DALHOUSIE UNIVERSITY

Date September 10, 1986

Author SHABBIR MOHAMMED MOOCHHALA

Title REGULATION OF MIXED FUNCTION OXIDASE BY INTERFERON

Department or School Department of Pharmacology

Degree Ph.D. Convocation Fall Year 1986

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.

THIS THESIS IS

DEDICATED TO MY

PARENTS,

MOHAMMED AND KHEROON

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS AND SYMBOLS USED IN THIS THESIS

ACD	actinomycin D
ADP	adenosine diphosphate
AHH	aryl hydrocarbon hydroxylase
ALA	δ-aminolevulinic acid
ALAV	amininolevulinic acid
BNF	β-naphthoflavone
BSA	bovine serum albumin
°C	degree centrigrade
Ci	curie
dextran SO_4	dextran sulfate
DNA	deoxyribonucleic acid
d.p.m.	disintegration per minute
E	extinction coefficient
ER	endoplasmic reticulum
g	acceleration due to gravity
g	gram
HCHO	formaldehyde
hr	hour
IFN	interferon
IL-1	interleukin-1
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous

KD	kilodalton
Kd	degradaton rate constant
Km	Michaelis constant
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M	molar
3-MC	3-methylcholanthrene
mol wt.	molecular weight
M.W. (Mr)	molecular weight
NADH	diphosphopyridine nucleotide, reduced form
NADPH	triphosphopyridine nucleotide, reduced form
NK	natural killer (cell)
nm	nanometers
PBS	phosphate buffered saline
PRD_{50}	units used to express the concentration of interferon that reduces virus plaques by 50%
Rf	relative mobility
RNA	ribonucleic acid
r.p.m.	revolution per min
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
$t_{\frac{1}{2}}$	half-life
TCA	trichloroacetic acid
t-RNA	transfer ribonucleic acid
UV	ultraviolet

Vmax	maximum velocity of uptake wavelength
XO	xanthine oxidase

Prefixes for units of measurement:

n	nano	(10^{-9})
μ	micro	(10^{-6})
m	milli	(10^{-3})
K	kilo	(10^{3})

ABSTRACT

IFN-αCON1 is a synthetic interferon (IFN) produced in Escherichia coli from a constructed gene, which incorporates the most frequently observed sequence seen in the naturally occurring human α-interferon subtypes.

Administration of IFN or poly IC (an interferon inducer) into hamsters caused an initial increase (3 hrs and 6 hrs after IFN or poly IC treatment respectively) in hepatic (as well as extrahepatic) cytochrome P-450 content, followed by a significant depression within 24 hrs.

The early increase in apocytochrome P-450 content by interferon was shown to be associated with increased protein synthesis de novo. This conclusion was based on the following. (a) Induction of cytochrome P-450 by IFN was prevented by protein synthesis inhibitors (b) increase in radiolabelled amino acid incorporation into hepatic microsomal apocytochrome P-450 fractions, (c) increase in in vitro translatability of hepatic mRNA into proteins, including apocytochrome P-450.

The decrease in apocytochrome P-450 content 24 hrs after IFN or poly IC treatment was due to a decrease in protein synthesis. This conclusion was based on the following: (a) decrease in radiolabelled amino acid incorporation into hepatic microsomal apocytochrome P-450 fractions (b) both rate of synthesis and degradation of apocytochrome P-450 was decreased, (c) decrease in in vitro translatability of hepatic mRNA into proteins, particularly to apocytochrome P-450. The decrease in cytochrome P-450 caused by IFN treatment was prevented by protein synthesis inhibitors, suggesting the involvement of protein intermediate(s).

Interferon-induced xanthine oxidase activity (XO), may be responsible for the depression of cytochrome P-450 via reactive oxygen intermediates. In this study, the activity of the (D) form of xanthine oxidase was increased by IFN (3 hr treatment) without any increase in the (O) form of XO (responsible for generation of free radicals). After 24 hrs of IFN treatment, the (O) form of XO was increased by 2-fold and cytochrome P-450 was depressed. The induction of XO and the depression of cytochrome P-450 were prevented by protein synthesis inhibitors and allopurinol (xanthine oxidase inhibitor) and α-tocopherol (free radical scavenger). This suggests XO may play a role in the IFN mediated loss of mixed function oxidase.

ACKNOWLEDGEMENT

I would like to express my thanks and sincere appreciation to my teacher and friend, Dr Kenneth W. Renton, for his guidance throughout the research work and preparation of this manuscript His frank discussions, valuable criticisms and above all, his encouragement has made this work possible and worthwhile.

I also wish to express my thanks to Dr P E. Dresel and Dr M.M Vohra for their invaluable support, not only in the area of research but also in many other areas during the course of my studies. Though it's not possible to list each and every faculty member by name, the author wishes to express his indebtedness to them as well.

I would like to thank Mrs. Myrna Espiritu for excellent technical assistance and advice which was in large measure responsible for the quality of the research described. I also wish to thank Dr. Nowell Stobbing who kindly supplied me with the interferon that made this study possible. I also thank Ms Shirley Dean for the EM studies.

Many thanks to Ms. Elaine Wright, Ms Allison Edwards and Mrs. Caroline Renton who helped in the preparation of this thesis. I would also like to thank Debbie Monminie and Marilee Matheson for the excellent typing of this thesis. Special thanks also go to Debbie Monminie for the ungodly hours she had to put up with in typing this thesis.

I would like to thank Ms. Janet Murphy, the "classic administrator", for solving my many administrative problems.

I gratefully acknowledge the financial support from Dalhousie University and the Canadian Heart Foundation which made these studies possible

Finally, I wish to thank the graduate students of the department for their friendship. I will never forget the "special friends" I have made over the years in the department.

INTRODUCTION

The onset, intensity and duration of drug effects depend to a large degree in achieving and maintaining an appropriate concentration of the agent at its site of action. This concentration is dependent on factors such as absorption, distribution, metabolism and elimination. The biotransformation of drugs plays a key role in elimination processes by converting lipophilic drugs to more polar metabolites which can be readily excreted in the urine and bile. Most drug biotransformation occurs in parenchymal cells of the liver, where the drug metabolizing enzyme system is located in the endoplasmic reticulum (ER) which consist of a network of interconnected channels present in the cytoplasm. Unlike some cellular organelles, the ER cannot be separated from cells as an intact structure, however, when liver cells are homogenized and then centrifuged, the tubular reticulum breaks up, and sections of the membranes are "pinched off" to form tiny vesicles, called microsomes. The microsomal fraction seperated from liver homogenates is a convenient source of the oxidative enzyme system and is used for the laboratory study of xenobiotic biotransformation by the mixed function oxidase system.

Section I of this introduction is a general review of hepatic mixed function oxidase. Included is a review of the cytochrome P-450 component of the mixed function

oxidase and factors affecting the steady state levels of cytochrome P-450 through inductive and inhibitive processes.

Section II reviews the regulation of cytochrome P-450 turnover.

Section III reviews the relationship between host defence mechanism and cytochrome P-450 system.

Section IV is a review of interferon with cadence on a particular synthetic alpha type interferon called interferon alpha consensus one (IFN-αCON$_1$).

Section V reviews the relationship between interferon and interferon inducers and cytochrome P-450 system.

Section VI is the formulation of the problem.

SECTION I

A. General review of hepatic mixed function oxidase system

Exhaustive study of mechanisms of drug oxidation at subcellular levels first began in 1948-1949 by Mueller and Miller (1949) who detailed the reductive cleavage of an azo dye by rat liver homogenates and demonstrated a requirement for reduced NADP and oxygen (Mueller and Miller 1953). Subsequently this enzyme system, in the microsomal fraction of the liver, was shown to be capable of oxidizing a wide variety of different substrates by several diverse reactions including N-dealkylation, aromatic hydroxylation,

deamination, sulphoxidation, O-dealkylation, S-oxidation and N-oxidation (Brodie, 1956; Gillette, 1966).

Presently, this oxidative drug-metabolizing enzyme system is known to consist of a flavoprotein reductase, a phospholipid fraction, and a hemoprotein, named cytochrome P-450, which functions as the terminal oxidase (Gillette et al., 1972). This system is also referred to as mixed-function oxidase or monooxygenase as it requires NADPH and molecular oxygen, and catalyzes the transfer of one atom of molecular oxygen to the substrate, forming hydroxylated intermediates or products, while the second oxygen atom appears in water. A schematic representation of microsomal electron transport is shown in Figure 1.

As indicated, the primary electron donor is NADPH and the flavoprotein reductase, NADPH cytochrome P-450 reductase (also referred to as NADPH cytochrome c reductase) catalyzes the electron transfer to cytochrome P-450. Drug substrate binding occurs with the oxidized form of this hemoprotein and the drug-cytochrome P-450 complex accepts the electrons from NADPH-cytochrome P-450 reductase to form the reduced cytochrome P-450-drug complex. The reduced complex combines with molecular oxygen and, after a second electron transfer to the oxygenated complex, the hydroxylated substrate, water, and oxidized cytochrome P-450 are generated. Electrons from NADH via cytochrome b_5, other than from NADPH via NADPH-cytochrome P-450 reductase

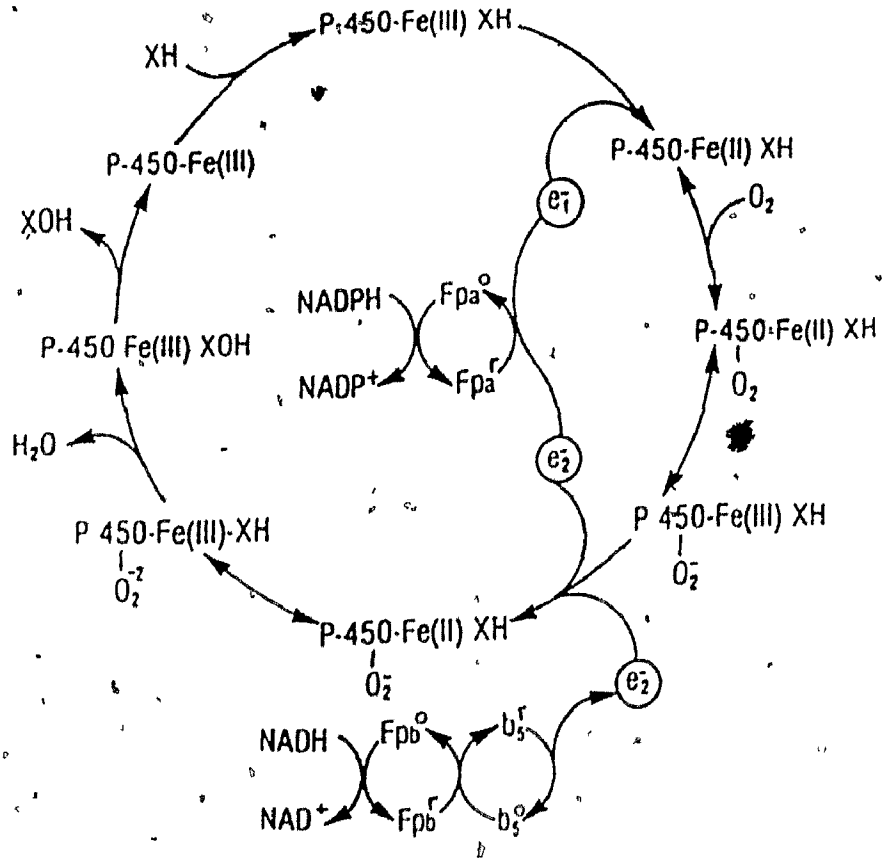


Figure 1

Redox cycle of cytochrome P-450 during oxidation of drugs XH: substrate; Fp_b: NADH-cytochrome b_5 reductase, Fpa: NADPH-cytochrome P 450 reductase, b_5 cytochrome b_5, P 450 (II): reduced cytochrome P-450; P-450 (III): oxidized cytochrome P-450. (Gander, J.E. and Mannering, G.J., 1980).

may contribute to the second electron input on the cytochrome P-450 complex (Gander and Mannering, 1980).

