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Paper Chromatography of Podophyllin

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A paper-chromatographic technique has been developed for the separation and recognition of previously recorded natural constituents of *Podophyllum peltatum* L. and *Podophyllum emodi* Wall. as well as of some artifacts.

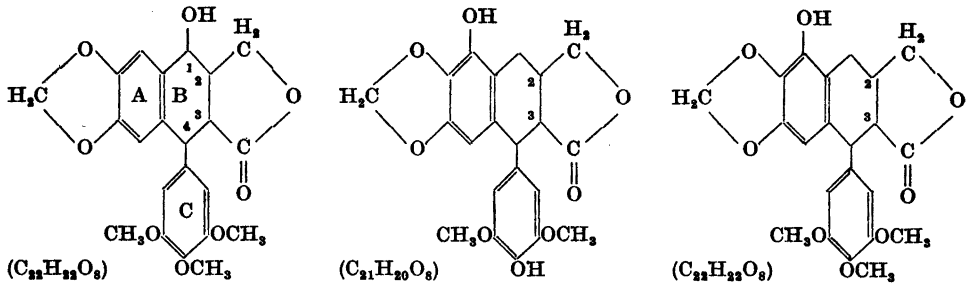
At least two spots in chromatograms of *Podophyllum peltatum* could not be accounted for on the basis of constituents at present on record. Their possible origin is discussed.

The influence of various factors on flow rates in the system formamide (sorbed stationary phase) / benzene (mobile phase) has been examined.

Podophyllin is an old cathartic, which in recent times has become of considerable interest, because it is a mitotic poison of a selective action against certain types of tumor tissue and hence is believed to be a promising point of attack in the search for a chemotherapy of cancer.

The drug podophyllin of most European pharmacopoeias and of the National Formulary USA is the alcohol-soluble resin from rhizome and roots of *Podophyllum peltatum* L. The natural antimitotic constituents so far discovered are podophyllotoxin, first isolated and named in 1881 by Podwyssotzki [1], and α- and β-peltatin, recently discovered by Hartwell and Detty [2]. The extensive structure studies by Hartwell and collaborators [2,3,6–9] lead to a revision of the original podophyllotoxin formula proposed independently by Borsche and Niemann [4] and by Späth *et al.*[5] in 1932. The latest formulations suggested by Hartwell *et al.*[6,7] are given in Fig. 1.

There are three asymmetric carbon atoms in the peltatins and four in podophyllotoxin. Hartwell and Detty [9] have prepared two optical isomers of each of the peltatins. They are denoted A and B and are shown to be C_3-epimers (Fig.1). The naturally occurring and tumor-active isomers A are readily and apparently irreversibly inverted to the tumor-inactive B-compounds when treated with basic reagents and reprecipitated with acid (opening of the lactone ring, followed by relactonization in the alternative configuration). Individual series of derivatives (esters, ethers) are formed by the four peltatins. The A-series is levorotatory, the B series is dextrorotatory or at least less levorotatory than the A series. The inversion of peltatin-A to peltatin-B has



Podophyllotoxin (PT)
trans-1:2-*trans*-2:3-*cis* 3:4

Picropodophyllin (PP)
trans-1:2-*cis*-2:3-*trans*-3:4

Epipodophyllotoxin (EPT)
cis-1:2-*trans*-2:3-*cis*-3:4

Epipicropodophyllin (EPP)
cis-1:2-*cis*-2:3-*trans*-3:4

α-Peltatin-A
trans-2:3-*cis*-3:4

α-Peltatin-B
cis-2:3-*trans*-3:4

β-Peltatin-A
trans-2:3-*cis*-3:4

β-Peltatin-B
cis-2:3-*trans*-3:4

Fig. 1. Natural constituents of Podophyllum peltatum L. *and their epimers. (Hartwell et al.)* *

been accomplished also for a great number of derivatives. Infra-red spectroscopy indicates less strain in the B-isomers. On this basis and from inversion and pyrolysis studies Hartwell and collaborators suggested that the A-series is *trans*, the B-series *cis* around the carbon atoms 2 and 3, and the complete configurations [7] are as indicated in Fig. 1.

