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HUMAN PLATELET PHENOLSULPHOTRANSFERASE M AND P: SUBSTRATE SPECIFICITIES AND CORRELATION WITH *IN VIVO* SULPHOCONJUGATION OF PARACETAMOL AND SALICYLAMIDE

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1 Human platelet phenolsulphotransferase exists in two functional forms, M and P. In this study the substrate specificity of the two forms has been further delineated by correlating activities in different individuals with various substrates. *m*-Tyramine, noradrenaline, adrenaline, 5-hydroxytryptamine, *p*-hydroxyamphetamine, isoprenaline, salbutamol and l-naphthol were all specific substrates for the M form of the enzyme.

2 Paracetamol, a mixed substrate, was predominantly metabolized by the M form. Salicylamide at 5 μM was a substrate for the P form but became an M substrate at higher concentration. Phenol itself, a specific substrate for phenolsulphotransferase P at 10 μM, also became an M substrate at 1 mM concentration. These substrate specificities were confirmed with the selective inhibitor, dichloronitrophenol.

3 In this study, we measured phenolsulphotransferase activity in platelets from 13 individuals selected on the basis of their wide variation in ability to sulphoconjugate paracetamol and salicylamide *in vivo*. There was no significant relationship between the *in vivo* pattern with either drug and the activity of platelet phenolsulphotransferase assayed with paracetamol or salicylamide respectively.

Introduction

Sulphate conjugation, catalyzed by phenolsulphotransferase (PST) (EC.2.8.2.1), is an important metabolic pathway, not only for phenolic monoamines and some of their metabolites but also for phenol and some phenolic drugs, including paracetamol and salicylamide (Williams, 1959). Although there is a great deal of inter-individual variation in ability to form both the glucuronide and sulphate conjugates of these drugs, some individuals are relatively unable to form the sulphate conjugate of one or other (Caldwell *et al.*, 1982). One possible reason for this finding is a decrease in PST activity. To investigate the possibility, we have measured the enzyme, using paracetamol and salicylamide as substrates, in platelets from a group of normal volunteers selected on the basis of their *in vivo* conjugation of these drugs.

Human platelet PST (Hart *et al.*, 1979; Anderson & Weinshilboum, 1980; Rein *et al.*, 1981) exists in two functional forms, PST P (specific for 30 μM phenol) and PST M (specific for the monoamines, dopamine and tyramine) (Rein *et al.*, 1982). These two forms differ in substrate specificity, tissue distribution and inhibitor sensitivity, the P form being selectively inhibited by 10^{-6}M 2,6-dichloro-4-nitrophenol (DCNP) (Rein *et al.*, 1982); they appear to be under separate control (Bonham Carter *et al.*, 1981). To determine which forms of the enzyme metabolize paracetamol and salicylamide, we have examined the effect of DCNP on platelet PST activity using these drugs as substrates. We have confirmed our findings by correlating individual platelet PST activity values from the group of normal volunteers, using paracetamol and salicylamide as substrates, with those

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obtained using *p*-tyramine (for PST M) and phenol (for PST P) as substrates. There is a high degree of correlation between activities in different individuals with different substrates acting at the same active site (Bonham Carter *et al.*, 1981). We have also tested eight other phenolic compounds in this way in order to characterize the substrate specificities of PST M and P further.

The efficiency of the assay procedure was assessed for each of the different substrates used as there is evidence that it can vary considerably with different phenolic acceptors (Foldes & Meek, 1973; Baranczyk-Kuzma *et al.*, 1981).

Methods

Materials

Materials for PST assay were essentially the same as previously described (Bonham Carter *et al.*, 1981) except for the following substrates: paracetamol (*N*-acetyl-*p*-aminophenol), salicylamide, 5-hydroxytryptamine creatinine sulphate complex (5-HT), adrenaline (free base) and noradrenaline (free base), which were all purchased from Sigma Chemical Co., Poole, Dorset, U.K. l-Naphthol was purchased from Hopkin & Williams, Chadwell Heath, Essex, U.K. *p*-Hydroxyamphetamine hydrobromide was a gift from Smith Kline & French Laboratories Ltd, Welwyn Garden City, Herts, U.K., isoprenaline hydrochloride was a gift from Pharmax Ltd, Bexley, Kent, U.K., salbutamol sulphate was a gift from Allen & Hanburys Ltd, Bethnal Green, London, E.2, U.K. and *m*-tyramine was a gift from Roche Products Ltd, Welwyn Garden City, Herts, U.K. (Ro 4-4657). DCNP was obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, U.K.

