THE BIOCHEMICAL TOXICOLOGY OF
SOME BETA-ADRENERGIC BLOCKING AGENTS

Being a Thesis presented for the Award of a
Degree of Doctor of Philosophy
in the University of Surrey

by

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January, 1981

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ABSTRACT

A number of beta-adrenergic blocking agents were examined for their in vivo effects on the rat hepatic microsomal mixed-function oxygenase system to determine their potential for microsomal enzyme induction and epigenetic carcinogenesis. The method is based on previous findings that pretreatment of rats with chemical carcinogens preferentially stimulates biphenyl 2-hydroxylase and ethoxyresorufin O-deethylase, mixed-function oxidase activities catalysed by cytochrome P-448, the form of microsomal cytochrome known to be formed by chemical carcinogens. None of the beta-adrenergic blockers studied with the exception of propranolol and pronethalol, stimulated these cytochrome P-448-mediated enzyme activities at very high dosage.

Mutagenicity studies of some of these beta-adrenergic blocking agents, using the Ames' bacterial and mammalian micronucleus tests, indicated that none of these agents give rise to significant and dose-dependent increases in mutations. The numbers of His\(^+\) revertant colonies produced with or without rat S-9 activation system and the number of micronucleated polychromatic cells formed in mice were not significantly increased over the spontaneous control levels.

Because of a suggestion that practolol toxicity, namely, ulceration of intestinal, nasal and oral mucosae, and the conjunctiva of the eyes may involve inhibition of mucus synthesis, the effects of several beta-adrenergic blocking agents were studied. Only practolol significantly inhibited mucus glycoprotein synthesis as measured by the rates of incorporation of radiolabelled amino acid and sugar precursors into rat gastrointestinal mucus glycoproteins. None of the other agents studied showed any effects similar to practolol.
It has further been suggested that some carcinogens, tumour-promoting agents and inhibitors of glycoprotein synthesis, preferentially stimulate tissue guanylate cyclase and cyclic GMP without concomitant increases in adenylate cyclase and cyclic AMP leading to a decrease in the ratios of adenylate/guanylate cyclases and C-AMP/C-GMP. None of the beta-adrenergic blocking drugs studied were shown to preferentially stimulate tissue guanylate cyclase and C-GMP. They did give rise to concomitant increases in tissue guanylate and adenylate cyclases and decreases in the C-AMP and C-GMP concentrations. However, there was no direct relationship between the ratios of adenylate/guanylate cyclases and C-AMP/C-GMP before and after treatment with the various agents.
"Irrationally held truths may be more harmful than reasoned errors".

T.H. HUXLEY (1825-1895)

(The Coming of Age of the Origin of Species)
DEDICATION

This work is dedicated to my wife Constance, and to my parents.
ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Professor D.V. Parke for providing me with a place in his department for this work, and for his candid advice, valuable suggestions and encouragement, during the course of this project. I am also greatly indebted to Dr. C. Ioannides for his help and moral support.

I would also like to thank Susan Hawkins, a technician in the department, and Julian Richardson, of Huntingdon Research Centre, for their help and advice respectively in the mutagenicity assays.

Finally, my sincere thanks go to Miss Surrey Lawes for typing this thesis.
CHAPTER ONE

GENERAL INTRODUCTION
INTRODUCTION

Beta-adrenergic blocking agents are currently widely prescribed in clinical practice for the treatment of many forms of congenital heart diseases. It is estimated that over 2 million patients are being daily treated with propranolol (Inderal) worldwide and similar numbers of patients are receiving other marketed beta-adrenergic blocking agents when they are considered together. Furthermore, since its introduction onto the market in 1965, there have been approaching 13 million patient years of experience with propranolol. There are about eleven beta-adrenergic blocking agents now available in the U.K.

Pronethalol was the first beta-blocking agent synthesized (Black and Stephenson, 1962) to be introduced into clinical practice. However it was found to produce thymic tumours in certain strains of mice (Paget, 1963; Alcock and Bond, 1964; Howe, 1965) and was subsequently withdrawn from further clinical investigations. Propranolol was introduced (Black et al, 1964) with a more potent beta-blockade effect and used successfully in the treatment of angina pectoris (Gillam and Pritchard, 1966).

The concept of $\alpha$- and $\beta$-adrenergic receptors was first introduced by Ahlquist (1948) and confirmed by Moran and Perkins (1958). This finding led to the introduction of the terms $\alpha$- and $\beta$-adrenergic blocking agents. A beta-adrenergic blocking agent was defined by Fitzgerald and Barrett (1967) as a substance which competitively antagonizes the effects of exogenous adrenaline and isoprenaline as well as antagonising stimulation of the cardiac sympathetic nerves and does not antagonise the effects of digitalis, calcium, aminophyline, acetylcholine, 5-hydroxytryptamine or histamine.

However, recent study by Preiksaitis and Kunos (1979) has demonstrated an
interconversion between \(\alpha\) and \(\beta\)-adrenergic receptors which is temperature dependent. The existence of two forms of \(\beta\)-adrenoceptors, namely \(\beta_1\) and \(\beta_2\) has been demonstrated by Lands et al. (1966, 1967). The former (\(\beta_1\)) is present in the heart muscle and the latter (\(\beta_2\)) in the smooth muscle. Propranolol was found to block both adrenoceptors and it therefore became necessary to develop new compounds that will be specific to \(\beta_1\)-adrenoceptors for use by patients with other conditions such as asthma (lungs possess \(\beta_2\)-adrenoceptors).

Practolol was the first specific \(\beta_1\)-adrenergic blocking agent to be developed (Dunlop and Shanks, 1968) and was later introduced into clinical practice. Practolol was first marketed in 1970, and by the end of 1975 the full extent of its toxic side-effects (oculomucocutaneous syndrome) had become apparent. At this time it had had some 300,000 patient years of use in the U.K. and around 1 million worldwide. It was later withdrawn from the market, generally, leading to the development of new \(\beta_1\)-specific adrenergic blocking agents such as atenolol, acebutolol and metoprolol, whose cardioselectivity has been described in animal experiments (Barrett et al, 1973, Basil et al, 1973, Ablad et al, 1973).

The dose-response relationships between beta-adrenergic blocking agents and beta-adrenoceptors is tissue and species dependent. In normal men an oral dose of practolol 1.5 mg/kg selectively blocks the \(\beta_1\)-adrenoceptors but a dose of 12 mg/kg blocks \(\beta_2\)-adrenoceptors as well (Lertora et al, 1975). Acebutolol has been found to be cardioselective in dogs but not in man (Briant et al, 1971; Basil et al, 1971; Cuthbert and Owusu-Ankomah, 1971).

Beta-adrenergic blocking agents differ in four respects according to their potencies, presence or absence of membrane-stabilizing activity (MSA), intrinsic sympathomimetic activity (ISA) and cardioselectivity (Waal-Manning, 1976b). Current evidence suggests, however, that relative differences in potency, cardioselectivity, ISA and MSA are probably of
little significance in determining the antihypertensive effectiveness of the various beta-adrenergic blocking agents (Hansson and Werko, 1977; Niarchos and Tarazi, 1976; Niess and Shand, 1975). Inspite of known differences in pharmacological activity, most of the drugs seem to induce similar antihypertensive responses if administered in appropriate doses. Moreover, patients who fail to respond to one beta-adrenergic blocking agent generally fail to respond to the others (waal-Manning, 1976a; Morgan et al, 1974; Doyle, 1974).

These agents represent an important therapeutic advance. Their widest use is in hypertension (Morgan et al, 1975; Skrabal et al, 1976) and ischaemic heart diseases such as angina pectoris (Pritchard, 1977). They have been used in arrhythmias (Pritchard et al, 1976; Gibson, 1974) phaeochromocytoma and certain forms of congenital heart diseases; myocardial infarction (Mueller et al, 1974). Beta-adrenergic blocking agents have also been assessed with varying degrees of benefit in a number of other conditions such as thyrotoxicosis (Schelling et al, 1973; Turner and Hill, 1968), anxiety states with somatic manifestations (Granville-Grossmann and Turner, 1966; Tyrer and Lader, 1974; Bonn et al, 1972), psychosis and schizophrenia (Bonn and Turner, 1971; Bainbridge and Greenwood, 1971; Weinstock and Scheckter, 1975; Green and Graham-Smith, 1976; Middlemiss et al, 1977), migraine (Packard, 1975), hyperthyroidism (Turner, 1974; Disteler et al, 1973; Murchison et al, 1976; Wartofsky et al, 1975; Goulding et al, 1976) and drug addiction withdrawal syndrome.

The choice of beta-adrenergic blocking agent for clinical use depends on their unwanted effects, ease of dosage and cost. In a patient liable to asthma a cardioselective drug; i.e. atenolol, diacetolol (active metabolite of acebutolol), metoprolol and practolol tend to be used. Patients on agents showing symptoms of CNS disturbances i.e. vivid dreams, can try sotalol, atenolol and nadolol (have lower lipid-solubility).
However in most circumstances propranolol remains the choice of drug since it is cheap and its long-term risks are clearly understood.

**Structure-Activity Relationship**

For a drug to act as an antagonist of β-adrenoceptor agonist (adrenaline or isoprenaline) it must have a structure similar to that of the agonist in order to compete for the receptor sites. The activity of a β-adrenergic blocking agent is dependent on three main structural factors.

**Substituent on the amino group and α-carbon atom**

There is a clear cut structural relationship between the β-adrenergic drug, isoprenaline and the various β-adrenergic blocking agents (Figure 1.1). The influence of variations in the N-alkyl substituent in β-adrenergic blocking agents has been intensively studied and there is a clear correlation between β-adrenergic agonist and β-adrenergic blocking agent. This especially holds for the change from hydrogen via the methyl to the isopropyl and tertiary butyl substituted derivatives. For the aryl substituted compounds, the relations are less clear; however, only a few examples have been studied.

A second structural variation is the introduction of alkyl substituents on the carbon atom next to the amino group, the α-carbon. This substitution on the α-carbon atom of isoprenaline and related potent β-adrenergic compounds is of importance (Lands and Tainter, 1953; Triggle, 1965; Barlow, 1964). α-methyl groups seem to be less compatible with β-adrenergic activity than the α-ethyl groups; larger groups are not tolerated. α-methyl substitution leads to a decrease in the activity (Corrodi et al, 1963). The introduction of the α-ethyl group in DCI has about the same influence as the α-methyl group (Corrodi et al, 1963).
Sterical configuration

This involves the sterical configuration of the carbon atom in the side chain bearing the OH-group. Also the compounds devoid of this OH group can be considered in this respect. High ratios are reported for the activities of the sterical isomers of various β-adrenergic activity (Ariens, 1963). The ratio of the activities of the sterical isomers of various β-adrenergic blocking agents which differ in the configuration of the carbon atom in the side-chain bearing the OH-group have been studied and as a rule large ratios are reported for the activities of the isomers (Burns et al., 1964; Burns and Salvador, 1967; Salvador et al., 1964; Levy, 1964; Kvarn et al., 1965; Howe, 1963). Where the absolute configurations of the β-adrenergic blocking agents have been studied, the R-isomers are found to have the highest activity (Howe, 1963). This strong dependency of the β-adrenergic blocking activity on the steric structure, which is comparable to that observed for the β-adrenergic activity, holds for the effects induced by these compounds on the β-receptors.

Catecholamine nucleus structure

Gradual elimination of the phenolic OH-groups of catecholamines shows that the meta-OH group is more important than the para-OH group for the action both on the α- and β-receptors. (Ariens, 1963; Corrodi et al., 1963; Bovet and Bovet-Nitti, 1948; Triggle, 1965). Elimination of both OH-groups results in a decrease in the intrinsic activity on the β-adrenergic receptors. The resulting phenylethanolamine act as weak β-adrenergic blocking agents. For instance, phenylethanolamine and its N-methyl and its N-isopropyl derivatives have a β-adrenergic blocking activity with respect to the tachycardia induced by β-adrenergic drugs (Ariens, 1963; Pratesi and Grana, 1967). Introduction of a halogen especially a chlorine atom or of a methyl group in the meta and/or para position results in an increase in the affinity of the β-adrenergic blocking agents to their
Figure 1.1 Structures of beta-adrenergic blocking agents used in study
receptors as mentioned, (Powell and Slater, 1958; Pratesi and Grana, 1967; Corrodi et al, 1963). Larger alkyl groups, such as ethyl groups, are found to be less suitable (Corrodi et al, 1963).

The structure-activity relationship outlined indicates that the chemical properties of the substituent on the nucleus are of primary importance for the intrinsic activity on the β-receptors. An interesting aspect of the structure-activity relationship, is the substitution of a phenolic OH-group by a methylsulphonamide group. This group behaves to a certain degree as a bio-isoteric group for the phenolic OH-group (Staton et al, 1965b; Larsen and Lish, 1964; Uloth et al, 1966). Some of the compounds with a para- or inter-methylsulphonamide group act as β-adrenergic or β-adrenergic blocking agents (Lish et al, 1965; Kvam et al, 1965; Staton et al, 1965a,b; Dungan et al, 1965).

Pharmacokinetics of Beta-adrenergic Blocking Agents

The pharmacokinetics of most β-adrenergic blocking agents have been extensively studied with respect to their absorption and mode of elimination, bioavailability, drug clearance and plasma half-life, metabolism and tissue distribution.

Absorption and mode of elimination

All the beta-adrenergic blocking agents with the possible exception of acebutolol and atenolol are well absorbed from the alimentary tract (GIT) following oral administration (Shand, 1974) as shown in Table 1.1. Peak plasma levels generally occur within 1-3 hours. The absorption of sustained release preparations of alprenolol and oxprenolol is more prolonged, and lower peak levels are achieved (Johansson et al, 1971). In man, the absorption of atenolol after oral administration is incomplete, but blood levels after oral administration is incomplete, but blood levels after 100mg dose are consistent and adequate (Graham et al, 1973, Kaye et al, 1976).
Table 1.1 Differences in Pharmacokinetic Parameters of some Beta-blockers

<table>
<thead>
<tr>
<th>Beta-blocker</th>
<th>Human oral dosage (mg)</th>
<th>Absorption of oral dose (%)</th>
<th>&quot;First-pass&quot; effect (%)</th>
<th>†Half-life (t½) (hr)</th>
<th>Oral bioavailability (%)</th>
<th>Excretion* (%)</th>
<th>Urine (%)</th>
<th>Biliary (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acebutolol</td>
<td>300</td>
<td>50</td>
<td>-</td>
<td>8</td>
<td>50</td>
<td>50</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Alprenolol</td>
<td>400</td>
<td>90</td>
<td>≈ 90</td>
<td>1-2</td>
<td>≈ 10</td>
<td>90</td>
<td>&lt;90</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Atenolol</td>
<td>200</td>
<td>50</td>
<td>≈ 15</td>
<td>6-9</td>
<td>&gt; 40</td>
<td>40-45</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>300</td>
<td>&gt;95</td>
<td>≈ 50</td>
<td>3-4</td>
<td>≈ 50</td>
<td>&gt;90</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Oxprenolol</td>
<td>160</td>
<td>70-95</td>
<td>30-50</td>
<td>2</td>
<td>24-60</td>
<td>65-90</td>
<td>5-10</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Pindolol</td>
<td>15</td>
<td>&gt;95</td>
<td>13</td>
<td>3-4</td>
<td>87</td>
<td>&gt;90</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Pratolol</td>
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<td>&gt;95</td>
<td>0</td>
<td>9-12</td>
<td>100</td>
<td>&gt;90</td>
<td>&lt;4</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Propranolol</td>
<td>300</td>
<td>&gt;90</td>
<td>≈ 60</td>
<td>3.5-6</td>
<td>30</td>
<td>&gt;90</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Sotalol</td>
<td>340</td>
<td>&gt;75</td>
<td>&lt; 15</td>
<td>13-17</td>
<td>&gt; 60</td>
<td>60-75</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Timolol</td>
<td>30</td>
<td>&gt;90</td>
<td>-</td>
<td>4-5</td>
<td>-</td>
<td>&gt;90</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

After Meier (1977)
†Drug and Therapeutic Bulletin (1980)
*Both metabolized and non-metabolized compounds
entirely by hepatic metabolism (Paterson et al., 1970; Johansson et al., 1971; Reiss et al., 1970; Bodem et al., 1976). Practolol and Atenolol are eliminated almost completely by renal mechanisms, largely as a function of glomerular filtration (Bodem and Chidsey, 1973; Bodem et al., 1976; Baker et al., 1974). Sotalol and acebutolol are mainly eliminated by renal mechanisms but metabolized to a lesser extent in the liver whereas timolol and pindolol are mainly metabolized by the liver and eliminated to a lesser extent by renal mechanisms, (Ohnhaus, 1973; Shand, 1974; Johnsson and Ragardh, 1976; Waal-Manning, 1976a). The clearance of some beta-adrenergic blocking agents by the liver and kidneys is shown in Figure 1.2.

Bioavailability

With some drugs which are extensively metabolized by the liver, some of the administered dose fails to reach the circulation after oral administration, despite complete alimentary absorption, because the drug in the portal vein is taken up and removed by the liver before it can appear in the systemic circulation, (Gibaldi et al., 1972). This presystemic hepatic elimination is referred to as "first-pass" effect. It is negligible in the case of

Figure 1.2 Clearance of β-adrenergic blockers Meier et al., 1977

<table>
<thead>
<tr>
<th>LIVER</th>
<th>KIDNEY</th>
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<tbody>
<tr>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>20</td>
<td>0 (%)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Timolol</th>
<th>Pindolol</th>
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</thead>
<tbody>
<tr>
<td>Oxprenolol</td>
<td>Metoprolol</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Acebutolol</td>
</tr>
<tr>
<td>Atenolol</td>
<td>Sotalol</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Alprenolol</th>
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</table>
practolol and pindolol and about 60% for propranolol, 90% for alprenolol, 50% for metoprolol and 30-50% for oxprenolol (Table 1.1). The relationship between bioavailability and oral dose is therefore not proportional; the availability of small doses being very low. As the dose increases, proportionately more drug reaches the systemic circulation. This kinetic situation applies to the disposition not only of propranolol and alprenolol (Shand, 1974; Johnsson and Ragardh, 1976) but probably also to oxprenolol, (Riess et al, 1974).

**Drug clearance and plasma half-life**

The drug clearance of various β-adrenergic blocking agents vary between 0.14 - 1.2 litres/min. Because of the very efficient removal by metabolism, the half-life of these drugs in the plasma is short (2-3 hours) despite their large volume of distribution, (Shand and Ragno, 1972; Johansson et al, 1971). Pindolol, however has a longer half-life of 3-4 hours because of its smaller volume of distribution (Hicks et al, 1972). Since practolol is eliminated differently from the other beta-adrenergic blocking agents, it is cleared less rapidly, at a rate approximating that of glomerular filtration and its half-life (9-12 hours) is much longer than that of other beta-adrenergic blockers (Bodem and Chidsey, 1973; Reeves et al, 1978). The half-life of atenolol is about 6 hours (Brown et al, 1976).

The duration of effect of β-adrenergic blocking agents is different from the plasma half-life. Although most β-adrenergic blocking agents have a relatively short plasma half-life, their duration of β-blocking or anti-hypertensive effect is relatively long lasting. Most compounds can therefore be given at much longer dose intervals than indicated by their plasma half-lives (Johnsson and Regardh, 1976). Above certain dosage levels, increasing the size of the dose leads to greater duration of effect, rather than a greater magnitude of response (Carruthers et al, 1973).
A correlation between the plasma level and therapeutic effect has been demonstrated for alprenolol (Collste et al., 1976), propranolol (Cleaveland and Shand, 1971), sotalol (Sundquist et al., 1974) and pindolol (Anavakar et al., 1975; Wal-Manning, 1976a), but studies thus far have failed to demonstrate a significant correlation for metoprolol (Bengtsson et al., 1974), oxprenolol (Brunner et al., 1975) and practolol (Sundquist et al., 1974). The difference in the time-course between the anti-hypertensive effect and the plasma level means that for most beta-adrenergic blocking agents, a twice daily (or in some cases once daily) dosage regimen can be used.

**Tissue distribution**

With the exception of sotalol, which shows a higher distribution in the peripheral tissues than the CNS, the other beta-adrenergic blocking agents show the reverse distribution pattern (Scales and Cosgrove, 1970; Garvey and Ram, 1975; Hayes and Copper, 1971).

The higher ratios obtained with propranolol and alprenolol compared to practolol with regard to brain/blood, lung/blood and heart/blood levels are probably related to the low solubility of practolol compared to propranolol and alprenolol, and this may have a bearing on the cardio-selectivity of practolol. However, it is likely that all beta-adrenergic blocking agents enter the CNS to some degree. (Scales and Cosgrove, 1970; Johnsson and Regardh, 1976).

**Metabolism**

The metabolism of most beta-adrenergic blocking agents have been extensively studied and some of the metabolites have been isolated and identified (Bond, 1967; Scales and Cosgrove, 1970, Bond and Hove, 1967; Hayes and Copper, 1971; Leinweber et al., 1971). However the biological significance of these metabolites have not been fully investigated.
Figure 1.3 Pathway of metabolism of acebutolol

Figure 1.4 Metabolic pathway of atenolol
Figure 1.5 Metabolic pathway of propranolol
Figure 1.6 Metabolic pathway of pronethalol
Figure 1.7 Metabolic pathway of practolol
Pronethalol is metabolized by two main pathways; side chain oxidation and ring hydroxylation and conjugation. Five metabolites have been identified, four being formed by degradation and oxidation of the isopropylaminoethanol side chain. The fifth metabolite, 7-hydroxy-analogue of pronethalol is partly present in the free form, but the major amount is present as the glucuronide (Bond and Howe, 1967). Howe (1965) proposed a probable production of an ethyleneimine derivative, although it has not been identified either as a minor or transient metabolite, of pronethalol as the proximate carcinogen responsible for its tumorigenic properties.

Propranolol undergoes similar modes of metabolism as for pronethalol. Two major metabolites were initially detected and the major pathway of excretion is the urine. The first metabolite is a product of oxidation of the side chain. The second is the hydroxylated derivative of propranolol which also exists in its glucuronide form (Bond, 1967). Recent human studies have however led to the identification, in urine, of O-methylated catechol-like metabolites (Walle et al, 1978).

The major metabolite of practolol accounts for 9% of the dose and has been identified as the hydroxylated derivative. One-third of this is excreted as the free compound and the remainder as a glucuronide conjugate. About 4% of the drug is metabolized by the removal of the acetyl side-chain thought to be responsible for its adverse side-effects, and 80-90% remain unchanged in the urine of dogs, rats and mice (Scales and Cosgrove, 1970). Recent metabolic studies in man has demonstrated that the major metabolites were 3-hydroxy and desacetyl practolol. Desacetylation is however a minor metabolic pathway representing 5% of the drug. 3-13% is metabolized and eliminated in the urine and about the same amount remained unchanged in animal studies.
Atenolol undergoes a 10% metabolism leading to the formation of hydroxy derivative and its glucuronide conjugate. 40 - 50% of the unchanged compound is recovered in the urine (Brown et al, 1976).

The major metabolite of acebutolol is one in which the ethyl group of the side chain para to the isopropylamino-ethanol side chain has been removed (Steyn, 1976). Recent study by Andersen and Davis (1979) has led to the identification in human urine, a new metabolite which correlates to that found in the rat.

The metabolic pathways of the five main beta-adrenergic blocking agents used in the present study and described above are shown in Figures 1.3 - 1.8.

The Microsomal Drug-metabolizing Enzyme System

The most important of the hepatic drug-metabolizing enzymes are the mixed-function oxidases which oxygenate drugs and are complex, multicomponent systems comprising NADPH, a phospholipid-protohaeme-sulphite protein complex known as cytochrome P-450 and a linking electron transport system of cytochrome P-450 reductase, NADPHcytochrome c reductase and possibly cytochrome b_{5} (Coon et al, 1973; Estabrook et al, 1973). The mechanism of oxygenation (hydroxylation) of drugs and xenobiotics is postulated in Figure 1.8. The mixed-function oxidases of the endoplasmic reticulum of the liver and of certain other tissues reticulum of the liver and of certain other tissues such as the gastrointestinal mucosa, the lungs and skin catalyse these hydroxylation reactions.

The hydroxylations catalysed by this system are frequently referred to as non-specific reactions, whereas in fact these enzymes show a remarkably high degree of specificity of the substrate and of the insertion of the oxygen. These enzymes are very versatile and are able to carry out oxygenation of a number of different substrates. This is made possible
by the presence of different types of cytochrome P-450, by conformational changes of this enzyme system which converts cytochrome P-450 to cytochrome P-448 and also by alternative methods of oxygen transfer (Stier, 1976). Oxygen may be inserted into the substrate when this is firmly bound to the substrate-specific protein of cytochrome P-450 in the proximity of the haem moiety, or alternatively a labile, metastable oxygenated species such as the superoxy anion or radical may be generated by this system which subsequently hydroxylates the substrate possibly without its binding to the active site of the cytochrome P-450 enzyme. A number of these drug hydroxylations are inhibited by the enzyme superoxide dismutase, which destroys the superoxide anion (Parke, 1978).

Cytochrome P-450 has been demonstrated in many organisms including fish (Chambers and Yarbrough, 1976), birds (Yawetz et al, 1968), insects (Wilkinson and Brattsten, 1972), yeast (Wiseman et al, 1975) and bacteria (Sato et al, 1973). In its oxidized form, the haemoprotein combines with foreign compounds, as well as endogenous substrates, to produce difference spectra of two general types, type I and type II. The type I spectrum has a peak at 385nm and a trough at 420nm, while type II has a peak at about 430nm and a trough at about 395nm (Remmer et al, 1966; Imai and Sato, 1966; Schenkman et al, 1967). In addition, a modified type II or reverse type I (415nm to 420nm peak, 390nm trough) spectral change has been demonstrated.

The affinity of binding of substrates to cytochrome P-450 can be estimated by measurement of the spectral constant (Ks). The Ks value, by definition, is the concentration of substrate required for half maximal spectrum development. Some workers found a good correlation between Ks value for binding and the Km value for metabolism of type I compounds, such as hexobarbitone and aminopyrine (Schenkman et al, 1967; Remmer et al, 1969). However, in general, these two values are not directly correlated (Mannering, 1971).
Type I compounds do not interact directly with the haem of cytochrome P-450 but reaction with a hydrocarbon residue of the apoprotein occurs (Yoshida and Kumaoka, 1975). Therefore, the extraction of phospholipids destroys the type I interaction (Leibman and Estabrook, 1971; Chaplin and Mannering, 1970). The reverse type I spectral change results from the displacement of an endogenous type I substrate from the oxidized enzyme (Turner et al, 1977) by the presence of a compound with a hydroxyl group, e.g. an alcohol (Yoshida and Kumaoka, 1975).

Compounds, which contain a basic amino group, induce the type II spectral change by interacting directly with the haem iron of the cytochrome (Yoshida and Kumaoka, 1975). Type II compounds also include compounds with oxygen or sulphur atoms containing a pair of non-bonded electrons.

Cytochrome P-450, in its reduced form, similarly interacts with ethyl isocyanide and characteristically has Soret peaks at 430nm and 455nm (Imai and Sato, 1966). The relative sizes of the two peaks are pH dependent, but the sum of the heights are about the same regardless of pH. Changes in the pH equilibrium point at which the peaks are of equal magnitude have been used in studies of the qualitative differences of cytochrome P-450 in microsomes from untreated and 3-methylcholanthrene-treated rats (Sladek and Mannering, 1966).

