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Comparative studies with the enantiomers of the glycol metabolite of propranolol and their effects on the cardiac β-adrenoceptor

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The two enantiomers ((*R*)- and (*S*)-) of propranolol glycol, a metabolite of propranolol, have been synthesized, and their effects upon the β-adrenoceptor studied by two methods. The ability of these compounds to antagonize the inotropic actions of isoprenaline was examined on spontaneously beating rat atrial preparations. Also, the effects of these enantiomers upon the binding of [^{3}H]dihydroalprenolol to β-receptors in rat cardiac ventricular membranes was studied. Experiments with the atria indicated that the (*S*)-glycol was a reversible competitive antagonist of isoprenaline with a potency approximately one thousand times lower than that of (±)-propranolol. In contrast, the (*R*)-glycol appeared to act as an irreversible antagonist, producing complex dose–response curves. The effects of these compounds to cause displacement of alprenolol binding were consistent with the organ bath data. The interaction of the (*S*)-glycol with the β-receptor binding site was reversible (K_i of 27·6 ± 4·2 μM) but less potent than that of (±)-propranolol (K_i of 0·99 ± 0·07 nM). On the other hand, pretreatment of ventricular membranes with the (*R*)-glycol, followed by extensive washing techniques, resulted in alprenolol binding which did not regain control values, providing further evidence for an irreversible effect upon the β-receptor. The possible significance of these pharmacological actions of the two enantiomers is discussed in terms of the in-vivo metabolic pathways for propranolol.

In medical practice, drugs are frequently used as racemic mixtures and the pharmacological effects of the individual enantiomers are often poorly defined. Often only one isomer is therapeutically active, but this does not mean that the other is really inactive. It may well contribute to the side effects (Ariens 1984). This may have important therapeutic consequences as is the case with propranolol (Barrett & Cullum 1968; Yamamoto et al 1978), warfarin (Breckenridge & Orme 1972) and thalidomide (Ockenfels & Kohler 1970). For example, (*R*)-(+)-propranolol has less than one hundredth the potency of the (*S*)-(−)-enantiomer. In addition, the formation of an intermediate aldehyde after *N*-dealkylation of propranolol gives rise to a number of metabolic products including propranolol glycol, 4-hydroxypropranolol glycol, naphthoxylactic acid and 1-naphthol (Walle & Gaffney 1972; Vu & Abramson 1980). Further investigation of the pharmacological properties of the propranolol glycol was considered of interest since previous workers had found that similar glycerol ethers showed sedative, hypotensive and negative chronotropic activites (Berger 1948; Saelens et al 1973). Consequently in the present work we have examined the possible interaction between the glycol and the β-adrenoceptor in cardiac tissue by two different approaches. The first involved a study of the effects of the glycol on the spontaneously beating rat atrial preparation, while the second examined its influence on the binding of [^{3}H]dihydroalprenolol (DHA) to the β-adrenoceptor in rat ventricular membranes. Throughout this work particular attention has been paid to possible differences in the properties of the two enantiomers of propranolol glycol which have been synthesized for the purpose of conducting this study.

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MATERIALS AND METHODS

Materials

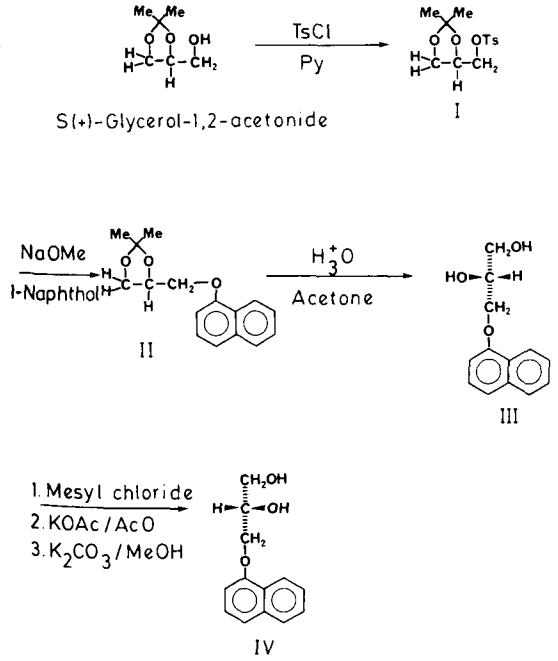
All chemicals and solvents were standard laboratory reagent grade supplied by the Aldrich Chemical Company (Gillingham, UK). (±)-Propranolol was supplied by Imperial Chemical Industries (Macclesfield, UK), (±)-isoprenaline HCl and (−)-alprenolol L-tartrate were obtained from Sigma Chemical Company (Poole, UK). The radioligand (−)-[^{3}H]dihydroalprenolol HCl (235 mCi mg^{-1}) was supplied by Amersham International plc. (Amersham, UK). The radiochemical purity before use was confirmed as not less than 98% by thin layer chromatography and radio-chromatogram scanning.