B. Cytochrome P-450

Historically, Klingerberg(1958) and Garfinkel (1958), were the first to describe the existence of a carbon-monoxide binding pigment in mammalian liver. Subsequently, Omura and Sato (1964) characterized this pigment and named it cytochrome P-450 due to the maximum absorption of the reduced hemoprotein carbon monoxide complex at 450 nm. Ryan and Engel (1957) had initially suggested the participation of cytochrome P-450 as the terminal oxidase in the hydroxylation of drugs and steroids and later Cooper et al. (1965) demonstrated directly that cytochrome P-450 was the terminal oxidase for the mixed function oxidase system.

Presently cytochrome P-450 is a generic term applied to a family of b-type cytochromes found in all phyla; bacteria, yeast, plants, insects and vertebrates. The reason for such ubiquity of cytochrome P-450 was reviewed by Nebert and Gonzalez, (1985), who reasoned for its existence by suggesting that early in evolution "primitive P-450 must have been required for intracellular biosynthesis and degradation of endogenous substrates critical to the organism's life function" (eg. steroids, fatty acids, biogenic amines, pheromones, leukotrienes and prostaglandins). Later in evolution, "by broadening their

substrate specificity, these proteins then began to metabolize foreign chemicals as an energy source; a "sensing device" also must have evolved about this time" (pseudomonads, fungi). Much later in evolution, cytochrome P-450 functions evolved to "metabolic activation and detoxification especially in organisms with a digestive tract and a liver".

The broad range of substrate specificity known to exist for the hepatic mixed function oxygenase systems led to the proposal that multiple forms of cytochrome P-450 existed. Evidence presented in support of the existence of multiple forms of cytochrome P-450 includes: alteration in substrate specificities (Powis et al., 1977); kinetic parameters (Alvares and Mannering, 1970), spectral properties (Sladek and Mannering, 1966), sensitivity towards inhibitors (Testa and Jenner, 1981) and electrophoretic banding patterns of microsomal cytochrome P-450 following the pretreatment of laboratory animals with various chemical inducing agents such as phenobarbital and 3-methylcholanthrene (Welton and Aust, 1974).

Subsequently, methodologies were developed for the purification of hepatic microsomal cytochrome P-450 in an active form (Van der Hoevein et al., 1974; Lu and Coon, 1968; Sato et al., 1973; Gibson and Schenkman, 1978), which enabled the purification and resolution of a number of cytochrome P-450 isozymes from the hepatic microsomes of

rats, rabbits, mice and humans (Lu and West, 1980; Guengerich, 1979). These hemoproteins generally have molecular weights ranging from 45,000 to 60,000 daltons as estimated on sodium dodecyl sulphate polyacrylamide gel electrophoresis. A number of cytochrome P-450 isozymes have been isolated in a highly purified state and shown to differ in their molecular weights, amino acid compositions, spectral properties, immunochemical properties and catalytic properties, including their substrate specificities and their regiochemical and stereo chemical selectivities for model substrates (Lu and West, 1980) These isozymes have also been shown to exhibit marked structural differences as demonstrated using limited proteolysis techniques (Cheng and Schenkman, 1982) and from protein sequencing of their respective amino termini (Bothello et al., 1982) and carboxy termini (Cheng and Schenkman, 1982).

Recent advances in the application of recombinant DNA techniques (Fujii-Kuriyama et al., 1984) and monoclonal antibodies (Sesardic et al., 1986) to specific cytochrome P-450 isozymes have certainly advanced our understanding of not only the existence of multiple forms of cytochrome P-450 but also the elucidation of the genetic, molecular and evolutionary mechanisms responsible for the existence of a multiplicity of forms of cytochrome P-450 and the induction

of distinct forms by specific inducing agents (Whitlock, 1986).

Although initial purification studies were performed using chemically induced animals, recent attention has focussed on the constitutive isozymes (Cheng and Schenklman, 1982) since they are apparently more numerous and probably have more pharmacological relevance. Several distinct forms of cytochrome P-450 which have low basal contents in uninduced animals can be substantially increased (30 to 50 fold) following the treatment of animals with classic inducers such as phenobarbital, 3-methylcholanthnene (or β-napthoflavone) or Aroclor 1254 (a mixture of polychlorinated biphenyls) (Guengerich et al., 1982). Presently there appears to be at least four distinct classes of inducers, which induce distinct subsets of P-450 isozymes suggesting that these isozymes are under separate genetic regulatory control (Goldstein, 1984). These classes include (1) phenobarbital and a wide variety of structurally unrelated drugs and environmental chemicals; (2) polycyclic aromatic hydrocarbons such as 3-methylcholanthrene or β-naphthoflavone; (3) pregnenolone 16-α-carbonitrile (PCN) and certain other steriods; and (4) ethanol. Clofibrate, a hypolipidemic drug, is another good contender to be the model for a fifth class of inducer. Unfortunately, there is no generally accepted nomenclature for the different isozymes of cytochrome P-450. The term

"cytochrome P-450" at the present moment is used in a generic sense to denote any or all forms of the hemoprotein. Derivation of specific forms of cytochrome P-450 have been designated for different species (Nebert et al., 1985) on the basis of their decreasing mobility and increasing molecular weights by SDS-PAGE.

Cytochrome P-450 is also found in extrahepatic tissues. These include the kidney, small intestine, lung, adrenal cortex, skin, spleen, testis and placenta (Gram, 1980). Only recently several studies are beginning to compare the cytochrome P-450 present in mammalian liver with those present in extrahepatic tissues. These studies have usually compared the content of specific cytochrome P-450s before and after treatment of animals with various inducing agents and by exploiting the immunological, electrophoretic, catalytic and physical properties of the various cytochromes P-450. Generally the enzyme activity in these extrahepatic sites is a small fraction of that localized in the liver. The extrahepatic metabolism of xenobiotics is, therefore, probably of minor significance with respect to the overall biotransformation rate and the capacity of the animal to excrete drugs. However, the intratissue metabolic activity may be important with respect to local drug concentration and thus to the intensity of local drug effects, or toxicity may play a physiological role in the [redacted]duction of fatty acids,

prostaglandins and steroids (Bend and Serabjit-Singh, 1984). Recent studies have shown that the cytochrome P-450 in extrahepatic organs often shows tissue specificity for the presence or inducibility of certain forms of cytochrome P-450.

C. Inducers and inhibitors that alter the steady-state levels of cytochrome P-450

The steady state level of cytochrome P-450 and the capacity of the liver to metabolize drugs can be altered by many endogenous chemicals, physiological factors and pathophysiological states. Alteration of cytochrome P-450 content will increase or decrease the elimination rate of a drug and result in plasma drug levels below therapeutic level or result in the accumulation of a drug to toxic levels. Many endobiotics and xenobiotics are known to modify cytochrome P-450 dependent pathways by induction or inhibition. As mentioned earlier, certain species of cytochrome P-450 are induced by agents such as phenobarbital, 3-methylcholanthrene (or β-naphthofavone), pregnenolone 16α-carbonitrile, ethanol and clofibrate. Induction of the enzyme system by different inducers can result in relatively specific increases in the types of substrate which are metabolized in the liver. For example, ethylmorphine and aminopyrine N-demethylase are best induced by phenobarbital and benzo(a)pyrene hydroxylation

and ethoxyresorufin O-deethylation are induced by 3-methylcholanthrene (or β-napthoflavone) The latter type of inducer produces distinct species of cytochrome P-450 commonly known as cytochrome P-448 and P1 450 (Nebert et al., 1980). Lauric acid hydroxylation is strongly induced by clofibrate without affecting other species of cytochrome P-450 (Tamburini et al., 1984). This agent induces another species of cytochrome P-450 named cytochrome P-452. Pregnenolone 16 α-carbonitrile (others e.g. includes glucocorticoids) and ethanol induce the metabolism of aminopyrine (or ethylmorphine) and ethanol oxidation respectively.

Inhibitors of cytochrome P-450 dependent mixed function oxidase as outlined by Netter (1980) include the following classes: a) Binding to the substrate binding site (e.g. cimetidine), b) Binding to the heme iron or in its vicinity, thereby diminishing binding of oxygen (e.g. carbon monoxide), c) Yielding a metabolite that binds firmly also under reducing conditions (e.g. piperonyl butoxide), d) Inactivating cytochrome P-450 by transformation to cytochrome P-420 (deoxycholate or steapsin), e) Inhibiting cytochrome P-450 reduction (e.g. antibodies to cytochrome c reductase), f) Diversion of electrons from NADPH (e.g. to cytochrome c), g) In vivo influences of nutrition (e.g. low protein diet), or toxicants (e.g. cobalt ions), or agents that stimulate or

depress host defence mechanisms or even inducers that produce a variant not as active towards a given substrate (e.g. cytochrome P-448).