Picropodophyllin (PP) has formerly been regarded as a structural isomer of podophyllotoxin (PT) with different positions of attachment of the lactone ring. Hartwell and Schrecker [6,9] have demonstrated that the substances are in fact C_3-epimers, analogous to the peltatins, podophyllotoxin belonging to the A-series, picropodophyllin to the B-series. They both contain one asymmetric carbon atom (C_1) more than the peltatins. The above authors have actually prepared, by a Walden inversion *via* the C_1-halides, two substances

* In a quite recent paper by J. Press and R. Brun (*Helv. Chim. Acta* **37** (1954) 190) these formulæ have been subjected to doubt. The alternative structures proposed by these investigators, mainly on the basis of elementary analyses and UV-spectra, differ fundamentally from those of Hartwell *et al.* even in the empirical formulæ. The "A-series" is claimed to be structurally different from the corresponding members of the "B-series" and not epimers as advocated by Hartwell.

The experimental results reported in the present paper are consistent with the formulations of Hartwell *et al.* At least one observation is incompatible with the Press-Brun formulæ. β-Peltatin-A and -B both give intense orange colourations with diazo-reagent in accordance with Hartwell's formula containing a phenolic hydroxy-group with a free *p*-position. The Press-Brun formula on the other hand cannot explain this reaction since there are no possibilities of coupling. The relative positions of the various substances on the paper chromatograms are equally well understood on the basis of any of the two sets of formulæ, both showing an increasing number of hydroxy-groups with decreasing R_F (Table 1).

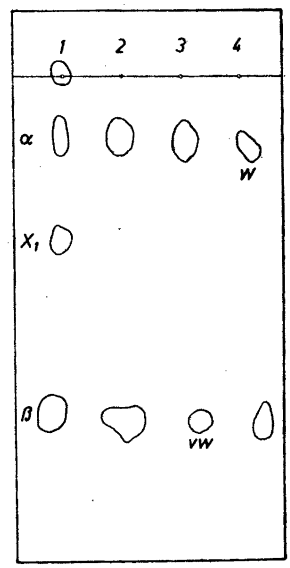


Fig. 2. Chromatogram of natural peltatins (diazo-reagent).

1. *Podophyllin ND I dep. (e peltatum).*
2. *Artificial mixture of natural podophyllotoxin, α-peltatin and β-peltatin.*
3. *Natural α-peltatin*
4. *Natural β-peltatin*

w = weak vw = very weak.

named epipodophyllotoxin (EPT) and epipicropodophyllin (EPP) (*cf.* Fig. 1) which are claimed to be C_1-epimers of the naturally occurring compounds.

For some research in another department of this institute pure podophyllotoxin and peltatins were desired. Whereas the former compound is easily prepared in a pure state by the chromatographic procedure of Hartwell and Detty [9] (activated alumina, benzene-ethanol) their method gave an imperfect separation of the peltatins. The criteria of identity and purity used by these authors were melting points and methoxyl determinations (α-peltatin contains two, β-peltatin three methoxy-groups).

In the search for a simpler and more sensitive purity- and identity test and an effective method for separation of the peltatins paper chromatography was tried. Since the substances are almost completely insoluble in water, the normal procedure involving a distribution between adsorbed water as the stationary phase and a suitable organic solvent as the mobile phase was impracticable. It was found, however, that a perfect separation could be obtained, when the paper was impregnated with formamide and benzene was used as the mobile phase. This technique has been succesfully employed in glucoside work by Zaffaroni [11] and later by Reichstein [12]. Spraying with diazobenzene-sulphonic acid solution was used for detection of the spots. The peltatins, containing one and two phenolic hydroxy-groups respectively, gave intensely coloured coupling products. Podophyllotoxin, being a secondary alcohol, did not respond to the reagent.

Fig. 2 shows a one-dimensional descending papyrogram of podophyllin alongside with the isolated components. No. 1 is a commercial podophyllin (*e peltatum*), further purified according to Hartwell and Detty [8] by precipitation of indifferent resins from an ethanolic solution on addition of benzene.

