Series 1: In order to evaluate the effect of temperature variations, Kaminski's apparatus was transferred to an air thermostat whose temperature varied less than 0.1°. It seems most unlikely that moving the apparatus into the thermostat could have changed any condition other than temperature, which may have been responsible for the Nutting effect observed by Kaminski. Deviations in flow rate up to 1.5% from the mean value were observed, but they showed no discernible consistent change after the capillary had been immersed in distilled water overnight, or after it had been allowed to dry out again for 3 days. The data are plotted in Fig. 1a.

Series 2: A more precise control of temperature was obtained by immersing the jacket containing a capillary in a water thermostat at 25° ±0.002°. The internal diameter of this capillary was 0.747 mm. In these experiments, the capillary was made part of a U-tube, enlarged at one point to form a small reservoir, in the manner of an Ostwald viscosimeter. Great care was taken to eliminate possible surface active impurities. The entire apparatus was flamed to incipient fusion while a current of dried filtered air was passed through it. Both entrance tubes were bent downwards and guarded by plugs of glass fiber. The Nujol had stood with 10 per cent of its weight of Florisil (an activated silica) for a week with occasional shaking, and was centrifuged before use.

Thirteen runs were made in the dry capillary, 9 after filling the jacket with water, followed by 5 more after drying again. The flow times varied within 0.4% of the mean value (between 360 and 363 sec) with no indication of a consistent increase during the period of wetting. The experimental errors were magnified somewhat by an uncertain drainage of the measuring reservoir, which was below the capillary in this apparatus.

Series 3: Since a small percentage of a polar long-chain compound might produce an oriented immobile layer under conditions where a nonpolar oil would not, lauryl alcohol was added to make a 0.69% solution. (The Nujol wetted the glass, the lauryl alcohol solution did not.) The flow times relative to the first value, plotted against the number of runs, are shown in the upper plot (Fig. 1b). The radius of the circle represents probable errors in the timing, but does not take into account the variable drainage of the tube. It will be seen that any retardation that may have occurred on wetting the outside of the capillary is less than the random fluctuations (about 0.5%). A small but definite retardation with time of about 0.5% may be seen, however. The film thickness necessary to account for this would be about 0.9 μ, instead of 6.5 μ, which although still high, is more nearly in accord with the observations of others in this general field. It may also be due at least in part to slight clogging of the capillary by traces of suspended matter.

References

1. Henniker, J. C. In press.
2. Kaminski, A. *Office of Naval Research*, Rep. no. 1. 1948.
3. Nutting, P. G. *Science*, 1943, **97**, 74–75.

Paper Chromatography of Flavonoid Pigments[1]

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Although the use of classical chromatographic adsorption methods for the separation of flavonoid pigments from plant extracts has been reported previously (*3*, *4*, *5*), such methods have not been successful in the separation of microquantities of these compounds. In a search for better methods of examining plant extracts for flavonoid pigments, we have applied the method of paper partition chromatography (*2*) to the problem. This preliminary report deals with the determination of R_f values for 11 flavonoid pigments in chloroform, ethyl acetate, phenol, and n-butanol-acetic acid; the separation of mixtures containing four to six of these pigments; and the use of color developing sprays to locate and identify the pigment zones.