SECTION II

A. The turnover of hepatic hemoproteins

Hemoproteins, which contain both a heme moiety and protein moiety (apocytochrome), can be broadly categorized into five major classes according to their functions (Granick and Gilder, 1947): (a) Oxygen transporting hemoproteins (e.g myoglobin and hemoglobin); (b) Oxygen activating hemoproteins (e.g. cytochrome oxidase, cytochrome P-450 and tryptophan pyrolase); (c) Peroxidases, enzymes that activate hydrogen peroxide; and (d) catalases, enzyme that decompose hydrogen peroxide. The biosynthesis of a functional hemoprotein or holoenzyme requires the coordinated synthesis of its prosthetic heme moiety and the apocytochrome. In addition, functional cytochrome P-450 (or holoenzyme) requires to be inserted into the membrane of endoplasmic reticulum. Hence the steady state level of functional cytochrome P-450 (holoenzyme) present in the cell depends on a) turnover of the heme moiety, b) turnover of the apocytochrome, c) followed by the assembly of the heme moiety and the apocytochrome into the holohemoprotein (functional cytochrome P-450) (Tait, 1978). The

biosynthetic pathway of hemoprotein is given in the following scheme (Figure 2).

(i) Turnover of heme in cytochrome P-450

The term heme refers to a complex of ferrous iron linked only to the four nitrogen atoms of a tetrapyrrole nucleus (Falk, 1964) It is commonly used in reference to b type heme, which constitutes the prosthetic moiety of hemoproteins including cytochrome P-450, hemoglobin, myoglobin, catalase, peroxidase and mitochondrial and microsomal cytochrome b_5. The other heme types such as type c and type a, corresponds to that found in mitochrondrial cytochromes. Unlike most other prosthetic groups which are derived from dietary sources, the heme moiety is biosynthesized entirely in the hepatocytes via the metabolic pathway illustrated in Fig. 3.

The formation of the protoporphyrin molecule requires 8 molecules of glycine and 8 molecules of succinic acid and is synthesized through a sequence of reactions involving a condensation, followed by decarboxylation, and then another condensation which is followed by a series of oxidations (Figure 3). These reactions are catalyzed by enzymes present in the mitochondrial as well as the cytosolic fraction of the hepatocytes. ALA synthetase (found in the inner mitochondrial membrane), the enzyme responsible for the condensation of glycine and succinyl CoA, is rate

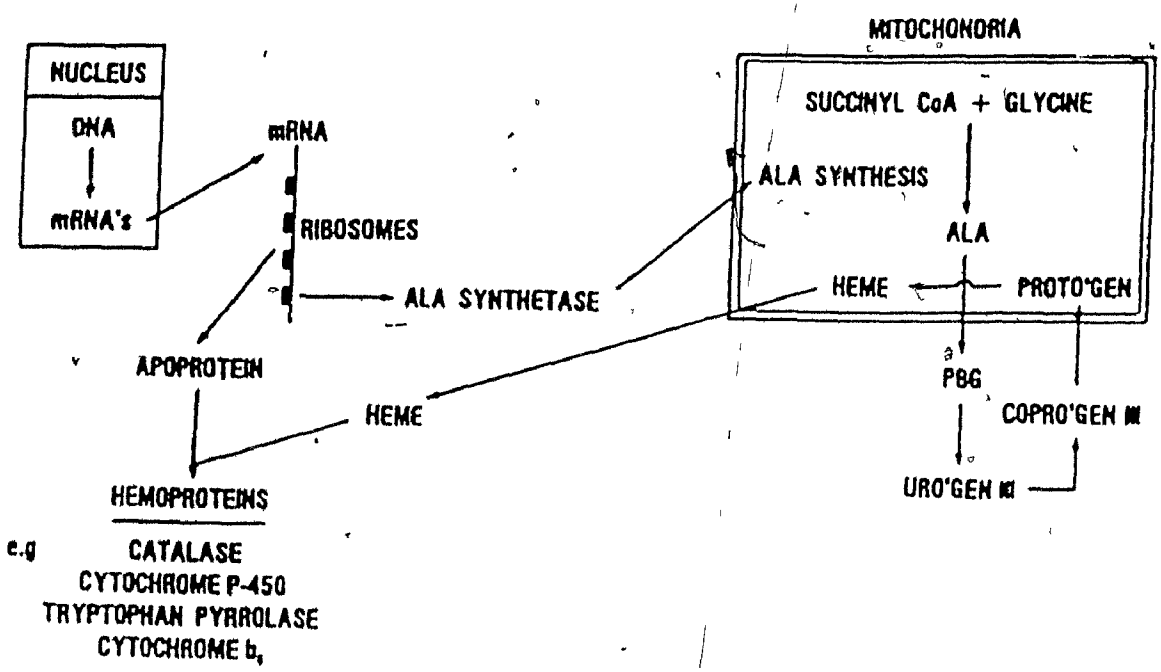
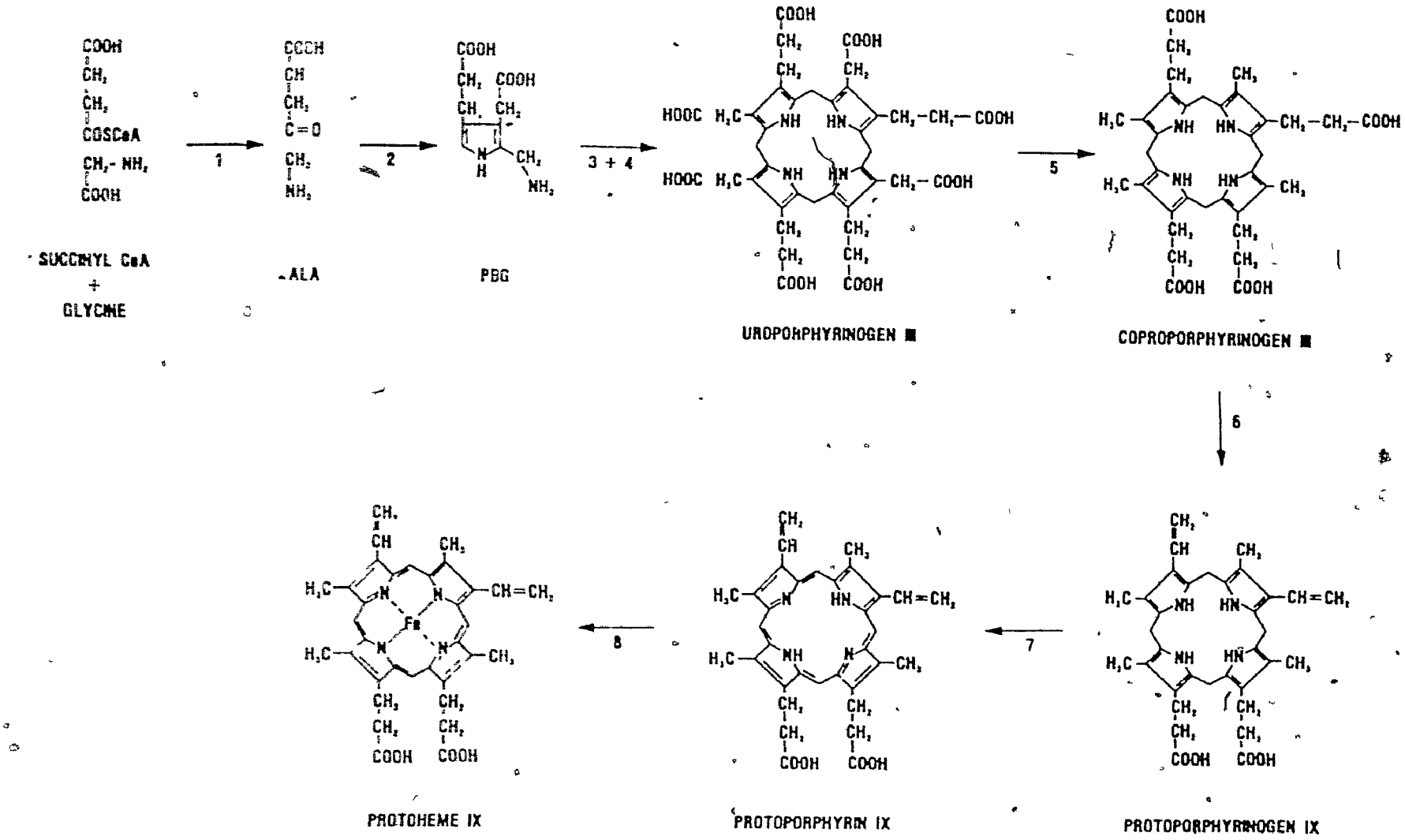


Figure 2

Biosynthesis of hemoprotein.
(Tait, G.H., 1978.)

Figure 3

Biosynthesis of heme enzymes catalyzing the interconversions are: 1. ALA synthetase. 2. ALA dehydratase. 3. PBG deaminase 4 Uroporphyrinogen III cosynthetase. 5. Uroporphyrinogen decarboxylase. 6. Coproporphyrinogen oxidase. 7. Protoporphrinogen oxidase. 8. Heme synthetase. (Tait, G.H., 1979).


SUCCINYL CoA
+
GLYCINE
1
ALA
2
PBG
3 + 4
UROPORPHYRINOGEN III
5
COPROPORPHYRINOGEN III
6
PROTOPORPHYRINOGEN IX
7
PROTOPORPHYRIN IX
8
PROTOHEME IX

limiting and is under negative feedback control (Tait, 1978).

Heme is degraded to the bile pigment, bilirubin, through a two step process involving an oxidation and a reduction. The reactions are catalyzed by the enzymes present in the microsomal membranes (heme oxygenase) and the cytosolic fraction (bilirubin reductase) in all mammalian cells tested to date (Maines, 1985). Newly synthesized heme effluxes into the cytoplasm, from the mitochondria, where it mixes with exogenous heme transported from plasma. The exogenous heme from plasma is transported into the hepatocyte by hemopexin, a heme binding serum protein (Smith and Morgan, 1979). In the cytosol heme accumulates in and enhances the activity of cytosolic tryptophan pyrrolase (Badawy and Evans, 1975), or incorporates into catalase in the peroxisomes (Lazarow and De Dure, 1973) besides incorporating into cytochrome c in mitochondria or cytochrome P-450 and b5 in the endoplasmic recticulum.