NMR spectra were obtained using a Varian EM360, 60 MHz spectrometer using tetramethylsilane as internal standard. Infrared spectra were obtained from a Pye Unicam SP1000 spectrometer using either Nujol mull or thin film samples.

Adult male Wistar rats (250–350 g) were obtained from our departmental breeding colony, Animal Services Unit, University of Dundee.

Chemical synthesis

Glycerol acetonide previously used as the chiral backbone in the synthesis of oxprenolol, practolol and timolol (Nelson & Burke 1978; Danilewicz & Kemp 1973; Weinstock et al 1976) has now been used to synthesize the two enantiomers of propranolol glycol (Scheme 1).



SCHEME 1. The synthesis of propranolol glycol enantiomers from the chiral starting material D-mannitol. Numbers refer to compounds prepared.

(R)-(−)-*3-Tosyloxy-1,2-propanediol acetonide (I)* (Baldwin et al 1978). (*S*)-(+)-Glycerol-1,2-acetonide was heated with tosyl chloride to yield (I) b.p. 78–88 °C, 17–18 mm (mercury); yield 66% (Lit. b.p. 80–90 °C, 20 mm; yield 91%).

(S)-(+)-*3-(1-Naphthoxy)-1,2-propanediol acetonide (II)*. Variant of the method of Danilewicz & Kemp (1973). Sodium (4·6 g; 0·2 mol) in methoxyethanol (40 mL) was heated with 1-naphthol (28·8 g; 0·2 mol) in methoxyethanol (50 mL) and the acetonide (68 g; 0·2 mol) added to the resulting solution which was stirred under reflux (4 h) in subdued light. The cooled solution was poured into water (500 mL) and exhaustively extracted with ether. The combined extracts were washed with brine and then dried ($MgSO_4$), and evaporated to yield the acetonide (II), (b.p. 180–190 °C, 2 mm; yield 45%) IR cm^{-1}: 725, 925, 1000, 1050, 1100, 1500, 3200 NMR δ (acetone d6): 8·4 (m, 1, Ar-H8), 7·7 (m, 1, Ar-H5), 7·3 (m, 4, Ar-H3, 4, 6, 7), 6·6 (dd, 1, Ar-H2), 4·7–3·9 (m, 5, CH_2CHCH_2), 1·6–1·3 (d, 6, $(CH_3)_2$).

(R)-(−)-3-*(1-Naphthoxy)-1,2-propanediol (III) ((R)-propanolol glycol)*. (Nelson & Burke 1978). To the acetonide (II) (65·3 g; 0·253 mol) in acetone (150 mL), hydrochloric acid (62 mL; 2 M) was added and the mixture refluxed (6 h). The acetone was removed under vacuum, water was added and the mixture exhaustively extracted with ether. The combined extracts were dried ($MgSO_4$), then evaporated to yield (*R*)-propranolol glycol (III), m.p. 108–109 °C, from toluene (charcoal), $[\alpha]_D = -6·76°$ (c. 1·0% in MeOH) (yield 52%) (M^+ 218, major peaks at 115, 124 and 127; analysis found: C, 71·5; H, 6·4; O, 22·1%. $C_{13}H_{14}O_3$ requires C, 71·6; H, 6·4; O, 22·0%). M.p. 108–109 °C, $[\alpha]_D = +10·2°$ (c. 1% MeOH) for (*S*)-enantiomer (Tucker 1973). M.p. 108–109 °C, $[\alpha]_D = +6·9°$ (c. 1% MeOH) for (*S*)-enantiomer. $[\alpha]_D = -8·1°$ for (*R*)-enantiomer (Nelson & Bartels 1982) (no concentration or solvent given).