In the following it will be named "Podophyllin ND I, dep.". There are three distinct spots visible after spraying. Two of these (marked α and β) are readily identified as being due to the natural α- and β-peltatins, *cf.* Nos. 3 and 4, representing authentic products of these substances prepared according to the method of Hartwell and Detty [9]. It is seen that neither α-, nor β-peltatin is pure. The α-peltatin preparation is contaminated with minute amounts of β-peltatin and the β-peltatin contains considerable amounts of α-peltatin. There is no doubt, however, about the identity of the spots and the separation is excellent. The method therefore constitutes a simple and adequate new means of analyzing these mixtures. In No. 1 and in several runs in the following figures there is a spot left at the starting point. It was visible *before* spraying, had a brownish colour, which altered somewhat upon spraying and represents some indifferent resin still left in the purified podophyllin, probably quercetin and related flavone derivatives, which on account of several phenolic hydroxy-groups (five in quercetin) are strongly retained by the formamide-phase and do not travel on the paper.

The spot X_1 in Fig. 2 has not so far been identified. It cannot possibly be podophyllotoxin, because this substance is non-phenolic and hence should not couple with the diazo-reagent. A decisive proof is given in Fig. 2, run No. 2 in which an artificial mixture of podophyllotoxin and the peltatins (Nos. 3 and 4) has been chromatographed. Only the peltatin spots are visible. The relative position of podophyllotoxin has been established by means of a different reagent, *vide infra*. The location of X_1 in Fig. 2 differs somewhat from that in the following chromatograms, the reason being that conditions in this exploratory experiment differed from the standard procedure adopted in the following work. Fig. 2 clearly demonstrates that the position of one compound is not influenced by the presence of another constituent in the moving spot. This was a general experience in all the work described in this paper.

Next it was investigated whether X_1 might represent an epimer of one of the peltatins. Samples of natural α- and β-peltatin were inverted to the corresponding B-compounds as described by Hartwell and Detty [2] by boiling with dilute sodium hydroxide and reprecipitating with acid. The inverted products were chromatographed as Nos. 4 and 5 in Fig. 3 together with the natural peltatin-A's, Nos. 3 and 6, and the podophyllin sample containing the unknown X_1 (No. 1). It is obvious from the relative positions and the colours of the spots, *cf.* Table 1, that the unknown X_1 is not identical with α-peltatin-B or β-peltatin-B. This was confirmed in a number of experiments from which data are included in Table 1. It may be noticed that the chromatograms, Figs. 3—6, corroborate the hypothesis of Hartwell *et al.* that the inversion of A-compounds to B-compounds is complete and not a racemization, since the chromatograms of the inverted peltatins-B are absolutely devoid of A-spots.

For general analytical purposes it was desirable to be able to detect also podophyllotoxin and picropodophyllin on the chromatograms. Antimony pentachloride was found fairly suitable for this purpose. This reagent forms coloured complexes with aromatic ring systems and in this manner podophyllotoxin (PT in Fig. 4) could be located between β-peltatin-A and β-peltatin-B. Picropodophyllin (PP) has a slightly smaller R_F, *cf.* Table 1 and Fig 6. The

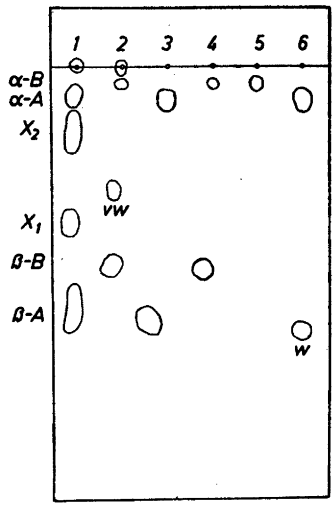


Fig. 3. Chromatogram of C_3-epimers of the peltatins (B-series of Hartwell et al., inverted according to the method of these authors [9]). Sprayed with diazo-reagent.

1. *Podophyllin ND I dep.*
2. *Podophyllin ND II dep., inverted*
3. *Natural β-peltatin*
4. *Inverted β-peltatin*
5. *Inverted α-peltatin*
6. *Natural α-peltatin.*

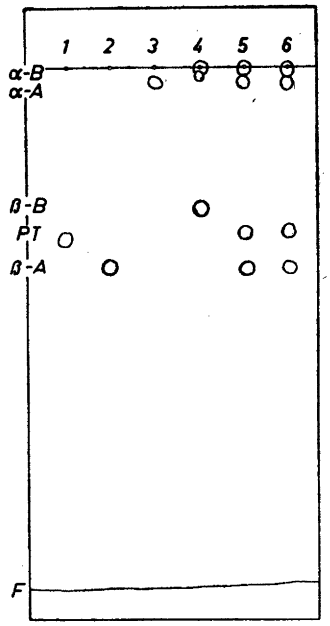


Fig. 4. Chromatogram showing the position of podophyllotoxin. Sprayed with antimony-reagent.

