recovered. The recovery of heme *b* determined by measuring [^{14}C]heme *b* was in good agreement with the recovery of heme *b* as determined by the pyridine hemochromogen method. The recovery of heme *a* is shown in Table 2. Approximately 55% of added heme *a* radioactivity was recovered in the acid acetone extract and approximately 40% of the heme *a* applied to the thin-layer plate was recovered. Essentially no heme *b* was found to contaminate heme *a* isolated from the thin-layer plate and only a small amount of heme *a* contamination was found in heme *b* isolated from the thin-layer plate (Table 3).

Glycine, δ-aminolevulinic acid, and uroporphyrins were not retained in the organic phase spotted on the thin-layer plate, and protoporphyrin IX, which was extracted from cardiac tissue, remained near the origin on the thin-layer plate in this system.

Table 3. Separation of heme *a* and heme *b*

Recovery of heme *b* in heme *a**	
Heme *b* added to cardiac homogenate	104,630 dpm
Heme *b* recovered in heme *a* fraction	732 dpm
% Contamination	<1%
Recovery of heme *a* in heme *b*†	
Heme *a* added to cardiac homogenate	9,569 dpm
Heme *a* recovered in heme *b* fraction	146 dpm
% Contamination	1.5%

* Radioactive heme *b* was added to cardiac homogenates and heme *a* was isolated as described in Materials and Methods.

† Radioactive heme *a* was added to cardiac homogenates and heme *b* was isolated as described in Materials and Methods.

Discussion

The investigation of the properties of cellular hemes, as well as the mechanisms of their synthesis and breakdown, requires the development of techniques to isolate these hemes. This report describes a technique for simultaneous isolation of hemes *a* and *b* from small amounts of cardiac tissue by employing a standard extraction procedure coupled with a thin-layer chromatography system using silica gel 7 in conjunction with a polyvinyl alcohol binder. The polyvinyl alcohol did not serve merely as a binder but also as an integral ingredient in the separation procedure. All commercially available silica gel thin-layer plates that were tested were not effective and could not be used for these separations. Commercial plates do not have polyvinyl alcohol as a binder. The utilization of this procedure for measuring the separate rates of synthesis and catabolism of hemes *a* and *b* in biological systems is implicit, and studies which have been devised to test the effects of perturbants on the synthesis and catabolism of hemes *a* and *b* will be the subject of a future report.

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Antiplatelet actions of trimetoquinol isomers: evidence for inhibition of a prostaglandin-independent pathway of platelet aggregation

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Trimetoquinol (TMQ) possesses both β-adrenoreceptor stimulant [1, 2] and antiplatelet activities [3, 4]. TMQ exists as two stereoisomers and it has been reported that the β-stimulant activity resides primarily with *S*(−)-TMQ [1, 5, 6], whereas antiplatelet activity is selectively exhibited by the *R*(+)-isomer [3, 7]. Our findings that *R*(+)-TMQ is more effective than *S*(−)-TMQ as an inhibitor of platelet aggregation induced by arachidonic acid, thromboxane A_2 and U46619 (a stable PGH_2 analog) suggest that TMQ stereoselectively inhibited the prostaglandin-dependent pathway of platelet activation [7]. Since *R*(+)-TMQ inhibits prostaglandin-mediated platelet aggregation [7] and is considerably less active than *S*(−)-TMQ as an agonist of the bronchodilator activity [2, 6], it is likely that the pharmacological activities of the TMQ isomers are mediated by different mechanisms. To further examine the antiplatelet

action of the TMQ stereoisomers, we have investigated the effects of *R*(+)- and *S*(−)-TMQ on platelet aggregation mediated by phospholipase C (PLC) and low dose thrombin in the presence of aspirin, a known inhibitor of prostaglandin biosynthesis [8]. Here we report that both *R*(+)-TMQ and *S*(−)-TMQ differentially inhibit platelet aggregation mediated by PLC and low dose thrombin, which are prostaglandin-independent pathways.

The methods and reagents used for the preparation of washed human platelets, aggregation and the secretion of [^{14}C]-5-HT from platelets were as previously reported [9]. Blood was collected from normal human volunteers who reported to be without medication for at least 10 days prior to blood drawing. Acid-citrate dextrose solution (ACD) was used as an anticoagulant in a ratio of 1 : 6 (v/v). Citrated blood was centrifuged at 120 *g* for 15 min at room temperature. Platelet-rich plasma (PRP) was centrifuged at 1100 *g* for 10 min and the platelet pellet was resuspended in modified Tyrode's solution without calcium, pH 6.5. Apyrase (EC 3.6.1.5), 34 μg/ml, was added to the platelet suspension to prevent accumulation of ADP. The platelet suspension was kept at 37° for 5 min and then centrifuged. Ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) (2%, w/v) was added (1 : 9, v/v) just before centrifugation during the first wash only. The washing procedure was repeated three times, and the final platelet pellet was resuspended in the modified Tyrode's solution, pH 7.4. Washed human platelets were used in all experiments. Platelet aggregation studies were performed according to the turbidometric method of Born as modified by Mustard *et al.* [10] using a Payton dual channel, model 600 aggregometer (Buffalo, NY).