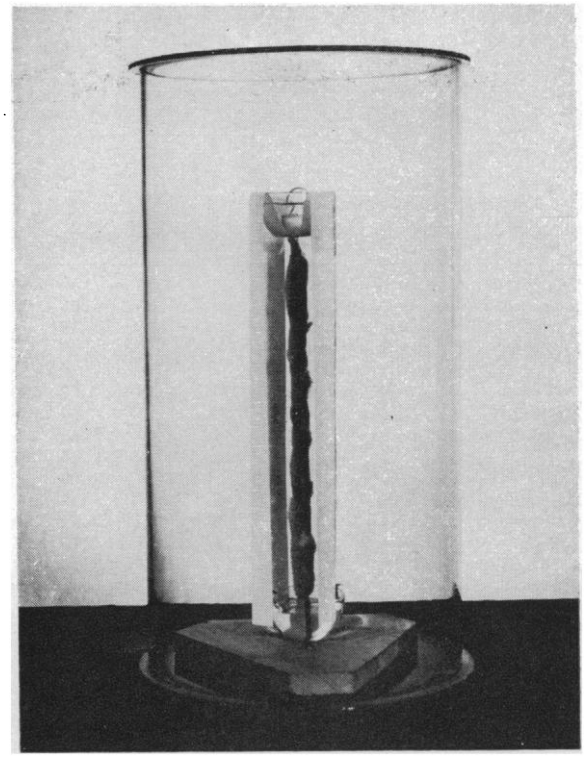

Fig. 1.

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Several forms of one-dimensional apparatus have been tried during the course of this study. The one which served best (Fig. 1) was a modification of an apparatus recently described by Winsten (*6*). Discarded gasoline pump cylinders, 12″ in diameter by 26″ in height and closed at each end by a ground glass plate, served as vapor chambers. For troughs, 3″ Pyrex evaporating dishes have been used in place of the Petri dishes recommended by Winsten. From four to six strips can be accommodated at one time in the upper dish. A small glass stopper was used to hold the end of the strip in the solvent, and the weight of the wet strip prevented its touching the walls of the trough. This eliminated the danger of capillary siphoning without recourse to any special supports to hold the paper away from the inner and outer walls of the trough. The lower dish was fastened to a wooden base by means of stainless steel clips.

TABLE 1

Pigment	Fluorescence	R_f values			
		Ethyl acetate	Chloroform	Phenol	n-Butanol acetic acid
Quercitrin	Brown	.36	.05	.50	.83
Isoquercitrin	Dark brown	.64	.05	.53	.62
Rutin	Orange-brown	.17	.08	.42	.34
Robinin	Pale brown (turns to yellow-orange)	.17	.14	.23	.78
Naringin	Blue-white	.53	.15	.84	.59
Xanthorhamnin	Brown	.11	.06	.42	.25
Rhamnetin	Yellow	.96	.96	.25 .68	.87
Homoeriodictyol	None	.97	.87	.95	.97
Quercetin	Yellow	.93	.05	.32	.80
Kaempferol	Yellow	.96	.12 to .17	.70	.95
D-Catechin	None	.87	.11	.36	.78

Chromatograms of the individual pigments were prepared, using phenol, chloroform, ethyl acetate, and n-butanol-acetic acid. The first three of these solvents were saturated with water before use, and water, saturated with the appropriate solvent, was used in the lower trough. The three-component system n-butanol-acetic acid-water (40–10–50 vol.%) was used as the fourth solvent in a similar manner.

Whatman No. 1 filter paper, 47 × 57 cm, was cut into strips, 2.5 cm × 56 cm, by means of a power-driven paper cutter. The strips were spotted 8 cm from one end with 12–18 μl containing 7–10 μg of flavonoid pigment, and then allowed to air-dry prior to development of the chromatogram. Development was allowed to proceed until the solvent had traveled 30–40 cm. This required 8–22 hours, depending upon the rate of movement of the individual solvent. The strips were then air-dried and the pigment zones located by their fluorescence in ultraviolet light. The solvent front could also be located by this method, due to the fluorescence of impurities in the paper which traveled with the solvent front.

In the case of homoeriodictyol and D-catechin, neither of which fluoresces in ultraviolet light, it was necessary to spray the strip with a chromogenic reagent in order to locate the pigment zones. D-Catechin was located by spraying the strip with ammoniacal silver nitrate solution. The treated strip was then washed with distilled water and allowed to dry. Homoeriodictyol was located by spraying the strip with alcoholic ferric chloride solution (1%). With ferric chloride solution, homoeriodictyol forms a red-brown spot which is visible on the strip in concentrations as low as 10 μg.