1. *Natural podophyllotoxin*
2. *Natural β-peltatin*
3. *Natural α-peltatin*
4. *Podophyllin ND II dep., inverted. The spot β—B covers the picropodophyllin spot, cf. Fig. 6.*
5. *Podophyllin ND I dep.*
6. *Podophyllin ND II dep.*

reaction is not very sensitive and rather large quantities of the unpleasant reagent are needed, but we have not so far succeeded in finding a more satisfactory colour-reaction, which was suitable for both the peltatins and podophyllotoxin and being at the same time indifferent to formamide. The conventional hydroxamic acid test for esters produced a distinct colour with the lactone ring, common to all the relevant compounds, but unfortunately formamide gives the same reaction and therefore had to be removed prior to spraying. On extraction of the formamide with water the spots were dissolved as well. Most of the formamide could be removed by heating the strip to 100° C for 24 hours, which did not affect the spots. The last traces of formamide were, however, difficult to get rid of and resulted in a strongly coloured background upon spraying.

Formamide can be replaced by propylene glycol, which does not interfere in the hydroxamic acid test. It is, however, rather expensive.

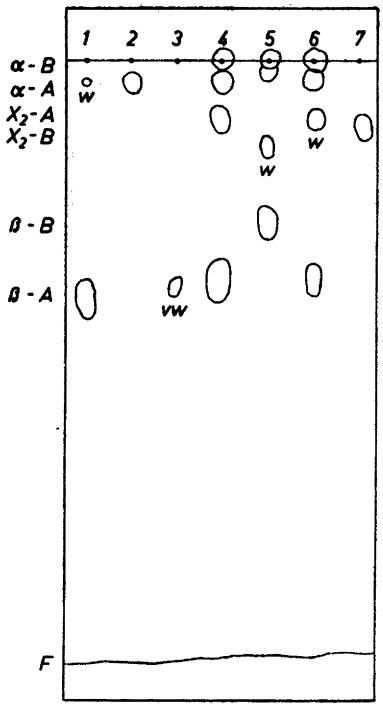


Fig. 5. Chromatogram II, Table 1.

1. *Natural β-peltatin*
2. *Natural α-peltatin*
3. *Natural podophyllotoxin*
4. *Podophyllin ND II dep.*
5. *Podophyllin ND II dep., inverted.*
6. *Podophyllum peltatum L., freshly prepared ethanol extract of fresh roots and rhizomes.*
7. *Fraction from column chromatography silicagel-formamide / benzene) containing the unknown X_2.*

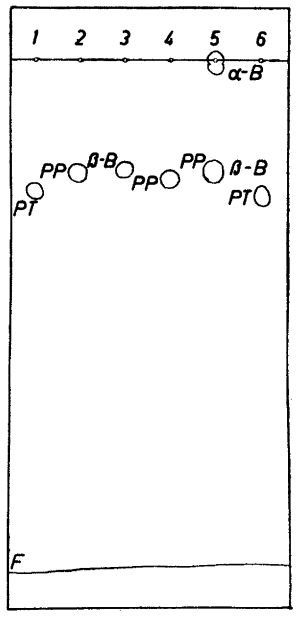


Fig. 6. Chromatogram (VIII, Table 1) showing the location of picropodophyllin.

1. *Natural podophyllotoxin*
2. *Natural podophyllotoxin, inverted*
3. *Natural β-peltatin, inverted*
4. *As No. 2*
5. *Podophyllin ND II dep., inverted*
6. *As No. 1.*

Examination of the chromatograms in ultra-violet radiation disclosed the well known fluorescence fronts due to impurities in the paper, but the spots could not be located in this manner.

A number of commercial samples of podophyllinum Ph. Dan. 48 have been investigated for the occurrence of the unknown X_1. It did not, however, appear in any of them save the one first examined (marked ND I).

