvent 6; column 7 in Table II). Here, all hydroxy derivatives gave well-defined spots; only pteridine itself yielded a rather elongated spot. In group II, 4,7-Pt and 6,7-Pt had identical $R_F$ values; all other members could be separated from this pair and from each other. In group III, excellent separation of all isomers is possible. However, in view of the small rate of migration, development has to be prolonged for more than 100 hours. It is also apparent that 4,6,7-Pt can not be distinguished from 2,4,6,7-Pt. This problem can, however, be solved with the aid of solvent 1 or 2. An important property of solvent 6 is the fact that 4,6,7-Pt can easily be distinguished from both spots characteristic for 2,6,7-Pt. This question actually arose in the elucidation of the oxidative pathway of 6,7-Pt[1].

It should be mentioned that an increase in the ammonia concentration did not produce any improvement over solvent 6. On the contrary, some of the derivatives of 6-Pt, which formed well-defined spots in solvent 6, started to trail again, when 5% ammonia was present. Solvent 6 seems to offer good prospects of solving analytical problems and it was used extensively by us in enzymic studies.

In view of the favorable results obtained by addition of ammonia, we also studied the simple combination of ethanol with this base, in the hope of being able to dispense with the use of the toxic DMF. With 50% ethanol and 2.5% ammonia (solvent 7; column 8 in Table II) all pteridines showed rather high $R_F$ values and very little differentiation. The results were in general very similar to those with solvent 2, but now all compounds formed well-defined spots. A much better separation was achieved by decreasing the percentage of water. Solvent 8, which contained 80% ethanol and 2.5% ammonia, led to well-concentrated spots—with the sole exception of pteridine

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(see Table II, column 9). In group I, 2-Pt exhibited a much smaller $R_F$ value than the other mono-hydroxy derivatives, which all migrated at about the same rate. In group II, two separate sections can be distinguished: 2,4- and 4,7-Pt possess a much higher $R_F$ than all other members, but these were again not clearly differentiated from each other. In group III, good separation can be effected by developing for a prolonged period. Solvent 8 thus revealed properties similar to those of solvent 6 and proved indeed very useful for special analytical purposes.

In Table II, we have also included the results, reported by ALBERT *et al.*[9-12] for a butanol–acetic acid–water mixture. Besides streaking, the appearance of more than one spot and the overlapping of $R_F$ values between different groups makes it difficult in most cases to use this solvent for the separation of mixtures containing pteridines with varying number of hydroxyl groups.

TABLE III

RECOVERY OF PTERIDINES FROM PAPER CHROMATOGRAMS

For these experiments, 80 $\gamma$ of each pteridine were spotted on the starting line over a length of 8 cm. After development with solvent 6, the spots were marked under ultraviolet light, cut out and extracted with 8 ml of 0.1 *M* phosphate buffer, pH 8.0. The extracts were read in a Beckman Model DU ultraviolet spectrophotometer at the absorption maxima given in column 2 of the Table. Paper blanks of the same size were treated in the same manner, and the optical density of the blank extracts at the relevant wavelengths was subtracted from the readings of the pteridine extracts. The derivatives marked with an asterisk showed—besides their individual $\lambda_{max}$—an additional peak at 313 m$\mu$. For pteridine and 2-Pt, solvent 2 was used for development, since in solvent 6 these two compounds do not concentrate satisfactorily (see Table II).

| *Substance* | $\lambda_{max}$ *(mμ) at pH 8.0* | *% Recovery* |
|---|---|---|
| Pteridine | 298 | 74 |
| *Group I* | | |
| 2-Pt | 310 | 83 |
| 4-Pt | 331 | 83* |
| 6-Pt | 289; 358 | 82 |
| 7-Pt | 328 | 77 |
| *Group II* | | |
| 2,4-Pt | 270; 328 | 90* |
| 2,7-Pt | 345; 361 | 56 |
| 4,7-Pt | 327 | 78* |
| 2,6-Pt | 298 | 53 |
| 4,6-Pt | 281; 359 | 89 |
| 6,7-Pt | 320; 336 | 88 |
| *Group III* | | |
| 2,4,7-Pt | 275; 328 | 90 |
| 2,4,6-Pt | 381 | 82* |
| 4,6,7-Pt | 317; 330 | 78* |
| 2,6,7-Pt | 354 | 94 |
| 2,4,6,7-Pt | 290; 332; 346 | 87 |

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*Recovery of pteridines from paper chromatograms*

For the evaluation of the results of enzymic reactions, it is of importance to determine individual pteridines quantitatively after their chromatographic separation. We have therefore extracted the spots with 0.1 *M* phosphate buffer of pH 8.0 and have measured the concentrations spectrophotometrically. As shown in Table III, in most cases a recovery of 70–90% was achieved. Notable exceptions are 2,6- and 4,6-Pt, where not much more than half of the amount applied to the paper, was found in the extract. Incomplete recovery of pteridines may be ascribed to two different factors: Some of the compounds are sensitive to air or light, as observed previously by ALBERT *et al.*[9–12] However, some derivatives of 4-Pt, which are characterized by their great stability, developed a new absorption maximum at 313 mμ. These compounds are marked in Table III by an asterisk. No explanation for this phenomenon has been found, since all substances involved form well-defined, single spots, without any indication of the presence of a common contaminant.

