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IN VITRO INHIBITION OF PHENOLSULPHOTRANSFERASE BY FOOD AND DRINK CONSTITUENTS

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Abstract—Several natural and synthetic food and drink constituents were tested *in vitro* for their inhibitory actions on phenolsulphotransferase P and M (PST P, PST M) and monoamine oxidase A and B (MAO A, MAO B). Cyanidin 3-rutinoside, a simple anthocyanin, (+)-catechin, a flavanol, and carmoisine, a synthetic food colorant, were found to be particularly potent, reversible inhibitors of PST P. All inhibited this enzyme by 100% at a concentration of 5 μM and had an IC_{50} in the μM range. The effects of these compounds on PST M and MAO A and B were less pronounced. There was a considerable difference in the inhibitory ability of different purified anthocyanins but all were selective for PST P. Several other phenolic food colorants were also found to be specific inhibitors of PST P, though less potent in their actions. Tartrazine, a non-phenolic food colorant, had little effect. The phenolic extracts from two red wines were also found selectively to inhibit PST P *in vitro*, suggesting that it is within this fraction that these inhibitors are to be found.

PST is an important enzyme involved in the inactivation of a wide range of exogenous and endogenous phenols. If such a degree of inhibition were to occur *in vivo*, potentially toxic concentrations of some phenolic substrates might result.

Phenolsulphotransferase (PST) (EC 2.8.2.1) inactivates a wide range of exogenous and endogenous phenols by catalysing their conjugation with sulphate [1]. In man, the enzyme exists in two forms [2, 3]: PST M acts preferentially on monoamines and their metabolites [4], and PST P conjugates low concentrations of phenol and certain exogenous phenolic compounds. Some drugs, such as paracetamol, salicylamide and, indeed, phenol itself, depending on concentration, are substrates for both [5]. Both forms of the enzyme are particularly active in the intestinal wall but are widespread in the body, including the platelet, adrenal gland [3, 6, 7] and placenta [8]. Activities are low in human brain, and sulphate conjugates of catecholamines and their metabolites only represent a small proportion of total metabolite concentration at this site. Sulphoconjugation thus appears to be more important peripherally than centrally in man.

Certain alcoholic drinks contain potent inhibitors of PST, particularly red wine [9]. Ethyl acetate extracts of red wine inhibit PST P by 90–100% at a dilution of 1/75, and PST M by 100% at a dilution of 1/10. In this study, we have attempted to identify the constituents of red wine that inhibit PST so potently and examined the action of various other food and drink constituents on PST activity. As an index of specificity, we have also examined the effect of these substances on monoamine oxidase (MAO), another enzyme important in the metabolism of amines.

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MATERIALS AND METHODS

Materials. [^{35}S]-3′-phosphoadenosine 5′-phosphosulphate (PAPS) and [^{14}C]-tyramine were obtained from New England Nuclear Corp., Boston, MA. Non-radioactive PAPS was purchased from PL Biochemicals Inc., Milwaukee, WI, tyramine hydrochloride from Sigma Chemical Co., Poole, Dorset, U.K., phenol from BDH Chemical Ltd., Poole, Dorset, U.K., and Instagel from Packard Instrument Co., Reading, U.K. Many of the samples tested were kindly donated by Dr C. F. Timberlake (Long Ashton Research Station, University of Bristol). The simple and acylated anthocyanins were extracted from leaves, fruits and flowers with a methanolic-acid solvent, concentrated and subjected to repeated preparative paper chromatography in several solvent systems. The compounds were defined as being chromatographically pure when they ran as a single spot or band and were found to be free of other u.v. and visible absorbing components. The "crude" samples were extracted with a methanolic-acid solvent as above, but separated by reverse-phase high performance liquid chromatography. While enriched with a mixture of anthocyanins, these samples contain other components derived from the original material.

