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# IRREVERSIBLE BINDING AND METABOLISM OF PROPRANOLOL BY HUMAN LIVER MICROSOMES—RELATIONSHIP TO POLYMORPHIC OXIDATION

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**Abstract**—Studies were performed to investigate the irreversible binding and oxidative metabolism of propranolol in human liver microsomes and the relationship of binding and metabolism to the polymorphic oxidation of debrisoquine. Incubation of microsomes with $^{14}$C-labelled propranolol in the presence of a NADPH-generating system gave rise to irreversible binding which increased linearly with time and became saturated at high substrate concentrations. The extent of binding was decreased by the exclusion of cofactors, boiling, anaerobic conditions, and the addition of reduced glutathione and SKF-525A. Trichloropropene oxide had a negligible effect on cofactor-dependent binding. However, debrisoquine, antipyrine and phenacetin decreased binding to a considerable extent. The latter compound abolished cofactor-dependent binding completely at the concentration used (1 mM). Electrophoresis of microsomes which had been incubated with tritiated propranolol revealed that binding was probably occurring to a large number of proteins particularly in the 40,000–90,000 molecular weight range. Glutathione, debrisoquine and antipyrine did not inhibit the 4′-hydroxylation and *N*-deisopropylation of propranolol. In contrast, phenacetin exerted a very potent inhibitory action on both routes of metabolism. It is concluded that (a) a product or products of propranolol oxidation bind irreversibly but non-selectively to human liver microsomal protein, (b) the enzyme system responsible for the activation of propranolol appears to be related more closely to the cytochrome P-450 system which metabolises phenacetin than to that metabolising debrisoquine, and (c) radiolabelled propranolol is not a sufficiently specific probe for studying these cytochrome P-450 systems.

Previous studies have shown that propranolol is a potent inhibitor of cytochrome P-450-mediated metabolism in rat and man [1–5]. Rats pretreated with propranolol *in vivo* showed a decreased ability to catalyse the further metabolism of the drug [5] and also that of lignocaine [4], despite the absence of significant residual unchanged propranolol. This work together with experiments using radiolabelled drug [6] provided strong evidence that a metabolic intermediate of propranolol binds irreversibly to the cytochrome P-450 system causing inhibition of further metabolism. Binding appeared to be selective for one or more cytochromes P-450 since 4′-hydroxylation of propranolol and 3-hydroxylation of lignocaine were inhibited yet the *N*-dealkylation of these drugs remained unaffected as did the activities of ethylmorphine *N*-demethylase and aryl hydrocarbon hydroxylase.

The 4′-hydroxylation of propranolol is impaired in subjects who are poor metabolisers of the polymorphically metabolised drug debrisoquine [7, 8]. Genetic polymorphism of the oxidation of debrisoquine and related compounds is thought to result from either the absence of or functional inactivity of one or more forms of cytochrome P-450 in the livers of poor metabolisers [9, 10]. Rat and human cytochromes P-450 with high specific activity for debrisoquine have been purified to electrophoretic homogeneity [11, 12]. The reactive intermediate of propranolol metabolism may therefore bind and inactivate selectively this cytochrome(s) P-450 and it may thus be possible to identify and isolate the enzyme system by covalent labelling following incubation with radioactive drug in the presence of a NADPH generating system.

In the present work we have confirmed that a product or products of propranolol oxidation binds irreversibly to microsomal protein from human liver but found that proteins were labelled in a relatively non-selective manner. Further experiments were performed to investigate the relationship between the irreversible binding and oxidative metabolism of propranolol and debrisoquine oxidation phenotype.

## MATERIALS AND METHODS

*Chemicals and drugs.* [1-$^{14}$C]Propranolol hydrochloride (specific activity 38.4 $\mu$C mg$^{-1}$) and [4-$^{3}$H]propranolol hydrochloride (74 mCi · mg$^{-1}$) were obtained from I.C.I. Pharmaceuticals (Macclesfield, U.K.) and Amersham International (Amersham, U.K.), respectively. Both were dissolved in 50 mM Tris–HCl buffer (pH 7.4) and diluted as necessary with unlabelled propranolol hydrochloride (I.C.I. Pharmaceuticals). Reduced glutathione was purchased from Sigma Chemical Co. (Poole, U.K.), trichloropropene oxide from Aldrich Chemicals (Gillingham, U.K.), antipyrine from B.D.H. Chemi-