Secretion of platelet contents from the dense granules was measured by monitoring the secretion of radioactivity from platelets prelabeled with [^{14}C]serotonin ([^{14}C]-5-HT). Platelets were incubated with [^{14}C]-5-HT (0.2 μCi/3 × 10^8 platelets) for 15 min during the third wash. Platelets were washed once more to remove the radioactivity from the medium and were resuspended in modified Tyrode's solution, pH 7.4. The secretion of [^{14}C]-5-HT from platelets was determined by centrifuging the samples at 1200 *g* for 1 min in a microfuge, and an aliquot of the supernatant fraction was counted by liquid scintillation spectrometry. Secretion data was calculated as the percentage of total ^{14}C in platelets.

Phospholipase C (PLC) from *Clostridium perfringens*, bovine thrombin, and apyrase were obtained from the Sigma Chemical Co., St. Louis, MO. Stereoisomers of TMQ were a gift from Dr. Y. Iwasawa, Tanabe Seiyaku, Ltd., Tokyo, Japan. [^{14}C]-5-Hydroxytryptamine creatinine sulfate (57 mCi/mmole) was supplied by the Amersham Corp. (Arlington Heights, IL).

The effects of TMQ isomers on prostaglandin-independent pathways were evaluated using low dose thrombin (0.03 units/ml) as an inducer of platelet aggregation and [^{14}C]-5-HT secretion. Since thrombin has been shown to cause platelet aggregation by three mechanisms, namely release of ADP, prostaglandin biosynthesis, and production of platelet activating factor (PAF) [11, 12], a low concentration of thrombin that does not evoke PAF production [12] was used, and all samples were treated with 1 mM aspirin to inhibit prostaglandin biosynthesis. Under these conditions, both *R*(+)-TMQ and *S*(−)-TMQ inhibited thrombin-induced aggregation and [^{14}C]-5-HT secretion in a concentration-dependent manner (Fig. 1). However, in contrast to the previously reported effects of the stereoisomers of TMQ on U46619-induced platelet activation [7], *S*(−)-TMQ was found to be 3-fold more effective as an inhibitor of thrombin-induced platelet activation than the corresponding *R*(+)-isomer. The 50% inhibitory concentrations (IC_{50}) for the *S*(−)- and *R*(+)-isomers against thrombin (0.03 units/ml)-induced aggregation were 5×10^{-5} M and 1.5×10^{-4} M respectively (N = 3).

Recently we have shown that PLC mediates platelet aggregation by a mechanism independent of prostaglandin biosynthesis and of released ADP [13]. To further explore the antiplatelet effects of TMQ on prostaglandin-independent mechanisms of platelet aggregation, the stereoisomers of TMQ were tested for their inhibitory effects on PLC-induced platelet activation (Fig. 2). Both isomers of TMQ displayed a concentration-dependent inhibition of PLC-induced aggregation in the presence of 1 mM aspirin. In other experiments, nearly identical results with the TMQ isomers were obtained in the absence of aspirin (data not presented). *S*(−)-TMQ was 3.5-fold more effective than *R*(+)-TMQ as an inhibitor of the PLC-mediated platelet aggregation. The IC_{50} values for the *S*(−)- and *R*(+)-isomers against PLC (0.05 units/ml)-induced aggregation were 5×10^{-5} M and 1.75×10^{-4} M respectively (N = 3).

These studies demonstrate, for the first time, that TMQ is an inhibitor of platelet activation mediated by prostaglandin-independent mechanisms. In a previous report [7], we showed that the concentration-dependent inhibition of U46619-induced aggregation and the secretion of serotonin in human platelets were virtually identical. By contrast, it is noteworthy that the blockade of PLC-induced aggregation by the TMQ isomers did not parallel their observed effects on the inhibition of serotonin release. For example, 100 μM *R*(+)- and *S*(−)-TMQ blocked the aggregation response to PLC by 50 and 80%, whereas [^{14}C]-5-HT secretion was inhibited by only 29 and 39% respectively. These results reinforce our previous proposal that PLC-mediated aggregation is independent of prostaglandin biosynthesis and of released ADP [13]. Moreover, if PLC-induced aggregation was mediated by liberation of arachidonic acid and subsequent release of ADP, then this pathway would be selectively inhibited by *R*(+)-TMQ [3, 7]. Since PLC-mediated aggregation was indpendent of the released ADP and of prostaglandin biosynthesis, it is reasonable to conclude that TMQ isomers also inhibit platelet aggregation by a mechanism independent of prostaglandin biosynthesis and/or action.

