synovial fluid contained 44 μg./g. of DNOC. Further, the spray operator reported by Pollard & Filbee (1951) had a value of about 0·9 μg. DNOC/ml. of cerebrospinal fluid. Preliminary investigations suggest that once DNOC has entered the blood it is attached to the albumin fraction. It seems very likely, therefore, that the chief internal stores of DNOC in the body are the extracellular fluids containing albumin. Examination of the accountable and non-accountable DNOC in the body 24 hr. after dosing shows that only about 40 % can be accounted for in the blood and in the urine. There remains 60 % which must include DNOC and its metabolites. The bulk of these are probably held by the albumin of the other extracellular fluids.

Regarding other stores of DNOC, attention must be drawn to the skin and to the hair. Men working with DNOC have heavily stained skins, particularly on the palmar and plantar surfaces and many have bright yellow hair. It is relatively easy to account for the staining of the skin but less easy to account for that of the hair; this may be due partly to excretion and partly to external contamination. The experiments of Harvey *et al.* (1952) and King & Harvey (1953) suggest that although the skin may form a permanent reservoir for DNOC it is unlikely that a dose large enough to give fatal results will penetrate. It seems beyond doubt, therefore, that although the keratins of the external epidermal tissues may prove to be 'binders' of DNOC, the internal albumins are of much greater practical and physiological significance.

The marked quantitative response towards DNOC exhibited by the three species studied in the two communications of this series emphasizes the need for careful and accurate interpretation of results obtained on experimental animals when it is the ultimate object to apply them to man (cf. *Brit. med. J.* 1951).

The slow excretion of DNOC by man emphasizes the need for the institution and maintenance of adequate protective measures in order that exposure to, and absorption of, DNOC will be reduced to a safe minimum (King & Harvey, 1953).

SUMMARY

1. The blood 4:6-dinitro-*o*-cresol (DNOC) decay curves have been studied in man, the rat and the rabbit.

2. The ability to eliminate DNOC appears to be in the order rabbit > rat > man.

3. The storage of DNOC is discussed, and it is suggested that the extracellular fluids are the principal internal stores of DNOC because albumin links with DNOC.

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The Identification of Coumarins and Related Compounds by Filter-paper Chromatography

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The need for a method of identifying small amounts of coumarins and related blue-fluorescing compounds became evident during the course of an investigation on the naturally occurring polyphenolic compounds involved in enzymic browning by potato polyphenolase. Previous workers (Lewis & Doty, 1948; Bowman & Hanning, 1951) have reported that ethanolic extracts of the potato tuber have an intense blue fluorescence, and this has been borne out by investigations carried out in this laboratory (Baruah & Swain, unpublished observations). Furthermore, scopoletin has been isolated from potato tubers taken from plants infected with leaf roll virus (Andreae, 1948), and has been shown

to be metabolized by both healthy and diseased plants (Andreae & Andreae, 1949). Since the closely related compound, aesculetin, was found to be acted upon by the polyphenolase isolated from *Atropa belladonna* (James, Roberts, Beevers & Kock, 1948), it seems probable that coumarins may be included among the compounds involved in enzymic browning.

Casparis & Manella (1943*a*) made an extensive study of the qualitative tests for a number of natural coumarin derivatives occuring in drugs, and recently, Goodwin & Kavanagh (1949, 1950) have shown that the pH/fluorescence curves of a large number of natural and synthetic coumarins are sufficiently distinctive to permit positive identification. Casparis & Manella (1943*b*) showed, however, that the tests they had devised were also given by a number of polyphenolic substances such as the tannins, and that it was necessary to separate the coumarins by vacuum sublimation prior to examination. Thus, while these methods are most probably unequivocal when coumarin derivatives can be isolated in a pure state, the presence of other compounds in natural extracts has to be avoided and a method of separating these compounds would be of value. It is likely, also, that the coumarins are present in natural sources as mixtures. Goodwin & Kavanagh (1949) obtained two coumarin-like fractions, besides scopoletin, from *Avena sativa*, and in many cases both the coumarin derivative and its corresponding glycoside have been isolated from the same plant.