Methods. Samples of food and drink constituents of known molecular weight were prepared as solutions with 0.01 M potassium phosphate buffer, pH 7.4, to result in final concentrations in the assays of 25, 5 and 1 μM. Crude anthocyanin extracts and samples of mixed anthocyanins were used at final assay concentrations of 10, 2 and 0.4 mg/l. Natural

fruit juices were adjusted to between pH 7.14 and 7.35 with 0.2 M potassium hydroxide. Platelet suspensions in sucrose were prepared as described previously [10] using blood obtained from four healthy volunteers. The individual platelet fractions were pooled and stored at −20° prior to use. PST was assayed using a modification [11] of the method of Foldes and Meek [12]. Phenol (final concentration 10 μM) was used as substrate for measuring PST P, and tyramine (final concentration 20 μM) for PST M. The incubation mixture contained 100 μl of 0.01 M potassium phosphate buffer (pH 7.4), 20 μl of the pooled platelet preparation, 20 μl of substrate, and 20 μl of the food or drink constituent (final concentrations 25, 5 and 1 μM for individual compounds and 10, 2 and 0.4 mg/l for mixtures); 20 μl [^{35}S]-PAPS (final concentration 0.66 μM) was added at successive intervals to tubes incubated at 37° in a water bath, and the reaction was terminated after 10 min by the addition of 200 μl of 0.1 M barium acetate.

A control containing buffer instead of the sample of food or drink constituent was used in each assay, except for the fruit juices which were replaced by a solution of potassium hydroxide diluted to the same concentration as in the sample and adjusted to the same pH using 2 M citric acid. Blanks, where phenol or tyramine had been replaced by 20 μl water, were also included. Substrate was removed by two precipitations with barium sulphate [12] and the [^{35}S]-labelled product was measured in a Packard "Tricarb" liquid scintillation counter after addition of 5 ml of "Instagel".

Inhibition of PST by the food and drink constituents was also assayed using human placenta as an alternative enzyme source. Fresh human placenta (10% w/v) was homogenised in ice-cold 0.01 M potassium phosphate buffer (pH 7.4) using an Ultra-Turrax homogeniser, and centrifuged at 10,000 *g* for 20 min. Aliquots of the final supernatant were stored at −20° prior to enzyme assay. PST P and M were assayed as described above, using 25 μl of the final supernatant in place of the platelet preparation.

Monoamine oxidase inhibition was measured using a standard radioenzymatic microassay [10, 13]. The enzyme source for MAO-A was human placental homogenate (final concentration 1% w/v) and for MAO-B pooled human platelets from four individuals, prepared as described above. The incubation mixture contained 120 μl 0.1 M phosphate buffer (pH 7.4), 20 μl of [^{14}C]-tyramine (final concentration 50 μM), 20 μl of enzyme preparation and 20 μl of the food or drink constituent in solution (final concentration 25 μM or 10 mg/l). A control containing 0.01 M potassium phosphate buffer, pH 7.4, instead of the samples was included, as well as an enzyme blank.

All assays were carried out twice, in duplicate.

Equilibrium dialysis was used to assess the reversibility of three of the compounds found to be inhibitors of PST P; 200 μl each of platelet preparation and inhibitor, either carmoisine (E122), (+)-catechin hydrate, or cyanidin 3-rutinoside (the former at a final concentration of 2 μM and the other two at a final concentration of 20 μM), or buffer as control, were dialysed overnight at 4° in one litre of 0.01 M potassium phosphate buffer, pH 7.4. Duplicate preparations were left undialysed at 4° overnight. Aliquots from each mixture were assayed for PST P activity using phenol as substrate (final concentration, 10 μM). Dialysis was carried out twice, in duplicate, for each inhibitor.

RESULTS

Table 1 shows percentage inhibition of both forms of PST and MAO by certain crude anthocyanin extracts and pure anthocyanins. The phenolic constituents extracted from two red wines (Nos 1 and 2) and elderberries (No. 3) were found to be potent and selective inhibitors of PST P and this selectivity of inhibition was apparent with all the anthocyanins tested. However, there was a considerable difference in the inhibitory ability of the different purified anthocyanins, cyanidin 3-rutinoside being the most potent.

The catechins were also found to be potent inhibitors of PST (Table 1). As with the anthocyanins, the two compounds tested belonging to this group, (+)-catechin and (−)-epicatechin, were more active against the P-form of the enzyme, inhibiting PST M by 64% and 37% respectively at a final concentration of 25 μM, and PST P by 100% and 63% at a final concentration of only 5 μM.

Eight synthetic food colorants were tested and several of these were also found to be powerful inhibitors of PST P (Table 2). Carmoisine (E122) was the most potent and gave 100% inhibition of PST P at a final concentration of 5 μM. Erythrosine (E127) was the least selective and inhibited PST M by 100% and MAO B by more than 60% at a final concentration of 25 μM, in addition to its action on PST P.