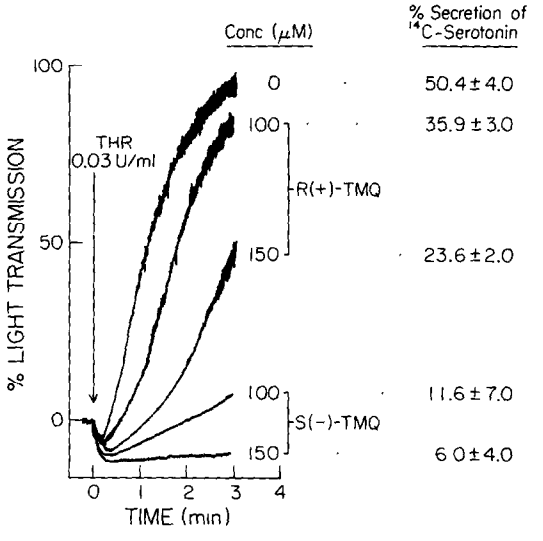

Fig. 1. Effect of TMQ isomers on thrombin-induced platelet aggregation and secretion of [^{14}C]serotonin (5-HT) from washed human platelets. Aspirin (1 mM) was added to all samples. Diluent, *R*(+)-TMQ or *S*(−)-TMQ was added to platelets 3 min before the addition of thrombin (0.03 units/ml). Superimposed tracings of platelet aggregation are representative of three or more experiments. The data on secretion of [^{14}C]-5-HT are expressed as a percentage of total [^{14}C]-5-HT in platelets. Each value is the mean ± S.E.M. of three or more experiments.

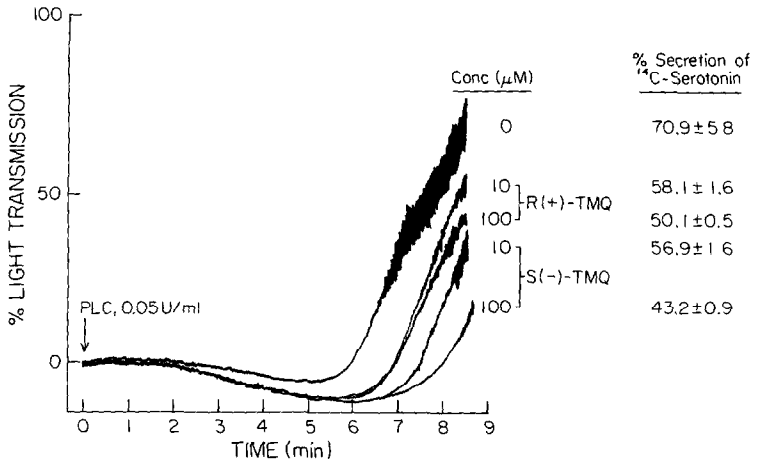

Fig. 2. Effect of TMQ isomers on PLC-induced aggregation and secretion of [^{14}C]serotonin (5-HT) from washed human platelets. Diluent, *R*(+)-TMQ or *S*(−)-TMQ was added to platelets 3 min before the addition of PLC (0.05 units/ml). All samples contained aspirin (1 mM). Superimposed tracings of platelet aggregation are representative of three or more experiments. The data on secretion of [^{14}C]-5-HT are expressed as a percentage of total [^{14}C]-5-HT in platelets. Each value is the mean ± S.E.M. of three to four experiments.

It has been reported that platelets aggregated by A23187 (a calcium ionophore), thrombin and collagen liberate a platelet activating factor (PAF) [12]. PAF has been shown to cause platelet aggregation by a mechanism which is independent of prostaglandin biosynthesis and of released ADP [12]. It is possible that PLC may induce aggregation by the liberation of PAF. To assess this possibility, we have determined that crude PAF extracts of A23187-treated platelets [12] produced aggregation of washed human platelets. However, the corresponding extract of PLC (0.05 units/ml)-treated platelets did not produce aggregation.* This suggests that PLC does not cause aggregation via release of PAF and, thus, the inhibitory action of the TMQ isomers cannot be attributed to an effect on PAF generation or action.

It has been shown that thrombin causes secretion from platelet granules by an activation of endogenous phospholipase C that specifically hydrolyzes phosphatidylinositol [14]. Recently, a relationship between the hydrolysis of phospholipids by thrombin or exogenous PLC and the phosphorylation of a 40,000 dalton protein in platelets has been linked to the secretory process [15]. Since the TMQ isomers stereoselectively inhibited platelet aggregation induced by both thrombin and PLC with a nearly equivalent potency, it is proposed that TMQ inhibits the endogenous phospholipase C pathway activated by thrombin. Finally, we suggest that the similar inhibitory potency and stereoselectivity of TMQ against thrombin and PLC-mediated aggregation may be indicative not only of the commonality in the mechanism of platelet aggregation by these inducers but, also, of a common site of inhibitory action for the stereoisomers of TMQ.