**Nature of hepatic microsomal enzyme inducers**

The drugs and foreign compounds which induce the microsomal enzymes have widely differing pharmacological activities, e.g. phenobarbital (hypnotic), butylated hydroxytoluene (BHT, food anti-oxidant), DDT (pesticide), 3-methylcholanthrene (carcinogen), and the only features they seem to have in common are:

a) Lipid-soluble and hence become localized in the endoplasmic reticulum
Figure 1.8 Diagrammatic representation of hepatic microsomal mixed-function oxidase system, microsomal electron transport and substrate hydroxylation
of the liver.
b) Substrates of or become bound to the microsomal drug-metabolizing enzymes.

The latter appears to be an important criterion for the microsomal enzyme induction, at least at the translational level. Study of the inductive effects of a series of barbiturate drugs on rat hepatic microsomal enzymes, showed an inverse correlation between the rates of metabolism of the barbiturates as determined by their plasma half-lives and the extent of induction that they produced (Ioannides, 1973). It is reasonable therefore to infer that the longer the substrate remains in the body and hence in contact with the enzyme, the greater will be the extent of induction, and indeed this is substantiated by the high level of induction that results with the methylenedioxyaryl compounds such as safrole and piperonyl butoxide which form stable ligand complexes with cytochrome P-450 (Parke and Rahman, 1970; Philpot and Hodgson, 1971).

The phenomenon of induction of the microsomal drug-metabolizing enzymes would thus appear to be an example of substrate-mediated enzyme induction where because of the unique multifunctional nature of the enzyme system involved, one substrate is able to increase not only the enzymic activity required for its own metabolism but also that for many others. Many naturally occurring anutrients e.g. terpenes, coumarins, flavones and caffeine have been shown to stimulate the hepatic drug-metabolizing enzymes thus confirming the fundamental nature of anutrients as natural substrates of these enzymes.

**Forms of cytochrome P-450**

Administration of 3-methylcholanthrene to normal rats led to the production of a P-450 haemoprotein which differed from that found in untreated animals (Sladek and Mannering, 1966). This new haemoprotein, in its
reduced form combined with CO had a maximum absorption at 448 nm instead of 450 nm (Alvares et al., 1967). Its binding to ethyl isocyanide also differed in that the peak size at 455 nm was specifically increased whilst the 430 nm peak remained relatively unaltered; also on the basis of the pH dependency of the ethyl isocyanide interaction, it was concluded that a new haemoprotein (cytochrome P-448) was formed (Mannering et al., 1969). Further studies, confirmed the existence of the new cytochrome by virtue of its spectral binding characteristics with various types of substrates (Hildebrandt et al., 1968; Remmer et al., 1969; Mannering, 1971) and its preferential specificity towards the metabolism of different substrates (Conney et al., 1969; Kuntzman et al., 1969). When animals are treated with phenobarbitone, cytochrome P-450 concentrations is greatly increased but remains qualitatively similar to that found in untreated animals.

Cytochrome P-450 from rats pretreated with phenobarbitone and cytochrome P-448 from rats pretreated with 3-methylcholanthrene have been solubilized from liver microsomes treated with sodium cholate and fractionated by ammonium sulphate, calcium phosphate gel and column chromatography on DEAE-cellulose with a further purification on a CM-cellulose column (Lu et al., 1976). The two haemoproteins were shown to have different molecular weight and different spectral, immunological and catalytic properties. This is consistent with the view that cytochrome P-450 in liver microsomes prepared from animals induced with drugs is different from that induced by polycyclic hydrocarbons.

The lack of substrate specificity of the microsomal mixed-function oxidase system has led to the suspicion that more than a single species of cytochrome P-450 may exist in the system. One of the advantages of the presence of several enzymes with a certain degree of specificity is that low levels of toxic environmental compounds may efficiently be
removed from the body. Also, the synthesis of an enzyme effective in the metabolism of a particular drug is biologically more economical than to continuously synthesize large amounts of a less efficient enzyme (Ullrich, 1977).

Recently, several workers have identified more than one form of cytochrome P-450 in liver microsomal preparations of untreated animals (Comai and Gaylor, 1973; Haugen et al, 1975a; Philpot and Arinc, 1976). Gibson and Schenkman (1978) obtained two forms of cytochrome P-450 (I and II) from liver microsomes of untreated rats by lauric acid affinity chromatography, with further purification by gel filtration giving an overall 50% yield of the haemoprotein. Marked differences in substrate specificities of the two fractions were observed; for example, high activity of cytochrome P-450 II towards the metabolism of ethylmorphine was obtained but cytochrome P-450 I could only poorly metabolize this compound. Their CO difference spectral maxima also differed in that cytochrome P-450 I had its peak between 449.5nm and 451nm whilst cytochrome P-450 II absorbed maximally between 448.5nm and 450nm. The two haemoprotein also responded differently towards destruction by linoleic acid hydroperoxide.

Multiple forms of cytochrome P-450 have similarly been demonstrated in microsomal preparations from either phenobarbital or 3-methylcholanthrene-treated animals (Guengerich, 1977; Gustafsson and Ingel-Sundberg, 1976; Thomas et al, 1976a 1976b; Ryan et al, 1975; Coon et al, 1975; Lu et al, 1976). Four distinct cytochrome P-450 fractions (A₁, A₂, C₁ and C₂) have been isolated from the liver microsomes of phenobarbital-treated mice (Haugen et al, 1975b) by solubilization with sodium cholate and purification through several stages, including ion-exchange chromatography. All four fractions exhibited different absorption maxima in the reduced CO difference spectrum ranging from 449nm to 451nm. Testosterone was hydroxylated in the 16β-position by C₁ and C₂, in the 16α-position by C₁ and 7αα-position by
A2. A1 and A2 fractions possessed high activities towards the metabolism of benzpyrene whilst A1 preferentially metabolized 7-ethoxycoumarin and coumarin. Benzphetamine was particularly well metabolized by C1. Therefore, the cytochrome P-450 fractions show preference towards the metabolism of various substrates although their substrate specificities are broad and overlapping (Lu et al, 1975).

The cytochrome P-450 population in liver microsomes from phenobarbital or 3-methylcholanthrene-treated rats is obviously different. Cytochrome P-450 preparations from different species of animals treated with the same inducer, however, are also different proteins although they may have similar catalytic specificity towards different substrates and have identical CO difference spectra. Thomas et al (1976b) found that antibodies produced from the purified cytochrome P-448 obtained from liver microsomes of 3-methylcholanthrene-treated rats, cross-reacted poorly with cytochrome P-448 purified from liver microsomes of 3-methylcholanthrene-treated rabbits.

The existence of multiple forms of cytochrome P-450 in a microsomal preparation may explain the species, strain, age, tissue and sex differences in mixed-function oxidation (Lu and West, 1978). It may also explain the kinetics and patterns of inhibition with different substrates, inducing agents and inhibitors (Haugen et al, 1975b; Ullrich and Kremers, 1977).

**Interactions of beta-adrenergic blocking agents with cytochrome P-450**

The ability of the liver to bind and metabolize "first-pass" drugs is a major factor responsible for their blood concentrations on oral administration. Although some beta-adrenergic blocking agents undergo "first-pass" metabolism, their binding to hepatic microsomal cytochrome P-450 has not been extensively studied.

Alprenolol has been shown to bind with high affinity to cytochrome P-450 giving a type I spectral binding characteristic (Grundin et al, 1974, 1975).
Propranolol has similar characteristics (Evans et al, 1973). The microsomes appear to have four classes of binding capacity for alprenolol. Its binding to, and gradual saturation of, the high affinity binding site of cytochrome P-450 is an important determinant of the low and dose-dependent availability of alprenolol in man (von Bahr et al, 1974). It has been suggested that not only the binding capacity but also the rate of oxidation catalysed by this site should be important for the "availability threshold" since this rate largely governs the degree of saturation of the site in a metabolically functioning system (von Bahr, et al 1976).

It has been well established that compounds which bind to the active site of the microsomal cytochrome P-450 (type I compounds) must possess a suitable degree of lipophilic character to penetrate the lipid environment in which the microsomal enzyme is located (Ito and Sato, 1969).

The role of lipid solubility in determining both the affinity (Ks) and the extent (% of P-450 involved in the binding) of interactions of a type I compound with liver microsomal cytochrome P-450 is still not clear (Martin and Hansch, 1971; Mazel et al, 1966; Jansson et al, 1972; Canady et al, 1974).

Recent studies by Facino and Lanzani (1979) led to the observation of a strong correlation between the lipid solubility of some beta-adrenergic blocking agents and their affinity constants (Ks) for cytochrome P-450. Other molecular properties were also found to be of great importance in determining the extent with which the drugs interact with cytochrome P-450. The kinetic constants (Km and Vmax) for the metabolism of alprenolol and propranolol by the rat liver microsomal monooxygenases are related respectively to the lipophilic character and binding affinities of the molecules and to the extent of cytochrome P-450 bound. The poorly lipophilic compounds, oxprenolol and methypranol, bind to cytochrome P-450 to a greater extent than the more lipophilic propranolol and this
of the molecule facilitates the attack of these compounds on the active site of the cytochrome.

**Microsomal chemical activation and carcinogenesis**

Attention has recently been directed towards the activation of toxic chemicals by enzymes of the endoplasmic reticulum, more especially, to the activation of carcinogens. The carcinogenic polycyclic hydrocarbons, benzo(a)pyrene and benz(a)anthracene, are characterized by the presence of the phenanthrene nucleus within the chemical structure and it has been proposed (Jerina and Daly, 1977; Jerina and Lehr, 1977) that activation of polycyclic hydrocarbons to proximate or ultimate carcinogens necessitates 'bay region' epoxidation of this phenanthrene nucleus. Other workers (Sims et al, 1974; Swaisland et al, 1974) have found that the epoxide is converted into the corresponding dihydrodiol, by epoxide hydrase, then further oxygenation to yield the diol epoxide. These diol epoxides do not appear to be suitable substrates for epoxide hydrase, and form stabilized internal ion-pair compounds which react as carbonium with DNA, even in the presence of glutathione and proteins, so that no threshold concentration of the diol epoxide need exist for DNA arylation and consequent damage of DNA and carcinogenesis (Hulbert, 1975). Microsomal oxygenation is also known to be involved in the activation of carcinogenic aromatic amines such as 2-naphthylamine (Radomski and Brill, 1970), carcinogenic amides, such as 2-acetamidofluorene, (Weisburger et al, 1972) and carcinogenic nitrosamines (Magee and Barnes, 1967).

**Microsomal enzyme stimulation**

True induction of the drug-metabolizing enzymes involves de novo protein synthesis, and may result from treatment of animals with drugs or certain carcinogens. Stimulation does not require the synthesis of more enzyme protein but probably results from a conformational change of the existing
Induced by drugs

Biphenyl

Induced by carcinogens

Cytochrome P-450

Activation by carcinogens

Cytochrome P-448

4-hydroxybiphenyl

2-hydroxybiphenyl

Figure 1.9 Metabolic hydroxylation of biphenyl
enzyme, cytochrome P-450.

These differences are well illustrated by the model substrate biphenyl. Microsomal oxygenation of biphenyl yields two major products namely 2-hydroxybiphenyl and 4-hydroxybiphenyl (Figure 1.9). This is of interest since both 2- and 4-hydroxylation of biphenyl are catalysed by cytochrome-P-448, with cytochrome P-450 catalysing only the 4-hydroxylation (Bridges et al., 1973; Atlas and Nebert, 1976). This specificity of the 2-hydroxylation by cytochrome P-448 and the known formation of cytochrome P-448 by carcinogens has suggested a correlation between the effect of carcinogens on the endoplasmic reticulum and an increase in the enzymic 2-hydroxylation of biphenyl (Creaven and Parke, 1966; McPherson et al., 1976). Furthermore, the irreversible displacement of ribosomes from the endoplasmic reticulum (Degranulation) by carcinogens (Williams and Rabin, 1971; Delaunay and Schapira, 1974) has suggested a further relationship between degranulation of the endoplasmic reticulum and the expression of the enzyme biphenyl 2-hydroxylase (Parke, 1977a).

The increased biphenyl 4-hydroxylase activity is the result of enzyme induction whereas the increase in biphenyl 2-hydroxylase activity is biphasic, comprising an initial stimulation phase followed later by true enzyme induction. The initial stimulation, which occurs within 0-15 minutes of treatment with the carcinogen, probably represents a conformational change of cytochrome P-450 to cytochrome P-448 and may occur simultaneously to the degranulation of the endoplasmic reticulum. The structure of biphenyl contains the spatial arrangement of the phenanthrene nucleus, and the 2-hydroxylation, but not the 4-hydroxylation, may be considered as 'bay-region' oxygenation.

Glycoprotein synthesis and malignancy

Another essential biological function of the endoplasmic reticulum is the
synthesis of glycoproteins which play a vital role in the overall economy of the cell and of the eukaryotic organism. Glycoproteins secreted into the plasma membrane glycocalyx regulate cell division, and glycoproteins secreted from the cell, such as immunoglobulins, play a major role in immune surveillance. The nature of the carbohydrate moieties of these glycoproteins appears to be the critical feature determining the rate of mitosis, the immune characteristics of the cell and the immune surveillance within the living organism. However, the mechanism by which the various glycosyl transferases within the endoplasmic reticulum, responsible for initiation of glycoprotein synthesis, are regulated is yet to be established (Schachter, 1974).

The degranulation of the endoplasmic reticulum which accompanies carcinogenesis (Delaunay and Schapira, 1974) must of consequence be accompanied by inhibition of glycoprotein synthesis (Parke 1977b). Indeed in malignantly transformed cells, one of the earliest changes observed is the loss of a high molecular weight glycoprotein from the cell surface (Warren et al, 1974) and changes in the nature of the terminal sugar moieties of the glycoproteins of the glycocalyx. Elevated levels of certain glycosyltransferases have been observed in the plasma of cancer patients, and high levels of fucosyltransferase with correspondingly low levels of sialyltransferase and galactosyltransferase were demonstrated in various human metastasizing tumours (Kessel et al, 1977). A marked inhibition of glycosyltransferase in the synthesis of gastric mucus in patients with gastric carcinoma has been observed (Parke and Symons, 1977).

It appears that both the quantitative and qualitative aspects of glycoprotein synthesis are profoundly affected by malignant transformation, and that the changes occurring in the glycoprotein of the cell surface are associated with many of the characteristics of malignant cell, namely, loss of adhesiveness, accelerated cell division and altered antigenicity
(Warren et al., 1974). Furthermore, degranulation of the endoplasmic reticulum might lead to increased synthesis of intracellular proteins by the cytoplasmic ribosomes which, as in the neonate, would lead to the growth of the cell and accelerated cell division. Hence, degranulation would result in a switchover of the economy of the cell from a predominance of synthesis of glycoproteins for export (mucus, immunoglobulins, serum albumin) to the neonatal state of predominantly intracellular protein synthesis, with accelerated cell growth and mitosis.

Cyclic Nucleotides and Malignancy

It has been suggested that many of the properties of malignantly transformed cells are due to low levels of cyclic AMP (Pastan et al., 1975). When transformed isolated cells or hepatoma cells in culture are treated with cyclic AMP, the transformed cells change in appearance to more closely resemble normal cells and grow more slowly (Pastan, 1975; van Wijk et al., 1972). On the other hand, dibutyryl cyclic AMP has been shown to increase the frequency of cell transformation by oncogenic viruses (Smith et al., 1973), and chemical carcinogenesis is associated with an increased responsiveness of adenylate cyclase to hormonal control (Boyd et al., 1974), so that a cascade effect magnifying the malignant transformation could result especially in stressful conditions when the circulating catecholamines are high (Figure 1.10). Although the level of cyclic AMP of established tumours is always low and the hormonal responsiveness of their adenylate cyclase is not high (Boyd et al., 1974) increased levels of cyclic AMP are characteristic of the initial malignant cell transformation. In keeping with this hypothesis is the observation that the tumour-promoting phorbol esters, applied to mouse epidermis also produce an initial increase in cyclic AMP followed by a marked depression, then a second period of elevation (Grimm and Marks, 1974). At the same time the hormonal regulation of adenylate cyclase
is largely lost, indicating that the tumour promoters have resulted in

Stress

Catecholamines

ATP Adenylate cyclase Cyclic AMP

Normal cell Oncogenic viruses

Chemical carcinogens Malignant transformed cell

Figure 1.10 Possible Cascade Effect of Chemical Carcinogens and Cyclic AMP in Malignant Cell Transformation

damage of the cell membrane, the site of the β-adrenergic receptor of adenylate cyclase.

Recent work has suggested that the regulation of DNA synthesis and cell division is controlled by the ratio of cyclic AMP to cyclic GMP rather than by cyclic AMP alone. The tumour-promoting phorbol esters result in a marked increase in cyclic GMP, and conversely, cyclic AMP reduces the promoting effects of the phorbol esters. An increase of DNA synthesis which follows a reduction of the cyclic AMP/cyclic GMP ratio, is considered to be the most important effect of the phorbol ester promoters, rather than inhibition of DNA repair mechanisms (Trosko et al, 1975).

Toxicity of Beta-adrenergic Blocking Agents

The toxicological effects of these agents is reflected in their adverse drug reactions and potential carcinogenicity.

Adverse-reaction of beta-adrenergic blocking agents

A characteristic immune reaction - the oculomucocutaneous syndrome,
affecting singly or in combination, eyes, mucous and serous membranes and the skin, often in association with positive antinuclear factor (ANF) - has been reported in practolol-treated patients (Wright, 1975; van-Manning, 1975; Brown et al., 1974; Zacharais, 1972; Felix et al., 1974; Cumberbatch, Padfield et al., 1975; Knapp and Galloway, 1975).

Many of the practolol reactions are reversible on withdrawal of the drug together with topical corticosteroids, artificial tear solutions, anti-biotic eye drops and oral corticosteroids. Polyserositis and fibrosing peritonitis may however progress inspite of withdrawal of the drug and have developed up to a year after discontinuation of practolol (Ratfrey and Denman, 1973; Rowland and Stevenson, 1972; Felix et al., 1975; Lloyd, 1975; Holt and Waddington, 1975; Wright, 1975; Nicholls, 1976).

It is not known whether the practolol reaction is specific for practolol or is the direct specific result of pharmacologically induced change by \( \beta \)-blockade (Gaylarde and Sarkany, 1975). There have been few convincing reports of the oculomucocutaneous reactions with oxprenolol (Holt and Waddington, 1975; Knapp et al., 1975) and one with propranolol (Cubey and Taylor, 1975).

The cause and mechanism of this adverse reaction of practolol has yet to be classified. It was earlier suggested that it may be due to the differences in metabolism amongst various individuals and possibly the long plasma half-life. However, recent studies by Reeves et al., (1978) led to the suggestion that the adverse reactions associated with practolol may be unrelated to metabolism or kinetics.

Two separate processes have been suggested for the lachrymal gland and conjunctival damage (Wright, 1975). Firstly, the drug may accumulate locally in the lachrymal tissue and may have a local toxic effect on
secreting cells. Secondly, it is likely that the conjunctiva is damaged by an antibody identified by Amos et al., (1975) as antinuclear antibody (ANA), which binds to the intracellular region of the squamous epithelial tissue. Mackie et al., (1977) have suggested that the dry eye observed in patients on beta-adrenergic blocking agents, particularly $\beta_1$-selective blocker, is indicative of a reduction in tear lysozyme formation suggesting an impaired lachrymal gland function.

Brenner et al., (1968) have shown that $\beta$-adrenergic blockade increases antibody formation in response to antigen challenge and that adrenaline suppresses the normal response to antigenic stimulation. Furthermore, $\beta$-adrenergic blockers interfere with the action of anti-inflammatory agents (Reesterer and Jaques, 1968) and propranolol prevents the response of lymphocytes to phytohaemagglutinin in vitro (Smith et al., 1971). Finally adrenaline activates epidermal adenylate cyclase activity and reduces epidermal cell division (Yoshikawa et al., 1975). $\beta$-adrenergic blockers antagonize this effect; suppressing cyclic AMP formation and encouraging cell division and probably predispose to the development of psoriasiform changes in the skin.

It has however been suggested by Ratfrey and Denman (1973) that since practolol acts as a partial agonist it will stimulate cyclic AMP synthesis. This in turn may have some direct effect on epidermal cells or impair the activity of T-lymphocytes population; resulting in the production of lymphocytes with autoimmune propensities.

**Carcinogenicity potential of beta-adrenergic blocking agents**

Pronethalol, the first $\beta$-adrenergic blocking agent to be put to clinical test, was reported to induce tumours of the lymphoreticular system in certain
strains of mice, after oral administration of 200 mg/kg for a period of 2-3 months, whereas long-term administration up to 2 years to rats and guinea-pigs failed to elicit any evidence of carcinogenicity (Paget, 1963; Alcock and Bond, 1964; Howe, 1965).

Practolol and alprenolol have been shown to give some indications of tumorigenicity in rodents. In addition a variety of other β-adrenergic blocking agents have proved to be tumorigenic or otherwise hazardous. Two cardioselective agents, pamatolol and talomolol, have produced carcinomas of the liver in rats and talomolol has produced mammary carcinomas in mice as well. Other drugs of this general class appear to have produced benign tumours although some of the results are equivocal, necessitating repeat studies (FDA report, Drug Bulletin, 1978).

Newberne et al, (1977) investigated the carcinogenicity potentials of oxprenolol and pronethalol in mice and rats and showed that neither compounds have any indications of tumour formation after an 18-21 month study at dose levels of 15, 50 and 150 mg/kg. These findings are at variance with those reported for pronethalol (Paget, 1963; Alcock and Bond, 1964; Howe, 1965). In attempting to clarify the disparity in response to pronethalol between the mouse and other species, Howe (1965) proposed a probable production of an ethyleneimine derivate of pronethalol which might be the proximate carcinogen of pronethalol in vivo in the mouse, although an ethyleneimine structure has not been identified as either a minor or transient metabolite of pronethalol.

Recent studies on the potential carcinogenicity of propranolol have cast doubts as to its safety. Propranolol administered in drinking water during treatment with a fixed concentration of the carcinogen 3-methyl-4-dimethylaminoazobenzene, for a period of 14-20 weeks, produced dose-
dependent increases in the incidence of tumours in rat livers (Boyd and Martin, 1977). However, it is not clear whether this effect of propranolol was due to specific beta-blockade or to non-specific actions. Nor is it clear whether this effect is due to direct action on the liver cells or to an effect on appetite (to alter intake of carcinogen) or on the immune system. Further studies with propranolol in mice administered with the drug, at low doses of about 0.7 mg/kg for 2-8 months, showed time-related changes in the salivary glands with epithelial necrosis and lymphocyte infiltration and the appearance of granulomas on the serosal surface of the colon and duodenum (Smith and Butler, 1978).

In a study of the carcinogenesis of sotalol hydrochloride which included propranolol, Wiekel and Kelly (1979) showed that propranolol administered to mice and rats in their diets, at daily dose levels of 100 and 37.5 mg/kg respectively for a period of 78 weeks, did not produce any changes in types and frequency of tumours as compared to control in either species. Sotalol hydrochloride administered to both species in their diets at daily doses of 300-600 mg/kg and 137 or 275 mg/kg respectively produced similar effects to propranolol. Toxicological evaluation of nadolol which included data on tumourigenicity revealed that this drug was not tumourigenic in either species (Sibley et al., 1978).

**Aims of Project**

Despite extensive research and safety evaluation carried out on β-adrenergic blocking agents, there are still problems associated with the potential toxicity of this class of drugs. Long-term animal carcinogenicity studies have led to the identification of tumours in rodents with some of these compounds, although some of these findings are equivocal and need further examination. There are however, no available data on short-term tests of their potential mutagenicity. The FDA has suggested that the whole class of drugs might be potential carcinogens possibly because of their expected
action on cellular adenylate cyclase/cyclic AMP and guanylate cyclase/
cyclic GMP levels. When the practolol toxicity arose, a new potential
manifestation of toxicity, possibly involving mucus and glycoprotein
synthesis became apparent. The aims of the present study were therefore
four-fold.

a) To investigate the potential carcinogenicity of some beta-adrenergic
blocking agents using a short-term carcinogenicity test involving the
measurement of their effects on the activities of biphenyl 2- and 4-
hydroxylase and ethoxyresorufin O-deethylase, catalysed by cytochrome
P-448 known to be formed by carcinogens (Bridges et al, 1973; Burke
et al, 1977; Atlas and Nebert, 1976; Burke and Mayer, 1975), as well as
other drug-metabolizing enzyme parameters.

b) To determine the potential mutagenicity of some of these agents using
short-term mutagenicity tests such as the Ames' bacterial (McCann et al,
1975) and mammalian micronucleus (Heddle, 1973; Schmid, 1975) tests.

c) To investigate whether the practolol toxicity is a direct result of
pharmacological effect of beta-blockade involving synthesis of mucus
glycoproteins measured by the effects of some of these agents on the
rates of incorporation of radiolabelled sugar and amino acid precursors
into rat gastrointestinal mucus glycoprotein as used by Johnston (1977)
for the study of the ulcer-healing effect of carbenoxolone.

d) To study their effects on cellular levels of adenylate cyclase/cyclic
AMP and guanylate cyclase/cyclic GMP to ascertain whether these effects
can be implicated in glycoprotein synthesis and the incidence of
malignancy as has been suggested for some carcinogens and tumour-
promoting agents (Macdonald et al, 1977; Delaunay and Schapira, 1974;
CHAPTER TWO

EFFECT OF SOME BETA-ADRENERGIC BLOCKING AGENTS ON RAT HEPATIC MICROSONAL MIXED-FUNCTION OXYGENASE SYSTEM
Long-term animal studies are the most widely used and generally accepted methods for the screening of chemicals for their carcinogenic potential. However, these studies have proved to be very expensive and time consuming making it much less practical to screen large numbers of compounds, and often results are equivocal due to high incidence of spontaneous tumours in control animals and ambiguous distinction of tumours, namely benign and malignant tumours, hyperplasia, metaplasia and neoplasia (Fishbein, 1976).

A wide variety of more economical short-term tests of high predictive value are currently developed to identify compounds with carcinogenic potentials. Many of these tests are designed to detect agents that cause damage to DNA (i.e. mutagens) in bacterial, mammalian or human cultured systems, since there appears to be a good correlation between mutagenesis and carcinogenesis (Miller and Miller, 1975; Ames et al, 1975b; Jenssen and Ramel, 1980). One of the most successful of these is undoubtedly the Ames test, which is an in vitro test system employing specially constructed strains of Salmonella typhimurium as sensitive indicators of DNA damage, and mammalian liver extracts fortified with the necessary cofactors for the metabolic activation of carcinogens to their mutagenic forms (McCann et al, 1975; McCann and Ames, 1976). An in vivo test system has also been developed for determining the DNA damage caused by mutagens and carcinogens known as the micromucleus test (Schmid, 1975). Although this test is less sensitive than the Ames test it has an almost parallel specificity and predictive value (Jenssen and Ramel 1980).