Sample collection and platelet preparation

Venous blood samples (10 ml) were collected from 13 normal volunteers and platelets prepared on the same day, as previously described (Bonham Carter *et al.*, 1981); the platelet pellet was washed with isotonic sucrose (1 ml/10 ml blood), resuspended in phosphate buffer (10 μM, pH 7.4; 1 ml/10 ml blood), divided into aliquots as required and stored deep frozen (−20°C). Care was taken not to allow plasma or platelet suspensions to heat up during centrifugation as PST M is thermolabile above 30°C (Bonham Carter *et al.*, 1981). The amount of glucuronide and sulphate conjugates of paracetamol or salicylamide excreted after administration of 1 g of either of these drugs had previously been estimated in 11 of the subjects for paracetamol and 9 of the subjects for salicylamide (Caldwell *et al.*, 1982).

PST assay

Platelet PST activities were measured by the same method as previously described (Bonham Carter *et al.*, 1981) based on that of Foldes & Meek (1973). A solution of ^{35}S-3′-phosphoadenosine-5′-phosphosulphate (PAPS) and non-radioactive PAPS in water was employed as sulphate donor (final concentration in incubation mixture, 0.6 μM). 10 μl (containing 18–35 μg protein) of platelet suspension was used with the following substrates: *p*-tyramine, *m*-tyramine, noradrenaline, adrenaline, 5-HT, *p*-hydroxyamphetamine, isoprenaline, salbutamol and salicylamide, and 20 μl (containing 35–70 μg protein) with the remaining substrates: phenol, paracetamol and salicylamide, at low concentration. Less than 20% of the PAPS was consumed during the assay.

Efficiency studies

In this method, excess (unreacted) PAPS is removed by 2 successive precipitations with barium hydroxide (200 μl, 0.1 M) and zinc sulphate (200 μl, 0.1 M). Foldes & Meek (1973) have demonstrated that this procedure removes at least 99.5% of unreacted PAPS in control experiments in the absence of enzyme and we have confirmed this. Any counts above a water blank level that remain in the supernatant after 2 precipitations in an assay are likely to be due to products of the PST enzyme reaction, i.e. sulphate esters. In order to assess the efficiency for different substrates, we carried out assays using the same batch of platelets and a range of phenolic acceptors with 6 tubes for each substrate. In each case, both supernatant and precipitate were counted, in duplicate for each substrate, after 1 precipitation, after 2 precipitations and after 3 precipitations. Blanks containing platelets, but water instead of acceptor substrate, were treated in the same way, and these counts were subtracted from those from the corresponding tubes containing acceptor substrate. In this way, the percentage radioactivity remaining in the third supernatant and that precipitated were calculated giving an indication of the solubility of the sulphate conjugates for each substrate.

K_m *determinations*

Human platelet PST activity was measured using different concentrations of the substrates for which apparent K_m values were not already known. The range of substrate concentrations used were as follows: for salicylamide 1–180 μM; for paracetamol 100–800 μM, for *m*-tyramine, *p*-hydroxyamphetamine and isoprenaline 1 μM – 1 mM. Apparent K_m values were calculated by the direct linear plot method (Eisenthal & Cornish-Bowden, 1974). Apparent K_m values for most of the remaining sub-

strates used have previously been reported (Rein *et al.*, 1981; Bonham Carter *et al.*, 1981; R. Sodha, personal communication).

Inhibitor studies

The effect of the selective inhibitor, DCNP, on platelet PST activities using several acceptor substrates was examined using different concentrations of DCNP as described previously (Rein *et al.*, 1982). PST assay conditions were as described above with the following acceptor substrate concentrations: *p*-tyramine, 30 μM; phenol, 30 μM and 1 mM; paracetamol, 850 μM and salicylamide, 5 μM.