During these experiments which are mainly of pharmaceutical interest (they will be published elsewhere) chromatograms were run with somewhat larger quantities of podophyllin on the paper, up to 50 μg. This resulted in the discovery of still another spot provisionally named X_2 (Fig. 5, *cf.* Table 1) in chromatograms of nearly all the podophyllin samples investigated. A

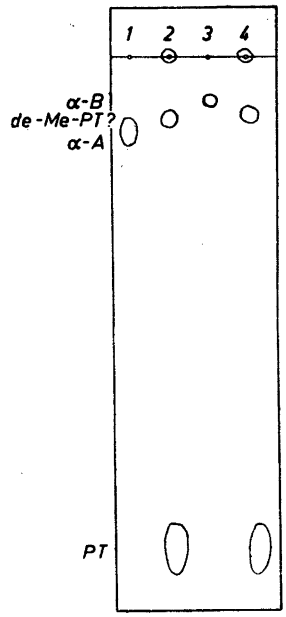


Fig. 7. Chromatogram showing the location of a constituent of Podophyllum emodi Wall., probably demethyl-podophyllotoxin.

1. *Natural α-peltatin*
2. *Emodi-podophyllin*
3. *Natural α-peltatin, inverted*
4. *As No. 2*

chromatogram of fresh roots and rhizomes of *P. peltatum* (freshly prepared ethanol extract), Fig. 5, No. 6, also showed the spot X_2, which is therefore to be regarded as a genuine constituent of this species. When podophyllin samples containing X_2 were boiled with alkali and reprecipitated with acid under conditions which convert "A-compounds" to "B-compounds" the R_F-value of X_2 was somewhat increased. Chromatograms before and after the treatment are shown in Fig. 5, Nos. 4 and 5 respectively (*cf.* Table 1). It is possible that X_2 is related to the compounds in Fig. 1 and analogously undergoes C_3-inversion. The lower spot X_2-B would then represent an epimer of the natural compound X_2-A. This obviously requires further investigation, particularly because the "inversion" causes a shift of the spot in the opposite direction to that found for the peltatins and for podophyllotoxin.

Fresh plant material of *Podophyllum peltatum* was found devoid of X_1. Since this spot has been found in one podophyllin sample only, it may be due to an artifact or to adulteration. Commercial podophyllin *e peltatum* may well be confused with resins of *Podophyllum emodi* Wall. (both are official in the British pharmacopoia). Alternatively it was not impossible that *P. peltatum* in fact contained small amounts of the inherent constituents of *P. emodi*, and one of these might be responsible for the spot X_2 found in all *peltatum* preparations. It was therefore decided to extend the paper-chromatographic work to *Podophyllum emodi*, in which Hartwell and collaborators [13] have discovered small amounts of demethylpodophyllotoxin and 1-O-(β-D-glucopyranosyl)-picropodophyllin besides the main constituent, podophyllotoxin.

Table 1. Approximate R_F-values of podophyllum constituents. Solvent system, sorbed formamide/ Sb = antimony

Chromatogram	I 12/12/2	II 15/12/1 fig. 5	III 14/12/1 fig. 4	IV 14/12/2	V 19/1/1	VI 20/1/2	VII 26/1/2
Temperature °C	20—23°	20—23°	20—23°	20—23°	21—22°	21—22°	20.8°
Spray-reagent	Diaz.	Diaz.	Sb	Diaz.	Diaz.	Diaz.	Sb
φ / Substance	0.33	0.44	0.46	0.46	0.51	0.54	0.55
α-Peltatin-B (α—B)	0.02_4	0.01_8	0.01_7		0.02_3	0.01_6	
Demethylpodophyllotoxin? (de-Me-PT)							
α-Peltatin-A (α-A)	0.04_6 0.05_1 0.04_9 0.05_1 0.04_9 } 0.05	0.03_1 0.03_6 0.03_8 0.03_8 } 0.04	0.03_2 0.03_2 0.02_9 } 0.03	0.04_5	0.03_6 0.03_9	0.03_0 0.03_0	0.02_2
Unknown (X_2)		0.11 0.10 0.10 } 0.10		0.09_6	0.11	0.09_0 0.09_0 } 0.09	
Epimer of X_2? (X_2—B)	0.15	0.15			0.15	0.12	
Unknown (X_1)	0.31						
β-Peltatin-B (β—B)	0.33	0.27	0.27		0.27	0.23	
Picropodophyllin (PP)							0.22 0.23
Podophyllotoxin (PT)			0.32 0.33 0.32 } 0.32				0.29 0.28 0.26
β-Peltatin-A (β—A)	0.41 0.41 0.41 0.41 } 0.41	0.37 0.37 0.37 0.39 } 0.38	0.38 0.39 0.38 } 0.38	0.41	0.39 0.40	0.35 0.35	0.35
β-Naphthole (N)							