The method of paper chromatography has been employed successfully for the separation and identification of the nearly related flavones and their glycosides (Bate-Smith, 1949; Lindstedt, 1950; Roberts & Wood, 1951*a*; Gage, Douglass & Wender, 1951) and the catechins (Bradfield & Bate-Smith, 1950) as well as for the simple phenols (Bate-Smith, 1949; Bray, Thorpe & White, 1950, etc.) and it appeared likely that the coumarins could be separated and possibly identified in this way. Bowman & Hanning (1951) have reported that the fluorescent compound they obtained from potato tubers migrated in the solvent systems used by Dent, Stepka & Steward (1947), and Gage *et al.* (1951) have reported the R_F values for aesculetin in several different solvent systems.

The R_F values of a number of natural coumarins and nearly related cinnamic acid derivatives in various solvent systems have been determined, and the visible and fluorescent colours produced by several chromogenic sprays when applied to the developed chromatogram have been ascertained. It is suggested that the results obtained will provide a means of identifying coumarins occurring in extracts from natural sources.

Over fifty solvent systems have been examined, but only those giving consistent and useful results are presented here. The use of single-phase solvent systems (Bentley & Whitehead, 1950; Kirby, Knowles & White, 1951; Gage *et al.* 1951) including water alone (Roberts & Wood, 1951*b*) has confirmed the fact that good separations may be obtained. In some cases, the aqueous layer of two-phase systems has been shown to give as good separations as the organic layer, and since the order is substantially different, these may be of use with other types of compounds. The results obtained with substances containing an ortho dihydroxy grouping on filter paper treated with sodium borate, and with those containing a carboxylic acid group on paper treated with sodium phosphate, bear out the results of Wachmeister (1951). The effect of the borate-buffered paper is especially striking and affords an easy method of demonstrating the presence of catechol-like groups.

EXPERIMENTAL

Materials. Most of the pure coumarins were kindly donated by Prof. R. H. Goodwin, Connecticut College, New London, Conn., U.S.A. These are marked in Table 1 with an asterisk. Scopoletin was obtained from Dr R. Best, Waite Agricultural Institute, Adelaide, South Australia. Caffeic and chlorogenic acid from Mr A. H. Williams, Long Ashton Research Station, Bristol. Aesculetin was prepared by hydrolysing aesculin, and the other coumarins are available commercially. Ferulic acid was prepared by a Doebner reaction from vanillin and malonic acid and had m.p. (uncorr.) 167–168° (Kurien, Pandya & Surange (1934) gave m.p. 168–169°). (Found: C, 62·0; H, 5·2. Calc. for $C_{10}H_{10}O_4$: C, 61·9; H, 5·2%.) *p*-Coumaric acid was likewise prepared from *p*-hydroxybenzaldehyde and malonic acid and had m.p. (uncorr.) 212–213°. (Kurien *et al.* (1934) gave m.p. 207°.) (Found: C, 65·8; H, 4·9. Calc. for $C_9H_8O_3$: C, 65·9; H, 4·9%.) All compounds were recrystallized, where necessary, until they were chromatographically pure. Since only small amounts of most of the natural coumarins were available, and their fluorescence differs widely (Goodwin & Kavanagh, 1950) solutions were made up, in ethanol, at the minimum concentration required to enable them to be readily detected on the developed chromatogram.

Apparatus. The apparatus used for determining the R_F values was, in general, the same as described by Consden, Gordon & Martin (1944). The cabinet and trough were of all-glass construction and the lid of the cabinet had a small hole fitted with a bung so that the solvent could be introduced with the least disturbance into the trough after equilibration (Jermyn & Isherwood, 1949).

Paper. Whatman no. 1 filter paper has been used throughout, and the sheets were all taken from one batch and run in the same fibre direction (Bate-Smith & Westall, 1950). The buffered papers (Wachmeister, 1951) were prepared by soaking batches of twenty-five sheets (size 18·5 × 12 in.) in 0·1 M-Na_2HPO_4 or 0·1 M-$NaBO_3$ solutions for 30 min. in a specially constructed rectangular Perspex Büchner funnel. The excess buffer solution was removed by suction and the papers allowed to dry overnight in a current of air. Other methods of preparing buffered paper led to uneven deposition or to difficulty of handling.