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cals (Poole, U.K.) and phenacetin from Hopkin and Williams (Chadwell Heath, U.K.). Debrisoquine was a gift from Roche Products Ltd. (Welwyn Garden City, U.K.), 4'-hydroxy- and *N*-desisopropylpropranolol from I.C.I. Pharmaceuticals (Macclesfield, U.K.) and 1-(4-butyramido-2-butyrylphenoxy)- 2- hydroxy- 3- isopropylaminopropane (HPLC internal standard) from May and Baker Ltd. (Dagenham, U.K.).

*Radiochemical purity.* The radiochemical purity of the carbon-14 and tritium-labelled samples were stated to be at least 99.7 and 98%, respectively. This was confirmed using thin-layer chromatography with the following systems: (a) 2-propanol/ammonium hydroxide/water (40:2:5 v/v) (b) 1-butanol/acetic acid/water (40:2:5 v/v) (c) 0.880 ammonia solution/methanol (1.5:100 v/v).

*Source of human liver.* Small samples of human liver tissue were obtained from renal transplant donors who had been maintained on life support systems until the kidneys were removed. The samples were taken as soon as possible after the kidneys were removed, cut into approximately 2 cm cubes, frozen in liquid nitrogen and stored at −80° until use. The period between cessation of organ perfusion and cooling of the tissue was about 30 min.

Samples of 3 livers were used in this study and all donors (subsequently referred to as 1, 2 and 3) were Caucasian males. Drugs given prior to or during organ removal were: (1) diazepam, dopamine, mannitol, pancuronium, nitrous oxide, oxygen, phentolamine, phenoxybenzamine, heparin, (2) ampicillin, cloxacillin, metronidazole, phenytoin (100 mg t.d.s. for 4 days prior to death), frusemide, phenoperidine, pancuronium, (3) dexamethasone hydralazine, dopamine, dobutamine, chlorpromazine, frusemide, phenoxybenzamine, phentolamine. Liver histology was characterised by (1) slight lymphocytic infiltration, (2) no abnormality, (3) slight lymphocytic infiltration. None of the donors had a known history of liver disease. Their smoking and drinking habits were not known.

*Preparation of microsomes.* Microsomes were prepared by ultracentrifugation as described by Boobis *et al.* [13], using either a 10 × 20 ml or a 10 × 100 ml rotor. Microsomal pellets were resuspended in 0.25 M potassium phosphate buffer (pH 7.25) containing 30% glycerol (v/v) at a concentration equivalent to 1 or 2 g tissue per ml and stored at −80° until use. Microsomal protein content, cytochrome P-450 content and NADPH–cytochrome *c* reductase activity were measured by the methods of Lowry *et al.* [14], Omura and Sato [15] and Mazel [16], respectively.

*Incubation conditions.* Liver microsomes were incubated with substrate and inhibitor in the presence of a NADPH generating system (NADP 1 mM, glucose-6-phosphate 20 mM, yeast glucose-6-phosphate dehydrogenase 0.24 units per ml, magnesium chloride 42 mM). Incubations were performed in duplicate at 37° and pH 7.4. In the experiments where it was necessary to produce anaerobic conditions the microsomal suspension and the propranolol solution were gassed separately with oxygen-free nitrogen prior to mixing and incubated under a nitrogen atmosphere.

*Measurement of irreversibly-bound material.* The method of Nakagawa *et al.* [17] was used. Microsomal protein was precipitated by the addition of trichloroacetic acid (25% w/v, 1 ml) to the incubation mixture, which was then centrifuged at 2000 *g* for 10 min. The precipitate was collected, resuspended in aqueous trichloroacetic acid (5% w/v, 5 ml) and centrifuged again. The resulting pellet was extracted successively with the following solvents: 80% (v/v) aqueous methanol (twice times 4 ml), 80% (v/v) hot aqueous methanol (twice times 4 ml), methanol:diethylether (1:1 v/v) (twice times 4 ml) and, finally, 80% (v/v) aqueous methanol (twice times 4 ml).