Fresh roots of *P. emodi* were worked up to emodi-podophyllin by precipitating an ethanolic extract with 10 volumes 0.3 *N* hydrochloric acid, washing with water and air-drying. A paper-chromatogram of this product is given in Fig 7, Nos. 2 and 4. Emodi-podophyllin gives two spots detectable with

benzene. φ = gram formamide per gram paper. Diaz. = diazobenzene sulphonic acid reagent. pentachloride reagent.

VIII 27/1/1 fig. 6	IX 20/1/1	X 16/12/1	XI 16/12/2	XII 27/1/2	XIII 17/1/1	XIV 19/1/2	XV 21/1/1	Colour of spot	
25.0	21—22°	20—23°	20—23°	25.0±0.2	9.5°	21—22°	20.8°		
Sb	Sb	Diaz.	Diaz.	Sb	Sb	Sb	Sb		
0.57	0.58	0.59	0.59	0.59	0.60	0.61	0.67	diazo-reagent	antimony reagent
	0.01_7				0.01_7			red	violet
	0.02_4				0.02_3			no reaction	violet
	0.03_1, 0.03_4 } 0.03				0.02_3, 0.02_9	0.02_9, 0.02_9	0.02_1	brownish red	violet
			0.11					yellow	
								yellow	
								bright yellow	
(0.22) 0.22	0.23	0.24, 0.24 } 0.24	0.24	(0.18) 0.19	0.23	0.24		orange	brownish yellow
0.23 0.22				0.19 0.19			0.19 0.18	no reaction	brownish yellow
0.26 0.27	0.29, 0.30, 0.31, 0.30, 0.29 } 0.30			0.24 0.23	0.27 0.28	0.28, 0.29, 0.28, 0.28, 0.27 } 0.28	0.22 0.22	no reaction	brownish yellow
	0.35, 0.36, 0.34 } 0.35	0.34, 0.35, 0.36 } 0.35	0.34, 0.34, 0.34 } 0.34		0.35	0.33 0.34	0.26	orange	brownish yellow
		0.46	0.43					red	

antimony pentachloride, neither responds to diazo-reagent in the concentrations in which the peltatins produce intense colouration. The lower spot is due to the main constituent, podophyllotoxin. The upper spot may well represent demethylpodophyllotoxin. It is located above α-peltatin, where

one might expect to find it, for the following reasons. Demethylpodophyllotoxin has a phenolic hydroxy-group in stead of a methoxy group in 4-position in the ring C (Fig. 1). It follows that α-peltatin and demethylpodophyllotoxin bear the same relationship to eachother as do β-peltatin and podophyllotoxin. Since in the latter pair the shift of a hydroxy-group from ring A (phenolic function) to ring B (alcoholic function) causes a decrease in R_F, demethylpodophyllotoxin should have a lower R_F-value than α-peltatin. The apparent rule that an increasing number of phenolic hydroxy-groups decreases the R_F value is supported by the observation that the indifferent resins, mainly quercetin (pentahydroxy-flavone) do not travel at all on the formamide paper (*cf.* p. 944).

The fact that the upper spot does not respond to the diazo-reagent, although this is by far the more sensitive, is also believed to support the hypothesis that it be due to demethylpodophyllotoxin. Coupling with the diazonium group never occurs in meta position to a hydroxy-group, so it is fairly certain that the positive reaction of the peltatins only involves ring A, and that the diazonium group enters the *p*-position to its phenolic hydroxy-group. As a consequence the spot, if due to demethylpodophyllotoxin, should not couple, as indeed we find. However, Hartwell and collaborators [13] report that demethylpodophyllotoxin gives an orange-red colour with diazotized *p*-nitroaniline. The spot under discussion does not respond to this reagent either. A rigorous proof of its identity by matching it with an authentic sample is therefore most needed. This is on our program. It is less probable that the picropodophyllin-glucoside, discovered in emodi-podophyllin is responsible for the upper spot in Fig. 7, Nos. 2 and 4, although this possibility cannot be quite excluded.

