THE COMPARATIVE EFFECTS OF GLYCERYL GUAIACOLATE AND ADENOSINE ON THE INHIBITION OF ADP-INDUCED PLATELET AGGREGATION*

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Clinical anticoagulant therapy has thus far approached the problems of thromboembolic disease through the use of heparin and coumarin types of drugs. While the exact mode of action of the heparin type of drug is not presently known, it appears to inhibit all stages of the clotting mechanism except surface activation (Hardisty and Ingram[11]) with its major effect on the thrombin-fibrinogen reaction reflected in a lengthened clotting time (Biggs and Macfarlane[1]). The coumarin group of drugs prolongs the 1-stage prothrombin time, initially producing a rapid fall in factor VII and a subsequent decrease in factors IX, X, and II (Biggs and Macfarlane[1]).

However, therapy based on these drugs fails to consider the importance of platelet aggregation in the production of *in vivo* clotting (Glynn, Murphy and Mustard[10]) and much research has been devoted to seeking out compounds effective in preventing platelet aggregation.

Since Gaarder *et al.* in 1961[9] showed that platelet aggregation is mediated by adenosine diphosphate (ADP), attempts have been made to prevent platelet aggregation by inhibition of ADP action. The aggregating effect of ADP has been stated to be specific, and the molecular structure of compounds capable of inhibiting ADP-induced aggregation in concentrations of the same order of magnitude as that of the ADP has also been thought to be specific (Born[2], Born *et al.*[4]). Adenosine, however, is a potent vasodilator producing a marked fall in arterial blood pressure (Born *et al.*[4]), and it is rapidly rendered ineffective *in vivo* by adenosine deaminase present in erythrocytes (Bunag *et al.*[5]). An even more potent inhibitor of ADP-induced platelet aggregation is 2-chloroadenosine but it was found to produce respiratory arrest in some rabbits (Born, Honour and Mitchell[3]). Competitive inhibition of ADP has also been demonstrated with certain substituted amino acids (Salzman and Chambers[14]). More recent studies have shown that dipyrimadole (Persantin), an arterial vasodilator, is able to inhibit ADP-induced

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platelet aggregation (Emmons *et al.*[7,8]) but not that produced by noradrenaline (Emmons *et al.*[7]). The mechanism of this action is unknown, but dipyrimadole has been shown to decrease the rate of disappearance of exogenous adenosine from whole blood (Bunag *et al.*[5]), and may act to prevent the entry of adenosine into erythrocytes by affecting the membrane permeability, thus slowing the usual rate of deamination and providing higher levels of adenosine. Presumably, a similar mechanism applies to endogenous adenosine.

In 1966, a different type of compound was found to have effects on platelet adhesiveness. Eastham and Griffiths[6] noted that a normal person who was taking glyceryl guaiacolate had a prolonged activated-plasma clotting time and that ADP-induced adhesive platelet counts were reduced both *in vivo* and *in vitro*. While the significance of inhibition of contact activation in the mechanism of *in vivo* clot formation is speculative (Biggs and Macfarlane[1]), the inhibition of platelet aggregation *in vivo* is most significant (Glynn, Murphy and Mustard[10]). Of further importance is the fact that glyceryl guaiacolate is not chemically related to adenosine or other previously tested inhibitors of platelet aggregation.

A study was, therefore, undertaken to determine the comparative effects of glyceryl guaiacolate and adenosine on the inhibition of ADP-induced platelet aggregation in an attempt to define the action of glyceryl guaiacolate.

Materials and Methods. Platelet aggregation was determined by a modification of the spectrophotometric method of Vainer and Caen[13]. Siliconized glassware and plastic syringes were used in all experiments, and whole blood was collected from normal volunteer donors. Nine milliliters of whole blood was placed in centrifuge tubes containing 1 ml. of 3.8% sodium citrate and centrifuged at 1500 rpm for 10 minutes at 4°C. in an International Centrifuge, Model PR-2. The platelet rich plasma (PRP) was aspirated and stored in a refrigerator for not more than one hour. Platelet-poor plasma (PPP) was made from the PRP by additional centrifugation at 3000 rpm under the previously described conditions. Prior to each experiment, the PRP, PPP and buffers were pre-incubated to 37°C.