Protein estimation

Protein concentrations of platelet suspensions were measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Results

Efficiency of the assay using different substrates

This assay procedure relies on removing excess PAPS by precipitating twice with barium hydroxide and zinc sulphate without precipitating the sulphate esters of the phenolic acceptors, i.e. the products of the reaction. In order to assess the efficiency of the assay with the substrates used, a third precipitation with barium hydroxide and zinc sulphate was carried out as described in the **Methods** section. The recovery in the third supernatant was 90% or more for *p*-tyramine, *m*-tyramine, 5-HT, *p*-hydroxyamphetamine, salbutamol, paracetamol, l-naphthol and phenol, and 80% or more for salicylamide and isoprenaline. However, for the catecholamines, adrenaline and noradrenaline, it was much less, 68% for adrenaline and only 48% for noradrenaline.

Apparent K_m *values*

The apparent K_m values found were as follows: *m*-tyramine, 5 μM; *p*-hydroxyamphetamine, 30 μM; isoprenaline, 2.5 μM; paracetamol, 850 μM and salicylamide, 5 μM. An apparent K_m of 7 μM has previously been reported for phenol (Rein *et al.*, 1981). In this study, by using a higher phenol concentration range a second, higher, apparent K_m of 560 μM was found.

Activity of platelet PST with different substrates

The mean specific activity of platelet PST from the 13 subjects, using the range of acceptor substrates described, is given in Table 1. The concentrations of acceptor substrates at which the specific activities were measured are also shown in Table 1. They were, in general, used at approximately their apparent K_m and in some cases at one-tenth, 10 times or one hundred times K_m, except for 1-naphthol where the K_m was unknown, and noradrenaline where the activity was not sufficiently greater than blank at 5 μM, presumably because of the precipitation problem described in the efficiency section. Each substrate was used at its determined K_m value in case it was also a substrate for the other form of the enzyme with a different K_m, as it is for phenol. The specific activities quoted in Table 1 are those that were actually obtained and have not been corrected for losses occurring during the precipitation procedure which vary from substance to substance. Table 1 also gives the mean activity for each substrate relative to that with *p*-tyramine at 13 μM. There was no measurable PST activity using paracetamol at 3 concentrations lower than the K_m (30, 60 and 90 μM).

Correlation of platelet PST activities with different substrates

There was a high degree of correlation between platelet PST activities in the 13 subjects obtained with *p*-tyramine as substrate (for PST M) and those using *m*-tyramine, noradrenaline, adrenaline, 5-HT, *p*-hydroxyamphetamine, isoprenaline, salbutamol and l-naphthol (noradrenaline, $P < 0.01$; all remaining substrates, $P < 0.001$) (Table 1) but no significant correlation of the activities using any of these substrates and those with phenol at 10 μM (for PST P) (Table 1). However, although the individual platelet PST activity values using paracetamol, at 850 μM and 8.5 mM correlated highly with those using *p*-tyramine, the correlation coefficients obtained between paracetamol values and those for phenol (at 10 μM) were higher than for the other substrates, just reaching significance ($P < 0.05$) at the lower paracetamol concentration (850 μM) (Table 1). Platelet activity values using salicylamide as substrate at 5 and 50 μM (but not 500 μM) concentration showed a highly significant degree of correlation with 10 μM phenol values (Table 1). However, the reverse was true when the salicylamide data were correlated with activities using 13 μM *p*-tyramine. There was a highly significant correlation with salicylamide at 500 μM, one which was just significant at 50 μM but no significant correlation at 5 μM (Table 1).

With 133 μM *p*-tyramine (approximately 10 times K_m), similar correlation coefficients were obtained with the other substrates as with 13 μM *p*-tyramine; *m*-tyramine at 500 μM (aproximately one hundred times K_m) gave similar correlation coefficients as it did at its lower concentration. However, although the activities with *p*-tyramine at 1.3 μM correlated highly both with *p*-tyramine at 13 μM (Table 1) and all the

Table 1 Specific activities of human platelet (PST) using different substrates, their activities relative to that using *p*-tyramine at 13 μM and the correlation coefficients of PST activities in 13 individuals with those using *p*-tyramine or phenol