Some experiments were made on the reproducibility of the R_F-values. They were found to be strongly dependent on the amount of formamide present in the paper. Some data are collected in Table 1. φ is the formamide content in g per g paper. On some chromatograms several spots occurred of the same substance. Individual R_F-values as well as the average are given in the table. The deviation between any two individual R_F's on the same strip was less than 5 % and usually much smaller. The mobile phase was benzene throughout the experiments and the temperature was 20—23° C. No significant variation of R_F with temperature was observed, *cf.* chromatogram XIII run at 9.5° C. When the formamide content was increased from about 0.3 to 1.3 g per gram paper, the R_F-values gradually decreased. High formamide contents diminished the rate of flow of the solvent, and the formamide phase was imcompletely sorbed by the paper tissue, which was noticeable as a slow downward drift of the initial spots during the period of equilibration. With long irrigation periods good separation, but large, diffuse spots were obtained. When the formamide content was decreased below 0.2, development went too rapidly, the R_F-values varied with φ in the opposite way, probably due to imperfect equilibrium, separation was poor and there was a marked tailing. Optimal conditions were at 0.4 to 0.7 g formamide per g paper (3 to 6 mg/cm^2). Although a certain desired amount of formamide could fairly easily be reproduced by the method given in the experimental section, and the R_F-values in periods were quite reproducible, other factors, difficult to control, seem to influence the

flow rates, for instance moisture variations *etc.* in the formamide phase. Fluctuations up to 10 % were noticed in experiments carried out under supposedly identical conditions. The use of absolute R_F-values for identification purposes therefore had to be abandoned, and reliable identification of individual spots was accomplished by running authentic samples simultaneously on the paper. The sequence of the various substances in the chromatograms was invariably that given in Table 1, but all R_F-values should be regarded as relative values, strictly comparable only within the individual paper strip (vertical columns). This still presupposes an even distribution of formamide on the strip. Experience showed that this could be secured by the impregnation method described in the experimental section.

The original aim at a more effective preparative separation of the constituents was now conveniently combined with a search for the unknowns X_1 and X_2 in quantity. Column partition chromatography on the principle outlined in this paper has been performed on a larger quantity of podophyllin. The paper-chromatographic technique described in this publication was used as an indicator for cutting the fractions. In Fig. 4, No. 7 is reproduced a chromatogram of the fraction containing X_2. Attempts to isolate and identify this substance — or these substances — are in progress, and will be reported in a following publication.

EXPERIMENTAL

Materials. A commercial formamide Merck was used without further purification. It had n_D^{20} 1.4472 and the density D_4^{22} 1.130 (lit. 1.4472 [14] and 1.1313 [15], respectively). Ordinary thiophene-free benzene was used. A chromatographic test proved that it was sufficiently pure for the present purpose.

Podophyllotoxin was prepared according to the method of Hartwell and Detty [2]. Recrystallized from benzene and air-dried it had a melting point 111.5—112° C. This product, which contained liquid of crystallization, was used in the experiments. Borsche and Niemann [16] report a melting point of 112—115° for a product of the composition 2 $C_{22}H_{22}O_8$, 2 H_2O,C_6H_6. Our preparation, after drying at 110° C and 0.01 mm Hg for 18 h had a m. p. 188—89° (lit. 183—84°).

The peltatins were also prepared according to the procedure of Hartwell and Detty [2]. α-Peltatin melted at 227—228° (lit. 230.5—232.5), β-peltatin at 214—228° (lit. 231—238). Each of them contained some of the other peltatin, as shown by the chromatograms.

Picropodophyllin was prepared by refluxing 0.1 g podophyllotoxin for 6 hours with 2 ml absolute ethanol and 0.02 g sodium acetate, precipitating the product with cold water and recrystallizing from ethanol. M. p. 230—231.5° C (lit. 224.5—226.5 [13]).

Podophyllin ND I, dep. and Podophyllin ND II dep. Two commercial samples meeting the requirements of Pharmacopoea Danica, ed. 1948, further purified by precipitation of indifferent resins from an ethanolic solution on addition of benzene.