Four or more cuvettes were used in each experiment, a diagrammatic representation of which is shown in Table 1. Cuvette 1 contained 0.8 ml. of PPP and 0.4 ml. of Michaelis veronal-acetate buffer, pH 7.4, and served as a plasma blank. Cuvette 2 contained 0.8 ml. PRP and 0.4 ml. of buffer and provided the platelet-rich plasma control. Cuvette 3 contained 0.8 ml. PRP, 0.15 ml. of buffer, and 0.25 ml. of buffer containing 200 μg. per ml. of ADP*, to give a final concentration of 41.6 μg. per ml. (8.8×10^{-5}M) ADP. Cuvette 4 and additional cuvettes contained 0.8 ml. PRP, 0.15 ml. of buffer containing either adenosine† or glyceryl guaiacolate‡ in varying concentrations, and 0.25 ml. of buffer containing 200 μg. per ml. ADP.

Buffer containing adenosine or glyceryl guaiacolate was added to Cuvette 4 and additional cuvettes, mixed by inversion, and incubated at 37°C. for exactly 5 minutes prior to the addition of ADP. Then ADP was added to Cuvettes 3, 4 and others, the contents of which were again mixed by inversion, and optical density readings were taken at 1 and 15 minutes using a Beckman DU Spectrophotometer at 610 mμ.

Calculations of the inhibition of ADP-induced platelet aggregation produced by adenosine or glyceryl guaiacolate were made as follows. The decrease in O.D. due to uninhibited ADP action occurring in Cuvette 3 was taken as the maximal obtainable decrease, and this reading was subtracted from the control O.D. value of Cuvette 2. The readings in Cuvette 4 and other cuvettes containing inhibitors were similarly subtracted from the reading of Cuvette 2. The percent inhibition was then calculated according to the formula:

$$100 - \frac{\text{O.D.}_2 - \text{O.D.}_4}{\text{O.D.}_2 - \text{O.D.}_3} \times 100 = \text{per cent inhibition}$$

*Adenosine-5′-diphosphate, tri-sodium salt, Control Number 6186209, C. F. Boehringer & Soehne GmbH, Mannheim.

†Adenosine (Free base), Lot R1554, Mann Research Laboratories, Inc., New York.

‡Glyceryl guaiacolate, 3-(o-Methoxyphenoxy)-1,2-propanediol, generously supplied by Drs. Roger Cooper and Arnold J. Singer, Reed and Carnrick Research Laboratories, Kenilworth, New Jersey.

TABLE 1.—MODIFIED METHOD FOR THE SPECTROPHOTOMETRIC DETERMINATION OF INHIBITION OF PLATELET AGGREGATION. FOUR OR MORE CUVETTES WERE USED IN EACH EXPERIMENT. X DESIGNATES ADDITIONAL CUVETTES CONTAINING EITHER GLYCERYL GUAIACOLATE OR ADENOSINE AS INHIBITORS

CUVETTE NUMBER	PLATELET POOR PLASMA (ml)	PLATELET RICH PLASMA (ml)	BUFFER (ml)	BUFFER CONTAINING INHIBITOR(ml)	ADP 200 γ/ml (ml)
1	0·8	—	0·4	—	—
2	—	0·8	0·4	—	—
3	—	0·8	0·15	—	0·25
4	—	0·8	—	0·15	0·25
X	—	0·8	—	0·15	0·25

Method:

→ Add buffer (Michaelis barbital-acetate, pH 7.4) at 37°C to plasma at 37°C in cuvettes 1, 2 & 3.

→ Add buffer containing inhibitor at 37°C to plasma in remaining cuvettes.

→ Mix by inversion; incubate exactly 5 min. at 37°C

→ Add ADP.

→ Read O.D. at 1 & 15 min. on Beckman DU spectrophotometer at 610 mμ at 37°C.

Results. The average effect of adding increasing amounts of adenosine to PRP samples from 5 donors, and of adding increasing amounts of glyceryl guaiacolate to samples from 7 donors is shown in Fig. 1. The greatest inhibition of ADP-induced platelet aggregation was obtained with adenosine in a concentration of 25 μg. per ml. (9.4×10^{-5}M) at 1 minute. Inhibition was not further increased by additional adenosine, and approximately 10% less inhibition was found at 15 minutes with the optimal concentration of adenosine. Glyceryl guaiacolate also showed its greatest inhibitory effect at 1 minute, but the maximum inhibition was less than that obtained with adenosine and required a concentration of 50 μg. per ml. (2.5×10^{-4}M). At 15 minutes, a concentration of glyceryl guaiacolate of 100 μg. per ml. (5.0×10^{-4}M) was needed to produce greatest inhibition and this inhibition was approximately 13% less than that obtained at one minute.