Substrate	*Concentration* (μM)	*Specific activity (nmoles product/ mg protein/10 min) Mean ± s.e. mean*	*Relative activity (p-tyramine 13 μM = 100)*	*Pearson correlation coefficient with p-tyramine* (13 μM)	*Pearson correlation coefficient with phenol* (10 μM)
p-Tyramine	13†	0.20 ± 0.01	100	—	0.14
p-Tyramine	133	0.49 ± 0.04	245	0.96***	0.23
p-Tyramine	1.3	0.03 ± 0.002	15	0.85***	0.50*
m-Tyramine	5†	0.19 ± 0.02	95	0.89***	0.30
Noradrenaline	20	0.05 ± 0.01	25	0.65**	0.20
Adrenaline	3†	0.09 ± 0.01	45	0.85***	0.16
5-HT	160†	0.09 ± 0.01	45	0.88***	0.16
p-Hydroxy-amphetamine	30†	0.14 ± 0.01	70	0.87***	0.17
Isoprenaline	2.5†	0.11 ± 0.01	55	0.92***	0.13
Salbutamol	150†	0.16 ± 0.01	80	0.82***	0.31
l-Naphthol	20	0.19 ± 0.02	95	0.94***	−0.01
Paracetamol	850†	0.07 ± 0.01	35	0.85***	0.48*
Paracetamol	8,500	0.19 ± 0.01	95	0.90***	0.46
Salicylamide	5†	0.04 ± 0.01	20	0.33	0.95***
Salicylamide	50	0.06 ± 0.01	30	0.51*	0.82***
Salicylamide	500	0.15 ± 0.01	75	0.88***	0.002
Phenol	10†	0.05 ± 0.01	25	0.14	—
Phenol	1,000	0.21 ± 0.01	105	0.81***	0.05

Significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
†approximate apparent K_m value

other PST M-type substrates, they also correlated significantly with PST P activities — phenol at 10 μM (Table 1) and salicylamide at 5 μM (correlation coefficient $r = 0.68$, $P < 0.01$).

Inhibition by DCNP

As shown in Figure 1a, PST activity with phenol at 30 μM was 98% inhibited in the presence of 10^{-6}M DCNP, but with phenol at 1 mM, it was only inhibited by 17%. Activity with *p*-tyramine was only inhibited by 7% in the presence of 10^{-6}M DCNP. PST activity with 5 μM salicylamide was 98% inhibited by 10^{-6}M DCNP, showing a similar pattern to 30 μM phenol; paracetamol sulphation on the other hand, whilst showing greater sensitivity to 10^{-6}M DCNP than that of *p*-tyramine, was still only 43% inhibited (Figure 1b).

Correlation of PST activities with the in vivo *sulphate conjugation of paracetamol and salicylamide*

The ratios of glucuronide to sulphate conjugates of paracetamol and salicylamide excreted by the group of normal volunteers after ingestion of 1 g of these drugs (Caldwell *et al.*, 1982) are shown in Table 2. There is a substantial degree of interindividual variation in relative conjugating ability, with some evidence, in the case of paracetamol, that the effect derives from the inability of certain individuals to form sulphate conjugates (Caldwell *et al.*, 1980, 1982). These ratios were ranked and correlated with platelet PST activities in the same individuals using these 2 drugs, and *p*-tyramine and phenol, as substrates. Spearman rank correlation coefficients are shown in Table 3. There was no significant positive correlation between paracetamol excretion data (11 individuals) and PST activities with paracetamol or *p*-tyramine (for PST M) as substrates. However, with phenol (for PST P) as substrate, there was a positive correlation which just reached statistical significance ($P < 0.05$, Table 3). There was no significant positive correlation between salicylamide excretion data (9 individuals) and PST activities with salicylamide, *p*-tyramine or phenol as substrates; in fact, all these correlation coefficients were negative, for reasons which are not immediately apparent.