The ratio of distance traveled by the pigment to distance traveled by the solvent (R_f value) is given in Table 1. The fluorescence of each pigment on the filter strip has also been included. The fluorescence of robinin changed from a pale brown to a yellow-orange color during the development of the chromatogram. This

TABLE 2

Pigment	Na_2CO_3		Alcoholic $AlCl_3$		Boric acid-Citric acid		n-Lead acetate		Basic lead acetate	
	a*	b*	a	b	a	b	a	b	"a"	"b"
Quercitrin	YB	YB	Y	Y	Y	Y	YB	OY	Y	O
Rutin	Y	OY	Y	OY	Y	Y	Y	OB	Y	O
Robinin	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Naringin	..	..	..	..	..	..	..	BlW	..	GY
Xanthorhamnin	Y	Y	Y	Y	Y	Y	YB	OY	Y	O
Rhamnetin	Y	Y	Y	GY	Y	Y	B	Y	B	O
Homoeriodictyol	..	..	..	..	..	..	..	..	..	..
Quercetin	YB	OB	Y	GY	Y	Y	B	OB	B	O
Kaempferol	Y	Y	Y	GY	Y	Y	Y	GY	Y	Y
D-Catechin	RB	B	..	..	..	..	B	Blk	Y	..
Isoquercitrin	Y	YB	Y	Y	Y	Y	YB	OY	Y	O

* Column a—color in ordinary light; column b—fluorescence in ultraviolet light; Y—yellow; B—brown; O—orange; G—green; W—white; Bl—blue; Blk—black; R—red; . .—none.

may have been due to a trace of impurity initially present and removed as development proceeded. Rhamnetin gives two zones when chromatographed with phenol.

Mixtures of four to six pigments have been separated on a one-dimensional chromatogram strip by selecting the solvent giving the greatest differences in R_f values for the particular pigments. The R_f value is lowered slightly by the presence of other flavonoid pigments but the relative values appear constant.

The color of the pigment zones in ultraviolet light is an aid in identifying the respective pigments on the developed chromatogram. In addition, many of the usual qualitative color tests for flavones, flavonols, flavonones, and chalcones can be applied to the pigment zones on the strip. This is of advantage in determining whether a particular spot from a plant extract is of flavonoid character and, in addition, is also of value in the tentative identification of a particular flavonoid pigment. Such reagents include: sodium carbonate, ammonium hydroxide, alcoholic ferric chloride, alcoholic potassium hydroxide, alcoholic aluminum chloride, normal lead acetate, basic

lead acetate, ammoniacal silver nitrate, antimony pentachloride in carbon tetrachloride, and boric acid-citric acid in acetone. The reaction products obtained by use of basic lead acetate, normal lead acetate, alcoholic aluminum chloride, sodium carbonate, and the boric acid-citric acid reagent give an intense fluorescence in ultraviolet light and the characteristic color test in ordinary light. This property has been used to locate and identify pigment zones on the developed chromatogram strips. Table 2 lists the visible and fluorescent colors obtained with the latter reagents.

By paper partition chromatography, coupled with the use of chromogenic sprays, one can quickly and easily obtain information as to the presence of one or more flavonoid pigments in a small quantity of plant extract and even tentatively identify the individual flavones, provided the R_f values have been previously determined for a pure sample of the pigment in question. The method makes it possible to separate mixed crystals of two or more flavonoids and to identify microquantities of an isolated pigment by running mixtures of the unknown pigment with samples of known composition on the same strip.

While the present study was in progress, E. C. Bate-Smith (*1*) reported the successful separation of anthocyanin pigments by paper partition chromatography and suggested the possibility of separating flavonoid pigments from plant extracts by similar methods. Further work with plant extracts is now in progress in this laboratory and will be the subject of a more detailed report in the near future.