It has been suggested that not all organic carcinogens produce tumours by the electrophilic theory of DNA attack (Pitot and Heidelberger, 1963; Weisburger, 1973). A mechanism has been suggested for such non-mutagenic
Carcinogens for the expression of their tumorigenic potential; called epigenetic mechanism (Williams, 1977). Tumorigenic compounds acting via this mechanism may act in a manner analogous to differentiation, where cells of demonstrably equivalent genetic constitution turn into types that become more increasingly diverse. Irreversible changes induced by specific molecular species in the cell such as a modification of regulator-gene activity for a short period of time could give rise to a variety of phenotypes in the absence of genetic damage and could result ultimately in the production of tumours (Weiss, 1968; Magee, 1977; Pitot and Heidelberger, 1963). This phenomenon may account for the tumours produced by chemicals such as saccharin, phenobarbitone, chloroform, carbon tetrachloride, dieldrin and DDT amongst others. The limited evidence available indicates that most of the possible epigenetic carcinogens (carcinogens acting through epigenetic mechanisms) are species specific in their effects and produce tumours only after prolonged exposure to the chemical at high dose levels. In these cases normal dose-response relationships may be inappropriate and the definition of a critical toxic effect level, threshold dose, may be necessary below which exposure to the chemical would not be hazardous and may be much higher than the 'safe level' suggested by analogy with mutagenic carcinogens operating via a genotypic mechanism (Cornfield, 1977; Graham et al, 1975).

Carcinogens are known to exert various effects on the endoplasmic reticulum and these appear to provide promising alternatives to carcinogen testing. The microsomal oxygenation of the model substrate biphenyl, leads to the production of two major products namely, 2-hydroxybiphenyl and 4-hydroxybiphenyl. Both products are catalysed by cytochrome P-448 whilst cytochrome P-450 catalyses only the 4-hydroxylation (Bridges et al, 1973; Atlas and Nebert, 1976). This specificity of the 2-hydroxylation by cytochrome P-448 and the known formation of cytochrome P-448 by chemical carcinogens has suggested a correlation between the effects of carcinogens on the endoplasmic
reticulum and an increase in enzymic 2-hydroxylation of biphenyl (Creaven and Parke, 1966; McPherson et al., 1976). Furthermore, the irreversible displacement of ribosomes from the endoplasmic reticulum (degranulation) by carcinogens (Williams and Rabin, 1971; Delaunay and Schapira, 1974) has suggested a further relationship between the degranulation of the endoplasmic reticulum and the enzyme biphenyl 2-hydroxylase (Parke, 1977a). Carcinogens have also been shown to produce changes in other drug metabolizing enzymes (Feuer and Granda, 1970) as well as ethoxyresorufin O-deethylase activity which is known to be catalysed by cytochrome P-448 (Atlas and Nebert, 1976; Burke and Mayer, 1975; Burke et al., 1977).

Significant interaction among cardiovascular drugs occur with induction or inhibition of hepatic metabolism of drugs. A number of different drugs can affect the hepatic microsomal enzyme system that are responsible for metabolism of various drugs (Burns and Conney, 1965; Gelehrter, 1976). Chemically important interactions due to induction and inhibition of hepatic microsomal enzymes have been observed with some cardiovascular agents. (Datta et al., 1976; Kiørbre, 1966; Kutt et al., 1968; Garrettson et al., 1969; Soda and Levy, 1975; Lumholtz et al., 1975). The induction or inhibition of drug-metabolizing enzyme activities by beta-adrenergic blocking agents may exert an important influence on their metabolism as well as on chemicals such as food additives that are generally present in human diet. This may lead to beneficial effects if the foreign compounds are rendered inactive, but harmful when toxic metabolites are formed.

Long-term animal carcinogenicity studies with beta-adrenergic blocking agents have produced equivocal results (Paget, 1963; Alcock and Bond, 1964; Howe, 1965; FDA report, Drug Bulletin, 1978; Boyd and Martin, 1977; Smith and Butler, 1978; Newberne et al., 1977; Wiekel and Kelly, 1979). However short-term studies of their potential carcinogenicity through epigenetic mechanisms have not been investigated. It was therefore of great interest
to investigate the effects of pretreatment of rats with beta-adrenergic blocking agents on the cytochrome P-450 and P-448 - mediated hepatic microsomal mixed-function oxygenase system as well as other drug-metabolizing enzyme parameters to ascertain their carcinogenic potential and possible implications in drug interactions.
EXPERIMENTAL

Materials

Biphenyl was twice crystallized from 96% ethanol (M.P. 60 - 70°C)
4-hydroxybiphenyl and 2-hydroxybiphenyl were twice recrystallized from
methanol (M.P. 166 - 167°C) and light petroleum (M.P. 56-57°C) respectively
(Burke, 1972). Glucose 6-phosphate (monosodium salt), NADP+, glucose
6-phosphate dehydrogenase (type III) and 3-methylcholanthrene (Sigma
Chemical Co. London). 7-Ethoxyresorufin and resorufin standard (Pierce
Chemical Co. Rockford 11, USA). Ethylmorphine hydrochloride (May and Baker,

The beta-adrenergic blocking agents were obtained from the respective
drug companies as gifts. Atenolol "free base" (Tenormin), pronethalol
hydrochloride, propranolol hydrochloride and practolol (Imperial Chemical
Industries (ICI) Ltd, Cheshire, U.K) Acebutolol hydrochloride (Sectral)
May and Baker. Phenobarbitone sodium [British Drug Houses (BDH) Ltd,
Poole, U.K] were used as purchased.

All other chemicals used were either of the analytical or general reagent
grade.

Animals

Male Wistar albino rats (150-200g) were kept in litter-mate cage groups
with a maximum of four animals per group. Water and food (Spratt's
laboratory chow) were provided ad libitum. The animals were killed between
10.00 and 11.00 am.
Treatment of animals

The beta-adrenergic blocking agents were administered daily as single oral doses for three consecutive days. They were administered in aqueous solution (1ml/100g wt) at three dose levels; 5, 50, 150 mg/kg. Controls for atenolol and practolol received equivalent volumes of vehicle (0.1M Citric acid/Na$_2$HPO$_4$ buffer, pH6.5) whilst the remaining controls received nothing. Phenobarbitone (75mg/kg) in saline 0.9%NaCl(w/v) and 3-methylcholanthrene (25mg/kg) in corn oil were administered intraperitoneally (i.p) [0.2ml/100g wt] and control animals received saline and corn oil respectively.

Preparation of Tissue Homogenate

The animals were killed by cervical dislocation 24 hours after administration of last dose. The livers were rapidly removed and placed in 1.15%(w/v) KCl, brought to pH7.6 by addition of NaHCO$_3$ solution, blotted with filter paper weighed and immersed in cold 1.15%(w/v) KCl. The weighed livers were homogenized in a Potter-Elvehjem homogenizer (Size C, A.H. Thomas and Co. Philadelphia P.A. USA) by using three strokes (10 seconds each) of the pestle, power-driven at about 2,500 rev/min. Homogenates (300mg=1ml) were poured into 50 ml polycarbonate centrifuge tubes and centrifuged at 11,000 rpm in an MSE high speed 18 for 20 minutes at 4°C using the 8 x 50ml rotor. The resulting supernatant was transferred into 25 ml polycarbonate tubes and further centrifuged at 50,000 rpm in a Beckman ultracentrifuge model LS-65 for 40 minutes at 4°C using the 8 x 25ml rotor. The supernatant (cytosol) was discarded and the microsomal pellet washed with the respective buffer for an assay and resuspended in buffer to give a 30% microsomal suspension.

Biphenyl Hydroxylation

The assay for biphenyl hydroxylation was according to the method of Creaven
et al (1965) as modified by McPherson (1975). The incubation system consisted of the following:

**Buffer**

\[ \text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4 \text{ buffer (0.3mM) pH7.6} \]  

<table>
<thead>
<tr>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.10</td>
<td>1.10</td>
</tr>
</tbody>
</table>

**NADPH regenerating system**

- Glucose 6-phosphate 50 µmoles
- NADP⁺ 10 µmoles
- Glucose 6-phosphate dehydrogenase 4 units
- MgSO₄ (3.3 mM) 0.30
- Microsomal suspension (30%) 0.20

**Substrate**

Biphenyl 14 mM in 1.15%(w/v)KCl containing 1.6%(w/v)Tween 80°

<table>
<thead>
<tr>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Total incubation volume** 2.00

* Addition after incubation

After an incubation period of 10 minutes at 37°C in a shaking water bath (100 cycles/min), the reaction was terminated by addition of 0.5ml 4NHCl. n-Heptane containing 1.5%(v/v) isoamyl alcohol (7ml) was then added to all the tubes, extracted for 15 minutes on a rotary shaker and centrifuged at 2,000rpm for 10 minutes. Aliquots (2ml) of the organic layer were withdrawn and added to tubes containing 5ml 0.1N NaOH. Further extraction was carried out for 10 minutes on the rotary shaker after which the tubes were centrifuged at 2000rpm for 15 minutes. After phase separation, the organic
layer was aspirated off and aliquots (2ml) of the aqueous layer put into fluorimetric cuvettes. 0.5N Succinic acid (0.5ml) was added to adjust the pH to 5.5.

The 4-hydroxybiphenyl standard incubation mixture was similar to the blank but the volume of buffer was reduced by 0.5ml and substituted with the same volume of 0.1mM 4-hydroxybiphenyl in 5% ethanol. In the case of the 2-hydroxybiphenyl standard 0.1ml of buffer was replaced by the same volume of 0.1mM 2-hydroxybiphenyl in 5% ethanol.

The 2- and 4-hydroxybiphenyls were measured using a Perkin-Elmer MPF 3 spectrophotofluorimeter at emission wavelengths 415nm and 338nm with excitation wavelengths 295nm and 275nm respectively.

From the reading at 338nm with $\lambda_{\text{exc}}^{275\text{nm}}$ 4-hydroxybiphenyl can be calculated since the 2-isomer does not interfere at this wavelength. From the reading obtained at 415nm with $\lambda_{\text{exc}}^{295\text{nm}}$, 2-hydroxybiphenyl can be determined after allowing for the contribution of the 4-isomer at this wavelength. This contribution was calculated as follows:

$$\text{Reading of 4-hydroxybiphenyl standard at 415nm} \times \frac{\text{amount of 4-hydroxybiphenyl in unknown}}{\text{amount of 4-hydroxybiphenyl in standard}}$$

7-Ethoxyresorufin O-deethylase

Hepatic microsomes catalyse the O-deethylation of ethoxyresorufin to resorufin. Both compounds are highly fluorescent, and the reaction can be monitored continuously in a fluorimeter.

The method used for this assay is that of Burke and Mayer (1974). The reaction mixture comprised 2ml sodium phosphate buffer (0.1M) pH7.8, 20 µl microsomal suspension (30%) and 10 µl ethoxyresorufin (50 µM in methanol).
The reaction rate was measured at 37°C in a Perkin-Elmer MPF-3 spectrophotofluorimeter at an emission wavelength of 586nm and at an excitation wavelength of 510nm. A baseline was initially drawn before addition of 10 μl NADPH (50 mM) to initiate the reaction which was followed for 2 minutes. A standard reference was prepared in which ethoxyresorufin has been replaced by 10 μl resorufin standard (0.1mM) and used in calculating the amount of product formed.

**Cytochromes b$_5$ and P-450**

The method employed is essentially that used by Ullrich (1969). Equal volumes of microsomal suspension (2.5ml containing approximately 2mg/ml protein in 0.3M Na$_2$HPO$_4$/KH$_2$PO$_4$ buffer pH7.6) were added to two matched cuvettes. 5 μl NADH (10mg/ml buffer) were added to test cuvette, mixed and the difference spectrum for cytochrome b$_5$ recorded between 390nm and 430nm wavelengths in a Pye Unicam SP1800 dual beam spectrophotometer. Few granules of sodium dithionite were added to both cuvettes to reduce cytochrome P-450. The test cuvette was gently gassed with carbon monoxide for about 30 seconds. The difference spectrum for cytochrome P-450 was recorded between wavelengths 430nm and 500nm. The amount of cytochromes b$_5$ and P-450 in samples were calculated using the extinction coefficients of 170 mM$^{-1}$ cm$^{-1}$ ($E_{424}$-$E_{409}$) and 91mM$^{-1}$ cm$^{-1}$ ($E_{450}$-$E_{500}$) respectively (Ullrich, 1969).

**NADPH Cytochrome c Reductase**

The principle of the assay is the measurement of the rate of reduction of cytochrome c. The method used in the assay was a modification of that of Masters et al (1967) and the incubation mixture is as follows:
## Ethylmorphine N-demethylase

The procedure of Holtzman et al., (1968) was used in which the formaldehyde produced was measured by the formation of a yellow complex by method of Nash (1953). The incubation mixture was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>TEST (ml)</th>
<th>BLANK (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (0.15M) pH 7.4</td>
<td>0.9</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>NADPH regenerating system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP⁺</td>
<td>1.5 μmoles</td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>30 μmoles</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>15 μmoles</td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>2 units</td>
<td></td>
</tr>
<tr>
<td>Microsomal suspension (30%)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethylmorphine hydrochloride (3mM)</td>
<td>1.0*</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total incubation volume</strong></td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Incubation mixture warmed for 5 minutes at 37°C before its addition.
The mixture was incubated at 37°C for 10 minutes in a shaking water bath (100 cycles/min). The reaction was terminated with 1ml 5%(w/v) ZnSO₄. Saturated Ba(OH)₂ (1.5ml) was added and shaken followed by addition of saturated sodium metaborate (0.5ml) with further mixing. The resulting mixture was centrifuged at 2,000rpm for 20 minutes in a Mistral 6L centrifuge and the formaldehyde formed determined by method of Nash (1953). To the supernatant(3ml) was added an equal volume of freshly prepared Nash reagent B (2mM ammonium acetate, 0.05M acetic acid and 0.02M acetylacetone). The mixture was shaken and incubated at 37°C for 40 minutes in the shaking water bath. The resulting yellow complex was measured at 412nm in a Cecil 20 spectrophotometer after bringing the solution to room temperature. The formaldehyde produced by reactions not involving drug substrate was corrected by preparation of tissue blanks in which only the substrate has been omitted. The concentration of formaldehyde formed was determined by using the extinction coefficient of 8.0mM⁻¹ cm⁻¹ (Nash, 1953).

**Protein Determination**

The method used was based upon the colourimetric determination of protein by Lowry et al, (1951).

Samples were diluted with 0.5N NaOH to give an amount of protein of about 0.5mg/ml. A 1ml aliquot of the diluted sample was then put into a test-tube. 5ml of a freshly prepared copper reagent* were added and mixed. This was allowed to stand for 10 minutes before adding 0.5ml Folin-Ciocalteau phenol reagent (diluted 1 in 3 with distilled water). The contents were immediately mixed and the resulting blue colour was read after 35 minutes in a Cecil 20 spectrophotometer at 720nm. The tubes
were run in duplicate. Suitable blank and standards 0-1 (mg/ml) bovine serum albumin were carried through the same procedure.

* Copper reagent comprised:

1% CuSO₄·5H₂O                  - 0.5 ml
2% Potassium-sodium tartrate   - 0.5 ml
2% Na₂CO₃                      - 50.0 ml
RESULTS

Effect of Phenobarbitone, 3-methylcholanthrene and some Beta-adrenergic Blocking Agents on some Hepatic Drug-metabolizing Parameters in the Rat

It appears from the study that none of the beta-adrenergic blocking agents significantly affected the liver wt/body wt, microsomal protein, cytochromes b5 and P-450 contents at any of the dose levels. In additionatenolol and practolol did not significantly affect the hepatic microsomal mixed-function oxygenases activities. However, propranolol, pronethalol and acebutolol at the highest dose level of 150mg/kg. The stimulatory effect on ethoxyresorufin O-deethylase by propranolol and pronethalol appear to be dose-dependent. Propranolol also significantly enhanced both biphenyl 2- and 4-hydroxylase activities whilst pronethalol only enhanced the biphenyl 4-hydroxylase activity at the highest dose level of 150mg/kg. None of the beta-adrenergic blocking agents at any dose level significantly changed the activities of ethylmorphine N-demethylase and NADPH2 cytochrome c-reductase (Tables 2.1 - 2.10).

Phenobarbitone (75mg/kg) and 3-methylcholanthrene (25mg/kg) non-carcinogen and carcinogen controls respectively, significantly increased liver wt/body wt without any changes in microsomal protein. However, 3-methylcholanthrene induced both cytochromes b5 and P-450 whilst phenobarbitone induced only cytochrome P-450. The activity of ethoxyresorufin O-deethylase activity was enhanced significantly (90-fold) and that of ethylmorphine N-demethylase inhibited by 3-methylcholanthrene. Phenobarbitone on the otherhand significantly enhanced ethylmorphine N-demethylase activity without markedly affecting ethoxyresorufin O-deethylase activity. 3-methylcholanthrene stimulated both biphenyl 2-hydroxylase (7-fold) and biphenyl 4-hydroxylase (2-fold) whereas phenobarbitone enhanced biphenyl 4-hydroxylase (2-fold) without significant stimulation of biphenyl 2-hydroxylase (Table 2.11).
Effect of Combined Administration of Propranolol and 3-methylcholanthrene on Rat Hepatic Drug-metabolizing Parameters

Administration of two different dose levels of propranolol (50,100mg/kg) together with 3-methylcholanthrene (20mg/kg) significantly increased liver wt/body wt and cytochromes b\textsubscript{5} and P-450 contents in the rat. It appears that these changes decreased with increasing propranolol concentration as compared to 3-methylcholanthrene control. The enhancement of biphenyl 2-hydroxylase and ethoxyresorufin O-deethylase activities by 3-methylcholanthrene were further augmented by combined administration with propranolol and these changes appear to increase with increasing propranolol concentration (i.e. combined effect appear to be additive). However the reverse is true for biphenyl 4-hydroxylase, ethylmorphine N-demethylase and NADPH\textsubscript{2} cytochrome c reductase activities; the enhancements shown by 3-methylcholanthrene for biphenyl 4-hydroxylase is slightly inhibited whilst the inhibition of ethylmorphine N-demethylase and NADPH\textsubscript{2} cytochrome c reductase is slightly counteracted by combined administration with propranolol (Tables 2.12, 2.13).
TABLE 2.1  Effect of Practolol Administration on some Hepatic Microsomal Mixed Function Oxygenase Activity in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>2-hydroxylation of Biphenyl</th>
<th>4-hydroxylation of Biphenyl</th>
<th>O-deethylation of Ethoxyresorufin</th>
<th>N-demethylation of Ethylmorphine</th>
<th>NADPH reduction of Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.10 ± 0.01</td>
<td>0.76 ± 0.04</td>
<td>0.15 ± 0.01</td>
<td>1.31 ± 0.02</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>Practolol</td>
<td>5</td>
<td>0.10 ± 0.01</td>
<td>0.76 ± 0.03</td>
<td>0.14 ± 0.01</td>
<td>1.31 ± 0.02</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.10 ± 0.01</td>
<td>0.75 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.10 ± 0.01</td>
<td>0.72 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>1.34 ± 0.03</td>
<td>9.1 ± 0.3</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min
### TABLE 2.2  Effect of Atenolol Administration on some Hepatic Microsomal Mix function Oxygenase Activity in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>2-hydroxylation of Biphenyl</th>
<th>4-hydroxylation of Biphenyl</th>
<th>O-deethylation of Ethoxyresorufin</th>
<th>N-demethylation of Ethylmorphine</th>
<th>NADPH&lt;sub&gt;2&lt;/sub&gt; reduction of Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.11 ± 0.01</td>
<td>0.62 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>Atenolol</td>
<td>5</td>
<td>0.12 ± 0.01</td>
<td>0.69 ± 0.04</td>
<td>0.12 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.12 ± 0.01</td>
<td>0.68 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.12 ± 0.01</td>
<td>0.65 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>1.35 ± 0.03</td>
<td>11.0 ± 0.3</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min
TABLE 2.3  Effect of Acebutolol Administration on some Hepatic Microsomal Mixed-function Oxygenase Activity in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>2-hydroxylation of Biphenyl</th>
<th>4-hydroxylation of Biphenyl</th>
<th>O-deethylation of Ethoxyresorufin</th>
<th>N-demethylation of Ethylmorphine</th>
<th>NADPH reduction of Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.14 ± 0.01</td>
<td>0.63 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>1.30 ± 0.02</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>Acebutolol</td>
<td>5</td>
<td>0.14 ± 0.01</td>
<td>0.68 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>1.29 ± 0.02</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.15 ± 0.01</td>
<td>0.68 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>1.29 ± 0.01</td>
<td>8.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.15 ± 0.01</td>
<td>0.67 ± 0.02</td>
<td>0.20 ± 0.01*</td>
<td>1.31 ± 0.03</td>
<td>8.3 ± 0.2</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min

Value significantly different from control;  *P<0.001
### TABLE 2.4  Effect of Propranolol Administration on some Hepatic Microsomal Mixed-function Oxygenase Activity in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>2-hydroxylation of Biphenyl</th>
<th>4-hydroxylation of Biphenyl</th>
<th>O-deethylation of Ethoxyresorufin</th>
<th>N-demethylation of Ethylmorphine</th>
<th>NADPH reduction of Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.11 + 0.01</td>
<td>0.65 + 0.02</td>
<td>0.10 + 0.01</td>
<td>1.22 + 0.03</td>
<td>9.5 + 0.2</td>
</tr>
<tr>
<td>Propranolol</td>
<td>5</td>
<td>0.12 + 0.01</td>
<td>0.66 + 0.02</td>
<td>0.11 + 0.01</td>
<td>1.20 + 0.03</td>
<td>9.6 + 0.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.12 + 0.01</td>
<td>0.65 + 0.02</td>
<td>0.13 + 0.01*</td>
<td>1.20 + 0.04</td>
<td>9.9 + 0.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.16 + 0.01*</td>
<td>0.83 + 0.02*</td>
<td>0.30 + 0.02*</td>
<td>1.22 + 0.05</td>
<td>9.9 + 0.1</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min

Value significantly different from control;  *P<0.001
TABLE 2.5 Effect of Pronethalol Administration on some Hepatic Microsomal Mixed-function Oxygenase Activity in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>2-hydroxylation of Biphenyl</th>
<th>4-hydroxylation of Biphenyl</th>
<th>O-deethylation of Ethoxyresorufin</th>
<th>N-demethylation of Ethylmorphine</th>
<th>NADPH reduction of Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.12 ± 0.01</td>
<td>0.67 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>Pronethalol</td>
<td>5</td>
<td>0.12 ± 0.01</td>
<td>0.71 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.13 ± 0.01</td>
<td>0.74 ± 0.03</td>
<td>0.20 ± 0.01**</td>
<td>1.30 ± 0.02</td>
<td>9.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.13 ± 0.01</td>
<td>0.78 ± 0.03*</td>
<td>0.36 ± 0.02**</td>
<td>1.31 ± 0.02</td>
<td>10.0 ± 0.3</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min
Value significantly different from control; *P<0.05, **P<0.001
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Liver wt./body wt. (x 100)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol/mg protein)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.2 ± 0.1</td>
<td>22.7 ± 0.8</td>
<td>0.49 ± 0.01</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>Practolol</td>
<td>5</td>
<td>5.4 ± 0.1</td>
<td>22.9 ± 0.8</td>
<td>0.51 ± 0.01</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.5 ± 0.1</td>
<td>20.8 ± 0.6</td>
<td>0.50 ± 0.01</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5.1 ± 0.1</td>
<td>20.1 ± 1.1</td>
<td>0.51 ± 0.01</td>
<td>0.71 ± 0.02</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals
### TABLE 2.7 Effect of Atenolol Administration on some Hepatic Drug-metabolizing Parameters in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Liver wt./body wt. (x 100)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol/mg protein)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.3 ± 0.1</td>
<td>22.7 ± 0.8</td>
<td>0.52 ± 0.02</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td>Atenolol</td>
<td>5</td>
<td>5.4 ± 0.1</td>
<td>23.0 ± 0.3</td>
<td>0.54 ± 0.02</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.4 ± 0.1</td>
<td>21.0 ± 0.3</td>
<td>0.55 ± 0.02</td>
<td>0.74 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5.2 ± 0.2</td>
<td>20.6 ± 2.0</td>
<td>0.56 ± 0.02</td>
<td>0.76 ± 0.03</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals
# TABLE 2.8 Effect of Pronethalol Administration on some Hepatic Drug-metabolizing Parameters in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Liver wt./body wt. (x 100)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol/mg protein)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.5 ± 0.1</td>
<td>25.2 ± 1.0</td>
<td>0.52 ± 0.02</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td>Pronethalol</td>
<td>5</td>
<td>5.3 ± 0.1</td>
<td>24.6 ± 0.5</td>
<td>0.53 ± 0.03</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.3 ± 0.1</td>
<td>24.2 ± 1.2</td>
<td>0.52 ± 0.03</td>
<td>0.77 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5.7 ± 0.1</td>
<td>23.9 ± 1.1</td>
<td>0.49 ± 0.01</td>
<td>0.79 ± 0.03</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals
## TABLE 2.9  Effect of Acebutolol Administration on some Hepatic Drug-metabolizing Parameters in the Rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Liver wt./body wt. (x 100)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>Cytochrome b$_{5}$ (nmol/mg protein)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.4 ± 0.1</td>
<td>22.8 ± 1.1</td>
<td>0.53 ± 0.02</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td>Acebutolol</td>
<td>5</td>
<td>5.2 ± 0.1</td>
<td>24.3 ± 0.5</td>
<td>0.55 ± 0.02</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.2 ± 0.1</td>
<td>23.3 ± 0.5</td>
<td>0.55 ± 0.02</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>4.9 ± 0.1</td>
<td>19.6 ± 1.0</td>
<td>0.54 ± 0.01</td>
<td>0.77 ± 0.02</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals


<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Liver wt./body wt. (x 100)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>Cytochrome b₅ (nmol/mg protein)</th>
<th>Cytochrome P-4₅₀ (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.2 ± 0.2</td>
<td>21.6 ± 0.9</td>
<td>0.51 ± 0.01</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>Propranolol</td>
<td>5</td>
<td>5.5 ± 0.1</td>
<td>22.8 ± 1.1</td>
<td>0.51 ± 0.02</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.6 ± 0.2</td>
<td>22.9 ± 1.1</td>
<td>0.52 ± 0.01</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5.3 ± 0.2</td>
<td>21.9 ± 1.1</td>
<td>0.54 ± 0.02</td>
<td>0.76 ± 0.02</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals.
**TABLE 2.11**

Effect of Phenobarbitone and 3-methylcholantherene on Hepatic Microsomal Drug-metabolizing Parameters in the Rat

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Phenobarbitone (75 mg/kg)</th>
<th>3-methylcholantherene (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver wt., Body wt. (X100)</td>
<td>4.9 ± 0.2</td>
<td>6.0 ± 0.2**</td>
<td>5.6 ± 0.2***</td>
</tr>
<tr>
<td>Microsomal protein (mg/gliver)</td>
<td>26.0 ± 1.4</td>
<td>29.4 ± 1.0</td>
<td>24.3 ± 0.6</td>
</tr>
<tr>
<td>Cytochrome b5 (nmol/mg protein)</td>
<td>0.54 ± 0.01</td>
<td>0.54 ± 0.02</td>
<td>0.67 ± 0.04**</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol/mg protein)</td>
<td>0.58 ± 0.04</td>
<td>1.26 ± 0.15***</td>
<td>0.98 ± 0.13*</td>
</tr>
<tr>
<td>NADPH2 Cytochrome c reductase</td>
<td>9.4 ± 0.9</td>
<td>12.2 ± 1.4*</td>
<td>8.1 ± 0.4*</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>0.12 ± 0.02</td>
<td>0.16 ± 0.04</td>
<td>0.88 ± 0.04**</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>0.68 ± 0.03</td>
<td>1.86 ± 0.08****</td>
<td>1.79 ± 0.06****</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>1.67 ± 0.02</td>
<td>1.99 ± 0.06</td>
<td>1.39 ± 0.03****</td>
</tr>
<tr>
<td>Ethoxyresorufin O-deethylase</td>
<td>0.13 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>12.3 ± 1.1****</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min where units are not indicated.