The exhaustively extracted protein was solubilised at 55° in 'Protosol' (1 ml) (New England Nuclear, Stevenage, U.K.) and then dispersed into 'Readisolv' liquid scintillation fluid (15 ml) (Beckman, High Wycombe, U.K.) to which was added glacial acetic acid (0.1 ml). The radioactivity in these samples was measured by liquid scintillation counting.

Since negligible quantities of radioactivity were detected in the supernatant from the last 2 extraction steps, any residual radioactivity in the protein was taken to reflect irreversibly-bound material.

*Electrophoresis experiments.* The microsomal reaction was quenched by the addition of 20% (w/v) sucrose containing 1% (w/v) sodium dodecylsulphate (40 μl) and followed by heating in a boiling water bath for 1 min. After cooling an aliquot (20 μl) was electrophoresed at 150 V on a 12.5%-acrylamide SDS polyacrylamide gel (prepared by the method of Hames and Rickwood [17]) using a 'Protean' slab gel electrophoresis system and a model 500/200 power supply (Biorad, Watford, U.K.). Electrophoresis was stopped when the tracker dye was 1 cm from the end of the gel. The gel was stained for 1 hr in 0.12% (w/v) Coomassie blue R250 (B.D.H. Chemicals Ltd.) in an aqueous solution of 45% (v/v) methanol and 9% (v/v) acetic acid and destained twice with 3.8 l of an aqueous solution of 7.5% (v/v) acetic acid and 5% (v/v) methanol in the presence of activated charcoal. A period of 12 hr was allowed for each destain.

Individual sample tracks were cut out longitudinally as strips and were sliced laterally into 2 cm blocks. The protein from each block was solubilised for 4 hr at 70° with 1% (v/v)·880 ammonia solution in 100 vol·strength hydrogen peroxide (0.4 ml) and, after cooling, dispersed into Optiphase "Safe" scintillation fluid (LKB, Croydon, U.K.) containing glacial acetic acid (0.1 ml). Samples were left in darkness for about 12 hr to allow chemiluminescence to subside before radioactivity was measured by liquid scintillation counting.

*Liquid scintillation counting.* Samples were counted in a Model 1211 Rackbeta counter (LKB Instruments). Correction for chemical quenching was made using a sample channels correction programme run on an Apple microcomputer linked to the counter.

*HPLC analysis of 4'-hydroxy- and N-desisopropylpropranolol.* A modification of the high performance liquid chromatographic method of Lo *et al.* [19] was used. After stopping the microsomal reaction with sodium carbonate (0.5 M, 0.5 ml) the

4′-hydroxypropranolol formed was stabilised with ascorbic acid (20 mg·ml$^{-1}$, 0.1 ml). This mixture, to which had been added internal standard, was shaken gently with diethyl ether (5 ml, 10 min) to extract the metabolites, which were then back extracted into dilute orthophosphoric acid (0.1 M, 0.1 ml) containing ascorbic acid (5 mg·ml$^{-1}$). An aliquot of the acid phase was injected into the HPLC, which consisted of a Model 6000A pump (Waters Associates, Northwich, U.K.), a Model 7125 Rheodyne injector (HPLC Technology, Macclesfield, U.K.), a Z-Module column system containing a cartridge packed with Nova-Pak $C_{18}$ reversed-phase packing material (Waters Associates) and a Model 970FS Schoeffel/Kratos fluorescence detector (HPLC Technology). A water–acetonitrile mixture (75:25) containing 1% (w/v) triethylamine and adjusted to pH 3 with orthophosphoric acid was used as the mobile phase. Chromatography was performed isocratically at a flow rate of 3 ml·min$^{-1}$ and at ambient temperature. The detector was operated at an excitation wavelength of 205 nm with an emission cut-off wavelength of 340 nm. Coefficients of variation for the assay were less than 5% for both metabolites.

## RESULTS

The protein content, cytochrome P-450 content and NADPH-cytochrome *c* reductase activity of microsomes from the 3 liver samples were 20, 21, 21 mg·g$^{-1}$ liver wet weight, 0.33, 0.62, 0.26 nmoles P-450 mg protein$^{-1}$ and 264, 368, 312 nmoles cytochrome *c* min$^{-1}$ mg protein$^{-1}$, respectively. As well as having the highest cytochrome P-450 content and reductase activity liver 2 was the most active catalytically (Table 1). This patient received phenytoin for 4 days prior to death and it is possible that some induction may have occurred.