Solvents (all proportions are v/v). All the solvents used were of the purest quality obtainable. In the case of two-phase systems the organic layer was used unless otherwise stated. In view of the findings of Lugg & Overell (1948) on the rate of esterification of alcohol-acid mixtures, *n*-butanol-acetic acid-water (4:1:5), amyl alcohol-acetic acid-water (4:1:5), and benzyl alcohol-acetic acid-water (4:1:5) were used after 12 hr. equilibration in the constant temperature room.

Procedure. The procedure described by Bate-Smith & Westall (1950) was found to be satisfactory in giving reproducible R_F values. The temperature used was $21 \pm 0{\cdot}5^{\circ}$. Phloroglucinol was used as a control substance and run on every chromatogram. The times for development of the chromatogram for each solvent are given in the results, and varied from 3 to 24 hr. The forward boundary of the solvent front was located after drying by viewing in ultraviolet light.

Developing reagents. The position of the spots on the dried paper chromatogram could be revealed in almost all cases by their fluorescence in ultraviolet light. In cases where the fluorescence was relatively weak it could be intensified by exposure of the paper to ammonia vapour, or by spraying the paper, prior to examination, with 2N-NaOH. In the case of coumarin itself the latter method was, in fact, always necessary. The use of chromogenic reagents to reveal the position of the compounds was found to be, in general, less sensitive than fluorescence. The following sprays were examined.

Diazotized p-*nitroaniline.* (*a*) *p*-Nitroaniline, 0·5% in 2N-HCl (5 ml.), and $NaNO_2$ (5% w/v) (0·5 ml.) were mixed before spraying and sodium acetate solution (20% w/v) (15 ml.) added; (*b*) Na_2CO_3 (20% w/v). The two were used successively, the colour reactions with each being noted.

Bisdiazotized benzidine. (*a*) This was prepared according to Linstedt (1950). After noting the colour reaction the chromatogram was oversprayed with (*b*) Na_2CO_3 (20% w/v).

Silver nitrate. This was modified from the directions given by Trevelyan, Procter & Harrison (1950). (*a*) The acetone solution of $AgNO_3$ was prepared according to the above authors and sprayed directly on to the paper followed by (*b*) a mixture of 2N-NaOH (5 ml.) and ethanol (15 ml.). The chromatogram was left for 10 min. and the excess silver oxide removed by spraying it with (*c*) glacial acetic acid.

RESULTS

The R_F values of the various coumarins (Table 1) and the related cinnamic acid derivatives in those solvent systems which proved most useful are given in Table 2. These represent the mean of several determinations under the carefully controlled conditions described above. The R_F values of the substances on unequilibrated paper at normal laboratory temperature (18°) usually differed from these by as much as $\pm 0{\cdot}04$, but the order of separation was substantially the same.

It was found that solvent systems containing light petroleum, benzene or chloroform (Bray *et al.* 1950; Lindstedt, 1950) or butyl ether (Stone & Blundell, 1951) gave excessive tailing, and the results are not presented here.

In most cases well-defined round spots were obtained, especially with the solvent systems containing acids. Butanol-water gave slightly elliptical spots and some tailing with the compounds having *o*-dihydroxy groups and with acids. Ethyl acetate-water gave noticeably elongated spots and more extensive tailing than butanol. The single-phase systems also tended to give tailing, especially with the more complex coumarins and those containing methoxyl groups. The times taken for the solvent front to travel about 35 cm. are shown (Table 2) for all the solvents used.

The visible and fluorescent colours given by the substances when the developed chromatogram is treated with the chromogenic sprays are shown in Table 3. All the compounds are revealed by examination of the chromatogram in ultraviolet light either with ammonia vapour or after lightly spraying the paper with 2N-sodium hydroxide.

DISCUSSION

The results presented in Table 2 show that the method of paper chromatography can be used for separating and identifying a large number of coumarins and related compounds from natural sources. The most useful solvents for a preliminary examination of plant extracts are *n*-butanol-acetic acid-water and amyl alcohol-acetic acid-water since the glycosides and aglycones which would probably be present are well separated in these systems (cf. Bate-Smith & Westall, 1950; Forsyth, 1949). The behaviour of the various types of compounds studied shows regularities between R_F value and structure which are probably characteristic and applicable to related phenols.