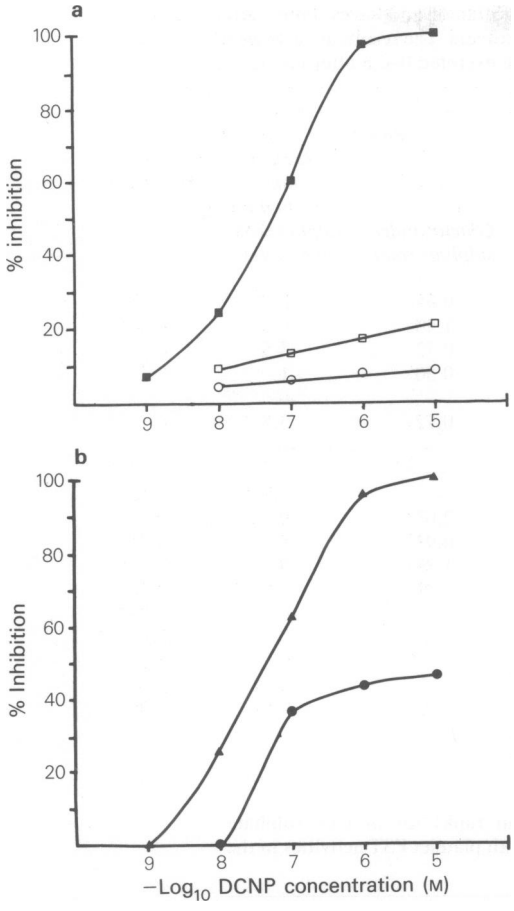

Figure 1 Inhibition of human platelet phenolsulphotransferase by various concentrations of dichloronitrophenol (DCNP) with (a) *p*-tyramine at 30 μM ○; phenol at 30 μM ■ and 1 mM □; (b) paracetamol at 850 μM ●; salicylamide at 5 μM ▲.
Results are expressed relative to values obtained in the absence of inhibitor.

Discussion

The results obtained in the recovery studies demonstrate that it is possible to use a wide range of neutral and basic phenols for this PST assay and obtain reasonably accurate specific activity values. Recovery values for the catecholamines, adrenaline and noradrenaline, were much poorer; the specific activities quoted have not been corrected and are therefore low. In their original paper, Foldes & Meek (1973) examined the recovery for a range of substrates by preparing the sulphate esters, dissolving them in phosphate buffer and then precipitating with barium hydroxide and zinc sulphate as in the assay procedure. They claimed an 82–100% range of recoveries for neutral and basic phenols, including noradrenaline. These values are somewhat higher than we have observed; however, we carried out our recovery experiments in the presence of barium acetate, which is used to stop the PST reaction in the assay. It is conceivable that the poor recoveries are due to low solubility of the barium salts of corresponding sulphate esters, and if this is so, then the barium acetate will provide an additional source of barium to form such salts. The fact that recoveries for acidic substrates in this assay are very poor (Foldes & Meek, 1973; Baranczyk-Kuzma *et al.*, 1981) may also be for this reason.

When different substrates are employed, variable recoveries in the assay will affect absolute specific activities but should not affect the correlation of individual values with those using either the PST M substrate (*p*-tyramine at 13 μM) or the PST P substrate (phenol at 10 μM). Rein *et al.* (1982) have shown that dopamine and *p*-tyramine are substrates for PST M, whilst 30 μM phenol is a substrate for PST P, using the selective inhibitor DCNP. We have previously confirmed that both dopamine and 4-hydroxy-3-methoxyphenylglycol (HMPG) are substrates for PST M by correlation of individual PST activities. That study also demonstrated that the 2 enzymes are under separate control (Bonham Carter *et al.*, 1981). Anderson *et al.* (1981) have also shown that individual PST activities with HMPG as substrate correlate significantly with values obtained using dopamine and *p*-tyramine. Using the same correlation procedure for the data obtained in this study, we have shown that *m*-tyramine, noradrenaline, adrenaline, 5-HT, *p*-hydroxyamphetamine, isoprenaline, salbutamol and l-naphthol are all substrates, for the M form of the enzyme only, at the concentrations we have used. However, paracetamol appears to be a substrate for both forms of the enzyme, although it shows more M than P type activity at the concentrations used, a conclusion confirmed by experiments with the selective inhibitor DCNP. Anderson *et al.* (1981) have shown similarly that individual platelet PST activity values, using 5-HT and paracetamol, correlate significantly with those for HMPG, indicating that both are substrates for PST M; they have not examined the relationship with any PST P substrate.