In the presence of a NADPH generating system and under aerobic conditions propranolol bound irreversibly to human liver microsomal protein (Fig. 1). Labelling occurred linearly with time and became saturated at high substrate concentrations. These data were used to select a substrate concentration of 100 μM and an incubation time of 1 hr for subsequent experiments. Omission of NADP, the boiling of microsomes, incubation in a nitrogen atmosphere, addition of reduced glutathione and addition of SKF-525A decreased binding, on average, by 75, 82, 92, 73 and 33%, respectively, (Fig. 2). It was thought that the 8–25% of "non-specific" i.e. cofactor and enzyme-independent binding may have arisen from the trapping of the drug within aggregates of the denatured protein. In an attempt to free any reversibly bound propranolol the exhaustively extracted microsomes were resolubilised in formic acid and the protein reprecipitated with trichloroacetic acid. However, the amount of protein-bound radioactivity was virtually unchanged by this treatment.

Trichloropropene oxide had a negligible effect on the cofactor-dependent binding. In contrast, debrisoquine, antipyrine and phenacetin caused a considerable decrease in binding (Fig. 2). Indeed, the latter compound, at the concentration used (1 mM), inhibited cofactor-dependent binding completely.

Electrophoretic separation of microsomal proteins after incubation with tritiated propranolol revealed that binding was probably occurring to a large number of proteins, particularly those in the 40,000–90,000 molecular weight range. Electrophoretograms from one liver are shown in Fig. 3. A similar pattern of labelling was observed in the other two livers. Inclusion of debrisoquine in the incubation mixture decreased the radioactive labelling of proteins in an apparently non-selective manner (data not shown).

Under the experimental conditions glutathione did not alter the rate of appearance of propranolol metabolites. Debrisoquine had only a small effect on the rate of 4′-hydroxylation and even increased the rate of *N*-dealkylation of propranolol (Table 1). Antipyrine inhibited 4′-hydroxylation in 1 of the 3 livers but was without effect on *N*-deisopropylation. In a similar manner to its effect on cofactor-dependent binding, phenacetin exerted a very potent inhibitory action on both routes of propranolol metabolism, causing total inhibition in some instances.

## DISCUSSION

Values for protein content, total cytochrome P-450 content and NADP-cytochrome *c* reductase

Table 1. Effect of glutathione (1 mM), debrisoquine (1 mM), antipyrine (1 mM) and phenacetin (1 mM) on the rate of appearance of 4′-hydroxy- and *N*-desisopropylpropranolol in human liver microsomes

| | Percentage of control | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4′-Hydroxypropranolol | | | | *N*-Desisopropylpropranolol | | |
| Liver | 1 | 2 | 3 | Mean | 2 | 3 | Mean |
| Control | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Glutathione | 102 | 111 | 95 | 103 | 97 | 95 | 96 |
| Debrisoquine | 77 | 92 | 103 | 91 | 173 | 168 | 171 |
| Antipyrine | 66 | 114 | 99 | 93 | 104 | 106 | 105 |
| Phenacetin | N.D. | N.D. | 28 | 7 | N.D. | 43 | 22 |

Substrate (100 μM) and inhibitors were incubated for 10 min with 10 mg protein in the presence of a NADPH-generating system and in a total volume of 1 ml. Control rates of appearance of 4′-hydroxypropranolol were as follows: liver 1, 1.29; liver 2, 3.17; liver 3, 0.85 ng·min$^{-1}$·mg protein$^{-1}$ and those for *N*-desisopropylpropranolol were: liver 1, not determined, liver 2, 13.44; liver 3, 3.93 ng·min$^{-1}$·mg protein$^{-1}$. N.D. = not detectable.