In summary, these findings demonstrate that: (a) *S*(−)-TMQ selectively inhibits platelet aggregation induced by PLC and low dose thrombin (in the presence of aspirin) and thus may represent a new class of antiplatelet agents that inhibit phospholipase C mediated aggregation of platelets, and (b) use of PLC as an inducer of platelet aggregation provides an excellent system to test antiplatelet agents that selectively inhibit prostaglandin-independent pathways of platelet activation.

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On the possible role played by hydrogen bonding in benzodiazepine-receptor interactions

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Recent studies [1–4] have singled out high affinity stereospecific binding sites for benzodiazepines (BDZs) in CNS. Several attempts have been carried out to isolate and identify endogenous ligands interacting with the BDZ receptor under physiological conditions and many compounds have been so far proposed as potential candidates, among which are the purines hypoxanthine and its nucleoside inosine, β-carbolines and nicotinamide [5]. The present uncertainties on the nature of the endogenous ligand make very difficult to hypothesize a reasonable scheme of the forces binding BDZs to their receptor. Although these forces can be generally identified in a combination of hydrogen bonding (HB), electrostatic and hydrophobic (or Van der Waals) interactions, only some evidence for a contribution of HB interactions has been so far obtained by Paul, Sapper and Lohmann [6] on the ground of the correlation observed between biologieal activities of BDZs and their free energies of HB interaction with nucleobase derivatives 1-ethyl thymine and 1,3-dimethyl uracil.

In view of the possible role played by HB in the mechanism of action of BDZs, we have thought worthwhile reviewing possible HB types formed by these compounds by an analysis of the crystal packing of all BDZs of known molecular structure. The results of such an analysis are reported in Table 1 and Fig. 1.

Most frequently BDZs appear to be self-associated in dimers, closing an 8- or 12-membered ring by means of two intermolecular HBs (types A, B, C and D of Fig. 1). Sometimes single HBs are observed (types E and F). Of particular interest is HB of type E, which does not correspond to a self-association but to the interaction between BDZ and a molecule of solvent (water or ethanol) and might describe the main interaction of BDZs in water solutions.

As regards the HB donor–acceptor properties of the different atoms or groups, the most frequently observed acceptor is the oxygen of the carbonyl group, while the most common donor is N_1H. The N_4 atom is found to be the acceptor only in two cases and the OH group at carbon atom 3, present in 3-hydroxylated BDZs, is found to act both as donor and acceptor. A particular scheme of HB formation is observed in chlordiazepoxide derivatives (type D), where the acceptor is the oxygen of the N-oxide group and the donor is the N_2H group of methylamine in position 2.

Assuming that HBs reported in Fig. 1 are representative of all possible HB interactions in BDZs, it may be wondered which ones are relevant to the BDZ–receptor interaction. A tentative answer may be sought considering what is known about the metabolic pathways of BDZs [7]. Most of the BDZs in clinical use are derivatives of the 1,3-dihydro-2H-1,4-benzodiazepin-2-one (e.g. nitrazepam; scheme A). A limited number of them are substituted 2,3-dihydro-1H-1,4-benzodiazepines (e.g. medazepam) or 3H-1,4-benzodiazepines (e.g. chlordiazepoxide; scheme D). However it has been proved that both medazepam and chlordiazepoxide are metabolized to the corresponding benzodiazepin-2-one derivatives, that the oxygen of the N — O group is lost and that dealkylation at N_1 is extremely fast in all BDZs tested. In a second time the N_1-dealkylated

Table 1. Hydrogen bond distances ($d_{X\text{-}Y}$) and types (HB type) observed in different crystals of benzodiazepines

	X	H—Y	$d_{X\text{-}Y}$(Å)	HB type	Reference
Nitrazepam	O_2	H—N_1	2.83	A	14
Clonazepam	O_2	H—N_1	2.87, 2.86	A	15
Lorazepam	O_2	H—N_1	2.88	A	16
	O_2	H—O_3	2.73	B	
	O_2	H—O—R	2.83	E	
	N_4	H—N_1	3.01	B	
Oxazepam	N_4	H—O_3	2.82, 2.87	C	17
	O_3	H—N_1	2.94	F	
Clordiazepoxide	O_4	H—N_2	2.85,2.91,2.80,2.79	D	18
Clordiazepoxide HCl	O_4	H—N_2	2.76	D	19
4,5-Dihydro-2'-fluoro-diazepam	O_2	H—O—R	2.81	E	20

X = acceptor and Y = donor in the hydrogen bond X-----H—Y; symbols in the HB type column refer to Fig. 1.