Value significantly different from control; *P<0.05, **P<0.02, ***P<0.01, ****P<0.001
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>2-hydroxylation of Biphenyl</th>
<th>4-hydroxylation of Biphenyl</th>
<th>O-deethylation of Ethoxyresorufin</th>
<th>N-demethylation of Ethylmorphine</th>
<th>NADPH_red of Cytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.13 ± 0.02</td>
<td>0.63 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>1.74 ± 0.05</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>3-methylcholanthrene</td>
<td>20</td>
<td>0.90 ± 0.03***</td>
<td>1.72 ± 0.08***</td>
<td>10.1 ± 0.40***</td>
<td>1.36 ± 0.04*</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>+ Propranolol</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methylcholanthrene</td>
<td>20</td>
<td>0.91 ± 0.05***</td>
<td>1.60 ± 0.11***</td>
<td>10.5 ± 0.20***</td>
<td>1.57 ± 0.04</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>+ Propranolol</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td>1.56 ± 0.05</td>
<td>6.9 ± 0.3</td>
</tr>
</tbody>
</table>

Propranolol given orally 1 hour after administration (i.p.) of 3-methylcholanthrene for 3 consecutive days

Results are Mean ± SEM for four animals expressed as nmol product/mg protein/min.

Value significantly different from control; *P<0.05, **P<0.01, ***P<0.001
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Liver wt./body wt. (× 100)</th>
<th>Microsomal protein (mg/g liver)</th>
<th>Cytochrome b$_5$ (nmol/mg protein)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>4.6 ± 0.2</td>
<td>18.9 ± 0.8</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>3-methylcholanthrene</td>
<td>20</td>
<td>5.8 ± 0.1**</td>
<td>18.9 ± 0.7</td>
<td>0.62 ± 0.03**</td>
</tr>
<tr>
<td>3-methylcholanthrene + Propranolol</td>
<td>20</td>
<td>5.8 ± 0.1**</td>
<td>19.2 ± 0.5</td>
<td>0.58 ± 0.02**</td>
</tr>
<tr>
<td>Propranolol</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methylcholanthrene + Propranolol</td>
<td>20</td>
<td>5.4 ± 0.2*</td>
<td>2.10 ± 0.5</td>
<td>0.61 ± 0.02**</td>
</tr>
</tbody>
</table>

Propranolol given orally 1 hour after administration (i.p.) of 3-methylcholanthrene for 3 consecutive days
Results are Mean ± SEM for four animals
Value significantly different from control; *P<0.02, **P<0.01, ***P<0.001
DISCUSSION

Long-term animal carcinogenicity studies with beta-adrenergic blocking agents such as pronethalol, propranolol, alprenolol, practolol, pamatolol and talomolol, have demonstrated their potential as tumour promoters although most results have been equivocal necessitating repeat studies. (Paget, 1963; Alcock and Bond, 1964; Howe, 1965; Smith and Butler, 1978; Boyd and Martin, 1977; FDA report, Drug Bulletin, 1978; Newberne et al, 1977). It has been suggested that this whole class of drugs may be potential tumour promoters because of their probable effects on cellular adenylate and guanylate cyclase and cyclic nucleotide levels.

Short-term studies of the effects of some beta-adrenergic blocking agents namely; acebutolol, atenolol, practolol, pronethalol and propranolol, on rat hepatic endoplasmic reticulum as potential epigenetic carcinogens have been investigated as measured by their effects in vivo on the in vitro rat hepatic microsomal mixed-function oxygenase system and other drug-metabolizing parameters.

The absence of any significant changes in liver wt./body wt., microsomal protein, cytochromes b5 and P-450 contents, NADPH2 cytochrome c reductase and ethylmorphine N-demethylase enzyme activities by any of the beta-adrenergic blocking agents studied, suggests that none of these agents is a potent inducer of rat cytochrome P-450-mediated microsomal mixed-function oxygenase system.

It appears that atenolol, practolol and acebutolol by their non-stimulatory effect on biphenyl 2-hydroxylase at any dose level studied behaved, at very high dosage, in a manner characteristic of phenobarbitone which does not significantly induce the formation of cytochrome P-448 known to catalyse both
the 2-and 4-hydroxylation of biphenyl (Bridges et al, 1973; Atlas and Nebert, 1976). However, the enhancement of biphenyl 2- and 4-hydroxylase activities by propranolol and to some extent pronethalol, at the highest dose level of 150mg/kg, suggest their induction of cytochrome P-448 formation. This is further supported by the apparent dose-dependent stimulation of the enzyme ethoxyresorufin O-deethylase by propranolol and pronethalol which is also catalysed by cytochrome P-448 (Burke et al, 1977; Burke and Mayer, 1975). However, the stimulation of this enzyme by acebutolol at the highest dose level of 150 mg/kg suggests the probable formation of cytochrome P-448 although this did not significantly affect the stimulation of biphenyl 2-hydroxylase. These findings suggest that propranolol and pronethalol are inducers of the rat hepatic cytochrome P-448-mediated microsomal mixed-function oxygenase system. At the normal human dosage (5mg/kg) however, none of the beta-adrenergic blocking agents significantly enhanced the hepatic microsomal mixed-function oxygenases activities, although it should be borne in mind that rates of oxidative metabolism of drugs and other xenobiotics in animals are many times higher than in man (Parke and Ioannides, 1980).

The observation that atenolol and practolol and to some extent acebutolol did not significantly affect both cytochrome P-450 and P-448-mediated hepatic microsomal mixed-function oxygenases activity suggests that their metabolic effects may not significantly affect their own rates of metabolism or of other chemicals such as food additives generally present in human diets. Hence they may not be involved in drug-interactions as inducers or inhibitors of the microsomal mixed-function oxygenase system as has been suggested for some cardiovascular agents (Data et al, 1976; Kiorbe 1966; Kutt et al, 1968; Garrettson et al, 1969; Soda and Levy, 1975; Lumholtz et al, 1975). However, pronethalol and propranolol by their induction of hepatic microsomal cytochrome P-448-mediated mixed-function oxygenase system may affect their
own rates and modes of metabolism as well as other chemicals leading to possible drug interactions and the formation of products with probable tumourigenic potentials.

The effects of beta-adrenergic blocking agents on ethoxyresorufin O-deethylase, biphenyl 2- and 4-hydroxylase activities appear to relate to their rates of gastrointestinal absorption, modes of metabolism and clearance which are a reflection of their polarities and hence lipid-solubilities. The more lipid-soluble beta-adrenergic blocking agents; pronethalol, propranolol and to some extent acebutolol, are mainly metabolized and eliminated by hepatic mechanisms (Bond and Howe, 1967; Kiechel and Meier, 1978) and enhance significantly these enzyme activities, whereas the less lipid-soluble ones; practolol and atenolol which are only slightly metabolized by the liver and eliminated by renal mechanisms (Kiechel and Meier, 1978) do not significantly affect these enzyme activities. These degrees of enhancements caused by the beta-adrenergic blocking agents are in the order, propranolol > pronethalol > acebutolol > atenolol and practolol (no induction) and are a direct reflection of their degrees of hepatic metabolism. The marked enhancement of biphenyl 2- and 4-hydroxylase activities by propranolol as opposed to pronethalol at the highest dose level of 150 mg/kg may probably be explained by its extensive hepatic "first-pass" effect (Howe, 1965; Bond and Howe, 1967; Kiechel and Meier, 1978).

The action of pronethalol and propranolol on cytochrome P-448 mediated biphenyl 2- and 4-hydroxylase as well as ethoxyresorufin O-deethylase activities show similar characteristics as suggested for epigenetic carcinogens such as saccharin and DDT, amongst others, which stimulate these enzymes to about the same extents [Tong, 1979; Tong, 1979 (unpublished results)]. The mutagenic carcinogen, 3-methylcholanthrene,
by its enhancement of these enzymic activities many orders of magnitude higher than those shown by propranolol and pronethalol suggests that it may be acting through a mechanism different from these beta-adrenergic blocking agents at the cellular level as at the genetic level. This is reflected in the augmentation of the activities of some hepatic microsomal mixed-function oxygenases on combined administration of propranolol and 3-methylcholanthrene over those caused by 3-methylcholanthrene alone as well as differences in their individual effects.

It may be inferred from these findings that propranolol and pronethalol, if administered at prolonged high dosage, may act as potential tumour promoters as has been observed in long-term animal studies with pronethalol (Paget, 1963; Alcock and Bond, 1964; Howe, 1965) and propranolol (Smith and Butler, 1978; Boyd and Martin, 1977), and may be involved in drug interactions by their induction of cytochrome P-448 which may lead to adverse drug reactions including tumour formation.
CHAPTER THREE

THE MUTAGENECITY TESTING OF SOME BETA-ADRENERGIC BLOCKING AGENTS
INTRODUCTION

A close correlation has been found between chemicals with a mutagenic activity and those that cause malignant tumours in experimental animals (McCann et al., 1975; Purchase et al., 1978), suggesting a common molecular mechanism for these two processes as proposed by Brusick (1977) and shown below. For that reason, short-term tests for mutagenicity can also be applied for the prediction of carcinogenic activity.

Since the number of chemicals identified or suspected as being human carcinogens is relatively small (Tomatis et al., 1979), most studies on the validation of short-term tests for the predictive value of carcinogenic effect have involved chemicals for which the presence (or absence) of a carcinogenic effect has been proved in laboratory animals (McCann et al., 1975; Purchase et al., 1978; Rosenkranz and Poirier, 1979; Simmon, 1979; Sugimura et al., 1976).

As increasing numbers of short-term assays are now available (Hollstein
et al., 1979), it seems important to define their reliability, and hence their sensitivity (the capacity to identify carcinogens), specificity (the power to discriminate between carcinogens and non-carcinogens) and predictive value have been proposed (Cooper et al., 1979; Malaveille, 1977; Purchase et al., 1978).

In validity evaluation reported by Bartsch et al. (1980), it was shown that the Ames test has a predictive value of 92% which confirmed an earlier finding by McCann et al. (1975). However, this value was strongly influenced by the way in which the chemicals were selected and by the proportion of carcinogens in the series of chemicals tested. Another limitation in the use of the Ames test is that it only reflects point mutations and therefore its combination with other tests that respond to other types of genetic damage is of great importance.

Recent publications (Friedman, 1977; Wild, 1980) have called attention to the value of the micronucleus test as a fast and cheap screening system for genetic hazards of environmental agents. This assay responds both to chromosome-breaking agents and to agents causing non-disjunction of chromosomes (Jenssen and Ramel, 1980; Maier and Schmid, 1975; Seiler, 1976). Furthermore it reflects the situation in vivo in mammals and can also be applied for investigations in man (Goetz et al., 1975, 1976; Jenssen and Huttel, 1976; Krough-Jensen, 1977).

The correlation between the micronucleus test and corresponding cancer data have been analysed to evaluate its usefulness as an economical short-term assay for the detection of carcinogens (Jenssen and Ramel, 1980).

A comparison of the micronucleus and Ames tests shows that they have an average specificity of about 80% and predictive value of about 90% and there
was significant difference in sensitivity in favour of Ames test. This is reflected in the data on evaluation of the Ames and micronucleus mutagenicity tests in the prediction of chemical carcinogens shown below.

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Definition</th>
<th>Ames test (%)</th>
<th>Micronucleus test (%)</th>
<th>Ames and micronucleus tests combined (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>$C^+M^+/C^+M^+ + C^-M^-$</td>
<td>80</td>
<td>58</td>
<td>86</td>
</tr>
<tr>
<td>Specificity</td>
<td>$C^-M^- / C^-M^- + C^-M^-$</td>
<td>82</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td>Predictive value</td>
<td>$C^+M^+/C^+M^+ + C^-M^+$</td>
<td>92</td>
<td>89</td>
<td>90</td>
</tr>
<tr>
<td>Proportion of carcinogens</td>
<td>$C^+M^+ + C^-M^- / Total$</td>
<td>71</td>
<td>73</td>
<td>72</td>
</tr>
</tbody>
</table>

$C^+M^+$ - Nos. of mutagenic carcinogens  
$C^-M^-$ - Nos. of non-mutagenic non-carcinogens  
$C^-M^+$ - Nos. of mutagenic carcinogens

After Jenssen and Ramel (1980)

The metabolizing system (S-9mix) used in the Ames test is an artificial system as compared with the situation in vivo for the micronucleus test (Greim, 1980). Since the co-factors for the deactivating enzymes tend not to be present in the S-9 fraction, mainly the activation part of the metabolism takes place in vitro, which might lead to an overestimation or false positive results in the Ames test. Besides the mutagenicity potency of a chemical in the Ames test may be influenced by at least fourteen major factors; from choice of bacterial strain to definition of counting colonies (Ashby and Styles, 1978).

However, of the compounds which give positive results in the Ames test, 52% of those requiring metabolic activation (S-9 activation) were positive in the micronucleus test and those that were directly mutagenic (not requiring metabolic activation) in the salmonella test, 63% were positive in the micronucleus test. The disparity (11%) may be explained by differences in metabolic...
capacities of the two test systems.

The sensitivity in the micronucleus test depends on the number of polychromatic cells counted as well as the criterion for a positive effect. In a study by Heddle and Bruce (1977) only a thousand cells/mouse were counted in three mice. Furthermore their criterion for a positive effect usually meant a 4-5 fold increase of the spontaneous level of micronuclei as compared to 2-3 fold increase in the Ames test. A count of four thousand cells per mouse led to a doubling effect which can be verified statistically.

Although long-term animal carcinogenicity studies have been reported for most beta-adrenergic blocking agents (Paget, 1963; Alcock and Bond, 1964; Howe, 1965; Smith and Butler, 1978; Boyd and Martin, 1977; Newberne et al, 1977; Wickel and Kelly, 1979; Sibley, 1978; FDA report, Drug Bulletin, 1978), there are no available data on their short-term mutagenicity studies. It was therefore of great interest to employ the Ames and micronucleus tests to screen a number of this class of drugs for their mutagenicity potentials.
EXPERIMENTAL

AMES TEST

Growing and Storage of Bacterial Test Strains

The bacterial strains were kept in frozen nutrient broth cultures in aliquots of 2ml at -70°C with 8.0% dimethylsulphoxide (DMSO) in 10ml sterile glass screw-capped vials with rubber-lined screw caps. Duplicate frozen cultures of each tester strain was prepared. One was stored as a master copy and was only used when there was need to regenerate frozen stocks. The other was used routinely to obtain fresh cultures from mutagenesis testing by scraping a sterile hypodermic needle over the surface of the frozen culture and inoculating 5ml of nutrient broth. This was incubated overnight for a maximum of 16 hours in a shaking water incubator at 37°C.

Checking out Tester Strains

All strains were tested for the presence of their mutations prior to their use in mutagenesis test:

a) The mutation in the histidine operon, basic to the test system was tested by checking for growth in the presence and absence of histidine on a minimal medium-agar base. Bacteria of the nutrient broth culture were streaked on the agar plate with/without supplementation with 0.1ml of a solution containing L-histidine and biotin in a final concentration of 0.1M and 0.5M respectively, as a growth requirement. The plates were incubated at 37°C overnight. Growth of all strains was seen only where histidine and biotin were present.

b) Sensitivity to crystal violet is a check for the presence of the deep rough (rfa) mutation, the loss of the lipopolysaccharide coat on the
bacterial surface, which permits large molecules such as polycyclic hydrocarbons to enter. A sterile filter paper disc containing crystal violet (10μl of 1mg/ml) is placed on a nutrient agar petri dish containing 0.1ml of the nutrient broth culture to be tested, in a thin overlay of agar (2ml) on the bottom minimal-agar. After 24 hours incubation at 37°C a clear zone of inhibition around the disc indicates the presence of the (rfa) mutation which permits large molecules such as crystal violet to enter the bacteria and inhibit growth.

c) Two of the tested strains (TA98 and TA100) contain a plasmid expressing resistance to ampicillin called R-factor. The R factors are somewhat unstable and can be lost from the bacteria. These factors make these strains more sensitive for detection of carcinogens not detected by TA1535 and TA1537. To check for the presence of R factor 10μl of 8mg/ml in 0.02 N NaOH of an ampicillin solution was streaked across the surface of a nutrient agar plate covered with a top agar layer (2ml). After the streak is dry, cultures to be checked are cross-streaked against the ampicillin, and after incubation for 12-24 hours at 37°C, strains which do not contain the R factor (TA1535 and TA1537) will show a zone of growth inhibition around the ampicillin streak, whereas R factor containing strains (TA98 and TA100) will not.

Checking Bacterial Growth in Broth Culture

The number of bacteria in the overnight broth culture was determined by the serial dilution technique (1:10^6). An aliquot (0.1ml) of the contents of the last dilution was added to a tube containing 2ml top agar, vortex-mixed and poured onto the bottom agar. This was incubated for 24 hours at 37°C after solidification. The presence of colonies of growth indicated that there were at least 10^8 bacteria/0.1ml of broth culture.
**Induction of Rat Liver Enzymes**

The induction procedure was similar to that used by Ames et al (1975a). Adult male Wistar albino rats weighing approximately 200g were each given an i.p. injection of a mixture of polychlorinated biphenyls, Aroclor 1254, in corn oil (200 mg/ml) at a dose of 500 mg/kg. Five days after the injection, the rats were killed by cervical dislocation and the liver homogenate (S-9 fraction) prepared as described below.

**Preparation of Liver Homogenates Fraction ("S-9" fraction)**

Basically, the procedure of Garner et al (1972) was used. All steps were at 0-4°C using cold, sterile solutions and glassware. The liver lobes not in contact with the gastrointestinal tract (GIT), to prevent liver contamination by bacteria of GIT, were placed in pre-weighed beakers containing 1.15% KCl (w/v) [approx. 1ml/g wet liver]. After weighing, the livers were transferred to a beaker containing 3 vol. of 1.15% KCl (w/v) [3ml/g wet] liver, minced with sterile scissors and homogenized in Potter-Ehlevehjem apparatus with a teflon pestle. The homogenate was centrifuged at 9,000g for 10 minutes in 8 x 25ml rotor of MSE high speed 18 centrifuge, and the supernatant (S-9 fraction) was decanted and saved. The protein concentration as determined by method of Lowry et al (1951) was found to average 40 mg/ml. The fresh S-9 fractions were distributed in 3ml portions into small plastic tube (5ml liquid nitrogen storage tubes/4-Shore U.S.A., La Jolla, California) and stored at -20°C. As required, sufficient S-9 fraction was thawed at room temperature and kept on ice; the unused portion was discarded at the end of the day.

**Materials**

Vogel-Bonner E minimal agar plates were obtained from Gibco Biocult, Paisley, Scotland. Oxoid nutrient broth and Difco-Bacto agar were obtained
from Oxoid Ltd, Hants, England and Difco Lab., Detroit, USA respectively. DMSO was spectrophotometric grade from British Drug Houses (BDH) Ltd., Poole, England.

NADP, glucose 6-phosphate and the carcinogens MNNG, 9-aminoacridine, 2-aminoanthracene and 2-nitrosofluorene were obtained from Sigma Chemical Co. Ltd., England. The beta-adrenergic blocking agents pindolol and sotalol hydrochloride were gifts from Sandoz Ltd., Leeds, England and Bristol Lab. Slough, England respectively. Oxprenolol hydrochloride and Metoprolol tartrate were obtained from CIBA-Geigy Labs., W. Essex, England. All other chemicals were obtained in the purest form available.

Preparation of Materials

Bottom agar

This was already prepared minimal-glucose agar medium called Vogel-Bonner 'E' medium and contains 2% glucose and 1.5% Difco-Bacto agar.

Top agar ("ordinary")

This was a 0.6% Difco-Bacto agar contained in 0.5% NaCl solution. The solution was autoclaved at 15lb/sq.in. pressure for 15 minutes (conditions for sterilization) in a glass dispenser. 10ml sterile 0.5mM L-histidine-0.5mM biotin were added per 250ml agar. 2ml of the resulting solution were dispensed into sterile capped plastic tubes and allowed to equilibrate at 45°C.

Top agar ("viability test")

This was 0.6% Difco-Bacto agar, contained in 0.5% NaCl solution used for dilution, ampicillin and crystal violet tests. 5ml sterile 0.1M L-histidine
-0.5M biotin were added per 100ml agar.

Nutrient broth

A preparation of a 2.5% (w/v) solution of nutrient broth in distilled water was used.

Test compounds

The compounds to be tested were prepared fresh daily in sterile DMSO or buffer. The appropriate dilutions were made from a stock of 10mg/ml so that not more than 100μl of each dilution was used to achieve a particular concentration. (A maximum of 100μl DMSO is known to have no significant effect on growth of revertant colonies). The concentrations of tests used were 1, 10, 100 and 1,000 μg/plate.

Positive controls

Stock solutions of positive carcinogen controls for the various bacterial strains were prepared in sterile universal glass tubes with sterile DMSO and a maximum volume of 100μl used to give concentrations/tube shown below in parenthesis.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Concentration (μg/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without S-9 mix</td>
</tr>
<tr>
<td>TA 98</td>
<td>2-nitrosofluorene (5.0)</td>
</tr>
<tr>
<td>TA 100</td>
<td>MNNG (2.0)</td>
</tr>
<tr>
<td>TA 1535</td>
<td>MNNG (2.0)</td>
</tr>
<tr>
<td>TA 1537</td>
<td>9-aminoacridine (40)</td>
</tr>
</tbody>
</table>

MNNG: N-methyl-N' nitro-N-nitrosoguanidine

"S-9" mixture

This was prepared fresh daily from sterile stocks of the following solutions
so that 0.5 ml contained the respective substances in the concentrations shown in the third column below.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Volume (ml)</th>
<th>Concentration/0.5 ml S-9 mix (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂ (80 mM)</td>
<td>0.05</td>
<td>4.0</td>
</tr>
<tr>
<td>KCl (0.33M)</td>
<td>0.05</td>
<td>16.5</td>
</tr>
<tr>
<td>Glucose 6-phosphate (50 mM)</td>
<td>0.05</td>
<td>2.5</td>
</tr>
<tr>
<td>NADP (40 mM)</td>
<td>0.05</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium phosphate buffer pH 7.4 (0.2M)</td>
<td>0.25</td>
<td>50.0</td>
</tr>
<tr>
<td>&quot;S-9&quot; fraction</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

**Assay System**

The assay system consisted of the following additions per tube in the order shown below.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Activation system &quot;S-9&quot; mix</th>
<th>Non-activation system &quot;S-9&quot; mix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume (ml)/tube</td>
<td></td>
</tr>
<tr>
<td>Top agar at 45°C</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Solution of test compound/vehicle/positive controls</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Bacterial suspensions</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>&quot;S-9&quot; mix</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Buffer</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The tubes were first mixed briefly, after addition of test compound/vehicle/positive control and then after addition of "S-9" mix/buffer
using a vortex mixer, and poured onto the minimal-medium agar base. The plates were incubated at 37°C for 48 hours, after solidification of top agar, in an Astell Hearson solid state constant temperature control oven. The colonies, the revertants of the wild type were counted manually using a Fisher colony counter. Each strain had a characteristic spontaneous rate.

MICRONUCLEUS TEST (M.T.)

Materials

Foetal calf serum was obtained from Gibco Biocult, Paisley, Scotland. Geimsa stain was from Fisons Ltd, Loughborough, England and Geimsa stain concentrated buffer pH6.8 was obtained from British Drug Houses (BDH) Ltd., Poole, England. Mitomycin c was obtained from Sigma Chemical Co., England.

Animals and Treatment

Adult male CFI outbred mice (6-8 weeks old) were kept in groups of four and fed ad libitum on Spratt's laboratory chow and water. They were given two i.p. injections (1ml/100g wt.) at three dose levels of test compound and an effective dose of positive control (Mitomycin c), at 24 hour interval. Control animals received equivalent amount of vehicle in which test compounds were dissolved; 0.1M citric acid/K$_2$HPO$_4$ buffer pH6.5 for atenolol, practolol and pindolol, and 0.9% NaCl (w/v) for all other compounds. The animals were killed by cervical dislocation 6 hours after administration of second dose.

Preparation of Smears

Essentially, the method of Heddle (1972) as modified by Schmid (1976) was used. The femur of the animals was removed in toto by cutting through the pelvic bones and below the knee. The bone was freed of flesh by means of
scissors and tissue paper. The knee-cap was peeled off to expose
the proximal end of the femur. The proximal end was then squeezed with
twizzers and rapidly touched many times in rows across a prewashed micro-
scope slide (washed overnight in absolute methanol and air-dried) that has
been wetted with a drop of foetal calf serum, at one end, to prevent too
many cells from sticking. The smear was produced by holding another slide
60° to the horizontal slide and moving it slightly backwards followed by
a forward movement to the other end of the slide. The smear was air-dried
and left overnight in absolute methanol to fix.

Staining and Mounting of Slides

The slides were treated as follows after removal from methanol:

Air dried
↓
Washed in buffered distilled water pH6.8
for 10 minutes
↓
Stained for 15 minutes in Geimsa stain
(1:10 of buffered distilled water)
↓
Excess stain removed by washing slides in
buffered distilled water
↓
Air-dried and underpart of slides cleaned
with methanol
↓
Cover-slips mounted with DPX

Scoring of Slides

The slides were first screened at 100X magnification for regions of
suitable technical quality. In such regions the cells are well spread
and well stained; vigorous red in mature erythrocytes and strong bluish
tint in polychromatic erythrocytes.

At higher magnification, 1000X, a thousand polychromatic erythrocytes were screened for the presence of micronuclei and the number of micronucleated polychromatic erythrocytes and not the number of micronuclei scored. As a safeguard against artifacts the number of micronucleated normocytes in the fields containing the thousand polychromatic erythrocytes was registered.
RESULTS

**Ames Test for Beta-adrenergic Blocking Agents**

None of the beta-adrenergic blocking agents at any concentration studied showed any marked increases in the number of His\(^+\) revertant colonies/plate over and above the spontaneous reversion level in the presence or absence of an activation system (S-9 mix). However, oxprenolol and propranolol at the highest concentration of 1,000 µg/plate showed marked bacteriocidal effects on the strains TA98, TA100, TA1537 and TA100, TA1537 respectively in the absence than presence of an activation system. Positive controls however showed marked increases in the number of His\(^+\) revertant colonies/plate with all four strains used (Tables 3.1 - 3.9).