A comparison of the R_F values of the compounds in both *n*-butanol-water and ethyl acetate-water systems on paper and on paper impregnated with phosphate or borate buffers affords, as shown by Wachmeister (1951), a simple method for the detection of acidic compounds and those containing two adjacent hydroxyl groupings. The lowering of the R_F values of scopoletin and ferulic acid (which have hydroxyl and methoxyl groups adjacent) on borate paper as compared with phosphate indicates that complex formation takes place with this grouping also although to a less extent than with the non-methylated derivative. The effect of borate-impregnated paper on the glucosides skimmin and aesculin may be due to the chelation of the glucose residue with borate as has been previously observed (Wachmeister, 1951).

The effect of the ethyl acetate-2N-ammonia system on the movement of the coumarins is also significant. All compounds having a free phenolic hydroxyl, except ostruthin in which the group is sterically hindered, have very low R_F values. A similar effect is shown to a less extent by *n*-propanol-concentrated ammonia. In ethyl acetate-2N-

Table 1. *Systematic names and formulae of various coumarins and related cinnamic acids in the present work*

Name	Systematic name	Formula	Examples of occurrence
1 Coumarin	—	CO O	*Melilotus officinalis* blooms, *Lavandula officinalis*, *Asperula odorata*, *Anthoxanthum odoratum*, etc.
2 Umbelliferone	7-Hydroxycoumarin	—	*Ferula assa-foetida*, *F. narthex*, *F. galbaniflua* and other Umbelliferae
3* Skimmin	7-Glucoxycoumarin	—	*Skimmia japonica*
4* Herniarin	7-Methoxycoumarin	—	Leaves of *Herniaria hirsuta* and *Lavandula officinalis*
5 Aesculetin	6:7-Dihydroxycoumarin	—	In bark of *Aesculus hippocastanum* and *A. turbinata*
6 Aesculin	7-Hydroxy-6-glucoxycoumarin	—	In bark of *Aesculus hippocastanum* and leaves of *Bursaria spinosa*
7 Scopoletin	7-Hydroxy-6-methoxycoumarin	—	In roots of *Avena sativa*, tubers of *Solanum tuberosum*, roots of *Scopolia japonica* and *Nicotiana tabacum*
8* Daphnetin	7:8-Dihydroxycoumarin	—	Bark of *Daphne alpina*, *D. mezereum* and *D. odora* as glycoside daphnin
9* Limettin	5:7-Dimethoxycoumarin	—	In rinds of *Citrus* spp.
10* Ostruthin	6-(3:7-Dimethylocta-2:6-dienyl)-7-hydroxycoumarin	—	Roots of *Imperatoria ostruthium* and *Archangelica officinalis*
11* Osthol	7-Methoxy-8-(2-*iso*pentenyl) coumarin	—	
12* Bergapten	5-Methoxyfurano-2′:3′-6:7-coumarin	OCH_3 CO O O	Bergamot oil, fruit of *Fagara xanthoxyloides* and *Heracleum sphondylium*
13* Xanthotoxin	8-Methoxyfurano-2′:3′-6:7-coumarin	—	Fruit of *Fagara xanthoxyloides*
14* Imperatorin	8-*iso*Pentenyl ether of 8-hydroxyfurano-2′:3′-6:7-coumarin	—	Root of *Imperatoria ostruthium*
15 *o*-Coumaric acid	*trans*-2-Hydroxycinnamic acid	—	*Melilotus officinalis* and *Lavandula officinalis*. *Angraecum fragans*
16 *p*-Coumaric acid	4-Hydroxycinnamic acid	—	*Prunus serotina*, *Thea sinensis*, *Trifolium pratense*
17 Caffeic acid	3:4-Dihydroxycinnamic acid	—	*Solanum angustifolium*, *Coffea arabica*, *Nicotiana tabacum*
18 Ferulic acid	4-Hydroxy-3-methoxycinnamic acid	—	*Opopanax hispidum*, asafoetida resin, *Pinus cembra*
19 Chlorogenic acid		$3{:}4(OH)_2C_6H_4.CH{:}CHCO.OCH$ (ring: CHOH.CHOH, CH_2, CH_2—$C(OH)(CO_2H)$)	*Coffea arabica*, *Strychnos nux-vomica*, etc.