Inversion of (R)-*propranolol glycol to* (S)-*(+)-3-(1-naphthoxy)-1,2-propanediol (IV)*. (Adapted from the method of Takano et al 1983.) (*R*)-Propranolol glycol (III) (2 g; 9·1 mmol) in dry dichloromethane (50 mL) and pyridine (4 mL) was purged with argon, then mesylchloride (1·8 mL; 23·3 mmol) was added to the stirred solution. The mixture was refluxed for 4 days (confirmation of completion of the reaction being determined by TLC) then diluted with ice/water (30 mL) and extracted with dichloromethane (3 × 20 mL). The extracts were combined, washed with brine (1 × 25 mL), HCl 5% v/v (2 × 30 mL), brine (1 × 25 mL), $NaHCO_3$ 5% w/v (2 × 30 mL), brine (1 × 30 mL), then dried ($MgSO_4$); evaporation gave the crude dimesylate.

Potassium acetate (3·14 g; 31·9 mmol) was added to the dimesylate which was stirred under reflux (2 h) with acetic anhydride (32 mL) under argon. Evaporation under vacuum gave a solid which was taken up in dichloromethane (5 × 30 mL). The combined

extracts were washed with brine (2 × 25 mL), Na-CO_3 5% w/v (2 × 30 mL), brine (1 × 30 mL) then dried ($MgSO_4$), filtered and evaporated. The residue was hydrolysed by stirring overnight with potassium carbonate (1·95 g; 14 mmol) in methanol (50 mL) under argon. Evaporation of the filtrate yielded a brown oil containing (*S*)-propranolol glycol (IV) (0·3 g; 15%) which on crystallization from toluene (charcoal) had m.p. 108–109 °C, $[\alpha]_D$ = +6·7° (c. 1·0% in MeOH) (Found: C, 72·1; H, 6·4; O, 21·5%. $C_{13}H_{14}O_3$ requires C, 71·6; H, 6·4, O, 22·0%). NMR: δ (acetone d6): 8·3 (m, 1, Ar-H8), 7·8 (m, 1, Ar-H5), 7·4 (m, 4, Ar-H3) 6·9 (dd, 1, Ar-H2), 4·1–4·4 (m, 5, CH_2CHCH_2), 4 (m, 2, OH).

Pharmacological experiments

The positive inotropic response to isoprenaline produced in the spontaneously beating rat atrial preparation was used to assess possible β-blocking activity of the two propranolol glycol enantiomers (III & IV) and (±)-propranolol. The tissue was mounted at 34 °C in a 10 mL organ bath with Krebs-Henseleit (Krebs-H) solution (mM; NaCl 122·5, KCl 4·7, $MgSO_4$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25, D-glucose 11 and $CaCl_2$ 2·5) and the twitch response recorded with an isometric transducer (Ormed) connected to a recorder (Devices MX212) with pre-amplifier (Devices M2P).

Both III & IV are only sparingly water soluble so stock solutions (10^{-3} M) were made using propylene glycol as a vehicle. This was prepared by dissolving the compounds in propylene glycol and dilution with Krebs-H to give a final propylene glycol concentration in the stock solutions of 40% (v/v). Lower concentrations for use in either technique were provided by further dilutions in Krebs-H. At the highest final concentration of propylene glycol used in the organ bath (0·4% v/v) the vehicle had no direct action on the force and rate of contraction of the tissue. After establishing a control dose–response curve to isoprenaline, then either (±)-propranolol, (III) or (IV) was added to the bath 1 min before the addition of isoprenaline. The change in force of contraction was determined over the next 90 s followed by washout before the next addition of drugs. In the absence of isoprenaline no direct action was detected using concentrations in the range 10^{-6}–10^{-11} M propranolol or 10^{-4}–10^{-7} M (III) and (IV). For each of the compounds four dose–response curves were constructed, the first with isoprenaline alone and the other three with varying concentrations of the particular compound under study in the experiment (Ogg et al 1985). From the resulting dose–response curves the ED50 values and dose ratios were determined where possible and used in the construction of Schild plots (Arunlakshana & Schild 1959) in order to determine the pA_2 values.

Radioligand binding

Male Wistar rats were killed by stunning and cervical dislocation and the entire heart excised, the atria removed and the ventricles (0·782 ± 0·027 g; n = 15) homogenized in Krebs-H solution at 0 °C. Membrane fractions from the tissues of individual rats were then prepared using the method of Kunos et al (1980). Total protein concentration of the membranes was assayed by the method of Lowry et al (1951) and found to be 34·6 ± 0·8 mg protein g^{-1} of wet tissues (n = 15).