Using placenta as an alternative human enzyme source for PST P and M, the same pattern of inhibition of these enzymes was seen with all the food and drink constituents tested.

Of the natural fruit juices examined (Table 3), pineapple juice showed almost no inhibition of PST, while apple juice selectively inhibited PST P by 86% at a final dilution of 1 in 14. At this concentration, orange juice increased the activity of PST when phenol was used as substrate compared with the control value, and thus seems to contain a substrate, presumably for PST M, rather than an inhibitor. Grape juice, with added "natural" colouring and preservatives, inhibited PST P by 100% and PST M by 71% at a dilution of 1 in 14.

Equilibrium dialysis showed that inhibition of PST P by carmoisine, (+)-catechin and cyanidin 3-rutinoside was reversible in each case. Less than 1% of the original inhibitory effect on PST P by catechin, 23% of that by cyanidin 3-rutinoside and 7% of that by carmoisine remained after overnight dialysis.

DISCUSSION

This study has shown that several natural and synthetic food and drink constituents are potent and specific inhibitors of PST P. Particularly potent in their inhibitory actions on this enzyme are cyanidin 3-rutinoside, (+)-catechin, carmoisine, erythrosine

Table 1. % Inhibition of phenolsulphotransferase (PST) and monoamine oxidase (MAO) activities by phenolic compounds

	Final concentration (mg/l) of phenolic compounds					
	10	2	0.4	10		
	PST P			PST M	MAO A	MAO B
Crude phenolic extracts						
1. Red wine 1	100	27	16	10	29	0
2. Red wine 2	100	43	10	17	14	1
Crude anthocyanin extracts						
3. Elderberry	100	61	23	4	7	0
4. Chicory leaf (largely cyanidin 3-glucoside acylated with malonic acid)	100	30	17	9	34	1
5. Red cabbage	33	24	18	0	13	11
Pure anthocyanins – acylated						
6. Mixed ternatins	15	7	5	0	11	7
7. Mixed acylated anthocyanins (coumaryl and caffeyl derivatives)	45	26	15	0	11	0
	Final concentration (μM) of phenolic compounds					
	25	5	1	25		
	PST P			PST M	MAO A	MAO B
8. Malvidin 3-p-coumaryl glucoside	44	11	12	0	0	0
Pure anthocyanins – simple						
9. Cyanidin 3-rutinoside	100	100	41	9	0	0
10. Peonidin 3-glucoside	54	16	17	0	0	5
11. Malvidin 3-glucoside	50	19	2	0	26	0
12. Cyanidin 3,5-diglucoside	48	24	20	0	0	0
13. Pelargonidin 3,5-diglucoside	40	16	13	0	18	4
14. Peonidin 3,5-diglucoside	36	15	8	0	9	11
15. Pelargonidin 3-rutinoside	12	0	0	3	0	1
Catechins						
16. (+)-Catechin	100	100	38	64	11	12
17. (−)-Epicatechin	100	63	26	37	26	7

All results expressed as mean values of two experiments carried out in duplicate, on a pooled human platelet preparation, as described in the Methods section.

Table 2. % Inhibition of phenolsulphotransferase (PST) and monoamine oxidase (MAO) by synthetic food colorants

Food colorant		Final concentration (μM) of food colorants					
		25	5	1	25		
		PST P			PST M	MAO A	MAO B
1. Carmoisine	E122	100	100	74	3	0	0
2. Erythrosine	E127	100	94	9	100	25	63
3. Amaranth	E123	100	91	27	3	0	13
4. Ponceau 4R	E124	100	39	11	1	0	7
5. Sunset Yellow	E110	100	55	17	0	0	6
6. Indigo Carmine	E132	80	13	17	2	0	0
7. Green S.	E142	65	27	0	34	0	5
8. Tartrazine	E102	6	0	0	0	0	0

All results expressed as mean values of two experiments carried out in duplicate, on a pooled human platelet preparation, as described in the Methods section.