Table 2. R_F *values of coumarins and related compounds*

Compound	Solvent system							
						n-Butanol-water		
	Phenol-water	*m*-Cresol-acetic acid-water (50:2:48)	*n*-Butanol-acetic acid-water (4:1:5)	Amyl alcohol-acetic acid-water (4:1:5)	Benzyl alcohol-acetic acid-water (4:1:5)	Plain paper	Borate paper	Phosphate paper
Coumarin	0·98	0·97	0·92	0·86	0·98	0·87	0·87	0·88
Umbelliferone	0·95	0·94	0·89	0·83	0·92	0·84	0·65	0·83
Skimmin	0·90	0·81	0·52	0·22	0·54	0·33	0·20	0·31
Herniarin	0·98	0·98	0·91	0·87	0·98	0·85	0·87	0·87
Aesculetin	0·72	0·81	0·79	0·65	0·80	0·32	0·11	0·29
Aesculin	0·80	0·64	0·49	0·21	0·44	0·30	0·03	0·12
Scopoletin	0·96	0·96	0·83	0·73	0·91	0·76	0·40	0·71
Daphnetin	0·84	0·83	0·81	0·68	0·83	0·60	0·10	0·40
Limettin	0·97	0·97	0·92	0·89	0·98	0·88	0·89	0·88
Ostruthin	0·96	0·98	0·93	0·95	0·97	0·95	0·92	0·93
Osthol	0·98	0·98	0·92	0·91	0·97	0·92	0·89	0·90
Bergapten	0·98	0·97	0·88	0·88	0·97	0·88	0·91	0·88
Xanthotoxin	0·96	0·97	0·87	0·88	0·96	0·87	0·85	0·86
Imperatorin	0·98	0·97	0·91	0·92	0·97	0·91	0·91	0·92
o-Coumaric acid	0·66	0·81	0·90	0·90	0·92	0·72	0·20	0·23
p-Coumaric acid	0·67	0·81	0·88	0·88	0·90	0·62	0·16	0·18
Caffeic acid	0·33	0·46	0·78	0·70	0·75	0·28	0	0·05
Ferulic acid	0·77	0·89	0·84	0·81	0·90	0·54	0·11	0·13
Chlorogenic acid	0·49	0·33	0·61	0·31	0·54	0·05	0	0·04
Phloroglucinol	0·32	0·16	0·69	0·56	0·52	0·70	0·60	0·65
Time taken for solvent front to reach 35 cm. (hr.)	20	36	15·5	22	20	15	15	15

Table 2 (*cont.*)