Presently it is generally accepted that there is an intracellular "heme pool" which contributes to the regulation of heme metabolism. This "heme pool" has been implicated in certain intracellular functions such as a) to inhibit the formation of ALA-synthetase as well as the transfer of pre-ALA-synthetase into mitochondria (Ades, 1983; Strivastava et al., 1983), b) to induce heme

oxygenase (Bissell and Hammaker, 1976), c) it may exchange between different molecular species of microsomally-bound P-450 (Sadavo and Omura, 1983). Contraction of this heme pool may occur during hepatic porphyrias and an expansion could occur in either of two states: namely, an increase in heme synthesis above that necessary for heme protein production (result of hemolytic anemia) or an increased influx of heme into cell (therapeutic administration of heme for treatment of hepatic porphyrias). Contraction of the heme pool would result in the induction of [redacted] synthetase (Maines, 1985), enhance the synthesis of heme-transporting proteins in an effort to seek heme extracellularly (Foidart, 1982) or to cause a shift in the compartmental heme distribution within the cell. Decrease in the heme pool has also been implicated to inhibit the synthesis of the apocytochrome P-450 in order to prevent utilization of heme (Guzelian et al., 1979; Ravishankar and Padmanaban, 1983). An expansion of the heme pool would lead to induction of heme oxygenase, increase or decrease in ALA-synthetase (Maines, 1984) and to an accumulation of heme in tryptophan pyrrolase. It is interesting to note that during chronic hemolysis in mice, hepatic ALA-synthetase and cytochrome P-450 levels are not decreased despite marked induction of heme oxygenase, suggesting the development of some adaptive mechanism after chronic exposure to heme (Sassa et al., 1979).

Presently the consequences of disturbed production or the utilization of hepatic heme is not fully known. Further, it is controversial whether heme oxygenase is capable of degrading heme while it is attached to the apoprotein.

(ii) Turnover of apocytochrome P-450

It is generally known that the apocytochrome P-450 is synthesized in the endoplasmic reticulum and combines with heme, supplied by the mitochondrial heme synthesis system, to form the holoenzyme. Thus it is appropriate to review the turnover of proteins in the endoplasmic membrane before proceeding to describe the regulation of turnover of apocytochrome P-450.

The study of turnover rates of cellular constituents of animal tissues in vivo by administering a single dose of a suitable labelled percursor compounds to animals and then measuring the loss of the label from those particular constitutents was first used by Reiner in 1933. An advanced modification of this method is the double labelling technique as proposed by Arias et al. (1969). This method was employed in this thesis utilizing [^{3}H] and [^{14}C] labelled leucine as the labelled precursor of protein. Various radioactive amino acids can be utilized, however it must be noted that serious problems inherent to all these methods are the reutilization of the label in the animal (Swick and Ip, 1974). It must also be noted that

the estimated turnover rates of microsomal proteins which have utilized isotopic tracers are slower than the real turnover rates when necessary corrections for unequal initial incorporations of the two isotopes are not made (Schimke, 1974). Recently, [^{14}C]-$NaHCO_3$ is used to label in vivo arginine to minimize reutilization. This is because arginase in hepatocytes can rapidly degrade arginine to urea and thereby minimize reutilization of this amino acid.

The membranes of the endoplasmic reticulum (ER) represents the most dynamic portion of the intracellular membrane system (Omura, 1980). The average half-life of ER protein in rat liver is 60 to 70 hours, which is considerably shorter than the half-life of proteins in other organelles. Investigation of individual proteins demonstrates that their turnovers differ and range between a few hours and several days (Table 1). Presently it is clear that individual enzymes are synthesized and degraded independently, indicating that membrane formation and degradation are complex processes that involve many synthetic and degradation reactions that occur simultaneously at different rates. Newly synthesized membrane proteins must be placed into an already existing membrane as single components and must also be removed in single reactions. Even enzymes which interact functionally and lie close to one another in the membrane, such as the

TABLE 1

Turnover rates of microsomal enzymes in rat liver

Enzymes	Half-Lives (hr)
Stearyl CoA desaturase	4
HMG-CoA reductase	4
Nucleoside diphosphatase	30
Cytochrome P-450 (protein)	40
Cytochrome P-450 (heme)	22
NADPH cytochrome P-450 reductase	70
Arylacylamidase	100
Cytochrome b_5 (protein)	100
Cytochrome b_5 (heme)	45
NADPH cytochrome b_5 reductase	140
Total microsomal membrane protein	70

From Omura, 1980.

electron transport enzymes (e.g. NADPH cytochrome c reductase), exhibit different turnover rates. Different parts of the same enzyme such as the apocytochrome and the heme prosthetic group of cytochrome P-450 may exhibit different turnover rates (Greim et al., 1970).

It is well known that the rate of both synthesis and degradation influences the steady-state level of an enzyme. Thus alteration of the rate of synthesis or of degradation or both (e g. by drugs or hormones) can affect the level of the enzyme. Recent studies utilizing highly specific antibodies to microsomal proteins from animals treated with [^{14}C]$NaHCO_3$ and ^{3}H-labelled ALA have aided in determining turnover rates of individual microsomal proteins. Pertaining to the proteins of the mixed functions oxidase, Sadano and Omura (1983) examined two cytochrome P-450 isozymes, NADPH cytochrome P-450 reductase and cytochrome b_5; Parkinson et al. (1983) examined three cytochrome P-450s and epoxide hydrolase and Shiraki and Guengerich (1984) examined seven cytochrome P-450 isozymes, NADPH cytochrome P-450 reductase and epoxide hydrolase. All of these studies have used specific inducers (such as phenobarbital, β-naphthoflavone and Aroclor 1254) of cytochrome P-450 isozymes except the latter study, in which cytochrome P-450 turnover rates were also determined in untreated rats. These studies have shown conclusively that a) the rate of synthesis of the apoproteins (Ks) follows a

zero-order process (i e. rate of synthesis is not dependent on the concentration of the protein present) and rate of the apoprotein degradation (Kd) follows first-order process (i.e. rate of degradation is dependent on protein concentration), b) the degradation rates of different cytochrome P-450 isozymes are similar, and cytochrome P-450 inducers (phenobarbital and β-napthoflavone) have no effect on these rates, c) rate of synthesis of individual cytochromes P-450 appear to be different when the specific isozymes are induced by specific inducers, d) the heme moiety of cytochrome P-450 is degraded more rapidly than the apocytochrome (Shiraki and Guengerich, 1984). The last viewpoint indicates that the heme of cytochrome P-450 can reversibly dissociate in vivo. This dissociation dictates that the rate of heme turnover cannot be used as accurate estimates for apocytochrome half-lives. Heme exchange in cytochrome P-450 is undoubtedly feasible since exogenous heme administered in vivo can reconstitute the apoprotein moiety of cytochrome P-450 formed either by chemically destroying the heme prostetic group of pre-existing cytochrome P-450 (Farrell and Correia, 1980) or by inhibiting heme biosynthesis during the induction of the apocytochrome (e.g. by phenobarbital).

The mechanism for the synthesis of apocytochrome P-450 has been a subject of considerable interest in recent years. Figure 4 shows a simplistic pathway of protein

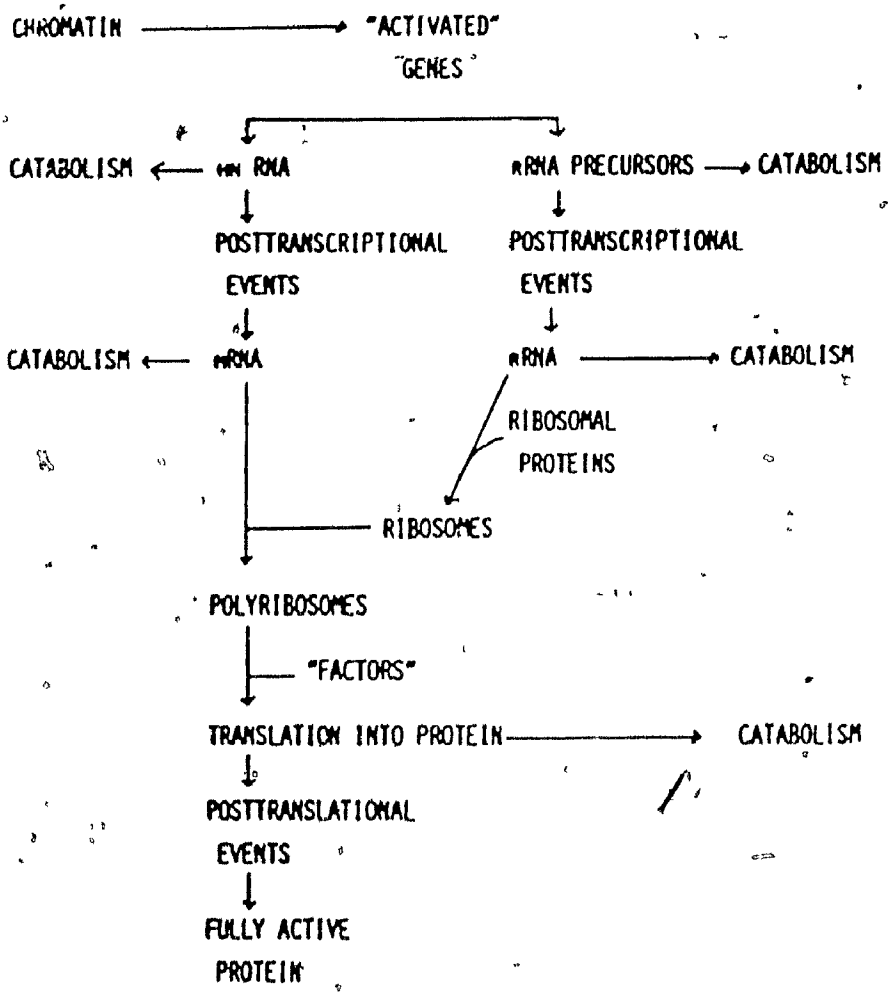


Figure 4

Biosynthesis of proteins.

synthesis. Recent reviews by Adesnik and Whitlock (1986) have presented some of the recent developments on the possible mechanisms of regulating cytochrome P-450 gene expression through application of recombinant DNA methods. Regulation of expression of genes can occur at different steps along the pathway of mRNA formation Control at the level of transcription occurs for some developmentalally regulated genes and hormone-inducible genes. In addition, foreign compounds such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), polycyclic aromatic hydrocarbons or phenobarbital can at the level of transcription induce, the genes coding for cytochrome P-450 and other drug-metabolizing enzymes (Israel and Whitlock, 1984). The mechanism for selective transcription for individual cytochromes P-450 require further study, although the mechanism for the activation of the TCDD-inducible cytochrome P-450 ($P_1$450 and $P_3$450) gene is nearly complete. Following transcription, the RNA precursor undergoes several postranscriptional events which involve in some cases, addition (e.g. polyadenylation and capping), trimming (e.g. endonuclase activity to remove intervening sequences material) and addition of proteins prior to exit from the nucleus into the cytoplasm. The amount of mature RNA extruded from the nucleus is usually less than that which was transcribed. Hence the rate of catabolism of the precursor RNA represents an area of metabolic regulation.