Preliminary experiments indicated that equilibrium binding was achieved at 20 min (37 °C) and this incubation time was used in all subsequent experiments. Assay tubes contained membranes (equivalent to 0·3 mg protein), [^{3}H]DHA (8·35 × 10^{-10} M), (−)-alprenolol (when appropriate) and varying concentrations of displacing agents, in a total volume of 1 mL. The range of specific binding obtained using (−)-alprenolol (10^{-5} M) as competing ligand was between 40 to 65% of total binding. IC50 values were determined from inhibition curves constructed over a range of displacing agent concentration and these were used to calculate the respective K_i values by the method of Cheng & Prusoff (1973) for reversible competitive inhibition, when appropriate. Kinetic constants for specific binding [^{3}H]DHA were estimated by Scatchard analysis (Scatchard 1949) of binding data obtained with varying concentrations (0·05–10 nM) of [^{3}H]DHA in control assays.

RESULTS

Rat atria

The dose–response curve to isoprenaline in the presence of (±)-propranolol was shifted to the right in a dose-dependent and parallel fashion.

(*S*)-(+)-Propranolol glycol (IV) displaced the dose–response curve to the right in a parallel fashion indicating a competitive type of antagonism of the isoprenaline effect (Fig. 1). (*R*)-(−)-Propranolol glycol (III) in doses of 10^{-7}–10^{-5} M on the other hand, inhibited the maximum response to isoprenaline and this could not be achieved again even after wash out (Fig. 2). With 10^{-4} M, not only a reduction in maximum response was observed but also a shift to the right of the dose–response curve was found. The results obtained with this enantiomer were therefore

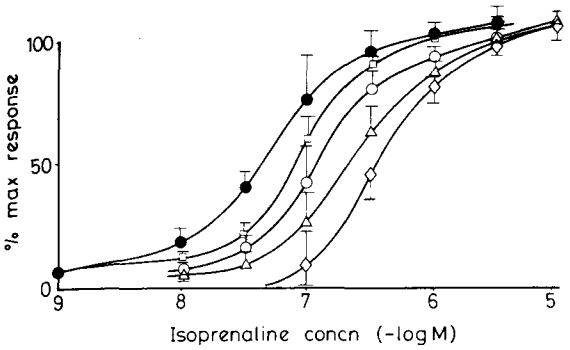


FIG. 1. Antagonism by (*S*)-propranolol glycol of the positive inotropic effects of isoprenaline upon spontaneously beating rat atria. ●— control (no glycol), □— (10^{-7} M), ○— (10^{-6} M), △— (10^{-5} M) and ◇— (10^{-4} M) (*S*)-propranolol glycol. Each point is the mean ± standard error of five experiments.

not found to fit classical plots and the antagonism with this metabolite is obviously more complex. Consequently the pA_2 values only for (±)-propranolol and its (*S*)-(+)-glycol metabolite (IV) were calculated from the intercepts of five Schild plots and are given in Table 1.

Radioligand binding data

The kinetic constants for [^{3}H]DHA binding were determined by regression analysis of the Scatchard data (Fig. 3) and gave values (n = 5) of Kd (1·36 ± 0·1 nM) and B_{max} (172·6 ± 52·5 fmol mg^{-1} protein). The isolated tissue experiments suggested that (III) showed irreversible antagonism and this was confirmed in binding experiments by preincubation (20 min) of the membranes with compounds (III) and (IV) (2×10^{-4} M). In our preliminary studies it

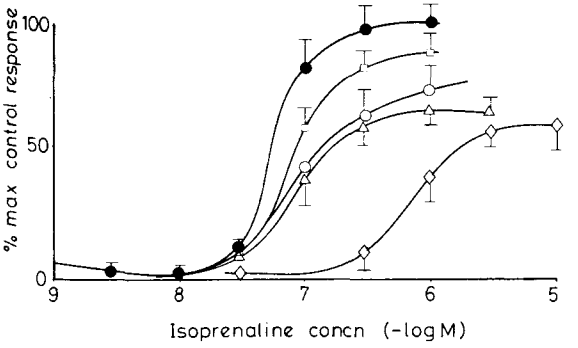


FIG. 2. Antagonism by (*R*)-propranolol glycol of the positive inotropic effects of isoprenaline upon spontaneously beating rat atria. ●— control (no glycol), □— (10^{-7} M), ○— (10^{-6} M), △— (10^{-5} M) and ◇— (10^{-4} M) (*R*)-propranolol glycol. Each point is the mean ± standard error of five experiments.