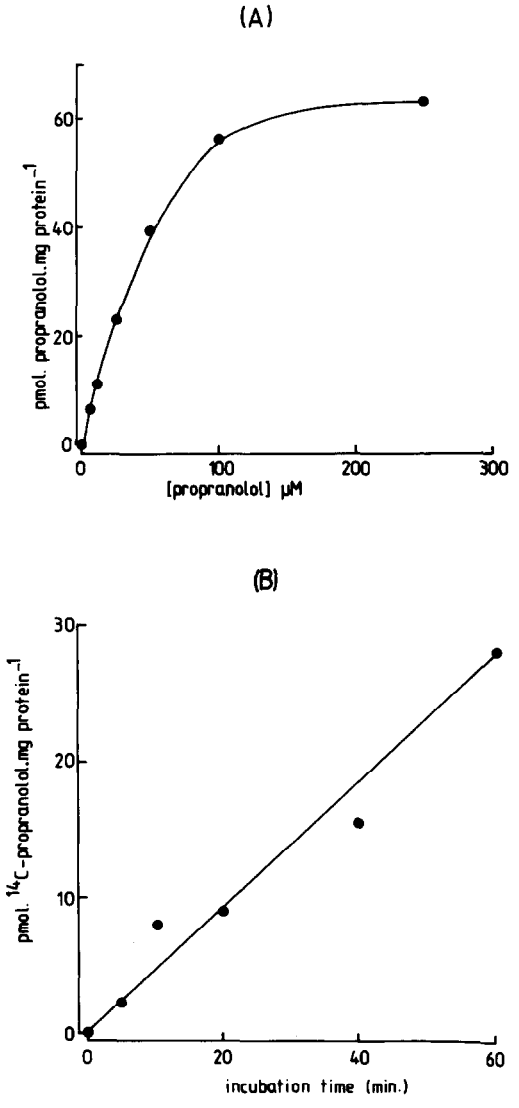


Fig. 1. Effect of (A) substrate concentration and (B) incubation time on the metabolism-dependent, irreversible binding of propranolol to human liver microsomal protein. (1-$^{14}$C)-Propranolol (0.484 μCi) was incubated with 34 mg protein from liver 2 in a total volume of 4 ml. Incubation time in (A) was 60 min and substrate concentration in (B) was 100 μM. The amount of propranolol-related material bound was calculated by subtracting the data from experiments with cofactors from those in which cofactors were excluded.

activity of the 3 liver samples studied agree well with published data from organ donor and surgical biopsy specimens [13, 20] suggesting that the components of mixed function oxidation had not degraded to any marked extent.

Incubation of radiolabelled propranolol with human liver microsomes was found to be associated with irreversible, probably covalent binding. The observations that non-extractable radioactivity was decreased substantially by omission of NADP, boiling the microsomes, incubation under anaerobic conditions or addition of SKF-525A are compatible with the hypothesis that binding is dependent upon the drug undergoing cytochrome P-450-catalysed metabolism to a reactive intermediate. Allowing for differences in protein concentration and incubation time the extent of binding to human liver microsomes was about 70 times less (0.15% of the dose at 60 min) than the value previously reported for rat liver microsomes (4% at 30 min) [6]. This may reflect a higher metabolic activity in rat compared to human liver.

Our data provide limited information on the mechanism of activation of propranolol and the structure of the reactive intermediate. Irreversible binding of propranolol to rat liver microsomes is associated with an impairment of 4′-hydroxylation [5]. Adduct formation may, therefore, lead to inhibition of 4′-hydroxylation in human liver. The microsomal 4′-hydroxylation of propranolol is thought to proceed via a 3,4-arene oxide intermediate [21]. The ability of glutathione to inhibit propranolol binding to human liver and, in a previous study, to rat liver [6] indicates that the reactive species is electrophilic. Since aromatic epoxides are potent electrophiles, it may be postulated that a fraction of the 3,4-arene oxide intermediate escapes conversion to 4′-hydroxypropranolol and reacts with nucleophilic centres such as cysteine residue sulphydryl groups on the surface of microsomal proteins. If an arene oxide was the reactive species then addition of trichloropropene oxide, a potent inhibitor of human liver microsomal epoxide hydrolase [22], might have been expected to increase binding by increasing the amount of epoxide available for nucleophilic attack. However, no consistent effect was observed, a finding that is compatible with other evidence that human epoxide hydrolase is not involved to any significant extent in the metabolism of propranolol 3,4-arene oxide [23].