With the components of translation in place in the cytoplasm (example mRNA and ribosomes), protein synthesis can then occur with positive control by protein factors of initiation, elongation and termination. Amino acids, ATP, GTP, monovalent (K^+) and divalent (Mg^{2+}) cations, transfer RNAs (tRNAs), mRNA, ribosomal units and many protein factors interact in a precise fashion to complete various steps

There are several ways in which the synthesis rate of a protein can be affected. (1) A transcriptional increase or decrease in the synthesis of precursor mRNA. Phenobarbital and 3-methylcholanthrene have been shown to increase selectively mRNA precursors of cytochrome P-450. This induction could be blocked by actinomycin D (a DNA dependent RNA synthesis inhibitor). Heme may also regulate the level of cytochrome P-450 gene expression. Using complementary DNA as a hybridization probe, Ravishankar and Padmanaban (1985) showed that inhibitors of heme biosynthesis (cobalt chloride and 1,2,4-triazole) inhibited the phenobarbital-stimulated increase in the rate of cytochrome P-450 expression. (2) An increase or decrease in the processing of precursor mRNA to mature mRNA (or perhaps an increase or decrease in selection of a particular mRNA from a pool of rapidly turning over RNA in the nucleus). (3) An increase or decrease in the rate at which the mRNA is translated into enzyme molecules. These

would depend on the above mentioned factors required for protein synthesis It is known that in eukaryotic systems, the number of ribosomes represent a rate-limiting component in the translational process. The steady state level of ribosomes is determined by the rates of catabolism and anabolism of these organelles. Argyris and Heinemann (1975) had reported that injection of 3 methylcholanthrene resulted in an increase in the amount of ribosomal RNA. Other workers (Hopkinson and Bresnik, 1975) have shown that 3-methylcholenthrene increased the half-life of liver polyribosomes resulting in their greater stability. This increase in steady state levels of ribosomes were also observed following phenobarbital administration (Smith et al , 1972) Increases in the activity of mRNA following phenobarbital administration was first observed by Kato et al, (1966). Later, Bresnick et al., (1981) demonstrated that 3-methylcholanthrene could increase the activity of mRNA. In the latter study, cytoplasmic RNA was isolated from livers of control and 3-methylcholanthrene-pretreated rats and the mRNA activity was assayed with an in vitro rabbit reticulocyte system with [^{35}S]-methionine as the amino acid precursor. Immunoprecipitation of the translated product followed by fractionation via gel electrophoresis gave a positive signal for an increase in activity of cytochrome P-450 mRNA. On the other hand, heme or protein kinase (activated by interferon) can inhibit

phosphorylation of the initiation factor and thereby inhibit protein synthesis.

The information on the mechanism for microsomal protein degradation, especially cytochrome P-450 apoprotein, is relatively unknown at present. The role of lysosomes in the degradation of cellular proteins is well recognized and morphological observation often confirms the engulfment of endoplasmic reticulum membrane by lysosomes in the liver cells (Furono et al., 1982). The heterogenous turnover rates of microsomal enzymes do not support the major contribution of the autophagic process to the degradation of endoplasmic reticulum in the liver cells, although degradation rates of cytochrome P-450 appear to be the same (Shiraki and Guengerich, 1983). These workers have argued against autophagocytic processes of cytochrome P-450 degradation and concluded that the level of the microsomal enzymes expressed in the liver are primarily a function of changes in rates of protein (and prosthetic group) synthesis as opposed to rates of degradation. Other workers (Omura, 1980) have assumed participation of specific non-lysosomal protease(s) in the membrane of microsomes or in the cytosol to explain heterogenous turnover of various microsomal enzymes.

iii) Assembly of cytochrome P-450

Cytochrome P-450 apoprotein is synthesized by the membrane-bound ribosomes of rough surfaced endoplasmic

reticulum as mature-size peptides as opposed to secretory proteins (albumin) which are synthesized as large precursor peptides (Bar-Nun et al., 1980). It is presently envisioned that the heme moiety from mitochondria would translocate to the apoprotein moiety which is embedded into the endoplasmic reticulum through a transient fusion of membranes of the two organelles (i.e. a direct transfer of heme) (Muller Eberhard and Vincent, 1985). The emerging hypothesis of a molecular mechanism involving the insertion of apoprotein of the endoplasmic reticulum and the heme into the apoprotein is still in its infant stage.

SECTION III

Relationship between host defence mechanism and cytochrome P-450 system

Recently it has become increasingly apparent that infectious disease can alter the biotransformation of drugs and chemicals in the liver. Many agents both biological and chemical which have the ability to alter the immune system or stimulate host defence mechanism also have the ability to impair processes involved in drug metabolism (Renton, 1983). Historically, Samaras and Dietz (1953) were the first to report that the elimination of drugs is impaired by a host defence mechanism. They suggested that the blockade of the reticuloendothelial system following the administration of trypan blue hampered detoxification

of pentobarbital. In this report, which was an abstract to a meeting, they said that "narcosis in dye injected animals were deeper and longer, reflexes were absent and the animals resembled carcasses". At this date the existence of cytochrome P-450 was unknown, and little was known about the regulation of xenobiotic metabolism.

Some non-specific immune modulators which have been reported to alter cytochrome P-450 mediated drug metabolism are shown in Table 2. The list includes vaccines, attenuated bacteria, non-specific immuno-stimulants, virus, compounds affecting the recticuloendothelial system and interferon inducers. All of these agents are effective in depressing the microsomal mixed function oxidase system when administered in vivo but have no effect when added in vitro to isolated preparation of hepatic microsomes.

For most of these agents, the mechanism by which they depress cytochrome P-450 is not known. Some of these agents have been shown to act via the production of interferon (Singh and Renton, 1980). The possible mechanism of action of interferon on the depression of cytochrome P-450 mediated drug metabolism will be discussed in subsequent sections. In addition to inducing interferon, immunomodulators also exhibit a broad spectrum of biological activities basically related to host immunity. These include a) alteration of reticuloendothelial activity, b) antiviral, antifungal,

TABLE 2

Nonspecific immune stimulants which have been reported to depress cytochrome P-450 mediated drug metabolism

Bordetella pertussis vaccine	Newcastle Disease Virus
Corynebacterium parvum	Dextran
Myobacterium butyricum	Dextran Sulfate
Bacillus calmette-guerin	Latex Beads
Freunds adjuvant	Trypan Blue
E.Coli endotoxin	Tilorone
Zymosan	Poly IC
Statolon	Interferon
Colloidal carbon	Maleic Anhydride Divinly-
Encephalomyocarditis virus	Ether copolymer

From Renton, 1983.

antibacterial, and antineoplastic activity, c) sensitization of bacterial endotoxin, and d) inhibition of adjuvant-induced arthritis e) depression of cytochrome P-450. Activation (e.g. by pyran copolymer) or depression (e.g. by methylpalmitate) of the reticuloendothelial system (RES) has been shown to decrease drug metabolism in the liver (Renton, 1983). Barnes and co-workers (1979) have shown that the ability of maleic anhydride ether copolymers to decrease drug biotransformation correlated with an increase in their molecular weight, their antiviral and antitumor activity and their ability to block phagocytosis. This copolymer or other immune modulating agents such as endotoxin do not stimulate the production of interferon to any great degree yet are among the most potent inhibitors of cytochrome P-450 mediated drug metabolism. Recent studies in our laboratory have suggested that humoral factor(s) released from the Kupffer cells of the RES following treatment with dextran sulphate (a sulphated, branched polysaccharide) or latex particles (a polystyrene compound) depresses drug biotransformation (Peterson and Renton, 1986a and 1986b). The effect is believed to be initiated as a consequence of phagocytosis by macrophages (e.g. Kupffer cells). The low molecular weight humoral factor (less than 12;000 daltons) excluded interferon as a possible factor released from Kupffer cells (Peterson and

Renton, 1984). The identity of this factor is unknown at the present time.

The mechanism for the depression of cytochrome P-450 elicited by inert modulators of RES may involve both the perturbation in cellular heme metabolism and/or protein metabolism. Recently, Barnes (1984) showed that a non-specific immunomodulator, maleic anhydride-divinyl ether copolymer, decrease hepatic microsomal cytochrome P-450 content by depressing the synthesis of apocytochrome P-450 without increasing its degradation On the other hand, the lipopolysaccharide (endotoxin) component of the gram-negative bacterial cell wall, an RES-activating agent has been shown to produce in vivo soluble mediator(s) that could be involved in the decrease in hepatic parenchymal cell mixed-function oxidase activity (Egawa et al., 1981). The mediator(s) was considered not to be endotoxin itself or endotoxin induced interferon. Ghezzi et al. (1986) have shown recently that tumor necrosis factor (TNF), a LPS induced macrophage product, depresses liver cytochrome P 450 drug metabolizing enzymes. These same workers (Ghezzi et al., 1985) and our laboratory (Peterson and Renton, 1985) have shown that another LPS-induced macrophage product, interleukin-1 ,also depresses hepatic cytochrome P-450. Interestingly, interleukin-1 from mitogen induced mononuclear cells has been proposed to induce the production of interferon (β-interferon) from fibroblast

cells (Damune et al., 1985). The intracellular mechanism elicited by LPS or its mediator(s) is thought to depress cytochrome P-450 content by initially causing a dissociation of heme from holocytochrome P-450 (Bissell and Hammaker, 1976a,b). The suppression of ALA synthetase, increased heme saturation of tryptophan oxygenase, and induction of heme oxygenase are all consequences of an increase in the heme concentration in a "free heme" regulatory pool. However, it is not known whether the depression of cytochrome P-450 content by LPS is due to alteration of heme metabolism or alteration in apocytochrome metabolism.

Presently it is thought that the inhibitory effects on cytochrome P-450 mediated drug biotransformation observed after administration of the various RES modulating agents including interferon may be due to the release of factors from LPS-induced peritoneal macrophages (Williams, 1985; Peterson and Renton, 1986a,b). It has not been established if the factor elaborated by the peritoneal macrophages exposed to LPS is also released by Kupffer cells and if it is identical to the factor released by dextran sulphate. Also, it remains to be seen if interferon can cause elaboration of similar factor(s) from peritoneal macrophages or Kupffer cells.

