group, the potency in inhibiting [^{3}H]diazepam binding decreased enormously, indicating the need for a methyl group at the 6-position for the inhibition of [^{3}H]diazepam binding. As shown in Fig. 2, the inhibition of [^{3}H]diazepam binding to rat brain membranes by 9-propyl-6-methylthioguanine was competitive in nature.

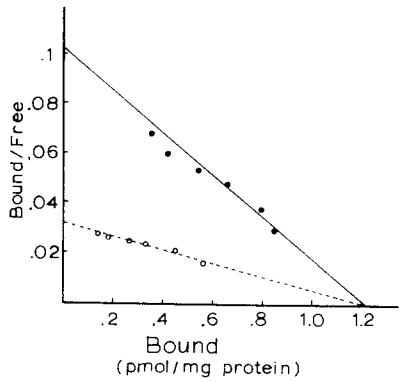

Fig. 2. Binding of [^{3}H]diazepam to rat brain cortical membranes as a function of concentration of [^{3}H]diazepam in the absence (●—●) and presence (○- - -○) of 0.25 μM 9-propyl-6-methylthioguanine.

We found previously that, among various purine derivatives, 6-methylthioguanine is potent in displacing the brain specific binding of [^{3}H]diazepam [4]. Later we observed that some of the more hydrophobic derivatives of 6-methylthioguanine have higher potencies than 6-methylthioguanine [5]. These results suggested that, in addition to hydrophobicity, there seems to be a steric effect by the alkyl derivatives as inhibitors of [^{3}H]diazepam binding.

In the present study we found that 9-alkyl derivatives of 6-methylthioguanine (e.g. 9-*n*-propyl and 9-*n*-pentyl derivatives) competitively inhibited [^{3}H]diazepam binding with K_i values in the nanomolar range. These compounds were several thousand times more potent than inosine and hypoxanthine. Since these 9-alkyl derivatives of 6-methylthioguanine inhibited [^{3}H]diazepam binding at nanomolar concentrations, these compounds could serve as useful tools for behavioral studies related to benzodiazepine function.

In summary, 9-alkyl derivatives of 6-methylthioguanine were tested for their abilities to displace [^{3}H]diazepam binding to rat brain membranes. The most active derivative, 9-*n*-pentyl-6-methylthioguanine, had an IC_{50} of 0.2 μM with a K_i of 0.082 μM. This is the first purine derivative that inhibits [^{3}H]diazepam binding at nanomolar concentrations.

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Formation of adenosine-5′-phosphosulfate from 3′-phosphoadenosine-5′-phosphosulfate in human platelets

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In a previous article [1] from this laboratory, it was demonstrated that ^{35}S-labeled 3′-phosphoadenosine-5′-phosphosulfate (PAPS*) was rapidly metabolized to inorganic sulfate when incubated in the presence of high speed supernatant solutions obtained from human brain. Hydrolysis of PAPS to inorganic sulfate was shown to be more rapid than the rate of sulfation of acceptor substrates by phenol sulfotransferase (PST) and, thus, the concentration of PAPS available for sulfoconjugation became limiting. Breakdown of PAPS to form free sulfate could possibly occur by the initial production of a sulfate-phosphate anhydride from PAPS [2] followed by the hydrolysis of the anhydride to sulfate and inorganic phosphate. Alternatively, PAPS may be broken down by 3′-nucleotidase [3] to form inorganic phosphate and adenosine-5′-phosphosulfate (APS) which may be subsequently hydrolyzed by APS-sulfohydrolase or some other nonspecific hydrolase to form inorganic sulfate and adenosine monophosphate (AMP) [4, 5]. Although the latter pathway is more likely, no APS was detected in the incubation mixtures of PAPS with human brain homogenates.