Compound	Solvent system											
	Ethyl acetate-water			Ethyl acetate-2N-HCl (1:1)	Ethyl acetate-2N-NH_3 (1:1)	*n*-Propanol conc. NH_4OH (7:3)	Aqueous phase of butanol-acetic acid-water (4:1:5)	Aqueous phase of benzyl alcohol-acetic acid-water (4:1:5)	Water	10% Acetic acid	*iso*-Propanol-water (1:4)	*iso*-Propanol-water (2:3)
	Plain paper	Borate paper	Phosphate paper									
Coumarin	0·92	0·94	0·94	0·93	0·95	0·87	0·84	0·81	0·67	0·76	0·74	0·87
Umbelliferone	0·90	0·73	0·89	0·90	0·28	0·66	0·75	0·73	0·57	0·60	0·66	0·84
Skimmin	0·09	0·04	0·10	0·12	0·07	0·61	0·84	0·85	0·72	0·82	0·73	0·80
Herniarin	0·92	0·94	0·94	0·94	0·95	0·87	0·79	0·76	0·50	0·67	0·63	0·84
Aesculetin	0·44	0	0·14	0·75	0	0·31	0·63	0·65	0·34	0·45	0·54	0·65
Aesculin	0·04	0	0·02	0·08	0	0·34	0·78	0·80	0·81	0·69	0·76	0·77
Scopoletin	0·85	0·36	0·79	0·83	0·06	0·59	0·70	0·70	0·50	0·51	0·60	0·78
Daphnetin	0·74	0	0·58	0·78	0·02	0·38	0·69	0·70	0·61	0·54	0·62	0·72
Limettin	0·94	0·95	0·95	0·96	0·96	0·88	0·67	0·40	0·11	0·39	0·44	0·87
Ostruthin	0·95	0·97	0·95	0·97	0·96	0·88	0·36	0·07	0·05	0·10	0·18	0·93
Osthol	0·96	0·96	0·94	0·89	0·96	0·89	0·72	0·71	0·17	0·53	0·66	0·91
Bergapten	0·94	0·96	0·95	0·94	0·96	0·86	0·65	0·59	0·19	0·45	0·38	0·81
Xanthotoxin	0·93	0·95	0·94	0·93	0·95	0·84	0·76	0·67	0·33	0·58	0·57	0·83
Imperatorin	0·91	0·96	0·94	0·96	0·97	0·90	0·65	0·50	0·22	0·47	0·59	0·90
o-Coumaric acid	0·78	0·01	0·18	0·93	0	0·39	0·74	0·69	0·86	0·58	0·81	0·81
p-Coumaric acid	0·77	0·01	0·12	0·90	0	0·32	0·69	0·65	0·81	0·49	0·79	0·78
Caffeic acid	0·21	0	0·03	0·80	0	0·19	0·60	0·57	0·78	0·38	0·78	0·69
Ferulic acid	0·55	0·01	0·13	0·90	0	0·26	0·68	0·63	0·78	0·48	0·77	0·74
Chlorogenic acid	0	0	0	0·31	0	0·10	0·78	0·76	0·89	0·64	0·81	0·75
Phloroglucinol	0·62	0·50	0·68	0·68	0·18	0·35	0·72	0·69	0·52	0·61	0·68	0·80
Time taken for solvent front to reach 35 cm. (hr.)	3	3	3	4	3·5	14	10	7	3	4	10·5	16·5

Table 3. *Colours produced by chromogenic sprays*

(f = faint, b = bright, l = light, d = dark, W = White, B = Blue, G = Green, V = Violet, Y = Yellow, Bl = Black, Br = Brown, R = Red, O = Orange, Gr = Grey.)