The result obtained with 2,6,7-Pt is especially interesting. After chromatography in solvent 6, two spots are obtained. From the lower one ($R_F$ = 0.035), almost 80% of the total amount was recovered, exhibiting $\lambda_{max}$ = 353 mμ. The extract from the upper spot ($R_F$ = 0.17) did not absorb at all at this wavelength, but showed a new maximum at 270 mμ, which was absent in the original solution. The optical density of the second extract at its maximum was, however, so small, that only a minor part of the total material could have been present in the upper spot. While these results indicate separation of 2,6,7-Pt into two stable entities, we can confirm the observation of ALBERT *et al.*[12] that each component, upon re-chromatographing, again produces a pair of spots.

## DISCUSSION

The results given in Table II demonstrate that no single solvent combination is suitable for the separation of all 15 hydroxylated pteridines. However, in actual problems only certain homologs and isomers occur together. Solvent 6 proved most suitable for the differentiation of such mixtures. The combined use of solvents 6, 2 and 8 has enabled us to solve all analytical problems encountered in enzymic reactions[1].

In the pteridine series, the $R_F$ values decrease regularly with an increase in the number of hydroxyl groups. This observation contrasts with the effect of non-polar substituents. For example, it is well-known that in homologous series the stepwise introduction of methyl or methylene groups *increases* the rate of migration[17,18]. When log $(1/R_F - 1)$ is plotted as a function of $n$, the number of identical substituents attached to a fundamental structure, a straight line should be obtained[19]. For methylated homologs this line exhibits a negative slope[18], *i.e.* the free energy of transfer from the stationary to the mobile phase *increases* regularly with each additional methyl group. In Fig. 1 we have plotted the above function for the $R_F$ values, measured in solvent 2, to demonstrate the *positive* slope of the straight line, *i.e.* the *decrement* of $\Delta F$, the free energy of transfer, for each additional hydroxyl group. Since $\Delta F$ is

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related to the distribution coefficient $\alpha$ by the equation $\Delta F = RT \ln \alpha$, the reversed slope in Fig. 1 is simply an expression of the fact that hydroxyl groups increase the affinity of a given structure for the stationary (*i.e.* aqueous) phase.

Fig. 1 also demonstrates that the linear relationship between log $(1/R_F - 1)$

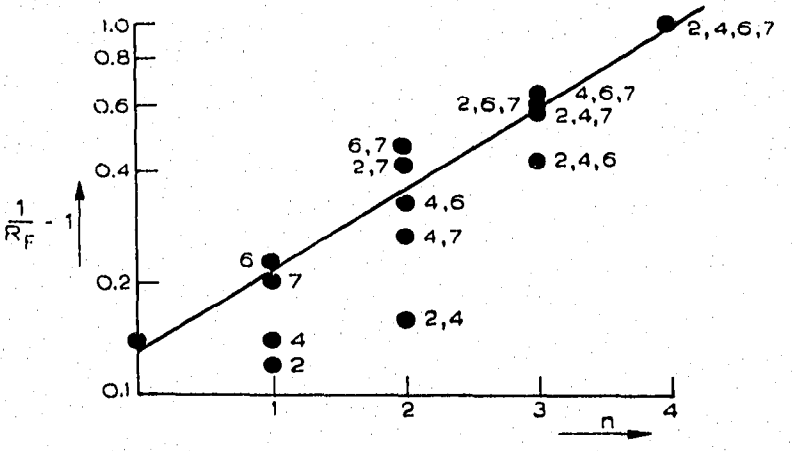


Fig. 1. Log $(1/R_F - 1)$ as a function of $n$, the number of hydroxyl groups attached to the pteridine nucleus. The values used in this graph refer to solvent 2 in Table II. Numbers indicate the position of the hydroxyl groups. Note the logarithmic scale on the ordinate. The line drawn shows that there is a linear relationship for the following two series: (a) Pteridine → 6-Pt → 4,6-Pt → 4,6,7-Pt → 2,4,6,7-Pt; (b) Pteridine → 7-Pt → 2,7-Pt → 2,6,7-Pt (or 4,6,7-Pt) → 2,4,6,7-Pt.

and $n$, the number of hydroxyl groups in the pteridine nucleus, holds only for certain derivatives, but is not of general validity. The spread of the $R_F$ values of isomers reveals again the profound influence of the position of hydroxyl groups on the physical properties of pteridines. This influence is analogous to the marked effect of the position of hydroxyls on the chemical behavior of pteridines[10–12].

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## SUMMARY

Methods for the paper-chromatographic separation of hydroxylated pteridines have been developed.

The dependence of the $R_F$ values of pteridines on the presence of organic acids or ammonia in the solvents used and on the number and position of hydroxyl groups in the heterocyclic nucleus has been discussed.

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