References

1. Bate-Smith, E. C. *Nature, Lond.*, 1948, **161**, 835.
2. Consden, R., Gordon, A. H., and Martin, J. P. *Biochem. J.*, 1944, **38**, 224.
3. Mager, A. *Z. physiol. Chem.*, 1942, **274**, 109.
4. Robeznicks, I. *Z. Vitaminforsch.*, 1938, **8**, 27.
5. Schoulties, Anna L., and Wender, Simon H. *Proc. Okla. Acad. Sci.*, 1947, **27**, 95.
6. Winsten, Walter A. *Science*, 1948, **107**, 605.

The Inhibitory Role of "Motor" Nerves

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It is well known that immersion of a nerve-muscle preparation in Ringer's solution, deficient in calcium, results in spontaneous activity which is evident in both the nerve and the muscle. The critical concentration of calcium for frogs' sciatic-biceps femoris preparations is approximately 1 millimolar, at which concentration the nerve commences to "fire" spontaneously. When the Ca^{++} concentration is further lowered, the muscle will show spontaneous fibrillation which persists *after* failure of indirect excitation.

We have shown that the failure of indirect excitation of such preparations is caused by a partial (or possibly complete) depolarization of nerve, as evidenced by a decrease in motor-axon resting potential. Restoration of calcium promptly restores the resting potential of the axon and indirect excitability of the muscle, at the same time arresting muscle fibrillation. When a comparison is made of the effects of degeneration of motor nerve following section with those produced by gradual withdrawal of calcium ions from the intact nerve-muscle preparation, the course of events is strikingly parallel. Shortly after nerve section the response of the muscle to indirect stimulation through the distal portion of the nerve is somewhat enhanced, as is the response of the muscle to intra-arterial injections of acetylcholine.

Both of these phenomena are observed in the nerve-muscle preparation when the calcium ion concentration is slightly lowered. As the distal portion of the cut nerve degenerates, indirect excitability of the muscle is lost, and the muscle shows greatly increased sensitivity to intra-arterial injection of acetylcholine. Again, both of these phenomena can be demonstrated in the nerve-muscle preparation when the calcium is lowered to the point where loss of polarization of the nerve is reached, accompanied by failure of indirect excitability. Many of the local anesthetics have been shown to prevent depolarization of axon membranes (*1*, *2*); therefore it was not surprising to find that a 1-millimolar solution of procainehydrochloride arrests fibrillation in an intact nerve-muscle preparation immersed in a calcium-free Ringer's solution. We have also shown that procaine, when added to calcium-free Ringer's solution, restores the resting potential of the immersed nerve. The cessation of muscle fibrillation accompanies this restoration and is not due to an effect of the procaine on the muscle itself because the intra-arterial injection of procaine (in concentrations sufficient to block indirect excitability in a *normal* muscle) into a *denervated* fibrillating muscle, failed to disturb its activity. It was also demonstrated that the intra-arterial injection of calcium-free solutions into denervated fibrillating muscles did not modify their activity. These findings indicate the validity of the hypothesis previously advanced by one of us concerning the inhibitory action of the normal polarized resting nerve upon muscle (*3*) and lend support to the belief that the polarized state of the terminal membrane of the normal motor axon, at rest, is inhibitory to the muscle.

Depolarization of the axon membrane or loss of the membrane following section and degeneration of the nerve results in spontaneous muscle activity. Thus passage of a "motor" impulse over a nerve does not, strictly speaking, stimulate a muscle to contract, but, as a result of the removal of the inhibiting effect exerted by the polarized end plate of the motor nerve axon, the muscle "automatically" contracts. Full details of these experiments will be published shortly.

References

1. Bennett, A. L., and Chinburg, K. G. *J. Pharm. exp. Therap.*, 1946, **88**, 72.
2. Bishop, George Holman. *J. cell. and Comp. Physiol.*, 1932, **1**, 177–94.
3. McIntyre, A. R. *Curare: its history, nature, and clinical use.* Chicago: Univ. Chicago Press, 1947. Pp. 290–211.