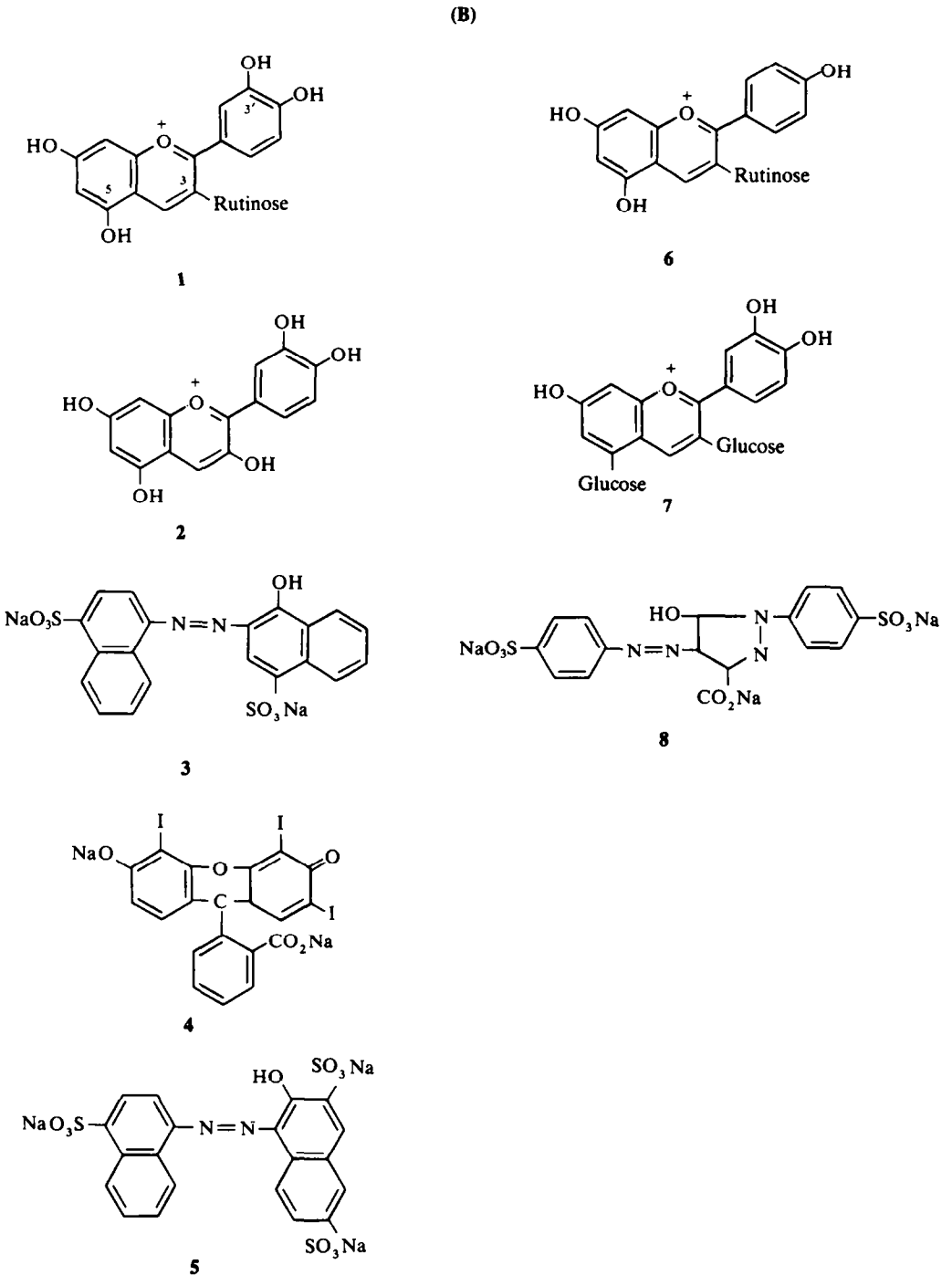

Fig. 1. Molecular structures found to inhibit PST P by 90–100% at a concentration of 5 μM (A) and those found to inhibit PST P by less than 25% at this concentration (B). (1) Cyanidin 3-rutinoside; (2) (+)-catechin; (3) carmoisine (E122); (4) erythrosine (E127); (5) amaranth (E123); (6) pelargonidin 3-rutinoside; (7) cyanidin 3,5-diglucoside; (8) tartrazine (E102).

and amaranth, all of which inhibited PST P by 90–100% at a final concentration of 5 μM and had an IC_{50} in the μM range. The structures of these compounds are shown in Fig. 1.