Subunit molecular weights of the cytochrome P-450 family of isozymes lie between 45,000 and 60,000 [25]. From the results of gel electrophoretic separations of microsomal protein labelled with tritiated propranolol it appears that much of the labelling was concentrated over this range. However, establishing whether one or more forms of cytochrome P-450 have been labelled was not possible because of the limited resolving power of this technique. Furthermore, a significant degree of adduct formation with many proteins outside this molecular weight range is also apparent. Thus, as well as inactivating the cytochrome P-450 responsible for its own formation, the reactive species may be stable enough to diffuse to other microsomal proteins where covalent binding could also occur. If this were the case the proportion of labelling resulting from "escape" of the active species should be suppressible by glutathione whereas the proportion causing suicide inactivation should not. Furthermore, glutathione should not influence the loss of 4′-hydroxylase activity. A fraction of metabolically-generated binding (mean of 3 livers = 21%) did remain after addition of glutathione and 4′-hydroxylation was unaffected by glutathione (Table 1). Thus, our findings are compatible with such a mechanism which has also been proposed for the cytochrome P-450-catalysed covalent binding of cyclopropylamines to liver microsomal protein [26].

To investigate the relationship between the cytochrome(s) P-450 responsible for the activation and

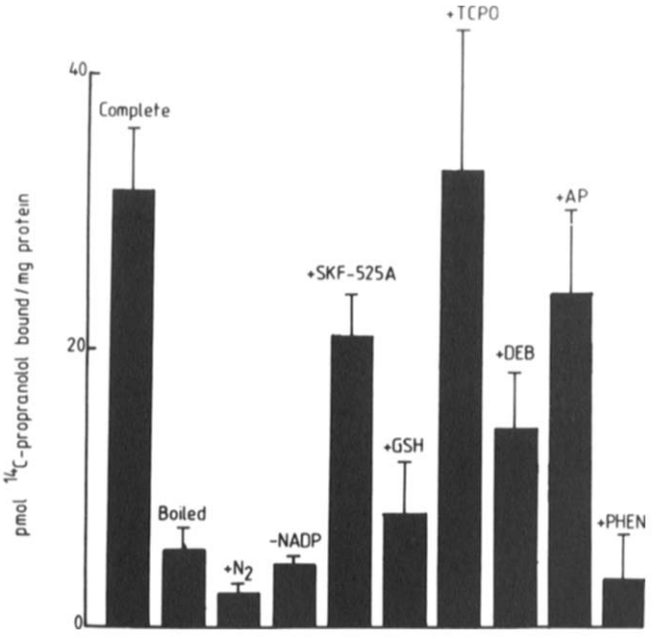


Fig. 2. Effect of various incubation conditions on the irreversible binding of propranolol to human liver microsomal protein (mean data ± SD). Microsomes from 3 livers containing 24, 24 and 13 mg protein, respectively, were incubated with (1-$^{14}$C)-propranolol (0.484 $\mu$Ci, 100 $\mu$M) in a total vol. of 4 ml. Abbreviations: Complete = complete system with NADPH-generating system and aerobic conditions; Boiled = microsomes denatured by boiling; −NADPH = absence of NADPH-generating system; SKF-525A = complete system with+SKF-525A (1 mM); +GSH = complete system with reduced glutathione (1 mM); +TCPO = complete system with trichloropropene oxide (100 $\mu$M); +DEB = complete system with debrisoquine (1 mM); +AP = complete system with antipyrine (1 mM); +PHEN = complete system with phenacetin (1 mM).