We have shown here that salicylamide is a PST P substrate at low concentration, becoming an M substrate at high concentration. Presumably the relatively low PST P activity is swamped at high concentration, because the PST M activity is so much greater. It is interesting that phenol itself becomes a substrate, again apparently exclusively for PST M at high concentration; this was confirmed by using DCNP and also by the second much higher K_m obtained for phenol sulphation. This finding may explain why Mulder & Scholtens (1977) found that

Table 2 *In vivo* excretion of conjugated paracetamol and salicylamide after administration of 1 g of either drug to normal volunteers. The results are expressed as the ratio of glucuronide to sulphate conjugate excreted 0–8 h after taking the drug

	Paracetamol		*Salicylamide*	
Subject	*Glucuronide/ sulphate ratio*	*Ranked data (1 = highest sulphate conjugate excretor)*	*Glucuronide/ sulphate ratio*	*Ranked data (1 = highest sulphate conjugate excretor)*
1	2.54	10	0.55	2
2	—	—	1.20	6
3	—	—	0.72	3.5
4	0.74	5	0.50	1
5	2.29	9	—	—
6	0.71	4	0.72	3.5
7	0.07	1	—	—
8	0.14	2	—	—
9	1.4	8	—	—
10	0.56	3	2.07	9
11	4.28	11	0.93	5
12	1.1	7	1.89	8
13	0.82	6	1.32	7

Table 3 Correlation coefficients (Spearman rank) for *in vivo* sulphate conjugation of paracetamol or salicylamide with platelet PST activities in the same individuals using different substrates

PST substrate (concentration)	*Paracetamol* (in vivo *sulphoconjugation*) (n = 11)	*Salicylamide* (in vivo *sulphoconjugation*) (n = 9)
Paracetamol (850 μM)	$r_s = 0.17$	—
Paracetamol (8,500 μM)	$r_s = 0.12$	—
Salicylamide (5 μM)	—	$r_s = -0.27$
Salicylamide (50 μM)	—	$r_s = -0.27$
Salicylamide (500 μM)	—	$r_s = -0.61$
p-Tyramine (13 μM)	$r_s = 0.10$	$r_s = -0.61$
Phenol (10 μM)	$r_s = 0.54^*$	$r_s = -0.20$

Significance: $^*P < 0.05$

sulphation of this substrate was not substantially inhibited by 10^{-6}M DCNP; they used phenol at 1 mM concentration. It is also noteworthy that *p*-tyramine sulphation at very low concentration (1.3 μM) showed a significant correlation with PST P activity, both with phenol and salicylamide (at 5 μM) as substrates, although the degree of correlation with M substrates is considerably greater. It is possible that other substrates of human platelet PST are substrates for both forms but that M activity has dominated at the substrate concentrations used for investigation.

It appears from this study that the consistently low excretion of the sulphate conjugates of paracetamol or salicylamide by some individuals in this group of volunteers (Caldwell *et al.*, 1980, 1982) cannot be explained by a generalized deficiency of PST. Most sulphate conjugation of orally administered substances occurs in the gut and since there are similarities between gut and platelet enzymes (Anderson & Weinshilboum, 1980; Rein *et al.*, 1981; Anderson *et al.*, 1981), the platelet seems to be a reasonable model to use for a study of this type where gut biopsies are not readily available. Whether the significant correlation between paracetamol sulphate excretion and PST P activity is a chance finding needs further investigation. Paracetamol is, to some extent, a substrate for PST P; however, at 850 μM and 8.5 mM it is predominantly a substrate for PST M and at the lower concentration of 90 μM there was no measurable PST activity at all.

There are other possible explanations for some individuals' relative inability to form sulphate conjugates of orally administered compounds, e.g. reduced drug availability at the conjugation site or modification of *in vivo* enzyme activity by the presence of endogenous inhibitors (Anderson & Weinshilboum, 1979). These may also be the reasons why patients suffering from depressive illness who excrete significantly low levels of tyramine-*O*-sulphate after oral tyramine administration (Sandler *et al.*, 1975; Bonham Carter *et al.*, 1978), have normal platelet PST activity (Bonham Carter *et al.*, 1981). It is interesting, however, that poor sulphate conjugators of paracetamol are not necessarily poor sulphate conjugators of salicyclamide and *vice versa* (Caldwell *et al.*, 1982). Whether either of these groups are also poor sulphate conjugators of tyramine or another phenolic compound must be determined by future investigation.

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