The presence of these PAPS catabolic enzymes in crude tissue homogenates has the potential to interfere with the assay of phenol and other sulfotransferases that require PAPS as the sulfate donor. For example, in a typical phenol

* Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PST, phenol sulfotransferase; APS, adenosine-5′-phosphosulfate; AMP, adenosine monophosphate; and TEA, tetraethylamine.

sulfotransferase assay utilizing a 100,000 *g* supernatant solution obtained from human brain, it was demonstrated that greater than 60% of PAPS (1.0 μM) was broken down to inorganic sulfate within 30 min, whereas less than 5% of the sulfate was actually utilized to conjugate acceptor substrates [6]. Inhibition of the PAPS degradation with the phosphate analog, phosphonoacetic acid, resulted in a corresponding increase in the linearity of sulfation of both dopamine and phenol [6]. This increase in sulfation was probably a result of both the preservation of PAPS in the reaction mixture and a decrease in the formation of dead-end product inhibitors such as PAP or other adenosine derivatives.

Human platelets have been found to possess PST activity similar to that observed in human brain [7–9]. Since human platelets are currently being used as a genetic marker to assess PST activity *in vivo* [8, 10–16], it was of interest to determine the metabolic fate of PAPS in this tissue.

Materials and methods

Platelet-enriched plasma preparations (500 ml) obtained from the American Red Cross were centrifuged at 10,000 *g* to obtain a platelet pellet. These platelet preparations contain approximately ten times the number of platelets normally obtained in a platelet-rich fraction. The resulting pellet was resuspended in isotonic saline and recentrifuged as above. This washing procedure was repeated a second time only using 10 mM tetraethylamine (TEA) buffer, pH 7.4. The final platelet pellet was resuspended in 20 ml of 10 mM TEA buffer, pH 7.4, and stored at −20°.

PST activity was assessed in crude homogenized (Teflon–glass homogenizer) platelet fractions by incubating the enzyme in the presence of 10 μM dopamine or phenol (M and P activity, respectively), 1 μM PAPS (approx. 2×10^5 dpm/assay), 1 mM pargyline and 0.05 M TEA buffer, pH 7.4 (total volume 0.5 ml). The phosphatase inhibitor, phosphonoacetic acid (PAA), was added to the reaction mixture as indicated in the Results to prevent enzymatic hydrolysis of PAPS to APS and inorganic sulfate. The methods to measure PST activity in high speed supernatant solutions of human brain frontal cortex have been reported previously [6].

PST activity was measured by a modification of the Ecteola cellulose ion exchange chromatography procedure [17] described previously [1]. In brief, Ecteola columns prepared in pasteur pipets were eluted in succession with 3 ml water to remove sulfated dopamine, 15 ml of 5 mM ammonium bicarbonate to remove sulfated phenol, 10 ml of 20 mM ammonium bicarbonate to remove inorganic sulfate, 10 ml of 60 mM ammonium bicarbonate to remove APS (see Results) and 10 ml of 140 mM ammonium bicarbonate to remove PAPS. Scintillation fluid (10 ml) was added to the appropriate Ecteola cellulose column effluents, and the radioactivity was measured in a Beckmann liquid scintillation spectrophotometer. As noted previously [6], recovery of radioactivity from the Ecteola columns was consistently greater than 95%. Where applicable, the values reported have been corrected for quench.

To identify the PAPS degradative product eluting from the Ecteola cellulose column in the 60 mM ammonium bicarbonate solution, this fraction was subjected to thin-layer chromatography using Baker-flex Silica Gel. The solvent system consisted of 1-propanol–ammonium hydroxide–water (6:3:1, by vol.). APS and the 60 mM ammonium bicarbonate fraction obtained by incubating PAPS with 3′-nucleotidase was also chromatographed on the same TLC plates.

Pargyline hydrochloride, dopamine hydrochloride, phosphonoacetic acid, triethanolamine hydrochloride, 3′-phosphoadenosine-5′-phosphosulfate, adenosine-5′-phosphosulfate, Ecteola cellulose and nuclease P1 (3′-nucleotidase) were obtained from the Sigma Chemical Co., St. Louis, MO; phenol, TEA free base and Baker-flex silica-gel TLC plates were obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. Formula 963 liquid scintillation fluid and [^{35}S]PAPS (1.0 to 5.0 μCi/mmole) were obtained through New England Nuclear, Boston, MA.

Results and discussion

As illustrated in Fig. 1, panels A and B, incubation of platelet PST in the presence of PAA increased the extent of sulfation of both dopamine and phenol respectively. Typically, at incubation times of 60 min, conjugation of dopamine and phenol was increased almost 75 and 33% respectively. The optimal increase in sulfation of both these substrates was found to occur at a concentration of PAA of approximately 0.5 mM.