SECTION IV

A. General review of interferon

The groundwork for the discovery of interferon was first laid by Henle and Henle (1943) who demonstrated interference between active and inactive influenza viruses in the developing chick embryo. Isaacs and Lindermann, (1957) eventually discovered interferon by demonstrating that the fluids from virus infected cell cultures contained a protein which could react with other cells to render them resistent to infection by a wide variety of viruses. As interferon consists of a heterogenous class of proteins and does not constitute the only inhibitor of virus replication, the following criteria have been proposed by an international committee to define a factor as interferon: (a) it must be a protein, (b) it exerts virus non-specific, antiviral activity against a wide range of unrelated viruses, (c) it must inhibit virus replication through cellular metabolic processes involving synthesis of both RNA and protein by the cells, (d) it must exhibit activity on a range of host cells, (e) it must induce non-antiviral alterations in cells such as priming, blocking, enhancement of double-stranded RNA toxicity and inhibition of cell multiplications (Stewart II, 1981). Table 3 shows the presently used nomenclature for the three major classes of interferon. There is a substantial amount of homology of amino acid sequences in the human α- and β-interferon (HuIFN), but almost none between Γ-interferon and α/β-interferon (Pestka 1983). Recent advances in the isolation

TABLE 3

Interferon nomenclature

Type	Other Designations	Source	Induced By	Number of Subspecies	pH Stablity
α (IFN-α)	leukocyte IF type I, IFNα, IFL, LIF, LIF ifnLe	β and null lymphocytes and macrophages: recombinant	virus: double-stranded RNA	15	+
β (IFN-β)	fibroblast IF, type I, FIF, INF, IfnF	Fibroblasts, lymphoblasts; epithelial cells: recombinant	virus: double-stranded RNA	1	+
γ (IFN-γ)	immune, type II, IFI, ImIF	T lymphocytes: recombinant	foreign antigens: mitogens	1-2	-

(Adapted from Mannering and Deloria, 1986).

and characterization of interferon has given rise to classification of subspecies of IFN-αs. Interestingly these multiple species of native HuIFN-αs demonstrate a range of antiviral activity and cell growth inhibition in a number of animal cell lines (Evinger et al , 1981).

Although most tissues have the capacity to produce IFN with appropriate inducing agents, the major production of three main types of interferon generally occurs in only few cell types. IFN-α, in B and T lymphocytes and macrophages, IFN-β in epithelial and fibroblast cells, and IFN-Γ in T lymphocytes with support of macrophages (Baron et al., 1984). A variety of natural and synthetic agents are capable of inducing interferon production. Baron et al (1984) have categorized interferon inducers into two major of classes: α/β inducers and Γ inducers. Class A α/β inducers are relatively potent (e.g. RNA viruses, DNA virus and synthetic polyribonucleotides). Class B α/β inducers are relatively weak (e.g. microbial products, pyran copolymers, cycloheximide). Inducers of IFN-Γ consist of antigens, antibody to OKT3 antigen on mature T lymphocytes and a number of mitogens.

It is now generally known that the process of induction of IFN-α and β by viruses and polynucleotides involves processes that can be inhibited by actinomycin D (inhibitor of DNA-directed RNA synthesis) i.e. new cellular RNA and protein synthesis. It therefore follows that

transcription of cellular genes is essential. The genes are inactive before treatment with virus or poly IC but following treatment with one of these agents, the genes are activated, messenger RNA is produced and this then is translated into proteins It is presently assumed that a repressor system exists in the cell for the interferon gene which can be depressed by the interferon inducer-receptor complex. Derepression of the interferon gene signals the formation of interferons (Marcus, 1983).

The mechanism by which IFN protects the cell against virus by interfering with nucleic acid and protein synthesis is only partially known. IFN is presently thought to exert its antiviral effect on specific receptors lying on the cell surface (Kushnaryov et al., 1983). Activation of this receptor has been reported to stimulate second messenger candidates such as cyclic AMP and GMP (Tovey, 1982), but the evidence for either of them playing a central role is not strong. After binding to the cell surface, however interferon triggers the synthesis of new cellular mRNAs and proteins which mediate the antiviral state. Both RNA synthesis and protein synthesis are needed after IFN treatment to develop the antiviral state. These conclusions were first drawn by using an RNA synthesis inhibitor (Taylor, 1964) and a protein synthesis inhibitor (Dianzani et al., 1969). For the several new proteins synthesized in interferon treated cells, at least three

have been associated with the antiviral state; these are (a) protein kinase, (b) 2'-5'-oligoadenylate (2-5A) synthetase which can catalyze the polymerization of ATP into oligonucleotides with the structure pppA2'p5'A2'p5'AOH, which then activates the endogenous inactive endoribonucleases normally present constitutively at low levels in the cell (Baglioni et al , 1978), (c) phosphodiesterase (in some systems). The protein kinase and 2-5A synthetase are both activated by double stranded (ds) RNA, which is a component of or is produced during the replicative cycle of many different types of viruses. Figure 5 illustrates the different enzyme pathways in interferon-treated virus infected cells that may be responsible for inhibition of virus-specific mRNA translation This inhibition is thus due to enhanced degradation of viral mRNA, inhibition of chain initiation, and an inhibition of chain elongation (Revel and Grover, 1978). How these above enzymes discriminate between viral and general host protein synthesis is not yet known, although it is presently thought that the antiviral enzymes (2-5A synthetase and protein kinase) may depress the cellular proteins which, like viral proteins, turnover most rapidly (Mannering and Deloria, 1986). It must be noted that enzymes other than 2-5A and protein kinase may be involved in the antiviral and other effects of interferon. Studies involving one and two-dimensional gel

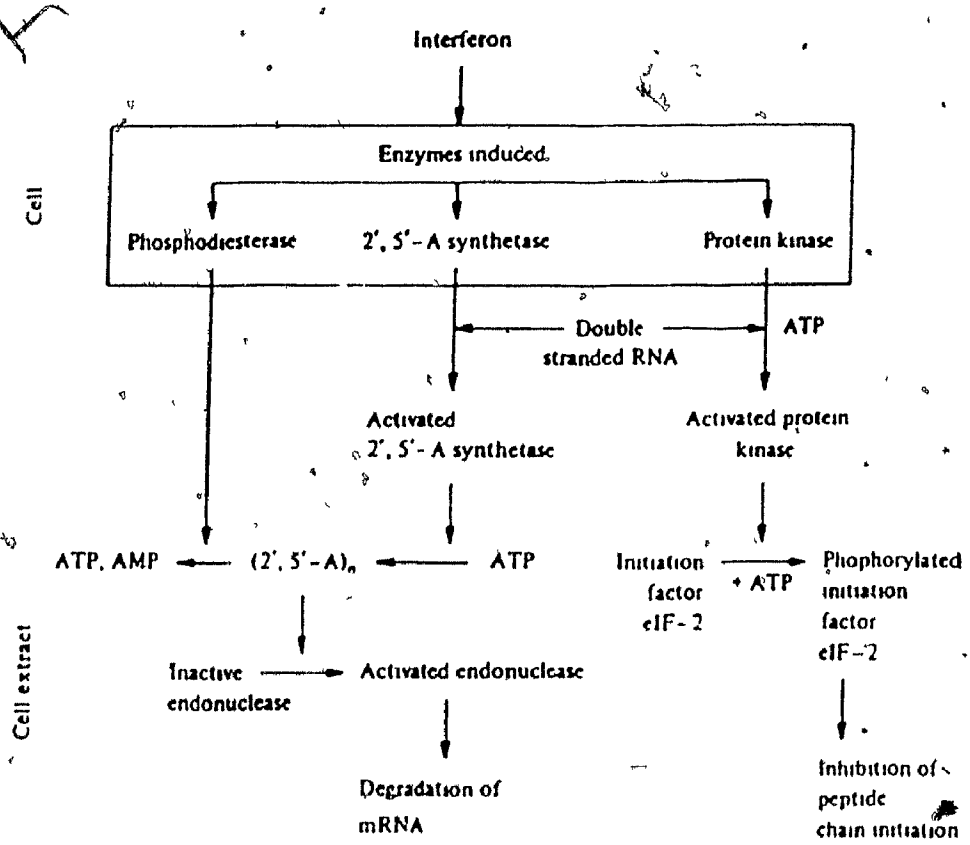


Figure 5

Role of interferon induced enzymes in the inhibition of translation.

electrophoreses have shown a number of unidentified polypeptides in cells that appear simultaneously with the antiviral state (Johnston and Torrence, 1983). In addition to their antiviral activities, interferons have many other effects on cellular structure and/or function, some of which may contribute to host defense against viral infection The non-antiviral effects of interferon includes (a) stimulation of natural killer cells, (b) enhancement of phagocytosis, (c) inhibition of cell motility, (d) cytostatic effect, (e) immunomodulating action, (f) cell surface modifications (Fc receptors, histocompatability antigens), (g) enhancement of ds RNA cytotoxicity (Lebleu and Content, 1982).

Presently it is thought that interferon probably mediates the antitumor activities by a combination of its antiviral activity, its cytostatic activity and immunomodulatory action. Furthermore, interferons have become recognized as a hormone-like messenger, which induces metabolic changes in distant cells. This is an interesting idea as many of the cellular processes which are incurred by interferon may not be directed against antiviral or antitumor activity. For example, high levels of IFN-α found in human amniotic fluid have been suggested to be involved in the regulation of fetal development and the immunoregulation of fetal acceptance rather than as an antiviral agent (Chang, 1983).

B. Properties of IFN-αCON1 interferon

Early clinical studies with purified human α type interferons proved disappointing as few positive responses were observed in cancer, and unexpected side effects occurred during treatment of cancer or viral infections. With molecular cloning of complementary DNA (cDNA) from human mRNA and specific probing of the human genes, a family of IFN-α subtypes (approximately 20) of 166 amino acids was derived. These subtypes are quite different in their potency and properties despite their close structural similarities (Zoon and Wetzel, 1984). With the knowledge of IFN-α amino acid sequences of different subtypes, a hybrid analog of human IFN-α called IFN-αCON1 (interferon alpha consensus one) was conceived by Amgen, California. This interferon (M.W. 19700) was derived by selecting the most frequently observed amino acid at each position in the different IFN-α subtypes (Alton et al., 1983). This interferon therefore has a high consensus of amino acid residues contained in the other subtypes.

Studies have confirmed that IFN-αCON1 demonstrates the following, in vitro: (a) stimulation of antibodies in peripheral blood leukocytes, (b) stimulation of natural killer cell activity; and in vivo (a) antiviral activity against EMC virus in squirrel monkeys, (b) antiviral activity against herpes virus infection in hamsters, (c) antitumor activity against the lymphosarcoma of hamsters

and the human breast carcinoma in nude mice (Stebbing et al., 1986). This interferon has potent antiviral activity in human, monkey and hamster cells. It has very little antiviral effect in mouse cell lines (Alton et al., 1983).

Because of the action of this interferon is limited to only a few species, we have confined the studies described in this thesis to the effect of IFN-αCON1 in hamsters.