**Micronucleus Test for Beta-adrenergic Blocking Agents**

In the micronucleus test, results show that none of the beta-adrenergic blocking agents with the exception of oxprenolol and propranolol, at any dose level studied, gave rise to marked increases in the number of micronuclei/1000 polychromatic erythrocytes over and above the spontaneous control levels. Oxprenolol appeared to show a dose-dependent increase in the number of micronuclei with a 3-fold increase at the highest dose level of 100 mg/kg. Propranolol on the otherhand did not show any dose-dependent increase in the number of micronuclei, but at the highest dose level of 60 mg/kg gave rise to a 2-fold increase. The positive control compound, mitomycin c, gave rise to a 5-7 fold increase in the number of micronuclei at the dose level of 3.5 mg/kg (Tables 3.10a-3.10c).
### Table 3.1 Ames' Mutagenicity Test for Acebutolol

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>His⁺ Revertant Colonies/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strain TA98 TA100 TA1535 TA1537</td>
</tr>
<tr>
<td>Spontaneous reversion (Buffer)</td>
<td>0</td>
<td>+</td>
<td>41  196  31  19</td>
</tr>
<tr>
<td>Acebutolol hydrochloride</td>
<td>1</td>
<td>+</td>
<td>42  196  20  17</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td>34  171  22  15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>46  190  29  16</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td>40  153  18  17</td>
</tr>
<tr>
<td>Positive control</td>
<td>+</td>
<td></td>
<td>888 2217 1417 2003</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>1553 3144 1868 1188</td>
</tr>
</tbody>
</table>

Results are Mean of triplicate determinations
Table 3.2 Ames' Mutagenicity Test for Atenolol

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>His⁺ Revertant Colonies/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strain TA98</td>
</tr>
<tr>
<td>Spontaneous reversion (DMSO)</td>
<td>0</td>
<td>+</td>
<td>41 159</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>26 208</td>
</tr>
<tr>
<td>Atenolol (Tenormin &quot;free base&quot;)</td>
<td>1</td>
<td>+</td>
<td>41 168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>31 203</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td>51 187</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>22 157</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>42 191</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>22 179</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td>40 195</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>23 214</td>
</tr>
<tr>
<td>Positive control</td>
<td>+</td>
<td></td>
<td>888 1866</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>1553 2745</td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations
## Table 3.3 Ames' Mutagenicity Test for Practolol

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>Strain</th>
<th>TA98</th>
<th>TA100</th>
<th>TA1535</th>
<th>TA1537</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous reversion (DMSO)</td>
<td>0</td>
<td>+</td>
<td></td>
<td>40</td>
<td>186</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>Practolol</td>
<td>1</td>
<td>+</td>
<td></td>
<td>49</td>
<td>185</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td></td>
<td>45</td>
<td>177</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td></td>
<td>43</td>
<td>188</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td></td>
<td>47</td>
<td>161</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>Positive control</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>888</td>
<td>2721</td>
<td>897</td>
<td>841</td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations
<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>Strain</th>
<th>TA98</th>
<th>TA100</th>
<th>TA1535</th>
<th>TA1537</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous reversion (DMSO)</td>
<td>0</td>
<td>+</td>
<td></td>
<td>61</td>
<td>195</td>
<td>43</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>30</td>
<td>159</td>
<td>62</td>
<td>14</td>
</tr>
<tr>
<td>Pindolol (&quot;free base&quot;)</td>
<td>1</td>
<td>+</td>
<td></td>
<td>65</td>
<td>180</td>
<td>49</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>31</td>
<td>190</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td></td>
<td>59</td>
<td>180</td>
<td>42</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>37</td>
<td>245</td>
<td>55</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td></td>
<td>48</td>
<td>170</td>
<td>46</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>35</td>
<td>254</td>
<td>45</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td></td>
<td>62</td>
<td>219</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>33</td>
<td>256</td>
<td>77</td>
<td>13</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>+</td>
<td></td>
<td>4155</td>
<td>2683</td>
<td>480</td>
<td>365</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td>1377</td>
<td>2051</td>
<td>1807</td>
<td>1690</td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>His&lt;sup&gt;+&lt;/sup&gt; Revertant Colonies/Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strain TA98</td>
</tr>
<tr>
<td>Spontaneous reversion</td>
<td>0</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>(Buffer)</td>
<td></td>
<td>-</td>
<td>24 *265</td>
</tr>
<tr>
<td>Metoprolol tartrate</td>
<td>1</td>
<td>+</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>+</td>
<td>1503</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>1007</td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations

* Unusually high spontaneous reversion value coincided with the shaving of mice treated with benzo(a) pyrene on the skin.
Table 3.6  Ames' Mutagenicity Test for Oxprenolol

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>His* Revertant Colonies/Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strain TA98 TA100 TA1535 TA1537</td>
</tr>
<tr>
<td>Spontaneous reversion</td>
<td>0</td>
<td>+</td>
<td>*200 *388 41 13</td>
</tr>
<tr>
<td>(Buffer)</td>
<td></td>
<td></td>
<td>*145 *332 55 12</td>
</tr>
<tr>
<td>Oxprenolol hydrochloride</td>
<td>1</td>
<td>+</td>
<td>188 436 44 10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>116 301 45 8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>192 429 31 11</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td></td>
<td>135 292 51 7</td>
</tr>
<tr>
<td>Positive control</td>
<td>+</td>
<td></td>
<td>175 348 44 10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>86 294 57 7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>120 192 44 10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>69 175 44 4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>1967 2065 769 142</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>1737 3629 975 409</td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations

* Unusually high spontaneous reversion values coincided with the shaving of mice treated with benzo(a) pyrene on the skin.
Table 3.7 Ames' Mutagenicity Test for Sotalol

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (μg/plate)</th>
<th>S-9</th>
<th>Strain TA98</th>
<th>TA100</th>
<th>TA1535</th>
<th>TA1537</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sotalol hydrochloride</td>
<td>1</td>
<td>+</td>
<td><strong>163</strong></td>
<td><strong>75</strong></td>
<td><strong>36</strong></td>
<td><strong>9</strong></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td><strong>171</strong></td>
<td><strong>48</strong></td>
<td><strong>31</strong></td>
<td><strong>8</strong></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td><strong>202</strong></td>
<td><strong>51</strong></td>
<td><strong>32</strong></td>
<td><strong>8</strong></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td><strong>196</strong></td>
<td><strong>54</strong></td>
<td><strong>52</strong></td>
<td><strong>15</strong></td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>+</td>
<td><strong>1944</strong></td>
<td><strong>845</strong></td>
<td><strong>715</strong></td>
<td><strong>150</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td><strong>1103</strong></td>
<td><strong>2209</strong></td>
<td><strong>1115</strong></td>
<td><strong>1163</strong></td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations

* Unusually high spontaneous reversion value coincided with the shaving of mice treated with benzo(a) pyrene on the skin.
Table 3.8 Ames' Mutagenicity Test for Pronethalol

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>His* Revertant Colonies/Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strain TA98 TA100 TA1535 TA1537</td>
</tr>
<tr>
<td>Spontaneous reversion</td>
<td>0</td>
<td>+</td>
<td>60 182 42 4</td>
</tr>
<tr>
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<td>-</td>
<td>72 242 36 6</td>
</tr>
<tr>
<td>Pronethalol hydrochloride</td>
<td>1</td>
<td>+</td>
<td>49 160 28 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>56 239 33 7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td>55 161 25 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>59 168 28 9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>57 156 32 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>53 251 36 8</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>+</td>
<td>45 132 50 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
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<td>+</td>
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</tr>
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<td></td>
<td>-</td>
<td>2152 2608 3448 1267</td>
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</table>

Results are Means of triplicate determinations
**Table 3.9  Ames’ Mutagenicity Test for Propranolol**

<table>
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<tr>
<th>Agent</th>
<th>Concentration (µg/plate)</th>
<th>S-9</th>
<th>Strain TA98</th>
<th>TA100</th>
<th>TA1535</th>
<th>TA1537</th>
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<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
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<td>211</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>+</td>
<td>48</td>
<td>211</td>
<td>40</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>-</td>
<td>31</td>
<td>165</td>
<td>34</td>
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<tr>
<td>Positive control</td>
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<td>888</td>
<td>2715</td>
<td>681</td>
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<td></td>
<td></td>
<td>-</td>
<td>1553</td>
<td>2519</td>
<td>2241</td>
<td>449</td>
</tr>
</tbody>
</table>

Results are Means of triplicate determinations
### Micronucleus Test for some Beta-adrenergic Blocking Agents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Nos. of micronucleated polychromatic erythrocytes/1000 polychromatic erythrocytes counted</th>
<th>Nos. of micronucleated normocytes/1000 polychromatic erythrocytes counted in the same field</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Acebutolol</td>
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<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9</td>
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</tr>
<tr>
<td></td>
<td>150</td>
<td>10</td>
<td>&quot;</td>
</tr>
<tr>
<td>Oxprenolol</td>
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<td>&quot;</td>
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<td></td>
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<td>&quot;</td>
</tr>
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<td></td>
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<td>&quot;</td>
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<td>Sotalol</td>
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<tr>
<td></td>
<td>100</td>
<td>7</td>
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</tr>
<tr>
<td></td>
<td>150</td>
<td>7</td>
<td>&quot;</td>
</tr>
<tr>
<td>Metoprolol</td>
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<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>150</td>
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Results are Means for four animals
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Nos of micronucleated polychromatic erythrocytes/1000 polychromatic erythrocytes counted</th>
<th>Nos. of micronucleated normocytes/1000 polychromatic erythrocytes counted in the same field</th>
</tr>
</thead>
<tbody>
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<td>None</td>
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<td>*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>4</td>
<td>*</td>
</tr>
<tr>
<td>Practolol</td>
<td>50</td>
<td>3</td>
<td>*</td>
</tr>
<tr>
<td></td>
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<td>5</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>Positive control</td>
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<td>25</td>
<td>*</td>
</tr>
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<td>Control</td>
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<td>5</td>
<td>None</td>
</tr>
<tr>
<td>Propranolol</td>
<td>20</td>
<td>8</td>
<td>*</td>
</tr>
<tr>
<td></td>
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<td>13</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>14</td>
<td>*</td>
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<td>Pronethalol</td>
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<td>*</td>
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<tr>
<td></td>
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<td></td>
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<tr>
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Results are Means for four animals
Table 3.10c Micronucleus Test for some Beta-adrenergic Blocking Agents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Nos of micronucleated polychromatic erythrocytes/1000 polychromatic erythrocytes counted</th>
<th>Nos of micronucleated normocytes/1000 polychromatic erythrocytes counted in the same field</th>
</tr>
</thead>
<tbody>
<tr>
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<td>None</td>
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<td>Pindolol</td>
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<td>n</td>
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<td></td>
<td>50</td>
<td>6</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- (Died)</td>
<td>n</td>
</tr>
<tr>
<td>Positive control</td>
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</table>

Results are Means for four animals
DISCUSSION

Many short-term tests have been developed for the screening of carcinogens as mutagens but by far the most rapid and economical methods are the Ames' bacterial and mammalian micronucleus tests which have been used in this study.

The absence of positive effects in the Ames test, (2-3 fold and dose-dependent increases in His\(^+\) revertant colonies) by any of the beta-adrenergic blocking agents, in the presence or absence of S-9 mix, suggests that neither the parent compounds nor their hepatic metabolites (produced by S-9 mix in vitro) caused any changes in the genetic structure of the Salmonella typhi-murium strains either by frame-shift or base-substitution mutations. The bacteriocidal action on strains TA100 and TA1537 by propranolol and strains TA98, TA100 and TA1537 by oxprenolol at concentration of 1000µg/plate, in the absence of S-9 mix and its absence in the presence of S-9 mix, suggests that oxprenolol and propranolol are metabolized to products non-toxic to the bacterial strains. Some beta-adrenergic blocking agents undergo gastrointestinal metabolism (Meier, 1978) and so the use of gastrointestinal mucosal homogenate would be of interest, but the problem of obtaining a sterile and active homogenate is enormous in view of the large numbers of bacteria present in the gastrointestinal tract.

The fact that there was no marked increases in the number of micronuclei above the spontaneous level by seven out of nine of the beta-adrenergic blocking agents studied as compared to the positive control, mitomycin c, indicates that neither the parent compounds nor their metabolites had any effect on the bone-marrow cells in the mouse. The increases in the numbers of micronuclei above the spontaneous level for propranolol (2-fold) and oxprenolol
At the highest dose levels suggest possible chromosomal damage to the bone-marrow cells by either the parent drugs or their metabolic products. However, these are below the 4-5 fold increase suggested by Heddle and Bruce (1977) to describe a positive effect in the micronucleus test.

The bacteriocidal action of propranolol and oxprenolol on the His$^+$ revertant colonies in the absence of 5-9 mix in the Ames test and their increases in the number of micronuclei above the spontaneous level in the micronucleus test, suggests that their action is at the chromosomal level. This indicates the possibility of the chromosomal damage being caused by the parent compounds rather than their metabolic products. However, the epoxidation of the allyloxy side-group of oxprenolol in the mice in vivo, leading to the production of reactive metabolites cannot be overruled. The combined result of Ames and micronucleus tests, for all the beta-adrenergic blocking agents, confirms the suggestion that the two tests complement each other and together provide a more sensitive short-term test system of higher predictive value as suggested by Jenssen and Ramel (1980).

These results suggest that none of the beta-adrenergic blocking agents studied, with the possible exception of oxprenolol and propranolol, may show properties characteristic of chemical mutagens although the levels of the effect of oxprenolol and propranolol on the bone-marrow cells in mice, at the chromosomal level, are far below that shown by known chemical mutagens such as mitomycin c. However, it must be borne in mind that, the dosage of mitomycin c used in this study is far below its sub-acute level which is the level represented by the highest dose levels of the beta-adrenergic blocking agents used.
CHAPTER FOUR

EFFECT OF SOME BETA-ADRENERGIC BLOCKING AGENTS ON RAT GASTROINTESTINAL MUCUS GLYCOPROTEIN SYNTHESIS
Gastrointestinal Mucus

Gastrointestinal mucus is secreted by the cells of the surface epithelium and those lining the crypts of the mucosa and pyloric glands. It is highly alkaline and viscous, covering the surface of the mucosa with a tenacious layer about 1mm thick.

It is firmly established that the molecules responsible for the viscous and gel-forming properties of mucus are glycoproteins (Spiro, 1970; Allen et al, 1976). Mucus glycoproteins are characterized by a high carbohydrate to protein ratio, the carbohydrate constituting usually more than 65% of the dry weight (Schrager and Oates, 1971; Allen and Snary, 1972; Jabbal et al, 1976; Gold and Miller, 1974; Marshall and Allen, 1977). This distinguishes them from many other glycoprotein secretions in the gastrointestinal mucus glycoproteins.

Mucus glycoproteins from all regions of the gastrointestinal tract have a very high molecular weight and consists of a large number of carbohydrate side chains attached to a protein core. The carbohydrate chains may contain up to five different monosaccharides; namely galactose, fucose, N-acetyl-glucosamine, N-acetylgalactosamine, and sialic acid, distinguished as either N-acetyl- or N-glycolyll-neuraminic acid. Trace quantities only of mannose and glucose have been reported for purified gastrointestinal mucus glycoproteins. The size of carbohydrate side chains and relative amounts of the different sugars are very variable in different gastrointestinal mucus glycoproteins (Schrager and Oates, 1971; Allen and Snary, 1972; Jabbal et al, 1976; Gold and Miller, 1974; Marshall and Allen, 1977).

The sequence of the sugar residues and the type of glycosidic linkage between
them are determined genetically by the expression of specific glycosyltransferase enzymes which catalyse the step-wise addition of sugar residues to the growing chain during glycoprotein biosynthesis (Watkins, 1972).

Sialic acid and ester sulphate, often together, are commonly found in glycoproteins from gastrointestinal mucus. Sialic acid residues are always terminal whereas ester sulphates residues occur in a more internal position within the sugar chains of the glycoprotein. Both sialic acid and ester sulphate have pKa values of less than 3. Thus any terminal N-acetyllneuraminic acid residue will mean a strongly negatively charged molecule. The function of this negatively charged residue is not entirely clear; although at least in some conditions, it is important in determining the tertiary structure of the glycoprotein. At low ionic strengths, when there is little charge shielding by counter ions, there will be increased repulsion between these negatively charged residues on adjacent carbohydrate chains resulting in an expansion of the tertiary structure of the molecule and a rise in its viscous properties. This is a feature of pig gastric mucus and rat intestinal mucus glycoproteins (Forstner et al, 1973a).

The linkage of the carbohydrate side chains to the central protein core is the same for all glycoproteins from gastrointestinal mucus. N-acetylgalactosamine is linked from the carbon at position one (the potential serine reducing group) to the hydroxyl groups of either/or threonine in the protein chain. This is reflected in the threonine and serine content of these gastrointestinal mucus glycoproteins which is considerably higher than other proteins (Schrager and Oates, 1971; Scawen and Allen, 1977; Jabbal et al, 1976).

A high proline content is also found in these gastrointestinal mucus glycoproteins, with smaller amounts of other amino acids, notably alanine, glycine, glutamic and aspartic acids (Schrager and Oates, 1971; Scawen and
Allen, 1977; Jabbal et al, 1976). Presumably, this large number of proline residues in the protein gives the necessary conformation for the sort of close packing of carbohydrate chains found in the glycoproteins.

**Biosynthesis of Glycoprotein**

It has been concluded from studies using classical inhibitors of protein synthesis (Spiro and Spiro, 1966) and from the autoradiographical demonstration of localization of growing polypeptide chains around the polysomes (Neutra and Leblond, 1966) that biosynthesis of the protein fraction of the glycoprotein takes place before and occurs independently of carbohydrate attachment. Once incorporated into the polypeptide chains of mucus glycoproteins, the amino acids serine and threonine may be glycosylated.

The addition of the first carbohydrate unit to the peptide chains and subsequent elongation of the carbohydrate chain is affected by a group of the enzymes known as the glycosyl transferases. These enzymes catalyse the transfer of sugar residues from sugar nucleotide donors (UDP-galactose, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, CMP-N-acetylgalactosamine, CMP-N-acetylneuraminic acid and GDP-fucose) to the polypeptide or growing carbohydrate chains as the glycoprotein moves from the rough endoplasmic reticulum and Golgi complex. Evidence exists that glycosylation may occur even before the polypeptide chain has left the ribosome (Lawford and Schachter, 1967; Louisot et al, 1967).

It has been suggested that subcellular distribution of the glycosyl transferases could play a role in the control of glycoprotein synthesis (Schachter, 1974). Thus the localization of N-acetylneuraminyl transferase in the Golgi complex could preclude N-acetylneuraminic acid from appearing in the carbohydrate chain anywhere other than in terminal positions (Schachter et al, 1970).

**The Effects of Drugs on Mucus Production**

Drugs may alter the rate of secretion of mucus, the chemical and physical
structure of mucus or its rate of synthesis. They may thus affect the normal physiological functions of mucus and its protective effect on the epithelium resulting in therapeutic benefit or adverse drug reactions. The sites of action of drugs on mucus may be central—via the hypothalamus or pituitary-adrenal axis, systemic—involving catecholamines or other hormonal control, or topical to the mucus-producing cell.

Phenylbutazone results in reduced synthesis of sulphated glycoproteins as determined by the rate of incorporation of labelled sulphate by rat gastric mucosa (Lambert et al., 1967) and aspirin produces the same effect in dogs (Levy et al., 1972). Mucus secreted by dogs after treatment with aspirin cortisone, corticotropin (ACTH) phenylbutazone or iodomethacin contained much less carbohydrate and was much more rapidly digested by pepsin than was control mucus (Menguy and Desbaillets, 1968), possibly because of the inhibitory action of these drugs on glucosamine synthetase (glucosamine phosphate isomerase) (Peery, 1968). In contrast the gastric-ulcer healing drug carbenoxolone causes the production of increased amounts of a carbohydrate-rich tenacious mucus both in human patients and experimental animals (Parke and Symons, 1977).

Most corticosteroids and anti-inflammatory drugs tend to cause gastrointestinal inflammation, associated with impaired mucus production, erosions of the mucosa and haemorrhage. These adverse effects occur in many different animal species and are produced by the following drugs in order of increasing gastrointestinal toxicity: salicylate < oxyphenbutazone < aspirin < phenylbutazone < dichlofenac < indomethacin (Ménessé-Gdynia and Drupp, 1974). The mechanism of gastrointestinal inflammations (impairment of mucus synthesis and loss of integrity of the gastric mucus barrier) has been studied most extensively with aspirin, a drug which clinical studies have
shown to be a major causative agent of gastric and duodenal ulcers (Rainsford, 1975). Dekanski et al, (1975) have shown that aspirin administered orally to rats results in significant reduction in the rate of gastric mucus synthesis, as measured by the incorporation of N-acetyl glucosamine, in those animals exhibiting gastric erosions. Aspirin has no significant effect on the individual hexose, hexosamine, fucose and sialic acid contents of glycoprotein, although there are significant differences in these carbohydrates between those aspirin-treated rats that show erosions and those that do not.

Aspirin stimulates the adenylate cyclase activity of rat gastric mucosa although the converse might have been expected (Mangla et al, 1974), but it is an inhibitor of prostaglandin synthetase and could exert its action on the gastric mucus synthesis by lowering endogenous prostaglandin activity (Rainsford, 1975). Kent and Allen (1968) demonstrated that salicylate inhibited the incorporation of $[^{14}C]$ glucose and $[^3H]$ threonine into glycoprotein synthesized by sheep colon and human stomach and Lukie and Forstner (1972) further demonstrated the inhibitory action of salicylate on mucus glycoprotein of rat small intestine by its reduction of the rates of incorporation of $[^{14}C]$ glucosamine and $[^{14}C]$ leucine.

Similar studies of the effects of indomethacin, phenylbutazone and steroid diuretic spironolactone on the synthesis of gastric mucus have been carried out in the rat and ferret (Shillingford, 1975; Dekanski et al, 1975). The rates of incorporation of both N-acetyl $[^3H]$ glucosamine and $[^{14}C]$ galactose into rat mucosal glycoprotein were inhibited by administration of each of these drugs. Aspirin and phenylbutazone also diminished the hexose content of the gastric mucus glycoprotein from which it may be inferred that these drugs inhibit the synthesis of mucus glycoprotein by impairment of glycosylation.
The drug carbenoxolone which has proved to be a useful treatment for healing gastric and duodenal ulcers has been shown to enhance the synthesis of mucus both in human patients and experimental animals (Johnston et al., 1975), and markedly enhances the rates of incorporation of a number of radioactively labelled monosaccharides into gastric mucosal glycoprotein. These effects were not seen with preparations of duodenal mucosa, however. Similar incorporation studies with $\left[^{14}\text{C}\right]$ threonine, $\left[^3\text{H}\right]$ serine, $\left[^3\text{H}\right]$ proline and $\left[^3\text{H}\right]$ asparagine have shown that carbenoxolone pretreatment of either rats or ferrets does not lead to any increased rate of amino acid incorporation into gastric glycoprotein and hence does not increase the rate of synthesis of the polypeptide precursor of the mucus glycoproteins. Carbenoxolone also increases the content of fucose and sialic acid and the activities of UDP-glucuronosyltransferase of the gastric mucosa but, again, the intestinal mucosa is unaffected by the drug. The increased fucose and sialic acid contents are indicative of increased glycoprotein and mucus synthesis. The increased UDP-glucuronosyl transferases are probably related to the mechanism of absorption of the drug from the stomach since glucuronic acid is not a normal constituent of gastric mucus, although Hietenan (1975) has shown that administration of salicylate to rats causes a marked reduction in the UDP-glucuronosyl transferase of both the gastric and duodenal mucosae.

It has consequently been suggested that the mode of action of carbenoxolone is to enhance the synthesis of gastric mucus by increasing the activities of the microsomal glycosyltransferases and thereby to increase the protection of the gastric mucosa against the corrosive actions of acid, pepsin and bile. There are, however, other suggested mechanisms of the ulcer-healing action of carbenoxolone (Avery-Jones and Parke, 1975). Amer et al., (1974) demonstrated that carbenoxolone inhibited 3' 5' cyclic AMP phosphodiesterase activity in
gastric mucosa and other tissues of the rat and other species. Peskar and Peskar (1976) have also shown that this drug inhibits gastric prostaglandin dehydrogenase and Δ-13 reductase, enzymes which deactivate the prostaglandins.

Drugs not generally associated with diseases of epithelial tissues or abnormalities of mucus secretion may nevertheless have an effect on mucus. This is particularly likely with drugs that affect cellular metabolism, such as adrenaline and other beta-adrenergic agents, beta-adrenergic blocking agents, inhibitors of adenylate cyclase or phosphodiesterase, prostaglandins and so on, and Forstner et al. (1973) have shown that beta-adrenergic agents, theophylline and dibutyryl cyclic AMP all stimulate glycoprotein synthesis in intestinal slices.

The site of mucus glycoprotein synthesis is the endoplasmic reticulum of the cell, which is also the location of enzymes concerned in drug metabolism and deactivation and in metabolic activation of carcinogens. The highly reactive metabolites of these carcinogens undoubtedly damage the endoplasmic reticulum in some way, impairing and changing the biosynthesis of mucus and other glycoproteins concerned in the characterization of the cell surface and in immune surveillance (Parke, 1977b). Such changes in glycoprotein synthesis may thus explain many of the biological anachronisms of cancer such as the rapid cell growth and division; the immune escape mechanisms and the synthesis of fetal protein. These changes in the endoplasmic reticulum may explain the observed correlations between changes in the nature of the plasma membrane glycoproteins of mucosal epithelial cells; changes in mucus synthesis and malignancy (Parke and Symons, 1977). Drugs that affect glycoprotein synthesis may, therefore, like anti-hormones eventually have a role in the treatment of epithelial cancer.

The beta-adrenergic blocking agent, practolol, has been shown to cause
ulceration of the cornea of the eye, nasal and oral mucosa (Wright, 1975; Rahi et al, 1976) as well as psoriasiform changes in the skin (Felix and Ive, 1974) in patients on long-term treatment, known as oculomucocutaneous syndrome. However, it is not known whether the practolol reaction is specific to practolol or is the direct specific effect of pharmacologically induced changes by beta-blockade (Gaylarde and Sarkany, 1975). There have been few convincing reports of the oculomucocutaneous reactions with oxprenolol (Holt and Waddington, 1975; Knapp et al, 1975) and one with propranolol (Cubey and Taylor, 1975) but these are very rare.

Recent observations in patients on long-term treatment with practolol indicates that it causes sclerosing peritonitis leading to intestinal adhesion and obstruction (Nicholls, 1976). This has led to the suggestion that practolol might be inhibiting the synthesis of gastrointestinal or peritoneal mucus which are essential for the lubrication and free movement of the gastrointestinal tract respectively.

Investigations were therefore carried out on the effects of administration of practolol and other beta-adrenergic blocking agents on the incorporation of radiolabelled sugar and amino acid precursors into rat gastrointestinal mucus glycoproteins to ascertain whether the adverse effects shown by practolol are peculiar to it or are a direct pharmacological result of beta-blockade, and thus to establish the possible mechanisms of its potential toxicity.
EXPERIMENTAL

Materials

Soluene-350 tissue solubilizer and dimilume-30 phosphor mixture were purchased from Packard Instrument Co, Reading, England.