Cyanidin 3-rutinoside, a simple anthocyanin, and (+)-catechin, a flavanol, are closely related, both belonging to subgroups of the C15 group of compounds known as flavonoids [14]. Not all anthocyanins are as potent as cyanidin 3-rutinoside in their inhibitory actions on PST P. In particular, pelargonidin 3-rutinoside showed negligible inhibition of this enzyme. As can be seen in Fig. 1, this anthocyanin only differs from cyanidin 3-rutinoside in having one hydroxy group less at the 3′ position.

Table 3. % Inhibition of PST P and PST M by fruit juices (final dilution of original 1/14)

	% Inhibition	
	PST P	PST M
Natural fruit juices		
Orange	0	5
Pineapple	0	17
Apple	86	7
Grape juice (added "natural" colorant and preservative)	100	71

Cyanidin 3,5-diglucoside, only a weak inhibitor of PST P, differs from the 3-rutinoside by two glucose molecules occupying positions 3 and 5. It thus may be that, for a flavonoid to be an inhibitor of PST P, it must possess an hydroxy group at the 3′ position, while a glucose molecule at the 5 position results in steric hindrance.

Carmoisine and amaranth are both red azo dyes, and erythrosine is a red xanthine derivative. As can be seen from Fig. 1, they all contain phenolic groups, and this probably causes them to bind to the active site of PST P. Three of the other synthetic food colorants which showed intermediate inhibitory effects on PST P, sunset yellow, ponceau 4R, and green S also contain phenolic groups. Indigo carmine, similarly of intermediate inhibitory potency, does not contain any phenolic group. However, a tautomeric form of this dye, with a hydroxyl group in place of a keto-group (by enolisation) might mimic a phenol. Tartrazine, an azo dye containing no phenolic groups, did not inhibit PST P to a significant extent (Fig. 1).

The finding that the phenolic constituents extracted from two red wines (samples 1 and 2, Table 1) selectively and potently inhibited PST P *in vitro* confirms and extends our earlier observations [9], and suggests that the inhibitors of PST P are to be found within this group of compounds. When red wine is made, the grape juice is left in contact with the skins and pips for several days after crushing, whereas it is separated immediately during white wine manufacture. Large amounts of phenolic flavonoids are leached out during the red wine making process, including catechins and anthocyanins, the latter giving the wine its colour. Whereas red wine typically contains 1200 mg/litre of flavonoids, only about 50 mg/litre are present in white wine. In an average red wine, anthocyanins comprise 10% of the flavonoid component and catechins 20% (a mixture of (+)-catechin and (−)-epicatechin) while most of the remainder is made up of large polymeric anthocyanogens [15]. Clearly, the catechins are potent inhibitors of PST P and are therefore likely to contribute to the *in vitro* inhibition of this enzyme by red wine. Some of the anthocyanins are also inhibitors of PST P and may be involved to some extent, but the most potent detected so far, cyanidin 3-rutinoside, is not present in grapes.

With much confectionery, food and beverages containing synthetic food colorants, many children consume large amounts of these substances; there is some evidence that they can exacerbate behavioural problems in susceptible children [16, 17]. Such an effect, at least from the red colours, might relate to their inhibitory actions on PST, resulting in a decrease in conjugated and a consequent increase in circulating free phenols, such as p-cresol, a substrate for the P-form of the enzyme [18–21]. It is important to note, however, that some fruit juices are also quite potent inhibitors of PST P *in vitro*, including "natural" apple juice, without added colorant or preservative.

Substantial interindividual variation in platelet PST P activity has been noted and activity at this site reflects that in other parts of the body [22–24]. It is possible that individuals with low values are more susceptible to the consequences of PST P inhibition, and it is thus of interest that patients with dietary migraine appear to have significantly lower mean levels of platelet PST P activity than non-dietary controls [25]. However, whether red wine, its constituent flavonoids or the synthetic food colours inhibit PST P *in vivo* will depend on the extent to which they are absorbed. The polymeric anthocyanogens are thought not to be [26] but the catechins and anthocyanins can be taken up from the gastrointestinal tract in man [14]. It is thus now important to determine whether compounds which inhibit PST so potently *in vitro* have a similar action *in vivo*.

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