Having determined the approximate concentrations at which glyceryl guaiacolate and adenosine produced their respective maximal inhibitions of ADP-induced platelet aggregation, studies were performed on PRP samples obtained from 15 donors, using glyceryl guaiacolate in a concentration of 100 μg. per ml. and adenosine in a concentration of 25 μg. per ml. Figure 2

shows that the average inhibition produced by glyceryl guaiacolate at 1 minute was 26 ± 6.4% (S.D.) and at 15 minutes 22 ± 5.9% (S.D.). Adenosine again produced greater inhibitions of 44 ± 5.7% (S.D.) at 1 minute, and 31 ± 9.0% (S.D.) at 15 minutes, respectively.

From these data it can be determined that glyceryl guaiacolate showed 59% of the inhibitory activity of adenosine at one minute and 71% of the inhibitory activity of adenosine at 15 minutes. However, the required molar concentration of glyceryl guaiacolate was approximately 5 times that of adenosine, while adenosine was found to produce its maximal inhibitory effect in a molar concentration (9.4×10^{-5}M) approximately equal to that of the ADP causing aggregation of the platelets (8.8×10^{-5}M).

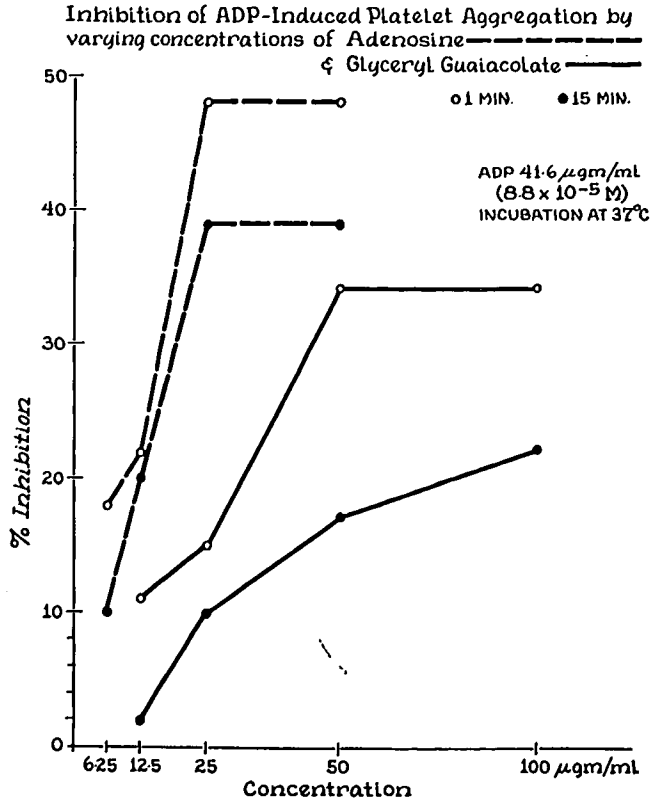

Fig. 1.—Inhibition of ADP-induced platelet aggregation by varying concentrations of adenosine and glyceryl guaiacolate. Increasing amounts of adenosine added to PRP from 5 normal donors and increasing amounts of glyceryl guaiacolate added to PRP from 7 normal donors.