N-Acetyl-D-[1-3H] glucosamine, D-[1-3H] fucose, [3H] and [14C] hexadecane standards and N-acetyl [4,5,6,7,8,9-14C] neuraminic acid were obtained from The Radiochemical Centre, Amersham, U.K. L-[3H] Serine was purchased from New England Nuclear, Boston, USA. All other chemicals were obtained in the purest possible forms from British Drug Houses (BDH) Chemicals Ltd, Poole, England.

Animals

Male Wistar albino rats (140-160g) were kept in cages in groups of six or less and fed ad libitum on Spratt's laboratory chow. Test animals received oral doses of the drugs (1ml/100g body wt) and controls received equivalent amount of drug vehicle, water in case of propranolol, pronethalol and acebutolol and 0.1M citric acid/Na_2HPO_4 buffer pH6.5 for atenolol and practolol. Animals were killed by cervical dislocation and stomach and intestine (15cm from stomach) removed immediately for preparation of mucosal homogenates.

Treatment of Animals

Two separate animal treatments were performed:

a) Administration of varying dose levels of drugs (5-200mg/kg/day) for 5 days and killing 15 hours after last dose (Tables 4.1-4.5, 4.10, 4.11).

b) Administration of fixed dose levels of drugs (150mg/kg/day) for 5 days and killing 5 hours after last dose. (Tables 4.6-4.10).
Mucosal Homogenate Preparation

The stomach and intestine were cut open along the greater curvature and longitudinally respectively, and the contents washed with chilled 1.15% KCl(w/v) solution. The washed stomach/intestine were kept in ice-cold 0.9% NaCl(w/v). The mucosae of the glandular area were scraped on a glass plate (25cm x 25cm) with a microscope slide and put into 10ml modified version of Krebs-Ringer bicarbonate solution (NaCl, 119mM; KCl, 5.6mM; CaCl$_2$, 3.0mM; KH$_2$PO$_4$, 1.4mM; MgSO$_4$, 1.4mM; NaHCO$_3$, 29.3mM; L-glutamine, 8.0mM). This was homogenized at 4°C in Potter-Elvehjem homogenized with teflon pestle to give a homogenous suspension.

Assay of Glycoprotein Synthesis

Duplicate samples (4ml each) of mucosal homogenate were pipetted into 10ml stoppered tubes. These were preincubated for 5 minutes at 37°C and radiolabelled precursors (10-40 μl) added (as shown), shaken and incubated for a further 1-3 hours in a shaking water incubator (100 cycles/min). The medium was gassed with 95% O$_2$: 5% CO$_2$ every 20 minutes.

The reaction was terminated with 5ml trichloroacetic acid:phosphotungstic acid 20%(w/v): 2%(w/v) and the tubes kept at 4°C overnight. The precipitate formed was spun down in a Mistral 6L centrifuge and washed twice with 5ml each of distilled water and twice with 5ml each of chloroform: methanol (1:1 v/v).

The tubes and contents were air-dried at 37°C overnight and cooled to room temperature. The glycoprotein was transferred into pre-weighed glass vials and reweighed. Distilled water (200 μl) and soluene -350 (1ml) were added. The vials were capped and allowed to stand overnight in an oven at 50°C to speed up the solubilization process.
Radiolabelled precursors and quantities used in experiments

<table>
<thead>
<tr>
<th>Radiolabelled Precursor</th>
<th>Specific activity (Ci/mmol)</th>
<th>Volume used (μl)</th>
<th>Quantity used (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl-D-[1-²H] glucosamine</td>
<td>3.0, 4.7†</td>
<td>25, 40†</td>
<td>72.5</td>
</tr>
<tr>
<td>D-[1-³H] Fucose</td>
<td>3.9</td>
<td>12.5</td>
<td>20.8</td>
</tr>
<tr>
<td>N-Acetyl-[4,5,6,7,8,9-¹⁴C] neuraminic acid</td>
<td>0.245</td>
<td>10.0</td>
<td>323.4</td>
</tr>
<tr>
<td>L-[G-³H] Serine</td>
<td>1.8</td>
<td>25.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>

† used where shown in tables

They were allowed to cool to room temperature before addition of 10ml dimilume-30 phosphor mixture and shaken for thorough mixing. The radioactivity in the glycoprotein was measured by LKB Wallac ultrabeta 2002 scintillation counter programmed for dpm calculations based on internal standard channel efficiency determination.

Experimental blanks were prepared by adding the trichloroacetic acid: phosphotungstic acid, immediately after addition of radiolabelled precursors, to mucosal homogenate.

Quench Curve Preparation

Quench curves were prepared for [¹⁴C]- and [²H]- hexadecane standards to enable direct conversion of cpm to dpm based on internal standard efficiency ratio (Fig. 4.1).
The samples for the quench curves contained the following:

- Dimilume-30 phosphor mixture - 10 ml
- Soluene-350 - 1 ml
- Water - 0.2 ml
- Chloroform as quencher - (0-0.5) ml
- $[^3H]$- or $[^{14}C]$- Hexadecane standard - 10 μl
Linearity of Incorporation of Radiolabelled Precursors into Rat Gastrointestinal Mucus Glycoprotein

The incorporation of radiolabelled precursors into rat gastrointestinal mucus glycoproteins at varying periods of incubation and concentration of radiolabelled precursors showed a linearity in respect of both the incorporation with time of N-acetylglucosamine, D-fucose and L-serine and of concentration for L-serine (Figs. 4.2 - 4.5).

Rates of Incorporation of Radiolabelled Precursors into Rat Gastrointestinal Mucus Glycoproteins After Pretreatment with Beta-adrenergic Blocking Agents

Pretreatment of rats with varying concentrations of beta-adrenergic blocking agents (50, 100, 150, 200 mg/kg) gave rise to changes in the rates of incorporation of radiolabelled precursors into gastrointestinal mucus glycoproteins. However the rate of incorporation was not significantly affected by the time of killing of animals. At the dose levels of 100, 150 and 200mg/kg, pronethalol stimulated the rate of incorporation of N-acetylglucosamine into rat gastric mucus glycoprotein whilst propranolol stimulated it at the dose levels of 150 and 200 mg/kg, without significant effects on the rates of incorporation of D-fucose, N-acetylneuraminic acid and L-serine. Acebutolol on the otherhand significantly enhanced the rate of incorporation of L-serine into gastric mucus glycoprotein at the dose level of 150mg/kg without significant effects on the rates of incorporation of N-acetylglucosamine, D-fucose and N-acetylneuraminic acid. (Tables 4.1 - 4.3, 4.6 - 4.8).

Atenolol showed some inhibition in the rate of incorporation of all the radiolabelled precursors into gastric mucus glycoprotein with the exception
of the stimulation shown for D-fucose at the dose level of 150mg/kg whilst practolol inhibited significantly the rates of incorporation of all the radiolabelled precursors at all four dose levels (50, 100, 150, 200mg/kg) into gastrointestinal mucus glycoprotein (Tables 4.4, 4.5, 4.9-4.11). However pretreatment with lower dose levels of practolol (5, 10, 20mg/kg) did not significantly alter the rates of incorporation of N-acetylglucosamine and D-fucose into gastrointestinal mucus glycoproteins (Tables 4.5, 4.10, 4.11).
Fig. 4.1 Quench Curves for $^{3}\text{H}$ and $^{14}\text{C}$ - hexadecane Standards

- [$^{14}\text{C}$] - Hexadecane Standard
- [$^{3}\text{H}$] - Hexadecane Standard

Per cent Counting Efficiency vs. Internal Standard Ratio
Fig. 4.2 Incorporation of N-acetylglucosamine into Gastric and Intestinal Mucosal Glycoproteins with Time

- intestinal mucosal glycoprotein
- gastric mucosal glycoprotein

Mole x 10^-15 N-acetylglucosamine/mg glycoprotein

Time (hrs)
Fig. 4.3 Incorporation of D-fucose into Gastric Mucosal Glycoproteins with Time
Fig. 4.4 Incorporation of L-serine into Gastrointestinal Mucosal Glycoproteins with period of Incubation

- ▲ - Gastric mucosa
- ● - Intestinal mucosa

Mole x 10^{-15} L-serine per mg glycoprotein

Time (hrs)
Fig. 4.5 Effect of Concentration on Rate of Incorporation of L-serine into Gastrointestinal Mucus Glycoproteins

- Intestinal mucus glycoprotein
- Gastric mucus glycoprotein

Concentration of radiolabelled L-serine (M x 10^-5)

Rate of Incorporation

Mole x 10^-15 L-serine/mg glycoprotein/hr
Table 4.1 Effect of Administration of Pronethalol on Rate of Incorporation of N-acetylglucosamine into Rat Gastric Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mole x 10^{-13} N-acetylglucosamine/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>10.1 ± 0.8 (6)</td>
<td>100</td>
</tr>
<tr>
<td>Pronethalol</td>
<td>50</td>
<td>11.1 ± 0.9 (4)</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15.7 ± 1.0 (6)*</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>16.5 ± 1.8 (6)*</td>
<td>163</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Value significantly different from control; *P<0.01
Table 4.2 Effect of Administration of Propranolol on Rate of Incorporation of N-acetylglucosamine into Rat Gastric Mucosal Glycoprotein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mole x 10^{-13} N-acetylglucosamine/ mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>10.5 ± 0.9 (5)</td>
<td>100</td>
</tr>
<tr>
<td>Propranolol</td>
<td>50</td>
<td>11.2 ± 1.2 (6)</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>11.0 ± 1.6 (4)</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>13.5 ± 0.5 (6)*</td>
<td>129</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Value significantly different from control; *P < 0.02
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mole x 10^{-13} N-acetylglucosamine/ mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>7.0 ± 0.7 (6)</td>
<td>100</td>
</tr>
<tr>
<td>Acebutolol</td>
<td>50</td>
<td>6.9 ± 1.0 (4)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.1 ± 0.6 (6)</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>7.7 ± 0.6 (6)</td>
<td>110</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.
Table 4.4 Effect of Administration of Atenolol on Rate of Incorporation of N-acetylglicosamine into Rat Gastric Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mole x 10^-13 N-acetylglicosamine/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>9.8 ± 1.4 (4)</td>
<td>100</td>
</tr>
<tr>
<td>Atenolol</td>
<td>50</td>
<td>7.9 ± 1.5 (3)</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.1 ± 0.9 (3)</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>8.6 ± 0.9 (3)</td>
<td>88</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.
Table 4.5  Effect of Administration of Practolol on Rate of Incorporation of N-acetylglucosamine into Rat Gastric Mucosal Glycoprotein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mole x 10^{-13} N-acetylglucosamine/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3.5 ± 0.1 (3)</td>
<td>100†</td>
</tr>
<tr>
<td>Practolol</td>
<td>5</td>
<td>3.3 ± 0.4 (3)</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.2 ± 0.2 (3)</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.5 ± 0.1 (3)</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>14.0 ± 1.2 (6)</td>
<td>100</td>
</tr>
<tr>
<td>Practolol</td>
<td>50</td>
<td>9.6 ± 0.9 (5)*</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6.5 ± 0.7 (6)***</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>8.0 ± 0.7 (6)**</td>
<td>57</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Value significantly different from control; *P<0.05, **P<0.01, ***P<0.001

Different control values are due to the use of N-acetyl-D-[1-3H] glucosamine with different specific activities (†Refer to table of radiolabelled precursors)
Table 4.6  Effect of Administration of Acebutolol on Rate of Incorporation of Radiolabelled Precursors into Rat Gastric Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Radiolabelled Precursor</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole x 10^{-15} radiolabelled precursor/ mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td></td>
</tr>
<tr>
<td>^N-Acetyl-D-[^3H]glycosamine</td>
<td>304 ± 42 (4)</td>
<td>378 ± 36 (4)</td>
</tr>
<tr>
<td>D-[^3H]Fucose</td>
<td>6.9 ± 0.7(6)</td>
<td>6.5 ± 0.5(6)</td>
</tr>
<tr>
<td>N-Acetyl[^4,5, 6,7,8,9-[^14C] neuraminic acid</td>
<td>4.5 ± 0.5(4)</td>
<td>5.0 ± 0.3(4)</td>
</tr>
<tr>
<td>L-[G-[^3H]Serine</td>
<td>16.8 ± 3.0(4)</td>
<td>26.9 ± 2.5(5)*</td>
</tr>
</tbody>
</table>

Dose: 150 mg/kg/day for 5 days

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Value significantly different from control; *P<0.05

† Refer to table of radiolabelled precursors.
Table 4.7  Effect of Administration of Propranolol on Rate of Incorporation of Radiolabelled Precursors into Rat Gastric Mucosal Glycoprotein

<table>
<thead>
<tr>
<th>Radiolabelled Precursor</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole x 10^{-15} radiolabelled precursor/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td></td>
</tr>
<tr>
<td>( ^{+}N)-Acetyl-D-[1-3H]glycosamine</td>
<td>304 ± 42 (4)</td>
<td>390 ± 60 (4)</td>
</tr>
<tr>
<td>D-[1-3H]Fucose</td>
<td>6.9 ± 0.7 (6)</td>
<td>5.8 ± 0.5 (6)</td>
</tr>
<tr>
<td>( ^{+}N)-Acetyl-[4,5,6,7,8,9-3C] neuraminic acid</td>
<td>4.5 ± 0.5 (4)</td>
<td>5.2 ± 0.6 (4)</td>
</tr>
<tr>
<td>L-[6-3H]Serine</td>
<td>16.8 ± 3.0 (4)</td>
<td>16.7 ± 1.5 (5)</td>
</tr>
</tbody>
</table>

Dose = 150mg/kg/day for 5 days

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

†Refer to table of radiolabelled precursors.
Table 4.8  Effect of Administration of Pronethalol on Rate of Incorporation of Radiolabelled Precursors into Rat Gastric Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Radiolabelled Precursor</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole x 10^{-15} radiolabelled precursor/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
</tr>
<tr>
<td>N-Acetyl-D-[1^{3}H] glucosamine</td>
<td>304 ± 42 (4)</td>
<td>425 ± 69 (4)</td>
</tr>
<tr>
<td>D-[1^{3}H] Fucose</td>
<td>6.9 ± 0.7 (6)</td>
<td>6.3 ± 0.4 (6)</td>
</tr>
<tr>
<td>N-Acetyl [4,5,6,7,8,9,^{14}C] neuraminic acid</td>
<td>4.5 ± 0.5 (4)</td>
<td>3.6 ± 0.5 (4)</td>
</tr>
<tr>
<td>L-[G-{3}H] Serine</td>
<td>19.8 ± 2.0 (4)</td>
<td>21.1 ± 1.5 (4)</td>
</tr>
</tbody>
</table>

Dose: 150 mg/kg/day for 5 days

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

†Refer to table of radiolabelled precursors.
### Table 4.9 Effect of Administration of Atenolol on Rate of Incorporation of Radiolabelled Precursors into Rat Gastric Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Radiolabeled Precursor</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole x $10^{-15}$ radiolabelled precursor/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Test</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-D-[1-3H] glucosamine</td>
<td>974 ± 141 (4)</td>
<td>880 ± 94 (4)</td>
</tr>
<tr>
<td>D- [1-3H] Fucose</td>
<td>4.5 ± 0.1 (5)</td>
<td>5.9 ± 0.4 (4)*</td>
</tr>
<tr>
<td>N-Acetyl[4,5,6,7, 8,9-14C] neuraminic acid</td>
<td>11.1 ± 2.5 (4)</td>
<td>6.4 ± 0.9 (4)</td>
</tr>
<tr>
<td>L-[G-3H] Serine</td>
<td>46.6 ± 3.9 (5)</td>
<td>37.1 ± 3.7 (5)</td>
</tr>
</tbody>
</table>

**Dose:** 150 mg/kg/day for 5 days

**Results:** Mean ± SEM of duplicate samples for number of animals given in parenthesis.

**Value significantly different from control:** *P<0.01
Table 4.10  Effect of Administration of Practolol on Rate of Incorporation of Radiolabelled Precursors Into Rat Gastric Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Radiolabelled Precursor</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mole x 10^{-15} radiolabelled precursor/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Test</td>
</tr>
<tr>
<td>N-Acetyl-D-[1-^3H] glucosamine</td>
<td>150</td>
<td>1085 ± 164 (4)</td>
<td>746 ± 57 (4)</td>
</tr>
<tr>
<td>D-[1-^3H] Fucose</td>
<td>5</td>
<td>5.2 ± 0.2(4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.9 ± 0.4(4)</td>
<td>5.5 ± 0.2(4)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.1 ± 0.4(4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>9.4 ± 1.4 (5)</td>
<td>5.7 ± 0.4(5)*</td>
</tr>
<tr>
<td>N-Acetyl [4,5,6,7,8,9-{^14C}] neuraminic acid</td>
<td>150</td>
<td>11.1 ± 2.5(4)</td>
<td>4.7 ± 0.3(4)*</td>
</tr>
<tr>
<td>L-[G-^3H]Serine</td>
<td>150</td>
<td>46.6 ± 3.9 (5)</td>
<td>29.8 ± 3.6(5)*</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Value significantly different from control; *P<0.05
Table 4.11 Effect of Administration of Practolol on Rate of Incorporation of Radiolabelled Precursors into Rat Intestinal Mucosal Glycoproteins

<table>
<thead>
<tr>
<th>Radiolabelled Precursor</th>
<th>Dose (mg/kg)</th>
<th>Glycoprotein Synthesis</th>
<th>% of control value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mole x 10^{-15} radiolabelled precursor/mg glycoprotein/hr</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-D-[1^-3H] glucosamine</td>
<td>150</td>
<td>511 ± 86 (4)</td>
<td>452 ± 51 (4)</td>
</tr>
<tr>
<td>D- [1^-3H] Fucose</td>
<td>5</td>
<td>4.6 ± 0.2(4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.0 ± 0.8(4)</td>
<td>4.9 ± 0.2(4)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.9 ± 0.3(4)</td>
<td>4.9 ± 0.3(4)</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>6.3 ± 0.9(4)</td>
<td>4.1 ± 0.3(4)</td>
</tr>
<tr>
<td>L-[6^-3H] Serine</td>
<td>150</td>
<td>4.9 ± 0.6(4)</td>
<td>3.7 ± 0.5(4)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.
DISCUSSION

Recent evidence has suggested the involvement of cyclic nucleotides in gastrointestinal function (Kimberg, 1974; Eichhorn et al, 1974). The possibility of the mediation of cyclic AMP and cyclic GMP in rat gastric mucus glycoprotein synthesis has been investigated, and Macdonald et al (1977) have shown that the inhibitory effect on glycoprotein synthesis on pre-incubation of rat gastric mucosal scrapings with dibutyryl cyclic AMP(10$^{-4}$M) in vitro was nullified at lower concentrations indicating an optimum requirement for cyclic AMP for an inhibitory effect to be shown. Beta-adrenergic blocking agents, by their probable effects on cyclic nucleotide levels may of consequence affect glycoprotein synthesis.

It appears from the results in this study that practolol and atenolol by their inhibition of glycoprotein synthesis, measured by the effects on rates of incorporation of radiolabelled amino acid and sugar precursors into rat gastrointestinal mucus glycoprotein, behaved in a way dissimilar to pronethalol, propranolol and acebutolol which stimulated the synthesis of mucus glycoproteins. These findings cannot be explained by the direct pharmacological result of beta-blockade, since if it were so all the beta-adrenergic blocking agents must act in a similar fashion. This is further supported by the fact that the expected probable decrease in cyclic nucleotide levels as a result of beta-blockade should be reflected in a probable decrease in glycoprotein synthesis (Solderling et al, 1973; Hepp, 1972) which was only shown by practolol and to some extent by atenolol but not pronethalol, acebutolol and propranolol.

The pharmacokinetic properties of these compounds which are a reflection chain of their characteristic ring structure and side if any, other than the isopropylaminopropoxy side chain (Fig 1.1) responsible for their beta-
blockade effect, may explain the observed differences in their effects on glycoprotein synthesis. Atenolol, practolol and acebutolol have similar structural characteristics so the differences in their effects on glycoprotein synthesis may be explained by differences in gastrointestinal absorption and metabolism (Kiechel and Meier, 1978) probably as a result of side chain structural differences. The stimulation in glycoprotein synthesis shown by propranolol and pronethalol may be a result of their naphthalene ring structure. However, the different degrees of their effect may be due to the extensive "first-pass" effect shown by propranolol as opposed to pronethalol (Bond and Howe, 1967; Kiechel and Meier, 1978).

The incorporation of the amino acid serine into gastrointestinal mucus glycoprotein is a measure of protein synthesis whilst the incorporation of the sugars; fucose, N-acetylglucosamine and N-acetylneuraminic acid, are a measure of glycosylation of proteins. The marked inhibition of glycoprotein synthesis by practolol may be explained in two ways. Firstly, by its inhibition of protein synthesis, less protein may be available for glycosylation suggesting that the inhibition of glycosylation is a direct result of inhibition of protein synthesis. Alternatively, it may be affecting protein synthesis and glycosylation separately. The latter may explain the observed inhibition of glycoprotein synthesis by atenolol since by its inhibitory and stimulatory effects on the rates of incorporation of serine and fucose respectively into gastric mucus glycoprotein, appears to indicate that protein synthesis is not the rate limiting step to glycosylation. The effect of propranolol and pronethalol on glycoprotein synthesis is probably at the glycosylation level without any effect on protein synthesis. However, acebutolol appears to stimulate protein synthesis without significantly affecting glycosylation.
Whereas the changes observed with pronethalol and propranolol on the rate of incorporation of N-acetylglucosamine into gastric mucus glycoprotein appears to be dose-dependent, those shown by practolol, atenolol and acebutolol are not directly dose-dependent. Acebutolol and atenolol, at the dose levels of 50, 100, 150 and 200 mg/kg did not have much significant effect on gastric mucus glycoprotein synthesis. However, the significantly marked inhibition of gastrointestinal mucus glycoprotein synthesis by practolol at these dose levels may probably be a result of a reaction in response to practolol on prolonged high dosage which appears to be in favour of the immunological basis for the adverse side-effects shown by practolol in man (Behan et al, 1976). The fact that lower dose levels of practolol (5, 10, 20 mg/kg) did not significantly inhibit the rates of incorporation of N-acetylglucosamine and D-fucose into gastrointestinal mucus glycoproteins, suggest a possible threshold dose, which is above the normal human dosage (50mg/kg), for practolol to show its inhibitory effects on glycoprotein synthesis.

Another possible cause of the inhibition of mucus glycoprotein synthesis by practolol and to some extent atenolol may be the degranulation of the mucosal cell endoplasmic reticulum as has been suggested for chemical carcinogens (Delaunay and Schapira, 1974; Parke, 1977b). However, against this background is the finding that atenolol and practolol did not enhance rat hepatic microsomal biphenyl 2-hydroxylase and ethoxyresorufin O-deethylase activities which are catalysed by cytochrome P-448 thought to be formed by chemical carcinogens probably through degranulation of the endoplasmic reticulum (Parke, 1977a; Burke and Mayer, 1975). The stimulation of mucus glycoprotein synthesis by propranolol, pronethalol and acebutolol is also at variance with earlier finding of their stimulatory effects on ethoxyresorufin O-deethylase activity in rat hepatic microsomes. These findings may be explained by the extent of gastrointestinal and
hepatic metabolism of the various compounds. Acebutolol and atenolol, by their 50% gastrointestinal absorption (Kiechel and Meier, 1978), may undergo gastrointestinal metabolism leading to products which may cause changes in the mucosal cell endoplasmic reticulum and hence changes in the mucus glycoprotein synthesis. Pronethalol and propranolol are almost completely absorbed from the gastrointestinal tract and extensively metabolized by the liver (Bond and Howe, 1967; Kiechel and Meier, 1978) to products that may stimulate mucus glycoprotein synthesis. Practolol on the other hand, does not show ready metabolism at either site (Kiechel and Meier, 1978). Hence its long plasma half-life (Bodem and Chidsey, 1973; Reeves et al, 1978) and the ability of it or its metabolites to bind irreversible to microsomal fraction (Case et al, 1978) may result in the inhibition of protein synthesis which could lead to the inhibition of glycoprotein synthesis.

These findings suggest that the inhibition of mucus glycoprotein synthesis by practolol is peculiar to this particular beta-adrenergic blocking drug, since none of the other agents studied showed similar effects, and therefore cannot be explained by probable changes in cyclic nucleotide levels as a result of direct pharmacological effect of beta-blockade. The probable mechanism of action of practolol, in causing intestinal adhesion and obstruction (Nicholls, 1976), the ulceration of the cornea of the eye, oral and nasal mucosae (Wright, 1975; Rahi et al, 1976) as well as psoriasiform changes in the skin (Felix and Ive, 1974), may be through qualitative and quantitative changes in glycoprotein synthesis, as a result of high plasma levels of the drug on prolonged high dosage.
CHAPTER FIVE

EFFECT OF SOME BETA-ADRENERGIC BLOCKING AGENTS ON RAT TISSUE ADENYLATE CYCLASE/CYCLIC AMP AND GUANYLATE CYCLASE/CYCLIC GMP LEVELS
INTRODUCTION

The properties of adenylate cyclase, the enzyme which catalyses the formation of cyclic AMP within the cell have been reported to differ in several malignant tissues (Ney et al., 1969; Brown et al., 1970; Dexter and Allen, 1971; Allen et al., 1971; Schorr and Ney, 1971; Emmelot and Bos, 1971) or cells (Nakran, 1971; Peery et al., 1971) from the activity of their normal counterparts. There are indications that the more anaplastic the cancer the lower is the activity of adenylate cyclase and the less it responds to the physiological stimulators such as hormones (Allen et al., 1971, Emmelot and Bos, 1971; Nakran, 1971). However, not all observations lend support to this scheme (Brown et al., 1970; Schorr and Ney, 1971; Peery et al., 1971) and the inter-relationships between adenylate cyclase levels and the onset of malignancy are not clear.

It has been suggested that many of the properties of malignantly transformed cells are due to low levels of cyclic 3',5' AMP (Pastan et al., 1975). When transformed isolated cells or hepatoma cells in culture are treated with cyclic AMP, the transformed cells change in appearance, resembling more closely the normal cells and grow more slowly (Pastan, 1975; Van Wijk et al., 1972). On the other hand dibutyryl cyclic AMP has been shown to increase the frequency of cell transformation by oncogenic viruses (Smith et al., 1973), and chemical carcinogenesis is associated with an increased responsiveness of adenylate cyclase to hormonal control (Boyd et al., 1974) so that a cascade effect magnifying the malignant transformation could result, especially in stressful conditions when the circulating catecholamines are high. Although the level of cyclic AMP of established tumours is always low and the hormonal responsiveness of their adenylate cyclase is not high (Boyd et al., 1974) increased levels of cyclic AMP
are characteristic of the initial malignant cell transformation. In keeping with this hypothesis is the observation that the tumour-promoting phorbol-esters, applied to mouse epidermis also produce an initial increase in cyclic AMP followed by a marked depression, then a second period of elevation (Grimm and Marks, 1974). At the same time the hormonal regulation of adenylate cyclase is largely lost, indicating that the tumour promoters have resulted in damage of the cell membrane, the site of the β-adrenergic receptors of adenylate cyclase.

Recent work has suggested that the regulation of DNA synthesis and cell division is controlled by the ratio of cyclic AMP to cyclic GMP rather than by cyclic AMP alone. An increase of DNA synthesis which accompanies a reduction of the cyclic AMP/cyclic GMP ratio, is considered to be the most important effect of the phorbol ester promoters, rather than their inhibition of DNA repair mechanisms (Trosko et al., 1975).