Compound	Spray reagent*												
	Ultraviolet fluorescence							Diazotized *p*-nitroaniline		Diazotized benzidine			
	Un-treated	+NH_3 vapour	2N-NaOH	2N-HCl	1% Ethanolic $AlCl_3$ +NH_3 vapour	1% Ethanolic $FeCl_3$	$AgNO_3$ +NaOH	+Na acetate	+Na_2CO_3	Alone	+Na_2CO_3	1% (aqueous) $KMnO_4$‡	2N-NaOH +diazotized *p*-nitroaniline +sodium acetate
Coumarin	—	—	*bG–Y*	—	—	—	—	—	—	—	—	*Y–G*	*lV–R*
Umbelliferone	*bB*	*bB*	*bB*	*lB*	*bB*	—	—	—	—	*R*	*Br*	*Y*	*B* or *Br*
Skimmin	*fV*	*fV*	*G–B*	—	*fV*	—	*fBr*	—	—	—	—	—	*lV–R*
Herniarin	*V–B*	*V*	*lB*	*fV*	*V*	—	—	*Y*	*V*	*O*	*R*	*Y–G*	*V*
Aesculetin	*blB*	*blG–Y*†	*bY*†	*lB*	*G–Y*†	*G*	*Bl*	*fBr*	*fY*	*Br*	*Br*	*fBr*	*Br*
Aesculin	*bB*	*bB*	*bB*	*B*	*bB*	—	*fBr*	—	—	*fGr*	*lV*	*fBr*	*fBr*
Scopoletin	*bB*	*bG–B*	*bG–B*	*lB*	*bG–B*	—	*Br*	*fGr*	*lG*	*Gr*	*V*	*G*	*lG*
Daphnetin	*fY*	*bY*†	*Br*†	—	*fdY*†	*G*	*Bl*	*Br*	*lG*	*O–R*	*dBr*	*YG*	*Br*
Limettin	*Y*	*dY*	*fdY*	*fBr*†	*B*	—	—	—	—	—	—	—	—
Ostruthin	*B*	*B*	*fB*	—	*fB*	—	—	—	—	—	—	—	—
Osthol	*fB*	*fB*	*fG–B*	—	*lB*	—	—	—	—	—	—	—	*fBr*
Bergapten	*fY*	*lB*	*fG*	—	*fY*	—	—	—	—	—	—	—	*fBr*
Xanthotoxin	*Y*	*Y*	*bY*	—	*fY*	—	—	—	—	—	—	*fYG*	—
Imperatorin	*Y*	*Y*	*Y*	—	*bG–Y*	—	—	—	—	—	—	*YG*	*lBr*
o-Coumaric acid	*bW–B*	*bG–Y*	*bG–Y*	*bY*	*bB*	*fY*	—	*Y*	*bV*	*Y*	*R*	—	*lV*
p-Coumaric acid	*fB*	*bB*	*bdB*	*fB*	*lB*	*fY*	—	*Br*	*GrB*	*O*	*V–R*	*fY*	—
Caffeic acid	*fB*	*lB*†	*fY*†	—	*Y–G*†	*dG*	*Bl*	*lBr*	*Br*	*lBr*	*lBr*	*YG*	*Br*
Ferulic acid	*B*	*B*	*B*	*bG–Y*	*B*	*Br*	*fBr*	*dR*	*dG–B*	*dBr*	*d–V*	*YG*	*dB–G*
Chlorogenic acid	*fB*	*dG–Y*†	*fY*†	—	*dY*†	*Gr–G*	*Bl*	*fBr*	*Y*	*fBr*	*lY*	*YG*	*lBr*
Phloroglucinol	—	*dB*	*dbB*	—	*dB*	*fBr*	*Br*	*O*	*O*	*dV–Br*	*dV–Br*	*YG*	*OBr*

* Other sprays: Limettin alone gives a dark brown with 2% ethanolic iodine.
† *Y* in visible light.
‡ On purple background.

hydrochloric acid, on the other hand, the R_F values of compounds of this type are not noticeably altered from those obtained in ethyl acetate-water, but the acids have a greatly increased value due to suppression of ionization. The very low R_F values obtained for the glucosides in ethyl acetate systems may be diagnostic since flavone glycosides behave similarly (Nordström & Swain, unpublished).

The degree of separation of the more complex coumarins and the methoxycoumarins in the commonly used solvents is slight, but the use of single phase systems containing large amounts of water (cf. Bentley & Whitehead, 1950; Kirby, Knowles & White, 1951) or the aqueous phase of two-phase systems has proved useful. The use of water alone as a solvent is successful in separating the aromatic amino-acids (Synge & Tiselius, 1949) and the polyphenols in tea leaves (Roberts & Wood, 1951*b*). The behaviour of the coumarin aglycones is notably different from that reported for the flavonols by the latter authors, who observed that these compounds had no movement in this system. The glycosides have a higher R_F value than the aglycones in all the aqueous systems used, but decreasing the amount of water tends to reverse this as shown by comparing the R_F values in the two *iso*propanol-water solvents. The behaviour of the compounds in water alone also affords support to the theoretical considerations put forward by Consden, Gordon & Martin (1944) on the relationship between the partition coefficient and R_F value. It has been pointed out to the author by Dr S. M. Partridge that if the ratio of A_L/A_S in the equation

$$\alpha = A_L/A_S \left(\frac{1}{R_F} - 1\right)$$

lies between 3 and 5, as found by Consden *et al.* (1944), and the partition coefficient, α, is taken as 1, then the R_F value should lie between 0·75 and 0·83. The results obtained indicate that in no case is the R_F value significantly higher than this. Although, as suggested by Hanes & Isherwood (1949), it is possible that the water imbibed on the cellulose and the water in the mobile phase are structurally different, it would seem likely that the separations achieved using water as a solvent are due to factors other than liquid partition.

