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HAEMANALYSIS OF TANNINS: THE CONCEPT OF RELATIVE ASTRINGENCY

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Abstract—The astringency of tannins, that is their efficiency as precipitants of proteins, is determined by their reaction with the proteins of haemolysed blood and colorimetric determination of residual haemoglobin. The method is accurate and sensitive because of the narrow range of concentration of tannin between that required to initiate precipitation (the threshold concentration) and that for complete precipitation. 'Relative astringency' (RA) is the ratio of the concentration of the tannic acid to that of the tannin which effects the same degree of precipitation. The RA of certain esters of hexahydroxydiphenic acid and of certain procyanidins of known constitution has been determined and the results applied to the ellagitannins and/or leucoanthocyanins present in species of *Shorea*, *Geranium* and other plants. The homology of each of these classes of tannins is discussed in the light of the results.

INTRODUCTION

FROM THE biological point of view, the importance of tannins in plants lies in their effectiveness as repellents to predators, whether animal or microbial. In either case the relevant property is 'astringency':[1,2] rendering the tissues unpalatable by precipitating salivary proteins or, by immobilizing enzymes, impeding the invasion of the tissues of the host by the parasite.

As regards the first of these effects, an obvious method of evaluation might seem to be the ability of an extract of the plant tissues to precipitate the mucoprotein of the saliva; but this is difficult to determine because the 'precipitate' is in the form of a gel which it is not easy to filter or centrifuge in a quantitative way. Other convenient protein solutions have, therefore, from time to time, been studied as test substances for the purpose, the foremost, of course, being gelatin, calf-skin or hide powder as employed by the leather chemists. The Official Method of the Society of Leather Trades Chemists[3] is, however, not only extremely laborious and demanding in regard to material, but the protein of skin, collagen, is far removed from the protein of saliva. It seemed better, therefore, to use a more appropriate source of protein, and with this aim milk protein and casein have been tested by several workers.[4] Kobert[5] and Gstirner[6] used the agglutination of blood corpuscles in rather elaborate methods. It seemed possible that haemoglobin in solution, as a chromoprotein, might have the especial advantage of being easily determined colorimetrically; but most commercial preparations are denatured and insoluble. There seemed no reason why haemolysed fresh blood should not be used, and its employment for the purpose is described in the present paper.

[1] BATE-SMITH E. C. (1954) *Food* **23**, 124.
[2] GOLDSTEIN, J. L. and SWAIN, T. (1963) *Phytochem.* **2**, 371.
[3] *Society of Leather Trades Chemists*, SL/2/3 (1965).
[4] (a) GSTIRNER, F. and KORF, G. (1966) *Arch. Pharm.* **299**, 763; (b) HANDLEY, W. R. C. (1961) *Plant Soil* **15**, 37.
[5] KOBERT, R. (1914) *Ber. Dtsch. Pharm. Ges.* **24**, 470.
[6] GSTIRNER, F., BOPP, A. and HOPMANN, H. (1956) *Arch. Pharm.* **289**, 188.

RESULTS

Desiderata for a Method of Determining the Protein-precipitating Ability of Extractable Plant Tannins

The first requirement is simplicity. The preparation both of an appropriate extract of the plant tissues and of the protein to be precipitated should be rapid and should only require equipment available in any botanical laboratory. The second requirement is adaptability to micro-quantities of material, since often only single leaves or seeds are available. The third requirement is that the determination itself should demand the minimum of specialized equipment.

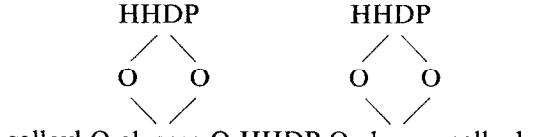
The method which has been worked out fulfils all these requirements. It is carried out with 1 ml of an aqueous extract containing the equivalent of 0·3–0·8 mg of tannic acid. The amount of blood required for four determinations (0·1 ml diluted to 5 ml) is obtained by pricking a finger as in the determination of blood sugar. The precipitated protein is centrifuged in an ordinary bench centrifuge and the depth of colour of the supernatant is measured and compared with that of the control.