subsequent 4′-hydroxylation of propranolol and that catalysing the 4-hydroxylation of debrisoquine we looked at the effect of debrisoquine, antipyrine and phenacetin on the irreversible binding and 4′-hydroxylation and *N*-deisopropylation of propranolol. Boobis *et al.* [10] have proposed an experimental approach which may serve to define whether one or more forms of cytochrome P-450 are involved in the monogenically controlled metabolism of two compounds. They postulate that substrates for such an enzyme should be mutually competitive inhibitors and several studies have been reported which support this hypothesis [27 and references therein]. Looking first at the 4′-hydroxylation of propranolol, it is clear from Table 1 that debrisoquine and antipyrine had a negligible effect on this and the parallel *N*-deisopropylation pathway whereas phenacetin caused profound inhibition. All 3 drugs were added in a ten fold excess to propranolol. The concentrations of debrisoquine and phenacetin used (1 mM) were well above their $K_m$ values for metabolism [13, 28] and should, therefore, be exerting maximum effect. Although the concentration of antipyrine used (1 mM) was less than its $K_m$ for metabolism [24], the lack of any inhibitory effect on propranolol metabolism is consistent with *in vivo* observations that antipyrine metabolism is essentially independent of debrisoquine oxidation phenotype [29]. However, the similar lack of inhibition by debrisoquine is somewhat at variance with earlier *in vitro* and *in vivo* studies linking the 4′-hydroxylation of propranolol with debrisoquine oxidation phenotype [7, 8, 12]. Studying the relationship between debrisoquine 4-hydroxylation and phenacetin O-deethylation Kahn *et al.* [30] found a similar discrepancy between *in vitro* and *in vivo* data and concluded that the 2 reactions were catalysed by different forms of cytochrome P-450, but that these enzymes are regulated by closely linked genes. Further studies are required to determine whether this is the case for propranolol. The potent inhibition of the 4′-hydroxylation of propranolol by phenacetin suggests that the same form of cytochrome P-450 catalyses phenacetin O-deethylation and propranolol 4′-hydroxylation. Furthermore, this isozyme may be one of the cytochrome P-448 family, since cigarette smoke is a known inducer of both pathways [31].

The question of which cytochrome(s) P-450 are involved in the metabolic activation of propranolol is not resolved satisfactorily by our data. Although phenacetin prevented totally the cofactor-dependent binding of propranolol, debrisoquine and antipyrine also caused decreased binding. The generation of more than one intermediate capable of covalent binding could explain these results.

In conclusion, inhibition studies have shown that the cytochrome P-450 system responsible for the activation and subsequent 4′-hydroxylation of propranolol appears to be related more closely to the enzyme system which oxidises phenacetin rather than to that metabolising debrisoquine. Owing to its non-selective binding to human liver microsomal protein,

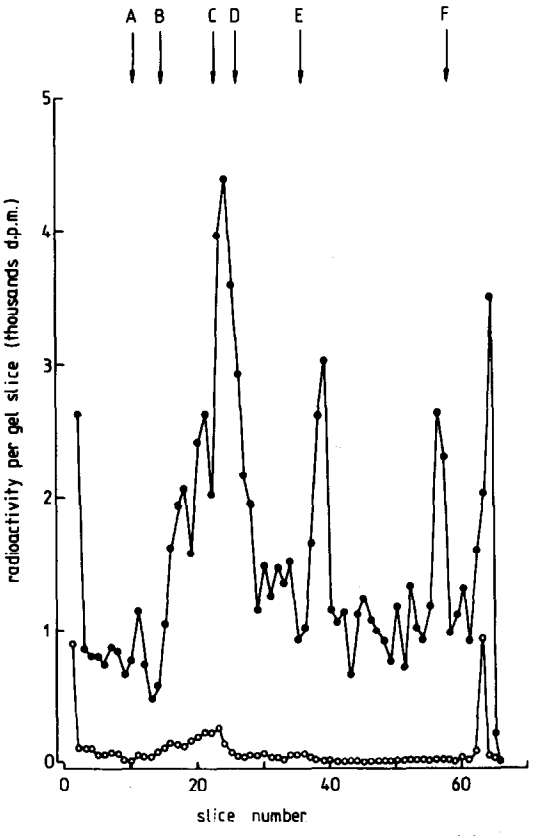


Fig. 3. Separation of (4-$^3$H)-propranolol-labelled human microsomal protein by SDS polyacrylamide gel electrophoresis. Microsomal protein (150 μg) from liver No. 2 was incubated with (4-$^3$H)-propranolol (44 μCi, 100 μM) for 60 min in the presence (closed circles) and absence (open circles) of a NADPH-generating system and in a total volume of 40 μl. Slices are numbered from the top (anode) of the gel. Lettered arrows indicate the following molecular weight markers: A. B-galactosidase (116,000); B. phospholipase (97,000); C. bovine serum albumin (66,000); D. ovalbumin (45,000); E. carbonic anhydrase (29,000); F. cytochrome *c* (12,400).

radiolabelled propranolol is not a suitable probe for studying these forms of cytochrome P-450.

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