Although the fluorescence of the compounds in ultraviolet light, especially after spraying the paper with 2N-sodium hydroxide, is useful for detecting their position, it is of little value in determining their probable nature since the fluorescence of most of the compounds is so similar. The use of chromogenic sprays to show certain structural characteristics is therefore of value. The production of green colours with 1% ethanolic ferric chloride and the ready reduction of silver oxide shows the presence of two adjacent hydroxyl groups (Bate-Smith & Westall, 1950) and the colours given with either diazotized *p*-nitroaniline or benzidine are sufficiently characteristic to distinguish between compounds of similar fluorescence and R_F value. The use of 1% aqueous potassium permanganate for detecting the compounds is of value if an ultraviolet lamp is not available, although many other compounds will react in the same way. The behaviour of the compounds with diazotized *p*-nitroaniline after prior treatment with sodium hydroxide is also almost as sensitive as fluorescence.

Finally it is felt that the colours produced by the chromogenic sprays considered in conjunction with the R_F values in different solvent systems make it possible to classify and in some cases identify coumarins occurring in natural extracts.

SUMMARY

1. The R_F values on filter-paper chromatograms of a number of coumarins and related cinnamic acids in various solvents have been determined.

2. The compounds are best detected on the chromatogram by their fluorescence in ultraviolet light.

3. The results obtained with different solvent systems and the colour reactions with various spray reagents are discussed in relation to the compounds, and suggest that definite indications of structure can be deduced.

4. It is suggested that the coumarins present in natural extracts could be readily separated and identified by this method.

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Studies in Carotenogenesis

6. THE EFFECT OF SOME POSSIBLE CAROTENE PRECURSORS ON GROWTH, LIPOGENESIS AND CAROTENOGENESIS IN THE FUNGUS *PHYCOMYCES BLAKESLEEANUS*

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A number of compounds which could theoretically act as primary intermediates (repeating units) in carotenogenesis have been examined using the fungus *Phycomyces blakesleeanus* as the test organism. Apart from the information obtained concerning the formation of β-carotene, interesting observations have also been made on the effect of the compounds tested on lipogenesis and growth in general. Many of the substances tested have proved to be extremely toxic. Some of the results described here have already been briefly reported (Glover, Goodwin & Lijinsky, 1951).

EXPERIMENTAL

Cultural conditions. Two types of experiments were carried out. In the first, well developed mats grown in Petri dishes on filter paper were transferred, when 3–4 days old, to fresh media also in Petri dishes. Details of this technique have already been given (Goodwin & Lijinsky, 1951). In the second type of experiment, *Phycomyces* was germinated and allowed to grow undisturbed on our standard medium (Garton, Goodwin & Lijinsky, 1951) modified only by the substitution of part of the glucose by an equivalent amount (calculated on C content) of the substance under examination.

Materials used. The materials used for the standard medium were as described previously (Garton *et al.* 1951). The following intermediates were synthesized: 3-methylbut-2-enoic acid (β-methylcrotonic acid; $Me_2C:CH.CO_2H$, m.p. 66·5–67·5°) from mesityl oxide (Vogel, 1948); DL-2-hydroxy-4-methylpentanoic acid (DL-α-hydroxy*iso*caproic acid; $Me_2CH.CH_2.CHOH.CO_2H$, m.p. 79–81°) and DL-2-hydroxy-3-methylbutanoic acid (DL-α-hydroxy*iso*valeric acid; $Me_2.CH.CHOH.CO_2H$, m.p. 84°) by the conventional method via the corresponding α-bromo acids (Vogel, 1948); 4-methylpentamide (*iso*caproamide, m.p. 118°) and 3-methylbutanamide (*iso*valeramide, m.p. 137°) by the usual method from the corresponding acid (Vogel, 1948) and 2-keto-4-methylpentanoic acid (α-keto*iso*caproic acid; $Me_2CH.CH_2.CO.CO_2H$, b.p. 83°/17 mm.) (2:4-dinitrophenylhydrazone, m.p. 146–148°) from *iso*valeryl chloride (Mauthner, 1909); and 3-methylbut-2-enal (β-methylcroonaldehyde; $Me_2C:CH.CHO$, b.p. 68–72°/95 mm.) (2:4-dinitrophenylhydrazone, m.p. 182°) by the method of

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