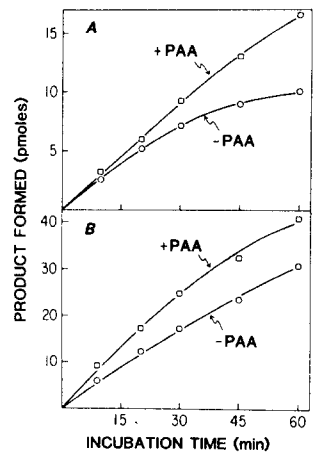

Fig. 1. Effect of phosphonoacetic acid (PAA) on sulfation of (A) dopamine and (B) phenol. Incubation mixtures (0.5 ml) contained a 10 μM concentration of either DA or phenol, 1 μM [^{35}S]PAPS, 100,000 *g* supernatant solution (6.5 μg protein), and 10 mM TEA buffer, pH 7.4. Reactions were terminated at the time specified by heating in boiling water for 1 min. Sulfated product was separated by ion exchange chromatography with Ecteola cellulose, as described in the text.

When PAPS was incubated with human brain homogenates as described previously, the majority of the radioactive label appeared as inorganic sulfate (20 mM ammonium bicarbonate fraction off of Ecteola cellulose columns) [1, 6]. In contrast, as indicated in Table 1, less than 2% of the PAPS was converted to inorganic sulfate when human platelets were incubated in the presence of [^{35}S]PAPS for 60 min. Upon appropriate fractionation of the platelet incubation mixture on Ecteola cellulose with various concentrations of ammonium bicarbonate, it was observed that a major portion (approx. 16% in the experiment illustrated) of the total radioactivity eluted from the column in the first 5 ml of a 60 mM ammonium bicarbonate wash.

As shown by the data presented in Fig. 2, appearance of the radiolabel in this fraction was both time and protein concentration dependent and independent of the presence of acceptor substrate in the reaction mixture. Although not shown, a similar ammonium bicarbonate fraction obtained from incubating PAPS with human brain homogenates had essentially no radioactivity associated with it. As illustrated in Fig. 2, panels A and B, when the phosphate analog, PAA, was added to the reaction mixtures containing PAPS and platelets, formation of the PAPS degradative product associated with the 60 mM ammonium bicarbonate fraction was greatly diminished. In the absence of PAA greater than 30% of the PAPS was converted to APS within 60 min, whereas only 8% of the PAPS was hydrolyzed to this

Table 1. Radioactivity in ammonium bicarbonate effluent from Ecteola cellulose column

Ammonium bicarbonate	Vol. (ml)	Radioactivity (cpm) Platelets	%	Brain	%
Water	3	41	<0.1	173	0.2
20 mM	5	1,293	1.4	65,783	71.8
20 mM	5			1,186	1.3
60 mM	5	11,353	12.4	714	0.8
60 mM	5	1,250	1.4	206	0.2
140 mM	5	34,989	38.2	17,850	19.5
140 mM	5	40,775	44.5	5,309	5.8
140 mM	5	1,894	2.1	401	0.4

[^{35}S]PAPS was incubated with human platelets and brain as described in Materials and Methods. An 0.3-ml aliquot of the reaction mixture was placed on an Ecteola column and eluted in succession with ammonium bicarbonate solutions as indicated in the table. Recovery of radioactivity from the columns was 103% for the platelets and 101% for the brain.

catabolite in the presence of PAA. Consistent with these findings was the observation that the decrease in this PAPS metabolite corresponded to a preservation of PAPS in the reaction mixture over the course of the incubation. At 60 min there was approximately a 44% increase in the PAPS concentration which represents almost a 2.5-fold decrease in the rate of utilization of the sulfate donor. It was also observed that incubating the PAPS metabolite appearing in the 60 mM fraction along with human brain homogenates resulted in complete conversion of the metabolite to inorganic sulfate.