Table 1. Antagonism of isoprenaline-induced inotropic effects in spontaneously beating rat atria by (III) and (IV).

Drug	Slope of Schild plot	pA_2	n
Propranolol	0·98 ± 0·15	9·12 ± 0·33	5
(*S*)-Glycol (IV)	1·01 ± 0·14	6·08 ± 0·17	5
(*R*)-Glycol (III)	These parameters could not be determined since the action appeared to be irreversible		3

All values are shown as means ± standard error.

was found that a concentration of the glycol (IV) (10^{-4} M) produced approximately 60% inhibition of [^{3}H]DHA binding and the same concentration of III was also used in the following experiments. Some samples of membranes were washed and recovered by centrifugation (3 times) before use in the binding assay. A control set of membranes preincubated without drugs was otherwise treated identically for comparison. In addition, control and drug-treated membranes which were not subjected to the washing procedure were also set up and assayed concurrently. It was found that inhibition produced by the glycol (III) in unwashed samples (63·8 ± 8·0% of appropriate controls) was similar to that after washing (52·6 ± 8·8%, n = 5). In contrast, inhibition by enantiomer (IV) (69·9 ± 11·8% of control) was largely reversed after washing of controls (89·9 ± 8% of control values, n = 3). These experiments therefore confirmed the reversibility of glycol (IV) found with the isolated tissue experiments.

Scatchard plots for alprenolol binding in the presence of glycol (IV) showed no evidence of multiple binding sites and therefore only one site binding was assumed. Typical inhibition curves for the enantiomers of the glycol and (±)-propranolol are shown in Fig. 4, the IC50 values were determined

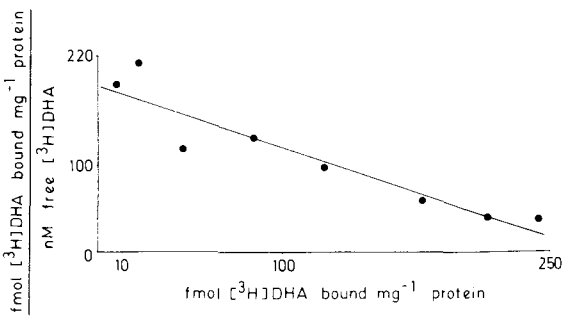


FIG. 3. A typical Scatchard plot for rat ventricular membranes and [^{3}H]DHA. Each point is the mean of duplicate assays on a single membrane fraction.

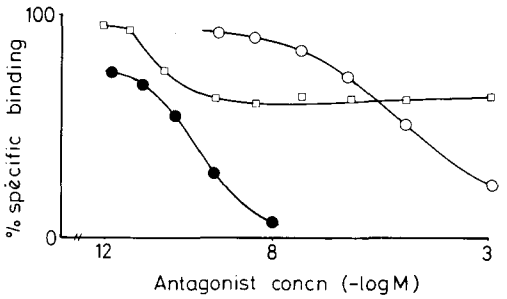


FIG. 4. Typical curves for inhibition of specific [^{3}H]DHA binding to rat ventricular membranes by propranolol (●), (*R*)-propranolol glycol (□) and (*S*)-propranolol glycol (○). Each point is the mean of duplicate assays on a single membrane fraction.

and the results from these binding studies are summarized in Table 2.

Table 2. Inhibition of [^{3}H]dihydroalprenolol binding to rat ventricular membranes by propranolol and its glycol metabolites.

Drug	IC50	K_i	n	Linear correlation Scatchard plot
Propranolol	1·54 ± 1·08 nM	0·994 ± 0·07 nM	5	0·933 ± 0·03 (n = 3)
(*S*)-Glycol	44·5 ± 0·065 μM	27·6 ± 4·2 μM	5	0·953 ± 0·03 (n = 3)
(*R*)-Glycol	These parameters could not be determined since the action appeared to be irreversible		3	

All values are shown as means ± standard error.