SECTION V

Effects of interferon inducers and interferon on drug metabolism

In 1972, Morahan et al. (1972) reported that pyran copolymer and poly IC, a weak and potent inducer of interferon respectively, decreased liver microsomal enzyme activity In 1976, Renton and Mannering, and Leeson et al. demonstrated that tilorone, an antiviral agent, then thought to produce its effect by inducing IFN, also depressed the hepatic cytochrome P-450 dependent mixed function oxidase system. Subsequently, Renton and Mannering (1976b) reported that 12 IFN-inducing agents, of widely different structure and molecular weight, depressed cytochrome P-450, ethylmorphine N-demethylase activity and aniline hydroxylase activity in hepatic microsomes. The authors suggested that the depression of the cytochrome P-450 system was a general property of interferon inducers. Interferon induction and release cannot however explain the

action of all immunoactive agents. Pyran copolymer and endotoxin produce very little interferon yet are the most potent inhibitors of cytochrome P-450 (Mannering et al., 1976). The possible reason for this was discussed in the previous section.

Indirect evidence to support the idea that interferon per se is involved in the depression of drug metabolism includes (a) time required for first indication of depression of drug biotransformation by interferon inducing agents such as poly IC or during infection with encephalomyocarditis virus (Renton, 1981), correlates with circulating serum level of interferon (b) when the nucleotides of poly C and poly I are administered separately in that order neither depression of cytochrome P-450 system ocurred nor are appreciable amounts of interferon produced. When the order of poly I and poly C is reversed, serum levels of interferon and depression of the cytochrome P-450 occurred (Mannering, 1980). This sequential administration of single-stranded polyribonucleotides has also been shown to result in antiviral activity (DeClercq and DeSomer, 1972). (c) Strains of mice which differ genetically in their responsiveness to induction of IFN levels elicited by Newcastle Disease Virus (NDV) have been used to examine whether IFN titers might be correlated with decreased cytochrome P-450 system. Animals that possess the high-

production allele responded to NDV injection with high circulating IFN titers and a depressed hepatic cytochrome P-450 level and decreased aminopyrine N-demethylase activity. Animals that possess the low-production allele show no significant effect on NDV injection to either increase IFN levels or decrease cytochrome P-450 system (Singh and Renton, 1981).

Proof for the direct involvement of interferon in the depression of cytochrome P-450 system had to await the availability of highly purified preparations of human interferon which were obtained by recombinant DNA techniques from Escherichia coli. HuIFN-AD, a recombinant hybrid of HuIFN-A and HuIFN-D, which possesses antiviral activity in the mouse, also depressed the cytochrome P-450 system in the mouse (Singh et al., 1982). HuIFN-A and HuIFNr-D which do not induce antiviral activity in the mouse, had little or no effect on cytochrome P-450 system (Singh et al., 1982). This experiment provides the first conclusive evidence to support the hypothesis that the production of interferon is a contributing factor in the depression of the cytochrome P-450 system and drug elimination that occurs during infection or following the administration of interferon inducing agents. Singh et al. (1982) and Parkinson et al. (1982) have also shown that a correlation exists between decrease in the drug-metabolizing activity and the antiviral activity of

purified recombinant human leukocyte IFN. In addition to IFN-α and IFN-β, pure recombinant mouse IFN-Γ also causes a depression of mouse cytochrome P-450 system (Franklin and Finkle, 1985), possibly by a mechanism different from that initiated by IFN-α and IFN-β (Renton, 1983). The mechanism for the depression of the cytochrome P-450 system by IFNs is the subject of this thesis.

Several properties of interferon or interferon inducers are known that may help to elucidate the mechanism of interferon-induced depression of cytochrome P-450 content. Firstly it is noted that the hepatic hemoproteins which are rapidly turning over (e.g catalase and cytochrome P-450) are depressed by interferon inducers but not hemoproteins which are turning over slowly (e.g. cytochrome bs and cytochrome a, b, c and ci) (Mannering et al., 1980). Further studies have also shown that not all types of cytochrome P-450 are depressed to the same degree (Zerkle et al., 1980). Secondly, the loss of hepatic cytochrome P-450 caused by interferon or interferon inducers never exceeds 50% of control values, even after repeated doses of interferon(Renton, 1983). This indicates that the cytochrome P-450 attains a new steady-state level rather than being completely depressed in a dose-dependent fashion, as is the case with cobaltous chloride or 2-allyl-2-isopropylacetamide (Renton,1983). As indicated in the previous section, changes in steady state levels of

cytochrome P-450 are regulated by the ongoing processes of synthesis and degradation of the enzyme.

The level of holocytochrome P-450 in a tissue is determined by both the rate of synthesis and degradation of the heme prosthetic group and apocytochrome P-450 (as discussed in the previous section) Using the incorporation of radiolabelled glycine and δ-aminolevulunic acid into cytochrome P-450, El-Azhary et al (1980) suggested that interferon-inducing agents (poly IC and tilorone) lowered the concentration of cytochrome P-450 by increasing heme degradation rather than affecting its synthesis. More recently, Singh (1982) disputed this finding and demonstrated, by measuring the expiration of CO_2 from the methene bridge carbon of the porphyrin ring of the heme, that interferon inducers increase both rate of heme degradation and decrease the rate of heme synthesis. By examining the relative time course of the levels of heme oxygenase (rate limiting enzyme for heme degradation), δ-aminolevulunic acid synthetase (rate limiting enzyme for heme synthesis) and holo- and apotryptophan 2,3-dioxygenase (enzyme regulating "free heme pool"), interferon-inducing agents could cause an increase in the regulatory heme pool that controls its rate of synthesis as well as rate of degradation of heme for the hemoproteins (El-Azhary and Mannering, 1979). This would occur if interferon inducers

decreased the content of the apocytochrome P-450 or increased the dissociation of heme from cytochrome P-450.

SECTION VI

Formulation of the problem

Although it is now generally accepted that interferon and interferon inducers depress the microsomal cytochrome P-450 dependent mixed function oxidase, these observations were only shown in mouse and rat (Mannering, 1986). Induction of interferon has also been correlated with a depression of hepatic mixed function oxidase in recent human studies (Meredith et al., 1985; Kramer et al., 1984; Renton, 1983).

The major objectives of this study were:

(a) To determine if IFN-αCON1, a constructed interferon which incorporates the most frequently observed amino acid sequences seen in human α-interferon subtypes depresses hepatic and certain extrahepatic mixed function oxidase in hamsters. This interferon has been shown to have higher antiviral and antitumor activity in human and hamster cell lines than any other α-interferon subtypes known.

(b) To investigate the mechanism by which interferon mediates depression of cytochrome P-450. This study has focussed on the overall effect on the synthesis and degradation of apocytochrome P-450.

During the course of this study, two separate groups of workers have proposed a novel mechanism for the depression of the cytochrome P-450 system by interferon and interferon-inducing agents (Ghezzi et al., 1985; Deloria et al , 1985) They observed that these agents increase xanthine oxidase activity in the liver and other tissues of mouse by several fold Because xanthine oxidase (a cytosolic enzyme) generates free oxygen radicals and oxygen radicals are known to destroy cytochrome P-450 (Paine, 1978), they proposed that interferon de resses the cytochrome P-450 system via the induction of xanthine oxidase activity.

(c) This study therefore also investigated the role of xanthine oxidase and reactive oxygen intermediates in the interferon mediated depression of hepatic drug metabolism in hamsters.

MATERIALS

A. CHEMICALS AND REAGENTS:

Throughout the entire study, standard reagent grade laboratory chemicals manufactured or supplied by Fisher Scientific Co., Fairlawn, New Jersey, or J.T. Baker Chemical Co , Phillipsburg, New Jersey or by Sigma Chemical Co., St. Louis, Missouri were used.

Non-standard reagents are listed below.

Reagents supplied by Sigma Chemical Co., St. Louis, MO:

acetylacetone
actinomycin D
allopurinol
aminopyrine
benzo(a)pyrene
bovine serum albumin
bromophenol blue
clofibrate
cytochrome c
D-glucose-6-phosphate
dalton Mark VII-L (molecular weight standards)
dithioerythritol
ethylene diamine-tetra-acetic acid (EDTA)
glucose-6-phosphate dehydrogenase
glycerol
glycine
guanidine hydrochloride

hemin (equine Type III)

HEPES (N-2-hydroxyethyl perazine-N'-2-ethanesulfonic acid)

lauric acid

leucine

2-mercaptoethanol

L-methionine

β-naphthoflavone

NAD (diphosphopyridine nucleotide)

NADH (Diphosphopyridine nucleotide; reduced form)

NADP (triphosphopyridine nucleotide)

NADPH (triphorphopyridine nucleotide, reduced form)

poly IC

puromycin

quinine hydrochloride

semicarbazide hydrochloride

sodium dodecyl sulphate

sodium potassium tartrate

TEMED (N,N,N',N'-tetramethyl-ethylene diamine)

α-tocopherol acetate

Triton X-100

Reagents supplied by other companies:

acetone: Fisher Scientific Co.

acrylamide: Bio-Rad Labs (Canada) Ltd , Ontario

ammonium persulphate: Bio-Rad Labs (Canada) Ltd., Ontario

Aquasol: New England Nuclear (NEN) Canada Ltd., Quebec

barium hydroxide: Fisher Scientific Co., Bethesda Research Labs (BRL)

in vitro translation kit: Bethesda Research Laboratories, GIBCO (Canada) Ltd., Ontario

BIS-acrylamide: Bio-Rad Labs (Canada) Ltd.

Biofluor: NEN Canada Ltd , Quebec

carbon monoxide: Union Carbide Canada Ltd., Halifax, N.S.

copper sulphate: Fisher Scientific Co.

diethyl ether: Fisher Scientific Co.

Enhance: NEN Canada Ltd.

ethoxyresorufin: Generously supplied by Dr. Richard Addison, Bedford Institute of Oceanography (B.I.O.), Dartmouth, N.S.

glacial acetic acid: BDH Chemicals, Burnside Industrial Park, Dartmouth, N.S.

hexane: Fisher Scientific Co.

hydrogen peroxide: Fisher Scientific Co.

Kodak X-OMAT AR (X-ray film): Picker International, Burnside Industrial Park, Dartmouth, N.S.

Kodak Developer (D-19) and fixer: Picker International

[1-^{14}C]lauric acid: Amersham (Canada) Ltd., Oakville, Ontario

[^{3}H]-leucine: NEN Canada Ltd.

[^{14}C]-leucine: NEN Canada Ltd

magnesium chloride: BDH Chemicals (Canada) Ltd.