Behaviour with Hydrolysable Tannins

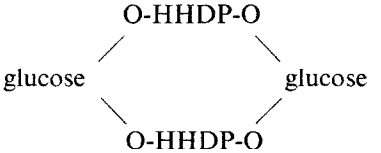
Under the above conditions, the precipitation of the blood proteins with tannic acid is linear with concentration in the final solution from a threshold value of 0·15 mg/ml of tannic acid up to 0·4 mg/ml at which concentration precipitation is complete. These values are reproducible from one experiment to another, providing freshly prepared solutions of tannic acid are used (0·1 % solutions slowly lose 'astringency' on standing at room temperature). The reaction is, therefore, very sensitive within this range, and it is desirable to include a fresh standard tannic acid solution in each series of experiments.

Apart from tannic acid, chebulagic acid was the only other constituent of hydrolysable tannins that has been available for detailed study. It behaved similarly to tannic acid, but its RA at 50% precipitation, was only 0·76.

With the limited amount of material available only exploratory determinations have been possible with other ellagitannins. These were some fractions of *Eucalyptus delegatensis* extracts supplied by Dr. W. E. Hillis, described by Seikel and Hillis[7] and used by the present author in work on the determination of ellagitannins.[8] Their RA was less than that of chebulagic acid, that of *D*3 and *D*6 being about 0·5, and that of pedunculagin (*D*2) and *D*13 about 0·25. There is no obvious connection between these values and what is known of their constitution. Pedunculagin is thought to be 2,3; 1,6-hexahydroxydiphenyl (HHDP)-glucose;[7] *D*3 possibly (I),[7] *D*6 (II),[8] and *D*13 a different type of ellagitannin, namely one with several glucose residues united to one HHDP group.[7]



(I)



(II)

Sufficient *D*13 was available for three determinations at different concentrations. The RAs at 23, 35 and 75% precipitation were, respectively, 0·24, 0·23 and 0·27.

[7] SEIKEL, M. K. and HILLIS, W. E. (1970) *Phytochem.* **9**, 1115.
[8] BATE-SMITH, E. C. (1972) *Phytochem.* **11**, 1153.

Behaviour with Condensed Tannins

Through the courtesy of Dr. E. Haslam, procyanidins of known constitution from avocado (*Persea gratissima*) seeds were available for examination. These are dimers, trimers, tetramers and higher oligomers originating from D-catechin and L-epicatechin.[9] They are readily soluble in water and form cyanidin, in amounts increasing with molecular size, when heated with butanolic HCl. Their relative astringencies are shown in Table 1.

TABLE 1. RELATIVE ASTRINGENCY AND *E* VALUES OF PROCYANIDIN FRACTIONS

Fraction	RA	LA *E* value*
Dimer B-2	0·10, 0·11	90–106
Mixed dimers B-1, B-3, B-4	0·085, 0·12	136
Mixed trimers D-1, D-2	0·23, 0·275, 0·33	170
Tetramer	0·35, 0·40	140–180
Higher oligomers	~0·50	200

* See Experimental: 1% solution in a 1 cm cell.

The values were less consistent than in the case of the hydrolysable tannins, and in the case of the tetramer and higher oligomers sometimes unreliable because the supernatant solutions were cloudy, The results show clearly, however, that the ability to precipitate protein increases regularly from MW 576 up to and beyond 1134. The above values were obtained with a slow-speed centrifuge (3000 rpm). With the higher oligomers more reliable results were obtained with a high-speed centrifuge (7000 rpm), RA 55, 56 and 56 being so obtained in three separate experiments.

DISCUSSION

Haemanalysis shows promise of being a useful tool in tannin analysis, not only as an indication of the probable effectiveness of the tannins as repellent constituents of foods and as inhibitors of digestive enzymes, but also as an indication of the molecular complexity of the tannins present in a particular tissue extract. A feature of the method is its accuracy, because the precipitation of the blood protein is so very sensitive between the threshold value and that of complete precipitation.

One of the main objects of this investigation was to obtain evidence with regard to the homology, or otherwise of the ellagitannins, on the one hand, and of the condensed tannins, on the other, as and when they occur in different kinds of plants. This is needed because theories have been made[12] regarding the phylogeny and taxonomy of the higher plants which depend on their presence or absence, on the assumption in the absence of evidence to the contrary, that they always have the same significance. The relative astringency is an index of the functional efficiency and molecular complexity of the tannins, and if the tannins from different sources have similar RAs, it is likely that they can be regarded as biologically related. This seems to be true at any rate of the ellagitannins; in all the species so far examined the RA is close to 0·4 (Table 2). There are, however, indications of systematic variability in the presence of precursors of the red reaction product with λ_{max} 530 nm. These

[9] THOMPSON, R. S., JACQUES, D., HASLAM, E. and TANNER, R. J. N. (1972) *J. Chem. Soc. Perkin Trans.* **I**, 1387.