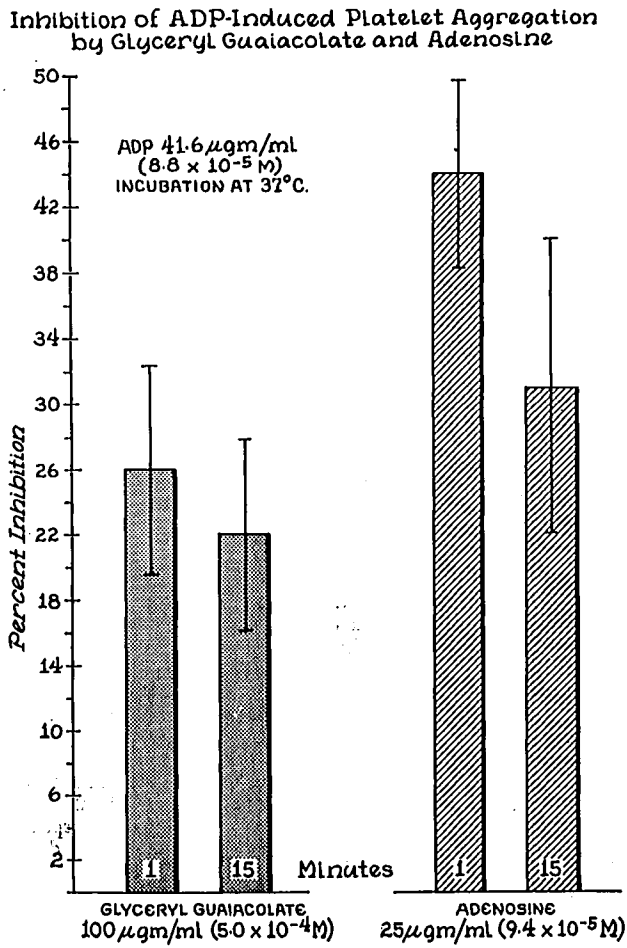

Fig. 2.—Inhibition of ADP-induced platelet aggregation by glyceryl guaiacolate and adenosine. Glyceryl guaiacolate added in concentration of 100 μg. per ml. and adenosine added in concentration of 25 μg. per ml. to PRP samples from 15 normal donors.

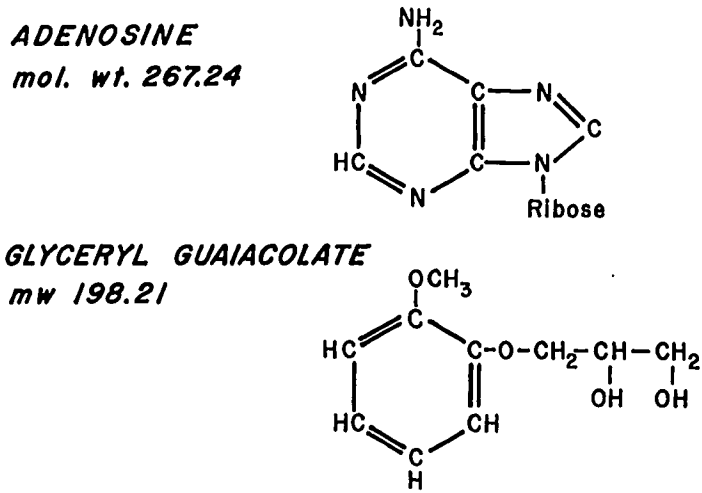

Fig. 3.—Chemical structure and molecular weights of adenosine and glyceryl guaiacolate.

Discussion. The molecular structures of glyceryl guaiacolate and adenosine, as shown in Fig. 3, would suggest that their respective mechanisms of action might differ, and this is borne out by the experimental data. Glyceryl guaiacolate is the orthoether of glycerol and guaiacol, and not an ester, as the common name would indicate. Adenosine, on the other hand, is adenine riboside. Adenosine lacks only the pyrophosphate group to be ADP, and produces maximal inhibition of ADP-induced platelet aggregation in a molar concentration approximating that of the ADP producing aggregation. Glyceryl guaiacolate does not produce as much inhibition of platelet aggregation as does adenosine, and requires approximately 5 times the concentration of adenosine to exert its maximal effect. Adenosine is probably a competitive inhibitor of ADP, and is sufficiently similar in structure to block the platelet binding sites of ADP. Glyceryl guaiacolate appears to work in a different manner.

Glyceryl guaiacolate is not likely to have an action similar to that of Persantin (Bunag *et al.*[5], Emmons *et al.*[7,8]) since it is not known to have a vasodilatory action. This effect would be expected if the deamination of endogenous adenosine were inhibited due to alteration of cell membrane permeability.

O'Brien has shown that a number of drugs[13] including certain antimalarials, antihistaminics and local anesthetics produce inhibition of spontaneous platelet aggregation. These drugs have in common the fact that they all have a hydrocarbon and lipophilic moiety and all carry a net positive charge. Glass treated with these drugs becomes hydrophobic and no longer attracts platelets. It has also been shown that non-ionic surface active agents such as Triton WR-1339 (Minor and Burnett[12]) prevent the formation of platelet aggregates. It is likely that this action occurs by the attachment of the lipophilic portion of the molecule to the

lipid-containing surface of the platelet, and the hydrophilic moiety forms hydrogen bonding with the surrounding aqueous solvent.