Guanylate cyclase, the enzyme which catalyses the conversion of GTP to cyclic GMP is found in virtually all mammalian cells (Hardman and Sutherland, 1969; White and Aurbach, 1969). A number of chemical carcinogens such as hydrazine (Vesely and Levey, 1977a,b), nitrosamines and nitrosamides (De Rubertis and Craven, 1976; Vesely et al., 1977; Claflin et al., 1978), sodium azide (Kimura et al., 1975) and butadiene diepoxide (Vesely and Levey, 1978) have been shown to stimulate guanylate cyclase activity. These findings are of significant importance in chemically induced carcinogenesis, since cyclic GMP has been associated with cell proliferation, cell growth and malignant transformation (Hadden et al., 1972; Kram and Tomkins, 1973; Vesely et al., 1976).

Recent studies have demonstrated that aromatic amines, o-halo esters, polycyclic hydrocarbons, azo dyes and aflatoxins significantly decreased
guanylate cyclase activity (Vesely et al., 1978) which is in contrast to the other carcinogens, mentioned earlier, which increased guanylate cyclase activity. Thus, among these broad groups of chemical carcinogens there appears to be divergent effects on guanylate cyclase activity and tissue levels of cyclic GMP. However, many carcinogens which stimulate guanylate cyclase activity, increase DNA synthesis (Banks et al., 1967; Saito and Sugimura, 1973), whereas many of the carcinogens which decrease guanylate cyclase activity decrease DNA or RNA synthesis (Alfred and Di Paolo, 1968; Clifford and Rees, 1967; De Recondo et al., 1966; Grunberger et al., 1973; Jenssen et al., 1963; Kidsom and Kirby, 1965; Price et al., 1949; Zieve, 1972).

In light of the evidence, it appears that a relationship between cyclic GMP, DNA and RNA synthesis and chemical carcinogenesis cannot be excluded.

Beta-adrenergic blocking agents are known to block the stimulating effect of adrenaline and other catecholamines by competing with them for the active site of the enzyme adenylate cyclase (Fitzgerald and Barrett, 1967). This has led to the suggestion that these agents may act at the adenylate cyclase level giving rise to a reduction in cellular cyclic AMP levels. It was therefore of interest to investigate the effects of some of these agents in vivo on adenylate cyclase/cyclic AMP and guanylate cyclase/cyclic GMP levels in various tissues of the rat.
EXPERIMENTAL

Materials

Theophylline, caffeine, creatine phosphokinase (EC 2.7.3.2), bovine serum albumin (BSA), creatine phosphate, alumina (grade 1) phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40) and cyclic GMP were obtained from Sigma Chemical Co, London. The radiolabelled compounds $[\delta^{14}C]ATP$, $[\delta^{3}H]cyclic$ AMP, $[\delta^{3}H]cyclic$ GMP, $[\delta^{14}C]GTP$ and the radioimmunoassay (RIA) kits of cyclic AMP and cyclic GMP were obtained from The Radiochemical Centre, Amersham, England. Insta-Gel and dimilume-30 were from Packard Instrument Company, Reading, England. Fluorescent cellulose coated thin-layer chromatography (TLC) plates (DC-Fertigplatten cellulose F) and all other chemicals were obtained from British Drug Houses (BDH) Ltd., Poole, England.

Animals and Treatment

Male Wistar albino rats (140 - 160g) were kept in groups of four and fed ad libitum on Spratt's laboratory chow. Test animals were dosed with compounds (150 mg/kg/day) for five days (1ml/100g body wt.) and control animals received equivalent amount of vehicle; water in case of propranolol acebutolol and pronethalol and 0.1M citric acid/Na$_2$HPO$_4$ buffer pH6.5 for practolol and atenolol. Animals were killed by cervical dislocation five hours after administration of last dose and tissues removed immediately and processed.

Enzyme Assays

The assay of adenylate cyclase and guanylate cyclase activities is based on the conversion of $[^{14}C]$-labelled ATP and GTP respectively into their
corresponding [¹⁴C]-labelled 3'5' cyclic nucleotides which are then isolated by column chromatography and determined by liquid scintillation counting.

**Tissue preparation**

The preparation of liver homogenates was according to the method of Vesely et al (1977) and that of gastric and intestinal mucosa by a modification of the method of De Rubertis and Craven (1977) used for the extraction of cyclic nucleotides (Figs. 5.1 and 5.2).

**Guanylate cyclase assay**

Guanylate cyclase activity was assayed by the original method of White and Zenser (1971) as modified by Vesely et al (1977) using [8-¹⁴C] GTP as the enzyme substrate.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume/tube (µl)</th>
<th>Wt/tube (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic GMP (13.35mM)</td>
<td>20.0*</td>
<td></td>
</tr>
<tr>
<td>Caffeine (200mM)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>0.1*</td>
</tr>
<tr>
<td>Creatine phosphokinase (11.5 I.U.)</td>
<td>-</td>
<td>0.1*</td>
</tr>
<tr>
<td>Creatine phosphate (50mM)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>Buffer (20mM Tris-HCl, pH7.6)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>[8-¹⁴C] GTP (0.152mM)</td>
<td>20.0*</td>
<td></td>
</tr>
<tr>
<td>MnCl₂ (60mM)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>Tissue homogenate (10-30mg/ml)</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0</strong></td>
<td><strong>0.2</strong></td>
</tr>
</tbody>
</table>
Livers

Homogenization 100mg/ml Tris-HCl buffer (30mM) pH 7.6

Centrifugation 10,000rpm for 10 mins. at 4°C

Supernatant discarded

Sediment washed and resuspended in same volume of buffer

Centrifugation (MSE 8 x 50 ml rotor) 10,000rpm for 10 mins. at 4°C

Supernatant discarded

Sediment resuspended in same volume of buffer

Supernatant used for Guanylate cyclase assay and protein determination

Fig. 5.1 - Preparation of liver homogenate for Adenylate and Guanylate cyclase assays
EDTA (0.133M) pH 7.6 - 10μl added after period of incubation at 37°C to stop reaction.

* Mixed together and 70μl used per tube
† Preincubated with mixture (*) at 37°C for 5 minutes prior to addition of tissue homogenate.

After stopping the reaction with EDTA, the tubes were placed in a boiling water bath for 3 minutes, to kill the enzyme, and kept at -20°C overnight. The contents were thawed at room temperature and distilled water (1 ml) was added to each tube. Approximately 50,000 cpm [β-3H] cyclic GMP were added, to monitor per cent recovery, vortex-mixed and centrifuged at 3,000 rpm for 15 minutes at 4°C. The supernatent was put onto a column (0.4 x 10 cm) containing 1g alumina prewashed with 50mM Tris-HCl buffer pH 7.6 (15 ml) and allowed to drain into the column. The eluate was collected into glass vials and 2 mls used for scintillation counting after addition of Insta-Gel (15 ml) as liquid scintillant. Blanks were prepared using equivalent volumes of denatured enzyme protein.

Adenylate cyclase assay

Adenylate cyclase activity was assayed by a modification of the method of Krishna et al (1968) using [β-14C] ATP as the enzyme substrate.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume/tube (μl)</th>
<th>Wt./tube (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (260mM)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>Theophylline (80mM)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td>0.08*</td>
</tr>
<tr>
<td>Pyruvate Kinase (4mg/ml)</td>
<td>5.0*</td>
<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate (18mM)</td>
<td>5.0*</td>
<td></td>
</tr>
<tr>
<td>Buffer (20mM Tris HCl pH 7.6)</td>
<td>10.0*</td>
<td></td>
</tr>
<tr>
<td>[8-14C] ATP (0.139mM)</td>
<td>30.0*</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (30mM)</td>
<td>10.0†</td>
<td></td>
</tr>
<tr>
<td>Tissue homogenate (10-20mg/ml)</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0</strong></td>
<td><strong>0.08</strong></td>
</tr>
</tbody>
</table>
**Fig. 5.2 - Preparation of gastric and intestinal mucosal homogenate for Adenylate and Guanylate cyclase assays**

1. **Stomach**
   - Cut along greater curvature
   - Contents washed with 1.15%(w/v) KCl (ice-cold)
   - Tissues put in Tris-HCl buffer (50mM) pH 7.6 (750 mg/ml)
   - Mucosae scraped into buffer
   - Homogenization
   - Centrifugation at 40,000 rpm for 40 mins at 4°C (Beckman Ultracentrifuge Model LS-65) 8 x 25ml rotor
     - Supernatant discarded
     - Sediment washed and resuspended in same volume of buffer
     - Centrifugation at 40,000 rpm for 40 mins at 4°C
       - Supernatant discarded
       - Sediment resuspended in same volume of buffer
       - Used for Adenylate cyclase Guanylate cyclase and protein determinations

2. **Intestine (15cm from stomach)**
   - Cut longitudinally
   - Contents washed with 1.15%(w/v) KCl (ice-cold)
   - Tissues put in Tris-HCl buffer (50mM) pH 7.6 (750 mg/ml)
   - Mucosae scraped into buffer
   - Homogenization
   - Centrifugation at 40,000 rpm for 40 mins at 4°C (Beckman Ultracentrifuge Model LS-65) 8 x 25ml rotor
     - Supernatant discarded
     - Sediment washed and resuspended in same volume of buffer
     - Centrifugation at 40,000 rpm for 40 mins at 4°C
       - Supernatant discarded
       - Sediment resuspended in same volume of buffer
       - Used for Adenylate cyclase Guanylate cyclase and protein determinations
EDTA (0.133 M) pH 7.6 - 10μl added after period of incubation at 37°C to stop reaction.

* Mixed together and 70μl used per tube

† Preincubated with mixture (*) at 37°C for 5 minutes prior to addition of tissue homogenate.

After stopping the reaction, the same procedure was followed as for guanylate cyclase except that 50,000 cpm [8-3H] cyclic AMP were added to measure per cent recovery. Blanks were prepared using equivalent volumes of denatured enzyme protein.

Cyclic Nucleotide Assay

The assay of cyclic AMP and cyclic GMP is based on the competition between unlabelled cyclic nucleotide and a fixed quantity of the tritium labelled compound for binding to a protein/antiserum which has a high specificity and affinity for cyclic nucleotides. The amount of labelled protein/antiserum-cyclic nucleotide complex formed is inversely related to the amount of unlabelled cyclic nucleotide present in the assay sample. Measurement of protein/antibody-bound radioactivity enables the amount of unlabelled cyclic nucleotide in the sample to be calculated. Typical calibration curves for cyclic nucleotides are shown (Fig. 5.4).

Tissue preparation and extraction

The tissues were prepared by the method of De Rubertis and Craven (1977) and the cyclic nucleotides extracted by a modification of the acidic methanol method described in the product information pamphlet, Radiochemical Centre, Amersham, England (1977) on cyclic AMP and cyclic GMP (RIA) kits (Fig. 5.3).

Cyclic AMP assay

The method used is as described in the pamphlet after Gilman (1970) and
Stomach and Intestine (15cm) → Addition to each stomach/intestine, 5ml Krebs-Ringer bicarbonate buffer pH7.4 containing 1mg/ml BSA and glucose and 10mM theophylline → Oxygenation $\left[95\%O_2:5\%CO_2\right]$ for 15 mins. → Mucosae scraped on filter paper soaked with ice cold saline → Mucosae of each stomach/intestine put in 2.5ml Krebs-Ringer bicarbonate buffer pH 7.4 and 5ml acidic ethanol → Homogenized and kept at room temperature for 10 mins → Centrifugation at 3,000 rpm for 20 mins at $4^\circ$C (MSE Mistral 6L centrifuge) → 1st supernatant saved → Sediment resuspended in aqueous ethanol (2.5ml) → Centrifugation at 3,000 rpm for 20 mins at $4^\circ$C → 2nd supernatant saved → Sediment Mixed and 2ml evaporated to dryness at $55^\circ$C under stream of $N_2$ → Addition of 0.5ml Tris-HCl buffer (0.05M) pH 7.5 containing 5mM EDTA → Used for tissue cyclic AMP and GMP determinations

Liver (0.25g) → Addition of 5ml Krebs-Ringer bicarbonate buffer pH 7.4 → Oxygenation $\left[95\%O_2:5\%CO_2\right]$ for 15 mins. → Addition of 2.5ml of fresh Krebs-Ringer bicarbonate buffer pH 7.4 → Addition of 5ml acidic ethanol $[1:2(v/v)\text{ in HCl:absolute alcohol}]$ → Homogenized and kept at room temperature for 10 mins → Centrifugation at 3,000 rpm for 20 mins at $4^\circ$C → 1st supernatant saved → Sediment resuspended in aqueous ethanol (2.5ml) → Centrifugation at 3,000 rpm for 20 mins at $4^\circ$C → 2nd supernatant saved → Sediment Mixed and 2ml evaporated to dryness at $55^\circ$C under stream of $N_2$ → Addition of 0.5ml Tris-HCl buffer (0.05M) pH 7.5 containing 5mM EDTA → Used for tissue cyclic AMP and GMP determinations

Fig. 5.3 - Extraction of tissue cyclic nucleotides
Brown et al. (1971).

<table>
<thead>
<tr>
<th>Assay tube</th>
<th>Buffer (µl)</th>
<th>Standards (µl)</th>
<th>Unknown (µl)</th>
<th>$[^3]$H cyclic AMP (µl)</th>
<th>Binding protein (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charcoal blank</td>
<td>150</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Zero dose</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Standard (0-16 pmol/tube)</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Unknown</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

Buffer = Tris-HCl buffer (0.05M) pH 7.5 containing 4mM EDTA
$[^3]$H cyclic AMP = 18 pmol/ml containing approx. 5uCi
Cyclic AMP Standard = 320 pmol/ml

The additions were done in the order shown above. After the last addition, the tubes were vortex-mixed for about 5 seconds, put in an ice-bath and placed in a cold-room at 4°C for 2 hours. Charcoal suspension (100µl) was added to each tube, vortex-mixed and replaced into ice-bath. The tubes were centrifuged 5 minutes after addition of charcoal suspension to the last tube at 2,000 rpm for 60 minutes at 4°C. Aliquots (200µl) were removed from each tube without disturbing the sediment, placed into plastic scintillation vials containing dimilume-30 (5mls) as liquid scintillant. The vials were counted in the LKB Wallac Ultrabeta 2002 scintillation counter for radioactivity. The cyclic AMP content of unknown was calculated using the calibration curve for cyclic AMP.
Cyclic GMP assay

The method was as described for cyclic GMP in the pamphlet of The Radiochemical Centre, Amersham, England (1977) on cyclic GMP (RIA) kit.

<table>
<thead>
<tr>
<th>Assay tube</th>
<th>[^{3}H] cyclic GMP (μl)</th>
<th>Buffer (μl)</th>
<th>Standard (μl)</th>
<th>Unknown (μl)</th>
<th>Blank (μl)</th>
<th>Antiserum (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero dose</td>
<td>50</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Blank</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Standard (0-8 pmol/tube)</td>
<td>50</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Unknown</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

Buffer - Tris-HCl (0.05M) pH 7.5 containing 4mM EDTA

\[^{8-3}H\] cyclic GMP - 8 pmol/ml H\textsubscript{2}O containing approx. 1.6 μCi

Cyclic GMP standard - 80 pmol/ml H\textsubscript{2}O

The additions were as shown above. After addition of the antiserum the tubes were vortex-mixed for 5 seconds and placed in ice-bath and kept at 2-4°C for 1½ hours. Ice-cold saturated (NH\textsubscript{4})\textsubscript{2} SO\textsubscript{4} solution (1 ml) was added to each tube, capped and vortex-mixed for a few seconds. The tubes were replaced in the ice-bath and allowed to stand for 5 minutes (timed from the addition of (NH\textsubscript{4})\textsubscript{2} SO\textsubscript{4} to the last assay tube). The tubes were then centrifuged at 2,000 rpm for 30 minutes at 4°C and the supernatent decanted. The tubes were made to stand upside down on tissue paper to drain. Any excess liquid from the neck of each tube was wiped carefully by a piece of tissue taking care not to disturb the precipitate. Distilled water (1.1 ml) was added to each tube, mixed, and 1 ml removed into glass vials and dimilume-30 (10 ml) added to each vial as liquid scintillant. The counting was done as for cyclic AMP and the cyclic GMP content of unknown calculated using the calibration curve for cyclic GMP.
Separate channels were set for $^3$H and $^{14}$C counting $[^3H]$- and $[^{14}C]$-hexadecane standards (10 μl each) were put into scintillation vials, in duplicate, containing Insta-Gel (15 ml) as liquid scintillant and 30 mM Tris-HCl buffer pH 7.6 (2 ml). The counting efficiencies in each channel for $^3$H and $^{14}$C were determined and used to calculate per cent recoveries of cyclic nucleotides formed in guanylate and adenylate cyclase assays. The mean per cent recoveries obtained in experiments were:

Cyclic AMP = 95 ± 15  
Cyclic GMP = 60 ± 10

Identification of $[^{14}C]$ cyclic nucleotide products

The $[^{14}C]$-containing products were identified as cyclic AMP and cyclic GMP as determined by TLC using 1 M formic acid, 1 M LiCl$_2$ as solvent system and developed with absolute alcohol and concentrated NH$_4$OH (5:2 v/v) using $[^3H]$ cyclic AMP and GMP as standards. The developed spots were identified under fluorescence.

Protein Assay

The protein content of the various tissue homogenates were determined by the method of Lowry et al (1951).

Calculation of Enzyme Activity

The activities of adenylate and guanylate cyclases were calculated according to the formula:

$$\frac{S - B}{SD} \times M \times \frac{100}{P} \times \% \text{ Recovery}$$

expressed as pmol cyclic nucleotide formed/mg protein/time of incubation.
Where

\[ S = \text{Sample cpm} \]
\[ B = \text{Blank cpm} \]
\[ SD = \text{Standard cpm} \]

\[ M = \text{Concentration of standard (pmol/tube)} \]
\[ P = \text{Enzyme protein concentration (mg/tube)} \]
RESULTS

Effect of Time of Incubation on Production of Cyclic Nucleotides

The production of cyclic GMP increased with incubation time in a linear fashion for all the tissues studied whilst the production of cyclic AMP increased non-linearly with time of incubation (Figs. 5.5 and 5.6).

Effect of Pretreatment with Beta-adrenergic Blocking Agents on Rat Tissue Adenylate and Guanylate Cyclase Activities

Practolol and atenolol gave rise to marked and concomitant increases in both adenylate and guanylate cyclase activities in all three tissues. This is reflected in the absence of any significant changes in the ratios of adenylate cyclase activity (ACA)/guanylate cyclase activity (GCA) before and after pretreatment with these agents. Pronethalol preferentially increased adenylate cyclase activity of the liver and gastric mucosa whilst causing concomitant increases in both adenylate and guanylate cyclase activities in the intestinal mucosa, as indicated by the ratios of adenylate/guanylate cyclases before and after pretreatment. Propranolol and acebutolol on the otherhand, preferentially increased guanylate cyclase activity in the tissues as shown by the decrease in the ratios of adenylate/guanylate cyclases after pretreatment with the compounds. (Tables 5.1 - 5.5).

Effect of Pretreatment with Beta-adrenergic Blocking Agents on Rat Tissue Cyclic Nucleotides Levels

The beta-adrenergic blocking agents, practolol and atenolol, did not significantly affect the levels of cyclic AMP and GMP in the three tissues. This is indicated by the similarities in the ratios of cyclic AMP (C-AMP)/
cyclic GMP (C-GMP) before and after pretreatment. Pronethalol on the other hand gave rise to concomitant decreases in cyclic AMP and GMP levels in gastric and intestinal mucosa as shown by the absence of any significant changes in the ratios of C-AMP/C-GMP on pretreatment. Propranolol markedly decreased the level of cyclic GMP in intestinal mucosa whilst acebutolol gave rise to a marked decrease in the cyclic AMP level of the liver (Tables 5.6 - 5.9).
Fig. 5.4 Calibration Curves for Cyclic Nucleotides

Counts bound in the presence of standard unlabelled cyclic nucleotide

Counts bound in the absence of unlabelled cyclic nucleotide

Concentration of cyclic nucleotide (pmole/tube)
Fig. 5.5 Effect of Time of Incubation of Cyclic GMP

Production by Guanylate Cyclase

Liver

Gastric Mucosa

Intestinal Mucosa

pmol cyclic GMP/mg protein

Time (Mins)
Fig. 5.6 Effect of Incubation Time on Cyclic AMP Production by Adenylate Cyclase

- ● ● Gastric mucosa
- ■ ■ Liver
- ▲ ▲ Intestinal mucosa

pmol cyclic AMP/mg protein

Time (Mins.)
Table 5.1  Effect of Practolol Pretreatment on Guanylate and Adenylate Cyclase Activities in Rat Tissues

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Guanylate Cyclase Activity (pmol C-GMP/mg protein/10 mins)</th>
<th>Adenylate Cyclase Activity (pmol C-AMP/mg protein/15 mins)</th>
<th>Ratio of ACA/GCA^</th>
<th>Before Treatment</th>
<th>After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>160 ± 10(5)</td>
<td>252 ± 16(4)**</td>
<td>1116 ± 89(4)</td>
<td>1442 ± 60(4)*</td>
<td>7.0</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>586 ± 45(6)</td>
<td>729 ± 52(4)</td>
<td>788 ± 96(5)</td>
<td>841 ± 61(4)</td>
<td>1.3</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>982 ± 79(6)</td>
<td>2208 ± 163(4)***</td>
<td>764 ± 113(5)</td>
<td>2298 ± 195(4)***</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis
Values significantly different from controls; *P<0.05, **P<0.01, ***P<0.001
Table 5.2  Effect of Atenolol Pretreatment on Guanylate and Adenylate Cyclase Activities in Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Guanylate Cyclase Activity (pmol C-GMP/mg protein/10mins)</th>
<th>Adenylate Cyclase Activity (pmol C-AMP/mg protein/15 mins)</th>
<th>Ratio of ACA/GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>160 ± 10(5)</td>
<td>285 ± 20(4)**</td>
<td>1116 ± 89(4)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>586 ± 45(6)</td>
<td>2259 ± 130(4)**</td>
<td>788 ± 96(5)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>982 ± 79(6)</td>
<td>1631 ± 79(4)**</td>
<td>764 ± 113(5)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Values significantly different from controls; *P<0.02  **P<0.001
Table 5.3  Effect of Pronethalol Pretreatment on Guanylate and Adenylate Cyclase Activities in Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Guanylate Cyclase Activity (pmol C-GMP/mg protein/10 mins)</th>
<th>Adenylate Cyclase Activity (pmol C-AMP/mg protein/15 mins)</th>
<th>Ratio of ACA/GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>164 ± 19(5)</td>
<td>208 ± 24(4)</td>
<td>1012 ± 82(5)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>503 ± 43(6)</td>
<td>624 ± 44(4)</td>
<td>698 ± 78(6)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>937 ± 70(6)</td>
<td>2304 ± 183(4)*</td>
<td>998 ± 93(4)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis. Values significantly different from controls; *P<0.001
Table 5.4 Effect of Propranolol Pretreatment on Guanylate and Adenylate Cyclase Activities in Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Guanylate Cyclase Activity (pmol C-GMP/mg protein/10 mins)</th>
<th>Adenylate Cyclase Activity (pmol C-AMP/mg protein/15 mins)</th>
<th>Ratio of ACA/GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>164 ± 19(5)</td>
<td>364 ± 38(4)**</td>
<td>1012 ± 82(5)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>503 ± 43(6)</td>
<td>1015 ± 46(4)***</td>
<td>698 ± 78(6)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>937 ± 70(6)</td>
<td>1460 ± 149(4)</td>
<td>998 ± 93(4)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.

Values significantly different from controls; *P<0.05, **P<0.01, ***P<0.001.
Table 5.5  Effect of Acebutolol Pretreatment on Guanylate and Adenylate Cyclase Activities in Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Guanylate Cyclase Activity (pmol C-GMP/mg protein/10 mins)</th>
<th>Adenylate Cyclase Activity (pmol C-AMP/mg protein/15 mins)</th>
<th>Ratio of ACA/GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>N D</td>
<td>N D</td>
<td>1012 ± 82(5)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>503 ± 43(6)</td>
<td>611 ± 112(3)</td>
<td>698 ± 78(6)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>937 ± 70(6)</td>
<td>1563 ± 115(4)*</td>
<td>998 ± 93(5)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.
Values significantly different from controls; *P<0.01
N D = Not determined
Table 5.6  Effect of Practolol Pretreatment on Cyclic Nucleotide Content of Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cyclic GMP Content (pmol/mg protein)</th>
<th>Cyclic AMP Content (pmol/mg protein)</th>
<th>Ratio of C-AMP/C-GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>0.40 ± 0.02(4)</td>
<td>0.33 ± 0.02(4)</td>
<td>2.2 ± 0.2(4)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>0.29 ± 0.02(4)</td>
<td>0.24 ± 0.02(4)</td>
<td>4.4 ± 0.3(4)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>0.27 ± 0.01(4)</td>
<td>0.24 ± 0.02(4)</td>
<td>2.1 ± 0.3(4)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cyclic GMP Content (pmol/mg protein)</th>
<th>Cyclic AMP Content (pmol/mg protein)</th>
<th>Ratio of C-AMP/C-GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.46 ± 0.02(3)</td>
<td>0.38 ± 0.04(4)</td>
<td>2.0 ± 0.1(4)</td>
<td>1.6 ± 0.1(4)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>0.32 ± 0.04(4)</td>
<td>0.23 ± 0.01(4)</td>
<td>3.8 ± 0.2(4)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>0.31 ± 0.03(4)</td>
<td>0.27 ± 0.03(4)</td>
<td>1.8 ± 0.3(4)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis.
Table 5.8  Effect of Pronethalol Pretreatment on Cyclic Nucleotide Content of Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cyclic GMP Content (pmol/mg protein)</th>
<th>Cyclic AMP Content (pmol/mg protein)</th>
<th>Ratio of C-AMP/C-GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>0.49 ± 0.02(4)</td>
<td>0.53 ± 0.03(4)</td>
<td>2.3 ± 0.1(4)</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>0.30 ± 0.01(4)</td>
<td>0.19 ± 0.01(4)</td>
<td>3.6 ± 0.3(4)</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>0.31 ± 0.02(4)</td>
<td>0.22 ± 0.02(4)</td>
<td>2.2 ± 0.1(4)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis. Values significantly different from controls; *P<0.02  **P<0.01,  ***P<0.001
Table 5.9 Effects of Propranolol and Acebutolol Pretreatment on Cyclic Nucleotide Content of Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cyclic GMP Content (pmol/mg protein)</th>
<th>Cyclic AMP Content (pmol/mg protein)</th>
<th>% of Control Value</th>
<th>% of Control Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Control: 0.52 ± 0.01(n)</td>
<td>Control: 2.7 ± 0.1(n)</td>
<td>108</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Propranolol: 0.56 ± 0.05(n)</td>
<td>Acebutolol: 1.9 ± 0.1(n)*</td>
<td>86</td>
<td>92</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>Control: 0.29 ± 0.03(n)</td>
<td>Control: 3.8 ± 0.3(n)</td>
<td>56</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Propranolol: 0.25 ± 0.04(n)</td>
<td>Acebutolol: 3.5 ± 0.1(n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>Control: 0.27 ± 0.02(n)</td>
<td>Control: 1.8 ± 0.1(n)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are Mean ± SEM of duplicate samples for number of animals given in parenthesis. Values significantly different from controls; *p<0.01.
DISCUSSION

Recent studies have shown that most chemical carcinogens produce marked increases in hepatic and gastrointestinal mucosal guanylate cyclase and cyclic GMP levels without concomitant increases in adenylate cyclase and cyclic AMP levels (De Rubertis and Craven, 1976; Vesely and Levy, 1977 a, b; Vesely et al, 1977). However the concomitant increases in guanylate and adenylate cyclase activities and decreases in cyclic nucleotide levels, in the various tissues studied, caused by the beta-adrenergic blocking agents is at variance with the above observations for chemical carcinogens.