[10] BATE-SMITH, E. C. (1972) *Phytochem.* **11**, 1755.

[11] ROSSI, J. A. and SINGLETON, V. L. (1966) *Am. J. Enol. Vitic.* **17**, 240.

[12] BATE-SMITH, E. C. (1972) *Nature, Lond.* **236**, 353; HARBORNE, J. B. (1972) *Rec. Adv. Phytochem.* **4**, 108.

appear to be of two kinds: one, present in *Geranium*, giving ellagic acid on hydrolysis; the other, present in *Psidium* and *Cornus*, associated with *O*-methylated derivatives of ellagic acids. These observations would be consistent with *O*-glycosylation in the former and *O*-methylation in the latter of the 3-OH group of HHDP. The evidence so far indicates that these are as functionally effective as tannins as the unsubstituted esters. The ellagitannins may, therefore, be regarded as homologous, and the dicotyledonous plants which contain them as phylogenetically related, until evidence to the contrary is forthcoming.

TABLE 2. APPLICATION TO SOME PARTICULAR CASES

Extract examined	Tannin % dry wt As HHDPG*	As LA†	RA
Ellagitannins only:			
Shorea gibbosa	33	0	0·39
Geranium lanuginosum Lam.	13·7	0	0·42
G. phaeum L.	1·3‡	0	0·53
	1·8	0	0·37
Condensed tannins only:			
Crataegus oxyacantha L. 'Pauli'	0	13·0	0·79
C. monogyna Jacq.§	0	3·9	0·33
Hedysarum multijugum Maxim.	0‖	10·0	0·30
	0	11·7	0·45
Both ellagitannins and condensed tannins:			
Rubus idaeus L.	4·0	1·5	0·42
Psidium guayava L.	7·0	11·3	0·65
Shorea macrophylla	2·6	0·4	1·2
Geranium platypetalum Fisch. et May	13·5	1·2	0·48

* HHDPG determined as described previously.[8]

† LA determined by heating in BuOH–HCl. $E^{1\%}_{1\,cm}$ is taken as 150 on basis of results with standard compounds (see Table 1).

‡ The two values quoted are for two sources which, while showing no differences botanically, differed in their response to the nitrite reaction, one giving a red reaction product max 530 nm in addition to the usual blue product. A similar red product is given by members of the Myrtiflorae (e.g. *Psidium guayava*) which are known to contain *O*-methyl ethers of HHDPG.[13] In these cases the ethers are present in the hydrolysates, but in *G. phaeum* only ellagic acid is present. It seems likely that in this case the red product is due to an *O*-glycoside of HHDPG.

§ The extraction of LAs from plant tissues by any of the solvents customarily employed is incomplete and variable. In the case of *C. monogyna* extraction was exceptionally low because of gel formation when the tissue was macerated with 50% MeOH, and the tannin was probably not representative of that actually present.

‖ Young leaves. Other herbaceous Leguminosae (which only rarely contain tannin) give RA up to 1·1. These (and also *Psidium guayava*) contain both LCy and LD.

With the leucoanthocyanins this appears to be less likely. In the first place, there is the systematically significant distinction between those yielding cyanidin and those yielding delphinidin when heated with mineral acid. In the second place, it is now recognized that there are two, if not more, kinds of leucoanthocyanins: those based on flavan-3,4-diols;

[13] LOWRY, J. B. (1968) *Phytochem.* **7**, 1803.

and flavan-3-ol dimers and higher oligomers, distinguished by Weinges *et al.*[14] as leucoanthocyanidins and procyanidins, respectively. The former appear to be the less common, and may, in fact, be represented only by the 5-deoxy series exemplified by leucofisetinidin, and the 3-deoxy series exemplified by leucoluteolinidin (luteoforol). There are, however, differences in behaviour on heating with mineral acid which suggest that the LAs present in aquatic monocotyledons such as *Pontaderia* are different in nature from those commonly present in terrestrial vascular plants[15] and it is not difficult to accept that these are systematic differences which it might be profitable to explore. As regards the monocotyledons, it has just been reported[16] for the first time that D-epicatechin occurs naturally in certain members of the Palmae—all reports hitherto having been of the L-epimer. The accompanying LAs are procyanidins, but, as would be expected, they differ from those described by Weinges *et al.*,[14] which are derived from D-catechin and L-epicatechin.