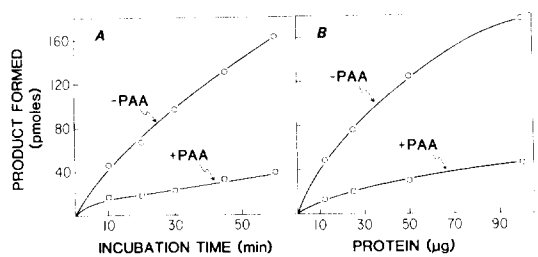

Fig. 2. Effect of (A) time and (B) protein concentration on the formation of the PAPS metabolite eluting from an Ecteola cellulose column in 60 mM ammonium bicarbonate. Incubation mixtures (0.5 ml) contained 100,000 *g* supernatant solution, [^{35}S]PAPS and TEA buffer, pH 7.4. Phosphonoacetic acid (PAA; 2 μM) was added to the reaction mixture as indicated in the figure. Reactions were terminated by heating for 1 min in boiling water, and 300 μl of the assay mixture was chromatographed over an Ecteola cellulose column as described in Materials and Methods. Protein concentration in part (A) was 130 μg and the time in part (B) was 60 min.

To identify the radioactive compound associated with the 60 mM ammonium bicarbonate fraction, an aliquot of this fraction was spotted on Silica gel thin-layer chromatograph plates along with [^{35}S]PAPS, APS and the 60 mM ammonium bicarbonate fraction obtained by incubating PAPS with nuclease P1 (3′-nucleotidase). A new spot of radioactivity at an R_f value = 0.35 appeared in both the 60 mM ammonium bicarbonate fractions obtained from incubating PAPS with human platelets or nuclease P1. This R_f value corresponded to the R_f value obtained with authentic APS and, accordingly, suggests that the radioactivity associated with the 60 mM ammonium bicarbonate fraction is ^{35}S-labeled adenosine-5′-phosphosulfate.

Studies in this manuscript demonstrate that PST activity in human platelets is dependent on the rate at which PAPS is broken down to APS since this catabolic process predominates over sulfation of acceptor substrates. The presence of PAPS degrading enzymes in tissue preparations has been noted [18], and previous investigators were forced to use very dilute platelet preparations to overcome this problem. The results of this study also suggest that platelets contain an enzyme (3′-nucleotidase) which is capable of rapidly hydrolyzing the 3′-phosphate moiety from PAPS to produce APS. The rate of PAPS breakdown appears to be greater under the *in vitro* conditions employed than the rate of conjugation of either dopamine or phenol by the M and P forms of PST respectively. In the case of platelets, the end product of PAPS degradation appears to be APS and little, if any, liberation of inorganic sulfate occurs. In contrast, PAPS catabolism in human brain results in the formation of primarily inorganic sulfate with no detectable production of APS. As demonstrated herein, human brain is capable of rapidly hydrolyzing APS to form AMP and inorganic sulfate, thus suggesting that platelets lack the enzyme(s) responsible for the formation of sulfate from either PAPS or APS.

A number of enzymes can be responsible potentially for degradation of APS to inorganic sulfate [4, 5, 19]. One such enzyme, APS-sulfohydrolase, has been identified previously in a number of animal species. In addition, the nonspecific enzyme, 5′-nucleotidase, can similarly hydrolyze APS or PAPS to inorganic sulfate, phosphate and adenosine [19]. Since little inorganic sulfate was formed during incubation of PAPS with human platelets, this tissue may be devoid of the enzyme, 5′-nucleotidase, as well as APS and PAPS specific sulfohydrolases.

The data presented in this manuscript also reveal for the first time that the phosphatase inhibitor, PAA, is an effective inhibitor of 3′-nucleotidase since, when PAA was added to the incubation mixture, formation of APS was greatly diminished. It is possible that the inhibitory process described previously [6] in human brain and attributed to PAA inhibition of phosphatases may, in fact, have been due to inhibition of the 3′-nucleotidase as well as phosphatase. Accordingly, formation of inorganic sulfate in human brain may first involve the formation of the intermediate APS which is then rapidly hydrolyzed to the final products, AMP and inorganic sulfate.

Because PAA prevents the breakdown of PAPS and the formation of potential dead-end inhibitors of PST, this phosphate analog should be added to all assays which attempt to determine both M- and P-PST activities in platelets *in vitro*.