Guaiacol (o-Methoxyphenol) is only sparingly soluble in water but is very soluble in organic solvents. Glycerol is soluble in all proportions with water but only slightly so in ether or benzene. It is probable that the combination of these compounds, in glyceryl guaiacolate, produces a molecule in which the methoxyphenol portion is lipophilic and the propanediol moiety is hydrophilic. No ionization of this compound should occur, but because of the two hydroxyl groups present, strong hydrogen-bonding forces would be expected with an aqueous solvent.

The action of glyceryl guaiacolate in producing inhibition of ADP-induced platelet aggregation is consistent with its being a surface active agent. This hypothesis might also explain Eastham and Griffiths' finding[6] that glyceryl guaiacolate interfered with contact activation of plasma since it may coat activating substances with a hydrophobic monolayer.

Further studies are in progress to determine the surface active properties of glyceryl guaiacolate and its therapeutic efficacy in thromboembolic disease, alone and in combination with other types of anticoagulants.

Summary. Recently, Eastham and Griffiths[6] noted that glyceryl guaiacolate produced reduction of platelet adhesiveness and prolongation of the activated plasma coagulation time in persons receiving this drug. Before this, Born[2] had shown that adenosine and a few compounds of similar molecular structure were inhibitors of ADP-induced platelet aggregation, and that this effect was specific. Since glyceryl guaiacolate is chemically unrelated to adenosine, a study was made to compare the effectiveness of these two compounds at various concentrations on the inhibition of platelet aggregation.

Glyceryl guaiacolate in final concentration of 100 μg. per ml. (5.0×10^{-4}M) produced maximal inhibition of aggregation at one and 15 minutes, approximating 26% and 22% inhibition, respectively. Adenosine in a concentration at 25 μg. per ml. (9.4×10^{-5}M) produced inhibition of about 45% at one minute and 30% at 15 minutes.

Since adenosine produced its maximal inhibition of aggregation in concentrations approximately equimolar to ADP, competitive inhibition is its indicated mechanism of action. Higher concentrations of glyceryl guaiacolate produced somewhat less, but still significant, inhibition of platelet aggregation suggesting a different action from that of adenosine. It is hypothesized that glyceryl guaiacolate may be a surface-active agent. Further studies of glyceryl guaiacolate as a possible therapeutic agent in thromboembolic disease are in progress.

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SUMMARIO IN INTERLINGUA

Le Effectos Comparative de Guaiacolato Glycerylic e de Adenosina Super le Inhibition de Aggregation Plachettal Inducite per ADP

Recentemente, Eastham e Griffiths ha notate que guaiacolato glycerylic produceva un reduction del adhesivitate plachettal e un prolongation del tempore de coagulation de plasma activate in subjectos recipiente ille pharmaco. Ante ille tempore, Born habeva monstrate que adenosina e varie compositos de un simile structura molecular esseva inhibitores de aggregation plachettal inducite per ADP que iste effecto esseva specific. In vista del facto que guaiacolato glycerylic es chimicamente non relationate con adenosina, un studio esseva interprendite pro comparar le efficacia de ille duo compositos a varie concentrationes in le inhibition del aggregation plachettal.

Guaiacolato glycerylic, in un concentration final de 100 μg per ml ($5{,}0 \times 10^{-4}$M) produceva inhibition maximal del aggregation a 1 e a 15 minutas, amontante a approximativemente 26 e 22 pro cento, respectivemente. Adenosina, in un concentration de 25 μg per ml ($9{,}4 \times 10^{-5}$M), produceva inhibition a 1 e a 15 minutas, amontante a circa 45 e 30 pro cento, respectivemente.

Viste que adenosina produceva su inhibition maximal del aggregation in concentrationes approximativemente equimolar con ADP, inhibition competitive es le indicate mechanismo de action. Plus alte concentrationes de guaiacolato de glyceryl produceva un minus marcate sed ancora significative inhibition del aggregation plachettal, suggestionante un action differente ab illo de adenosina. Es postulate le hypothese que guaiacolato glycerylic es un surfactante. Studios additional de guaiacolato como possibile agente therapeutic in morbo thromboembolic es in progresso.