As regards the method itself, there are several points that have to be made. (a) The method does not at present provide specifically for gallotannins, such as tannic acid itself. These do not occur so frequently as ellagitannins and leucoanthocyanins, but are common in certain families such as the Fagaceae, those in the Myrtiflorae, Polygonaceae, Betulaceae and Cornaceae; and sporadically elsewhere in the dictoyledons. Gallotannins will contribute to the astringency of the tissues, and will increase the overall RA of extracts when referred to the HHDPG and LA analytical data. Their presence is readily detected in acid hydrolysates by the behaviour of gallic acid in Forestal solvent: R_f 0·65, appearing dark in UV, and blackening when exposed to NH_3. (b) The method has so far only been applied to tissue extracts, and it is sometimes difficult to prepare these because of viscous gel formation (cf. the case of *Crataegus monogyna* cited above; *Nyssa sylvatica* provided an even more difficult example). It may in fact be impossible to extract the tannin, as in the case of *Onobrychis viciifolia* also cited above, although it is known that the tannins are functionally effective in impeding digestion. It is possible that the method may be applicable to finely-milled suspensions of the dried tissues. (c) The fact that there is a threshold value before any precipitation occurs is extremely important from the point of view of methodology and interpretation. A concentrated solution of tannin added to the protein will cause precipitation even though the ultimate concentration is below threshold; the tannin must therefore be diluted before injection into the protein. If this is done, the results are completely reproducible and stoichiometrical.

The existence of a threshold value also accounts for the subjective experience of astringency in the mouth. At concentrations lower than that at which the 'puckery' sensation is experienced, there is a sensation of 'substance' or 'body' in the item—e.g. a wine or fruit—being tasted. The little further increase before complete precipitation of the protein is achieved accounts for the subtle balance between too much and too little astringency in tannin-containing foodstuffs. This point is especially well exemplified in the ageing of wines and the ripening of fruits (see Goldstein and Swain[2]).

EXPERIMENTAL

The tannic acid used as a standard contained 8% H_2O. This sample gave no reaction for ellagitannin with HNO_2.

[14] WEINGES, K., BAHR, W., EBERT, W., GORITZ, K. and MARX, H.-D. (1969) *Fortschr. Chem. Org. Naturstoffe* **27**, 158.

[15] BATE-SMITH, E. C. and SWAIN, T. (1965) *Lloydia* **28**, 313.

[16] DELLE MONARCHE, F., FERRARI, F., POCE-TUCCI, A. and MARINI-BETTOLO, G. B. (1972) *Phytochem.* **11**, 2333.

Preparation of extract. Leaf material was extracted with 50% MeOH as described previously,[10] the extract concentrated *in vacuo*, and the residue taken up in the minimum vol. H_2O.

Determination of ellagitannin. This was determined as described previously.[8,10]

Determination of leucoanthocyanin. 0·5 ml of extract is heated with 3·0 ml. 5% *n*-Butanolic HCl for 2 hr at 90–95°. The absorbance of the anthocyanidin formed is measured at 550 nm in the case of cyanidin, at 565 nm in the case of delphinidin. In the absence of precise information with regard to the degree of polymerization of the LA, the value $E^{1\%}_{1cm}$ is assumed to be 150.

Determination of astringency. 0·1 ml of blood is drawn into a graduated capillary pipette from a fingertip (as in the determination of blood sugar) and added into 5 ml H_2O. 1·0 ml of the extract is injected with a hypodermic syringe into 1·0 ml of the haemolysed blood and the mixture immediately shaken. If precipitation is complete at the lowest dilution, the extract is diluted further as appropriate. The mixture is centrifuged for 5 min at 3000 or 7000 rpm. Haemoglobin has two spectral peaks, 578 and 541 nm; the former is used because it is less likely to be affected by irrelevant absorption. The absorptivity of the supernatant is then compared with that of a suitable control.

Drawing of blood samples. The first, or middle, finger is rinsed with Et_2O and a tourniquet applied immediately above its junction with the palm. A prick is made with a sterilised, preferably triangular section needle at the base of the nail, the finger is bent hard, and the blood allowed to flow by capillarity into a clean pipette. The treatment with Et_2O has the triple action of sterilizing the skin, stimulating the capillary circulation, and preventing coagulation of the blood.

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