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Metabolism of benzoic acid by stimulated polymorphonuclear cells*

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Polymorphonuclear cells (PMNs) are capable of generating reactive oxygen species (ROS) such as superoxide radical anion and hydroxyl radicals when stimulated [1–5]. These transient species are highly reactive and can interact both with macromolecular constituents of the cell and xenobiotic materials.

When stimulated by opsonized zymosan particles, human PMNs respond with a burst of oxidative activity [2]. We have shown previously that stimulated PMNs are capable of decarboxylating benzoate, although the exact nature of the attacking species has been debated [3, 4]. We have also shown that the decarboxylation reaction can be inhibited by specific radical scavengers such as mannitol and dimethyl sulfoxide (DMSO) [3, 6]. Others have shown, using both ionizing radiation and nonbiologic chemical reactions, that the aromatic hydroxylation of benzoate appears to be hydroxyl-specific [7–9].

In this paper, we report the results of experiments testing the benzoate hydroxylation ability of stimulated PMNs *in vitro*. Human blood granulocytes were obtained from the venous blood of human volunteers [10, 11]. Approximately 40 ml of blood was drawn by syringe from a peripheral arm vein into a mixture of EDTA and dextran solution. After mixing, the erythrocytes were allowed to sediment under normal gravity for 55 min. The upper plasma layer containing PMNs was carefully removed, mixed with cold Seligman's balanced salt solution (SBSS), and layered over Ficoll–Hypaque solution in 50 ml plastic centrifuge tubes. After centrifugation, the solutions were aspirated from the PMN pellet. The cells were subjected to shock lysis with distilled water to remove residual erythrocytes [10, 11]. The cells were suspended in Dulbecco's phosphate-buffered saline with 5 mM glucose, to a final concentration of 20×10^6 cells/ml [11]. Zymosan was opsonized by incubation with autologous serum for 30 min in a 37° bath [10, 11]. The zymosan particles were recovered by centrifugation and resuspended in the same medium used for the cells. An aliquot of the cell suspension (1 ml) was placed in a plastic tube containing a small magnetic stirring bar. The cells were preincubated for 10 min with 20 mM benozate and either mannitol (20 mM), DMSO (30 mM), azide (0.1 mM), superoxide dismutase (SOD) (15 μg/ml) (Sigma 2500 units/mg) or catalase (20 μg/ml) (Sigma 20,000 units/mg). Zymosan was then added to initiate the reaction (final concentration 5 mg/20×10^6 cells), and the cells were incubated at 37° for 60 min with constant stirring. Control incubations of cells were performed in each case with benzoate and zymosan alone, to establish full activity levels, and with benzoate alone, to correct for any activity in unstimulated cells. In the latter, no activity was ever observed. Incubations were halted by immersion of the tubes in an ice-water bath for 10 min. The material was then stored at −20° until analysis could be performed.

The sample were analyzed by high performance liquid chromatography (HPLC). A 400-μl aliquot of the incubate in a small test tube was acidified with phosphoric acid and spiked with *o*-methoxybenzoic acid as internal standard. This was extracted with 4 ml of diethyl ether. After transferring the ether to a second tube, 20 μl of 1 N sodium hydroxide was added, and the solvent evaporated under nitrogen. The addition of base was to prevent sublimation of benzoic acid during the evaporation process. The sample was then reconstituted with the chromatographic mobile phase and injected onto the column. The chromatograph used was an Altex MP 322 dual pump system operated in isocratic mode. The mobile phase was a mixture of isopropanol and 5% aqueous acetic acid containing 2.0 g/l ammonium acetate (5:95). The flow rate was 1.0 ml/min using a Beckman Ultrasphere C-18 4.6 × 250 mm column. Detection was by ultraviolet at 240 nm, using a Beckman model 165 variable wavelength detector (Beckman Instruments, Irvine, CA).

As seen in Fig. 1, PMNs incubated with benzoate alone showed no evidence of benzoate hydroxylation ability. However, when cells were stimulated with opsonized zymosan in the presence of benzoate, analysis of the 60 min supernatant fraction showed the presence of hydroxylated products. All three possible monohydroxybenzoate isomers could be detected. Typically, the final concentration of ortho-, meta- and para-hydroxybenzoate (HBA) totalled 5 μM, corresponding to production levels of 3.8 nmoles/

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