Emodi-podophyllin. Roots and rhizome of *Podophyllum emodi* Wall, grown in Denmark were worked up to podophyllin immediately after having been digged up. The material was washed, ground in a laboratory mill with 2 parts of 96 % ethanol, and macerated for 2 days, then for 8 days with 2 parts of absolute ethanol and finally for 14 days with two parts of a mixture of equal amounts of benzene and ethanol. The extracts were mixed, evaporated to one third at a temperature not exceeding 40° C and poured into 10 volumes of 0.3 *N* hydrochloric acid. The yellow, crystalline precipitate was washed with water and dried.

Apparatus. The chromatography chamber was made from an all-glass battery case, 21 by 30 cm and 60 cm high. It was closed with a heavy glass-plate, sufficiently tightened by means of a thin velvet tape, glued to the plane-ground upper edge of the case. In the uppermost part of the chamber was fastened a stainless-steel rack, supporting the usual

glass-trough for the descending technique. During the runs the glass chamber was placed in a closed wooden cabinet, provided with glass windows for inspection of the strips. This arrangement gave a sufficient temperature-constancy for the present purpose; Chromatograms were run at 20—23° C.

Paper strips. Whatman paper No. 1 was used. The strips were usually 22 × 55 cm (net weight 10 g), requiring approximately 5 g formamide. A desired and evenly distributed formamide content could fairly easily be obtained by spraying the previously weighed paper strip as uniformly as possible with 100 % excess of formamide, leaving the strip between filter paper sheets for at least 18 hours and until the formamide content was reduced to the desired value.

Procedure. The substance was applied to the impregnated paper strip as circular spots along a marked starting line in the usual way. When a single substance was chromatographed, normally a 1 % ethanolic solution was used; of this 1—2 μl was required, *i. e.* the quantity in each spot was approximately 10—20 μg; 1 μg still gave a detectible spot with the diazo-reagent, whereas the lower limit with antimony reagent was *ca.* 10 μg. When natural or artificial mixtures were chromatographed the quantity applied to the paper was increased *pro rata* (up to 400 μg). After evaporation of the solvent the strips (two were run simultaneously) were suspended in the chamber, which contained a layer of formamide-saturated benzene on the bottom. Two opposite walls of the chamber were lined with filter paper, soaked in and dipping into the bottom liquid. The solvent vapour pressure in the chamber was essentially that of benzene, since at 20° C formamide has a very small vapour pressure and is practically insoluble in benzene. Equilibration of the strips in the solvent atmosphere were extended to 18 h prior to development. The solvent used for development was benzene saturated with formamide. The normal time of development on paper containing 0.5 g formamide per gram was *ca.* 4 hours (travelling distance 40 cm from starting line to solvent front). The strips then should be removed from the chamber and the solvent-front quickly marked. In this connection the volatility of benzene was a great nuisance. Some investigators have proposed to develop to a previously marked pencil-line, which means continuous watching of the chromatograms towards the end of the development period, and limits the number of chromatograms, run simultaneously, to one only. An automatic and accurate location of the solvent-front was obtained in the following way. Before equilibration and development 4—6 narrow, equidistant and vertical bands of "sudan blue-G" were applied to the paper as a 1 % solution in benzene and beginning approximately 5 cm from the lower edge of the strip and extending right to the edge. The development could then be terminated at any convenient time after the benzene front had reached the indicator-bands, and the front would be clearly and permanently indicated by a concentration of the dye. Although sudan blue-G (1-methylamino-4-(*p*-toluidino)antraquinone) is completely insoluble in benzene-saturated formamide and very soluble in the benzene phase, the substance usually moves slightly behind the actual front, probably due to adsorption on the paper. For this reason vertical bands *in situ* were preferred to spots travelling with the solvent.

After development the strips were air-dried for some minutes and then sprayed in a fume-cupboard with either antimony pentachloride (3.5 ml dissolved in 10 ml tetrachloromethane) or with an alkaline diazo-reagent prepared in the following manner. 0.5 g sulfanilic acid was dissolved in 12.5 ml concentrated hydrochloric acid and 75 ml water. Immediately before use 4 ml of this stock-solution was mixed with 1 ml 0.1 *N* aqueous sodium nitrite and 5 ml 2 *N* sodium hydroxide. The boundaries of the spots were traced with a pencil, because the colour gradually faded.

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