It has been suggested that beta-adrenergic blocking agents may competitively inhibit the effects of catecholamines by acting at the adenylate cyclase level. The stimulation of tissue adenylate cyclase by all the compounds studied, appear to suggest the involvement of beta-blockade in the activation of adenylate cyclase. A plausible explanation is that the beta-blockade of a membrane beta-adrenergic receptor dependent GTP-ase may prevent the conversion of GTP to GMP which is an important step in the deactivation of adenylate cyclase as has been suggested for propranolol with plasma membrane (Levitzki, 1977; Cassel and Selinger, 1976, 1977).

The stimulatory effect of these agents on tissue levels of adenylate cyclase was in the order, pronethalol>atenolol>practolol>propranolol>acebutolol. The trend shown by the less lipid-soluble beta-adrenergic blocking agents, practolol, atenolol and acebutolol, may be due to a culmination of three factors namely; beta-blockade potency (waal-Manning, 1976b), degree of GIT absorptivity (Meier et al, 1977) and elimination
half-life (Bodem and Chidsey, 1973; Reeves et al, 1978; Brown et al, 1976; Steyn, 1976). The propranolol effect may be the result of its extensive liver metabolism and "first-pass" effect and shorter elimination half-life (Shand, 1974; Meier et al, 1977) leading to a reduction in its beta-blockade effect. The marked stimulation by pronethalol may be due to a culmination of its beta-blockade effect and the formation of products with a stimulatory action on adenylate cyclase. Although practolol, atenolol and to some extent acebutolol act preferentially on the $\beta_1$-adrenoceptors, at the dosage of 150 mg/kg used in this study they also affect the $\beta_2$-adrenoceptors (Lertora et al, 1975) and hence their effects on the peripheral tissues.

One of the characteristics of malignantly transformed cells is that they contain reduced levels of cyclic AMP and elevated levels of cyclic GMP leading to reduction in C-AMP/C-GMP ratio. (Stevens et al, 1978). There are conflicting reports on the influence of cyclic AMP and GMP phosphodiesterase activities on the levels of these cyclic nucleotides (De Rubertis et al, 1976; Stevens et al, 1979). The increased levels in tissue adenylate and guanylate cyclase after administration of the beta-adrenergic blocking agents, should of consequence give rise to increases in cyclic AMP and GMP levels. However, the parallel decreases in the levels of these cyclic nucleotides after pretreatment with these agents suggests the regulation of their levels through increased cyclic AMP and GMP phosphodiesterase activities of which the former has been suggested to be increased by the tumour-promoting agent, 1,2-dimethylhydrazine (Stevens et al, 1979). There was also no direct relationship between the ratios of adenylate/guanylate cyclases and C-AMP/C-GMP or their individual levels before and after pretreatment with the agents. These findings indicate that none of the agents displayed the characteristics of tumour-promoting agents as suggested for the

It has been suggested that carcinogens may cause the inhibition of glycoprotein synthesis through degranulation of the endoplasmic reticulum (Parke, 1977b). The lowering of tissue cyclic AMP or ratio of C-AMP/C-GMP has been associated with the inhibition of glycoprotein synthesis (Hepp, 1972; Solderling et al, 1973; Macdonald et al, 1977) and hence may lead to increased incidence of tumours. The effects shown by the beta-adrenergic blocking agents on ratios of adenylate/guanylate cyclases and C-AMP/C-GMP in the various tissues, did not cause marked inhibition of glycoprotein synthesis with the exception of practolol (Chapter 4). These observations suggest that the ratios of adenylate/guanylate cyclases and C-AMP/C-GMP alone may not cause the inhibition of glycoprotein synthesis. Hence the tumour-promoting potential of these agents does not involve the inhibition of glycoprotein synthesis through changes in tissue adenylate cyclase/C-AMP and guanylate cyclase/C-GMP levels.

It thus appears that the effects of the beta-adrenergic blocking agents on the various parameters studied are to some extent the result of direct pharmacological effect of beta-blockade and are uncharacteristic of those exhibited by known tumour-promoting agents, suggesting that their mechanism of action as possible potential tumour promoters does not involve changes in tissue levels adenylate cyclase/C-AMP and guanylate/C-GMP.
CHAPTER SIX

GENERAL DISCUSSION
Beta-adrenergic Blocking Agents in Short-term Mutagenicity Test

The deactivation of drugs and detoxication of environmental chemicals is brought about by microsomal mixed-function oxygenase system (Parke, 1968). The microsomal oxygenation, by the mixed-function oxygenases, of many compounds e.g. polycyclic aromatic hydrocarbons lead to the formation of reactive metabolites (Grover and Sims, 1968; Gelbion, 1969; Oesch et al, 1972). These metabolites are electrophilic in character and attack the nucleophilic centres of DNA and other biological macromolecules (Miller and Miller, 1969, 1971; Weisburger, 1973; Heidelberger, 1973). These reactions are believed to form the basis of the mutagenicity of these reactive metabolites.

There is considerable evidence in support of a correlation between carcinogenesis and mutagenesis (Miller and Miller, 1975; Ames et al, 1975; McCann et al, 1975; Purchase et al, 1978; Bartsch et al, 1980; Jenssen and Ramel, 1980), suggesting a common molecular mechanism for these two processes (Brusick, 1977). This has led to the development of fast and less expensive in vitro assay systems demonstrating mutagenic activity, for the identification of potential chemical carcinogens. However there are some carcinogens which are not mutagenic probably because they are not metabolized to reactive intermediates in these test systems or act via a different mechanism of carcinogenesis i.e. epigenetic mechanisms.

A combination of the in vitro Ames' bacterial and the in vivo mammalian micronucleus tests has been shown to provide a more sensitive system with higher predictive value for the identification of mutagenic carcinogens (Jenssen and Ramel, 1980). The in vivo assay system is able to detect also, the
breakage and non-disjunction of chromosomes. It also helps to overcome the imbalance between the activating and deactivating metabolism in vitro with the S-9 mix in the Ames test which affects its sensitivity (Ashby and Styles, 1978).

The fact that none of the beta-adrenergic blocking agents studied produced positive results in the Ames and micronucleus tests, indicates their non-mutagenic character. However, the bacteriocidal action of oxprenolol and propranolol, in the absence of S-9 mix in the Ames test, together with the increases in the number of micronuclei, above the spontaneous control level in the micronucleus test, may involve the breakage or non-disjunction of chromosomes which could result from mechanisms other than chemical interaction with DNA as suggested for saccharin by Bateman and Epstein (1971). The epoxidation of the allyloxy side chain of oxprenolol in mice, may be responsible for its chromosomal effects.

**Beta-adrenergic Blocking Agents and Short-term Carcinogenicity Tests**

Some carcinogens exert their action at sites other than DNA and these may explain why compounds such as DDT, chloroform and saccharin, amongst others, gave negative results in the Ames test (Uehleke et al, 1977; McCann and Ames, 1976; Marshall et al, 1976; Ashby et al, 1978). These compounds are termed epigenetic carcinogens (Williams, 1977) and the investigation of their sites of action coupled with the development of other test systems, which do not involve chemical interaction with DNA, is of great importance for the detection of such carcinogens.

One of the sites of action of carcinogens is the rough endoplasmic reticulum culminating in the degranulation of the endoplasmic reticulum (Gustafsson and Afzelius, 1963). This offers a possible means of carcinogenicity testing of epigenetic carcinogens, at the cellular level. This
effect has been observed in vivo by electron microscopy (Posner et al., 1961; Orrenius, 1965) and demonstrated in vitro by the measurement of disulphide rearrangease, which increases in activity with the degree of degranulation (Williams and Rabin, 1971; Williams and Parry, 1975; Dani et al., 1976). The pattern of protein synthesis would be affected as a result of extensive degranulation e.g. glycoprotein synthesis which normally occurs only in the endoplasmic reticular bound ribosomes (Halliran et al., 1968) would become inhibited as a result of degranulation of the endoplasmic reticulum and at the same time an increase in intracellular protein production may occur resulting in cancer formation and major physiological changes (Parke, 1977c, 1979).

The in vitro "degranulation" technique has been developed and a good correlation exists between various in vivo carcinogenicity tests and the degree of degranulation obtained (Wright et al., 1977). The use of human liver preparations for this test and the Ames test could provide more meaningful information as to the action of a variety of suspect carcinogens in man.

The biphenyl system offers an alternative possibility as a screening test for epigenetic carcinogens since the action of carcinogens on this system is also at the endoplasmic reticulum level. The degranulation of the endoplasmic reticulum and the stimulation of biphenyl 2-hydroxylase activity, caused by carcinogens, have been suggested to be a related process (Parke, 1976). However, a direct relationship does not exist since the stimulation of biphenyl 2-hydroxylase required a much shorter period to attain maximal effect (McPherson et al., 1976). Also agents such as EDTA, which cause extensive degranulation of the endoplasmic reticulum (Rabin et al., 1971; Lin and Farber, 1977), failed to have an effect on biphenyl.
2-hydroxylase (McPherson et al, 1974), although the cytotoxic effect of EDTA may lead to complete disruption of the endoplasmic reticulum and its constituent enzymes.

Pretreatment of animals with carcinogens, e.g. 3-methylcholanthrene, has been shown to stimulate the cytochrome P-448-mediated microsomal enzymes such as biphenyl 2- and 4- hydroxylases and ethoxyresorufin O-deethylase (Burke and Mayer, 1975; Bridges et al, 1973; Burke et al, 1977; Atlas and Nebert, 1976). Hence the measurement of these enzymic parameters after pretreatment with test compounds can provide useful information as to their carcinogenicity potential. Also the problem of interference of fluorescent metabolites of test compounds in the in vitro system in the measurement of 2- and 4-hydroxybiphenyls, as shown for 3-methylcholanthrene, 3,4, benzo(a) pyrene and safrole (Tong, 1979), would be reduced or eliminated in the in vivo situation.

The stimulation of the cytochrome P-448-mediated enzymic parameters, particularly of ethoxyresorufin O-deethylase activity, by beta-adrenergic blocking agents depends on the lipophilicity of the agents with the most lipid-soluble ones showing the most stimulation in the order, propranolol > pronethalol > acebutolol > atenolol and practolol. The levels of stimulation of these cytochrome P-448-mediated enzymic parameters by propranolol and pronethalol at very high dosage are similar to those shown by DDT and saccharin (Tong, 1979; Tong, 1979 (Unpublished results)) which are classified as epigenetic carcinogens. These results compare similarly to those of long-term animal carcinogenicity tests for pronethalol (Paget, 1963; Alcock and Bond, 1964; Howe, 1965) and propranolol (Smith and Butler, 1978; Boyd and Martin, 1977), suggesting the possibility of propranolol and pronethalol acting via epigenetic mechanisms as potential tumour promoters.
Microsomal Enzyme Induction by Beta-adrenergic Blocking Agents and Chemical Interactions

Short-term administration of pronethalol and propranolol at very high dosage, induced cytochrome P-448 resulting in the stimulation of the mixed-function oxidations it catalyses in the rat liver. The possibility of the marked induction of cytochrome P-448 by these agents on prolonged high dosage is not overruled and this may have long-term implications in man. High drug-metabolizing enzyme activities as a result of enzyme induction may be beneficial if the foreign compound is rendered inactive, but harmful when toxic metabolites are produced. Thus the induction of cytochrome P-448 by propranolol and pronethalol on prolonged high dosage in man may exert a profound influence on their own rates and modes of metabolism as well as other chemicals, such as food additives, that are generally present in human diet, leading to production of adverse reactions including the formation of tumours.

Apart from the mixed-function oxygenase system which metabolizes xenobiotics, there are a number of other enzymes in the cell e.g. epoxide hydrase and glutathione S-transferase, which exert a significant influence on the fate of foreign compounds in the animal body. Epoxide hydrase (Oesch et al, 1972) which transforms epoxides into dihydrodiols, is located in the endoplasmic reticulum whilst glutathione S-transferase (Hayakawa et al, 1975) which metabolizes diols or phenols into soluble conjugates, is found in the cytoplasm of the cell. These enzymes therefore convert mutagenic electrophilic intermediates into non-toxic substances that do not react with biological macromolecules. Thus if epoxide hydrase activity is greater than the rate of conversion of a carcinogen to its reactive intermediate by the mixed-function oxygenases, toxic effects may not be produced. Hence any evaluation of the safety of compounds such as beta-adrenergic blocking agents must take into account the implications of microsomal enzyme induction.
Adenylate Cyclase/Cyclic AMP and Guanylate Cyclase/Cyclic GMP Levels and Toxicity of Beta-adrenergic Blocking Agents

The properties of adenylate cyclase have been reported to differ in several malignant tissues of cells from their normal counterparts (Ney et al, 1969; Allen et al, 1971; Emmelot and Bos, 1971; Makman, 1971; Peery et al, 1971). The competitive inhibition of the stimulating effects of catecholamines by beta-adrenergic blocking agents at the adenylate cyclase level (Fitzgerald and Barrett, 1967). However, the activation of tissue adenylate cyclase after pretreatment of rats with these agents, may be explained by their possible inhibition of the membrane beta-adrenergic receptor GTP-ase which converts GTP to GMP; an important step in the deactivation of adenylate cyclase.

This has been proved with plasma membrane using propranolol (Levitzki, 1977; Cassel and Selinger, 1976, 1977). This stimulatory effect of these agents depends on their beta-blockade potency (waal-Manning, 1976b), degree of GIT absorptivity (Meier et al, 1977) and elimination half-life (Bodem and Chidsey, 1973; Reeves et al, 1978; Brown et al, 1976; Steyn, 1976). However this stimulatory effect is at variance with the decreased levels of this enzyme in anaplastic cancer cells, although the inter-relationship between the levels of this enzyme and the onset of malignancy is not clear (Allen et al, 1971; Makman, 1971; Brown et al, 1970; Peery et al, 1971).

Recent studies have shown that some carcinogens preferentially stimulated tissue guanylate cyclase activity as well as cyclic GMP level without significantly affecting the adenylate cyclase and cyclic AMP levels, such that the ratios of adenylate/guanylate cyclases and C-AMP/C-GMP are decreased (De Rubertis and Craven, 1976; Vesely and Levy, 1977 a,b; Vesely et al, 1977). One of the characteristics of malignantly transformed cells is that they contain decreased levels of cyclic AMP and elevated levels of cyclic GMP and hence reduced ratio of C-AMP/C-GMP(Stevens et al, 1978).
There are conflicting reports on the levels of these cyclic nucleotides (De Rubertis et al, 1976; Stevens et al, 1979). The absence of any direct relationship between the ratios of tissue adenylate/guanylate cyclases and C-AMP/C-GMP after pretreatment with the beta-adrenergic blocking agents implicates phosphodiesterase activity in the regulation of tissue cyclic AMP and GMP levels.

The role of adrenergic mechanisms in relation to gastric mucosal cyclic nucleotide levels have been investigated (Ruoff and Sewing, 1975) and has led to the suggestion that the rise in tissue cyclic AMP level on adrenaline administration is mediated by beta-adrenoceptors whilst that in tissue cyclic GMP level is mediated by alpha-adrenoceptors. Hence the lowering of tissue cyclic AMP levels is a result of direct pharmacological effect of beta-blockade as demonstrated by the beta-adrenergic blocking agents.

The activation of gastric mucosal adenylate cyclase appears to be an essential event during stimulation of acid secretion. This view and the finding of Ruoff and Sewing (1975) that no changes in phosphodiesterase activity of gastric mucosa occur after feeding and pentagastrin (adenylate cyclase stimulator) administration, is in contrast to proposals that gastric mucosal cyclic AMP levels are regulated by the changes in activity of this enzyme (Amer, 1972; Amer and McKinney, 1972). The question as to whether activation of adenylate cyclase by gastric secretagogues lead to the elevation of gastric mucosal cyclic AMP concentration has been raised.

The effect of beta-adrenergic blocking agents on gastric acid secretion are conflicting. They have been shown to stimulate (Konturek and Oleksy, 1969; Evans and Lin, 1970), inhibit (Pradham and Wingate, 1962; Bass and Patterson, 1967; Okabe et al, 1970; Guemei et al, 1972; Danhof and Guemei, 1972) or have no effect (Haigh and Stredman, 1968; Misker et al, 1969). Similar controversy
exists with regard to production of gastric ulcers. An increasing (Rosoff et al, 1968; Kohcut et al, 1970; Pfeiffer and Sethbhakdi, 1971) and decreasing (Okabe et al, 1970; Danhof and Guemei, 1972; Takagi et al, 1964) incidence of gastric ulcers have been reported. In recent study by Debnath et al (1974) it was reported that the stimulatory and inhibitory effects of propranolol on gastric acid secretion and ulceration in pyloric ligated rats was dose-dependent. Comparative study of the effects of propranolol, and practolol on basal gastric secretion (Goel et al, 1977) showed a dose-dependent effect on gastric secretion and ulceration. Smaller doses increased ulceration and higher doses decreased it. The probable action of these beta-adrenergic blocking agents may be that at high dosage, the activation of adenylate cyclase is counteracted by decreased levels of cyclic AMP resulting in decreased gastric secretion and hence reduced ulceration, whereas at low dosage there may be no significant change in cyclic AMP levels and thus increased adenylate cyclase activity leads to increased gastric secretion and hence increased ulceration.

A relationship has been shown between the inhibition of the rate of gastric mucus synthesis and the incidence of gastric erosions (Dekanski et al, 1975; Johnston et al, 1975). The possibility of the involvement of cyclic nucleotides in mucus glycoprotein synthesis has been investigated by Macdonald et al (1977). After in vitro incubation of mucosal homogenate with dibutryl analogues of cyclic AMP and GMP, a significant reduction of glycoprotein synthesis as measured by a reduction in the rate of incorporation of N-acetylglucosamine was observed with $10^{-4}$ M dibutyryl cyclic AMP. Dibutryl cyclic GMP gave no significant effects, while theophylline at a concentration of $10^{-4}$ M gave similar effects to dibutryl cyclic AMP. At concentrations of dibutryl cyclic AMP lower than $10^{-4}$ M, there were no significant effects. The levels of tissue cyclic AMP and adenylate cyclase,
as a result of beta-blockade, has no direct bearing on the rate of mucus glycoprotein synthesis as demonstrated by the beta-adrenergic blocking agents studied. This is at variance with the relationship of cyclic nucleotides and the incidence of gastric erosions, caused by increased gastric secretion as suggested by Goel et al (1977).

Practolol Toxicity and the Inhibition of Glycoprotein Synthesis

Drugs may alter the rate of secretion of mucus, the chemical and physical structure of mucus or its rate of synthesis. They may thus affect the normal physiological functions of mucus and its protective effect on the epithelium resulting in therapeutic benefit or adverse drug reaction. Drugs not generally associated with diseases of epithelial tissue or abnormalities of mucus secretion may nevertheless have an effect on mucus. This is particularly likely with drugs that affect cellular metabolism, such as adrenaline and other beta-adrenergic agents, beta-adrenergic blocking agents, inhibitors of adenylyl cyclase or phosphodiesterase, prostaglandins and so on (Parke, 1978). Most drugs having effects on mucus e.g. carbenoxolone and indomethacin have been shown to cause qualitative and quantitative changes in mucus glycoprotein synthesis as measured by the rates of incorporation of radiolabelled amino acid and sugar precursors into rat gastrointestinal mucus glycoproteins (Shillingsford, 1975; Dekanski et al, 1975; Johnston et al, 1975).

The practolol reaction, oculomucocutaneous syndrome in man, culminated in the ulceration of the cornea of the eye, nasal and oral mucosae (Wright, 1975; Rahi et al, 1976) psoriasiform changes in the skin (Felix and Ive, 1974) as well as intestinal adhesions and obstructions (Nicholls, 1976). Few observations to this effect have been reported for other beta-adrenergic blocking agents such as oxprenolol and propranolol (Holt and Waddington, 1975;
Cubey and Taylor, 1975). However whether these effects are a direct result of beta-blockade or non-specific reactions is not known.

The peculiar inhibition of mucus glycoprotein synthesis by only practolol amongst the beta-adrenergic blocking agents studied rules out the general involvement of beta-blockade in mucus synthesis. Practolol has been shown to be metabolized in vitro to metabolites which bind irreversibly to liver microsomes and this binding was highest with hamster microsomes (Case et al. 1978). Recent study by Lindup et al. (1979) on the in vivo tissue binding of practolol and its metabolites in hamsters has led to the observation of a linear dose-dependent irreversible binding to the eye. However there was no observation of ocular toxicity after chronic administration to several animal species including hamsters. This binding of practolol or its metabolites to microsomes could result in cellular changes which may lead to the reduction of mucus glycoprotein synthesis probably by inhibiting the synthesis of protein which is the rate-limiting step in the glycosylation process. The observed inhibition of gastrointestinal mucus glycoprotein synthesis by practolol suggests its possible degranulation of the mucosal cell endoplasmic reticulum and hence the probable formation of gastrointestinal tumours which may explain the intestinal obstructions observed in humans by Nicholls (1976). The qualitative changes in glycoprotein synthesis by practolol, in response to its long plasma half-life on prolonged high dosage, may result in the formation of immunoglobulin Es (IgEs), which are glycoprotein in nature, culminating in the psoriasiform changes in the skin.

Extrapolation of Toxicological Study of Beta-adrenergic Blocking Agents to Man

Different animal species can on occasions vary markedly in their response to drugs. This is important because laboratory animal species are commonly
used for the preclinical pharmacology testing and in particular for the safety evaluation of a new drug. Such species variations raise disturbing problems, particularly where the most serious types of toxicity are concerned such as carcinogenicity and teratogenicity. Thalidomide, is teratogenic in the rabbit (Fabro and Smith, 1966) and the rat (King and Kendrick, 1962) but not in the hamster (Hague et al, 1967). The polycyclic aromatic hydrocarbon, 3-methylcholanthrene, is carcinogenic to various species such as the rat, mouse and tree shrew, but not in the rhesus monkey (Adamson, 1972). DDT has been reported to be a weak carcinogen in the mouse but not in the hamster (Wallcave et al, 1973). These species variations therefore raise questions as to the adequacy of the animal model for the human situation.

Interspecies variations in response to toxic substances originate in differences in one or more of the following factors: rate of absorption, binding to plasma proteins and other tissue macromolecules, the mode of excretion, the rate and route of metabolism and the responsive nature of the receptor. Of these, one of the most important is the metabolic factor; many cases of species differences in response to toxic substances in mammalian species arise from metabolic differences, particularly in the nature of the metabolic pathway. These species differences often arise because of interspecies variations in the amounts of active or toxic metabolites formed during the course of biotransformations.

Extensive metabolic studies of most beta-adrenergic blocking agents have been carried out in man and most animal species. This has led to the identification in rats of products that are identical to those found in man suggesting identical pathways of metabolism (Scales and Cosgrave, 1970; Reeves et al, 1978; Bond, 1967; Hayes and Copper, 1971; Leinweber et al,

Recent studies into the safety evaluation of industrial and environmental chemicals have indicated that many compounds are carcinogenic only in the mouse. Many of these chemicals are negative in the Ames test for mutagenicity, implying their function as promoters of carcinogenesis (Epigenetic carcinogens).

The reason why carcinogenesis by this type of compound is so often seen only in mouse liver is probably because of some fundamental species abnormality in microsomal metabolism. A number of such abnormalities are seen with the mouse, which therefore makes it a most unsuitable species for monitoring carcinogenic potential, especially of promoters. Firstly, there is a high rate of hepatic microsomal metabolism in the mouse leading to greater generation of the active metabolite and hence greater damage to the endoplasmic reticulum, and greater potentiation of carcinogenesis, for a given dose of promoter. Secondly, there is a greater predominance of the rough to smooth endoplasmic reticulum in the liver of mouse than in rat, guinea pig or monkey liver (Gram et al, 1971). Also, mouse liver contains much higher activities of mixed-function oxygenase in the rough endoplasmic reticulum than occurs in other species (Gram et al, 1971). These features would suggest that the extent of degranulation of the endoplasmic reticulum for a given amount of active intermediate would be much greater in the mouse than in other species.

A further factor which may make the mouse more vulnerable to the action of promoters is the possible greater level of carcinogenesis initiation activity.
The high rate of microsomal metabolism, consequent degranulation and hence carcinogen activation, would potentiate the effect of environmental mutagens so that a given dose would lead to greater DNA damage than occurs in other larger species. Furthermore, there is evidence that there is a much higher level of oncogenic viruses present in the mouse than in most other species. All these factors would explain the anomalous behaviour of the mouse in chemical carcinogenesis, and would indicate that the formation of hepatocellular carcinoma in this species, is a spurious index of the tone carcinogenic potential of these chemicals in other animal species including man.

Long-term carcinogenicity studies of pronethalol in rats, mice and guinea-pigs, have led to the identification of thymic tumours in mice but not in the other species (Paget, 1963; Alcock and Bond, 1964; Howe, 1965). This led to the postulation of a hypothetical ethyleneimine derivative of pronethalol in mice as the proximate carcinogen responsible for the tumorigenic properties of pronethalol. This tumorigenicity potential observed in the mouse by pronethalol, and not in the other species, may be the result of the abnormalities in metabolism in the mouse.

Thus, although there is no evidence of tumorigenicity potential of beta-adrenergic blocking agents in man their probable induction of the mixed-function oxygenases, after prolonged high dosage, as demonstrated in the rat by pronethalol and propranolol, necessitates further studies in man since this may lead to chemical interactions resulting in adverse reactions including tumour formation. Also the anomalous inhibition of rat gastrointestinal mucus glycoprotein synthesis, irrespective of changes in cyclic nucleotide levels as a result of beta-blockade, by practolol may help explain the ulceration of the nasal and oral mucosae, the intestinal obstructions and adhesions, and probably the ulceration of the cornea of
the eye, in man after prolonged treatment. Hence the development of in vitro systems for the investigation of mucus glycoprotein synthesis with various human tissues would be of great interest.

It may be said that the mutagenic and epigenetic or enzyme induction studies have shown no major implications of the group of beta-blockers as a whole for mutagenicity or tumorigenicity, although there are indications for certain of these drugs. The cyclic nucleotide studies reveal no major adverse effects and the glycoprotein studies show that practolol is anomalous in its inhibitory effect, which may have some bearing on the mechanism of its known toxicity. In summary, the group of beta-blockers as a whole show no pattern of toxic or mutagenic effects, but specific drugs do show tendencies which at high tissue concentrations that may result from overdosage or impaired metabolism or excretion, might give rise to adverse side-effects in susceptible populations, especially those where genetic impairment of metabolism is present.
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