DISCUSSION

The (*S*)-(+)-glycerol acetonide was prepared by well-established methods from D-mannitol (Chittenden 1980) and its conversion into (*R*)-(−)-propranolol glycol proceeded in good overall yield. A similar synthesis could not be used for the (*S*)-enantiomer as this would have required the use of the unnatural L-mannitol. An attempted synthesis using vitamin C as chiral starting material (Jung & Shaw 1980) proved unsuccessful and an adaptation of the method of Takano et al (1983) was used in the present study. This produced the (*S*)-enantiomer (IV) although in low chemical yield, but this, being a 'one pot' reaction, was very much simpler than an alternative synthetic route. The importance of this for stereochemical synthesis is that there is now an inversion reaction giving sterically hindered glycerol ethers of high optical purity and this may obviate the necessity of carrying out a second synthesis.

In the assessment of the pharmacological activity of the two enantiomers, as well as the parent compound, (±)-propranolol, pA_2 values were used as an index of their β-blocking activity. In the present experiments glycol (IV) in both the rat atrial preparations and radioligand binding experiments appeared to inhibit in a reversible, competitive manner whereas its enantiomer (III) showed an irreversible effect and gave complex dose–response and inhibition curves. No pA_2 values were therefore obtainable for this enantiomer. The validation of rat atrial preparations was confirmed by Hughes & Stone (1983) who assessed β-blocking activity of (±)-propranolol and found that it produced a parallel shift to the right in the appropriate dose–response curves to isoprenaline.

With the rat atrial preparations it was shown that glycol (IV) had a potency some 1000 times lower than the parent compound (±)-propranolol and with the binding experiments some 10 000 times less potent. These differences between orders of potency in the two assessment procedures are probably not unexpected in view of the grossly different nature of the systems. Because of such differences in potency, in comparison with racemic propranolol, the β-adrenoceptor blockade produced by this metabolite is probably not significant especially in-vivo when in competition with propranolol. The irreversible antagonism shown by enantiomer (III) in these experiments is clearly complex. The antagonism of the effects of isoprenaline on the rat atrial preparation was unusual in that at low doses of III it involved a decrease in the maximal response to the agonist, with little apparent change in the ED50 of the agonist. However, a higher dose (10^{-4} M) also shifted the curves to the right. In the radioligand binding experiments, compound (III) only appeared to bring about partial displacement of [^{3}H]DHA from the β-receptor, with this effect occurring only over the glycol concentration of 10^{-12}–10^{-10} M. Although no effects at these concentrations were noted with isolated tissue experiments, it is possible that higher concentrations of the drug are required to inactivate irreversibly a significant fraction of the larger pool of β-receptors likely to exist in the intact tissue, compared with the small amount of tissue used in individual binding assays. In addition, it is possible that other interactions, possibly non-specific, upon the cardiac tissue may contribute also to the drug's activity. Further experiments would be required to investigate this possibility.

A recent report by Nelson & Bartels (1984) has now confirmed that the intermediate aldehyde (Walle & Gaffney 1972; Vu & Abramson 1980) found in in-vitro metabolism studies is chiral and that no racemization occurs during the formation of the

glycol and acid metabolites. They have also shown that reduction of the (*R*)-aldehyde gives exclusively the (*S*)-glycol (from the precursor (*S*)-propranolol). Similarly, oxidation of the (*R*)-aldehyde yields (*R*)-naphthoxylactic acid. (*S*)-Propranolol is found in lower concentrations in plasma than the (*R*)-enantiomer (Walle & Walle 1979) which suggests that there is a greater removal from the plasma of the (*S*)-compared with the (*R*)-enantiomer. One mechanism which may contribute to this could involve the preferential metabolism of (*S*)-propranolol to the (*S*)-glycol, via the (*R*)-aldehyde intermediate. If so, it is possible that much lower plasma concentrations of (*R*)- than of (*S*)-glycol may result, although to our knowledge, this has not yet been investigated. There will possibly be little contribution of (*R*)-glycol to the overall effect exerted by (*S*)-glycol in-vivo.

Now that the stereochemistry of propranolol distribution and metabolism has been partially described (Barrett & Cullum 1968; Yamamoto et al 1978; von Bahr et al 1982; Walle et al 1983) it should now be possible to screen for possible activity in particular tissues. With the glycol metabolite, effects in the central nervous system may be worthy of consideration.

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