[^{35}S]-methionine: NEN Canada Ltd.

phenobarbital sodium (Nembutal): Abbotts Labs, Montreal, Canada

phenol reagent: Fisher Scientific Co.

resorufin: Generously supplied by Dr. Richard Addison, B.I.O., N.S.

sodium dithionite: Fisher Scientific Co

Thin layer chromatographic plates (TLC): Mandel Scientific Co., Quebec, Canada

trichlororacetic acid (TCA): Fisher Scientific Co.

zinc sulphate: Fisher Scientific Co.

METHODS

B. ANIMALS:

Male Golden Syrian hamsters (100-120 gm) were obtained from the Canadian Hybrid Farm, Nova Scotia, and were used throughout the study. To avoid overcrowding ,not more than 4 hamsters were kept in a single cage. Randomly bred male Swiss Webster mice, 25-30 gm, were obtained from the Jackson Laboratories, Bar Harbour, ME, and were used in a single experiment. All animals were allowed to acclimatize for a period of at least one week following receipt from

the supplier, and were kept on clay chips bedding for the duration of the experiment. Diet consisted of Purina rat chow and water ad libitum.

C. TREATMENT OF ANIMALS:

(i) POLY IC:

A single dose of Poly IC (sodium salt) was administered i.p. (10 mg/kg in 0.9% sterile saline) and the animals were killed at various times, as indicated in the Results section.

(ii) INTERFERONS:

The interferon preparations (IFN-αCON1 & buffy coat) were diluted in 0.9% sterile saline, and were administered i.p. The two interferons were supplied as a part of a collaborative study with Dr. Nowell Stebbing of Amgen Inc , 1900 Oak Terrance Lane, Newbury Park, CA. IFN-αCON1 , a cloned interferon, was derived using recombinant DNA procedures, and was expressed in E. coli. The purity of this interferon exceeded 95% as assessed by polyacrylamide gel electrophoresis. The specific activity varied from batch to batch but was usually in the order of 2×10^8 to 2×10^9 units/mg protein (as calibrated against the NIH standard Ga-23-902-530). IFN-αCON1 is stable in solution at 0-4°C. The buffy coat interferon was derived from human leukocytes, and the single batch utilized had a specific activity of 1×10^8 units/mg protein. The buffy coat

interferon which is known to be labile was shipped on dry ice and was utilized immediately.

D. EXPERIMENTAL DESIGN TO ASSESS THE IFN-αCON$_1$ INDUCED DEPRESSION OF MIXED FUNCTION OXIDASE IN ANIMALS TREATED WITH PHENOBARBITAL, β-NAPHTHOFLAVONE OR CLOFIBRATE.

(i) PHENOBARBITAL/INTERFERON

Animals were treated with a single dose of IFN-αCON$_1$ ($1x10^6$ units, i.p) at day 0, and 3 doses of phenobarbital: 80 mg/kg, i.p. on day -2, 40 mg/kg, i.p. on day -1, and 40 mg/kg, i p. on day 0, and were killed 24 hr following interferon treatment. Groups of animals were also treated with saline, interferon or phenobarbital alone at corresponding times.

(ii) β-NAPHTHOFLAVONE/INTERFERON

Animals were treated with a single dose of IFN-αCON$_1$ ($1x10^6$, i.p.) at day 0, and three doses of β-naphthoflavone (40 mg/kg in soya oil, i.p.) on day -2, -1, and day 0, and were sacrificed 24 hr following interferon treatment. Groups of animals were also treated with saline, interferon or β-naphthoflavone alone at corresponding times.

iii) CLOFIBRATE/INTERFERON

Animals were treated with a single dose of IFN-αCONI$_1$ (1×10^6 units, i.p.) on day 0, and 4 doses of clofibrate

(250 mg/kg, i.p.) on days -3, -2, -2, and 0, and were sacrificed 24 hr following interferon treatment. Groups of animals were also treated with saline, interferon or clofibrate alone at corresponding times.

E. EXPERIMENTAL DESIGN TO ASSESS THE EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON THE IFN-αCON1 INDUCED STIMULATION AND DEPRESSION OF HEPATIC DRUG OXIDATION

(i) PUROMYCIN:

For the 3 hr IFN-αCON1 treatment study, animals were given a single dose of IFN-αCON1 ($1x10^6$ units, i.p.) at 0 hr, and 4 doses puromycin (2.72 mg in 0.17 ml sterile phosphate buffered saline (PBS), pH 7.4, i.p.) at -2, 0, 1.5 and 2 hr, and were sacrificed 3 hr following interferon treatment. For the 24 hr IFN-αCON1 treatment study, animals were treated with a single dose of IFN-αCON1 at 0 hr and 6 doses of puromycin at -2, 0, 1.5, 3, 4, and 5 hr, and were sacrificed 24 hr following interferon treatment. Control and interferon only treated animals received corresponding volumes of PBS at the same times.

(ii) ACTINOMYCIN D:

For the 3 hr IFN-αCON1 treatment study animals were given a single dose of IFN-αCON1 ($1x10^6$ units, i.p.) at 0 hr, and 3 doses of actinomycin D (19.8 ug in 0.17 ml of sterile PBS, pH 7.4, i.p.) at -2, 0, and 2 hr, and were sacrificed 3 hr following interferon treatment. For the 24

hr IFN-αCON1 treatment study animals were treated with a single dose of IFN-αCON1 at 0 hr and actinomycin D at -2, 0, and 4 hr, and were sacrificed 24 hr following interferon treatment (0 hr). Control and interferon-only treated animals received corresponding volume of sterile PBS at the same time.

F. EXPERIMENTAL DESIGN TO ASSESS THE INVOLVEMENT OF FREE RADICALS IN THE DEPRESSION OF HEPATIC DRUG OXIDATION CAUSED BY POLY IC:

(i) XANTHINE OXIDASE INHIBITOR:

Animals were treated with a single dose of poly IC (10 mg/kg) at 0 hr and 2 doses of the xanthine oxidase inhibitor allopurinol (either 7 mg/kg or 17 mg/kg in sterile saline, p.o.) at -1 hr and 6 hr, and were sacrificed 24 hr later. Control and poly IC-only treated animals received corresponding volume of sterile saline at the same times and same route of administration of either treatments.

(ii) FREE RADICAL SCAVENGER:

Animals were treated with a single dose of poly IC (10 mg/kg) 24 hr after a free radical scavenger, α-tocopherol (419 mg/hamster, i.p.), and were sacrificed 24 hr later. Control and poly IC-only treated animals received corresponding volume of sterile saline at the same times.

G. PREPARATION OF HEPATIC AND EXTRA-HEPATIC MICROSOMES:

The liver from each animal was separately rinsed in ice cold 1.15% KCl solution and weighed. Livers were then homogenized separately in 10 ml of 1.15% KCl in a glass homogenizer (10 strokes with a loose-fit pestle; and 1 stroke with a tight-fit pestle) and centrifuged at 10,000 x g for 10 min in a Beckman (J2-21) refrigerated centrifuge. The pellet, obtained from the 10,000 x g centrifugation, contained unbroken cells, cell wall fragments, nuclei, and mitochondria. The floating fatty layer was removed with a Pasteur pipette. The supernatant was recentrifuged at 125,000 x g for 40 min in an IEC/B-60 refrigerated ultracentrifuge to obtain a microsomal pellet. The supernatant from the 125,000 x g centrifugation was used to measure xanthine oxidase activity in some studies (see below). The microsomal pellet was resuspended in ice-cold 50 mM K_2HPO_4/KCl buffer, pH 7.5, containing 20% (v/v) glycerol, using a glass homogenizer (7 strokes of tight-fit pestle), to yield a 50% suspension (i e. 2 x volume of buffer/gm of liver weight). The microsomal suspension was stored at -70°C. Levels of cytochrome P-450 and microsomal enzyme activities were stable for several weeks at this temperature. Microsomes from extra-hepatic tissues (lungs, adrenals, kidneys and spleen) were prepared in a similar manner as above except the organs were pooled from different animals which had received the same treatment and

microsomal fractions were suspended in 100% weight per volume buffered solution. Special care was taken to remove fatty tissues surrounding the adrenals during dissection

VII. DETERMINATION OF PROTEIN IN MICROSOMAL AND CYTOSOLIC FRACTIONS:

Protein was determined by a modified version of the method described by Lowry et al. (1951). One ml aliquots of diluted microsomal or cytosolic fraction (1/100 for microsomal fraction and 1/200 for cytosolic fraction in deionized water) was added to 5 ml of solution containing: 49 mls of 2% sodium carbonate in 0.1 N NaOH; 0.5 ml 1% copper sulphate; 0.5 ml 2% sodium potassium tartrate and incubated at room temperature for ten minutes. Deionized water was used as blank. Phenol reagent (2 N) was then added to the incubation mixture, vortexed and incubated at room temperature for thirty minutes. Absorbance was determined at 700 nm using the blank as a zero absorbance reference in a Turner model 350 spectrophotometer. Bovine serum albumin was used as the standard protein.

I. DETERMINATION OF MICROSOMAL CYTOCHROME P-450 AND CYTOCHROME b_5

Microsomal cytochrome P-450 and cytochrome b_5 were determined by the method of Omura and Sato (1964). The microsomes were buffered with 1 M phosphate buffer pH 7.5

and diluted to 1 mg/ml protein concentration with 1.15% KCl. The microsomes were then divided equally between two spectrophotometer cuvettes and a baseline spectrum from 500 nm to 400 nm was determined with a Pye-Unicam SP8-200 spectrophotometer. A pinch of sodium dithionite was then added to the sample cuvette and a spectrum was obtained from 500 nm to 400 nm. The molar concentration of cytochrome b_5 was calculated from the difference in absorbance at 425 nm using the extinction coefficient of 171 nm^{-1} cm^{-1}.

The reference cuvette was then reduced with dithionite and carbon monoxide was bubbled into the sample cuvette and the spectrum redetermined. The peak at 450 nm and the extinction coefficient of 91 nm^{-1} cm^{-1} were used to determine cytochrome P-450 content. In the case of lung microsomes, cytochrome P-450 was determined after CO was bubbled into both cuvettes and the sample cuvette only was reduced with dithionite. This method permitted quantitation of cytochrome P-450 in the presence of large quantities of hemoglobin. Results are expressed in nmoles/mg protein for cytochrome P-450 and cytochrome b_5.

J. DETERMINATION OF MICROSOMAL AMINOPYRINE N-DEMETHYLATION:

The N-demethylation of aminopyrine in microsomes was determined by measuring the amount of formaldehyde formed

(Sladek and Mannering, 1969). Formaldehyde formed was trapped as semicarbazone by semicarbazide and measured by the method of Nash (1953).

The reaction mixture contained 1.2 mg microsomal protein, 0.8 mg magnesium chloride, 1 6 mg neutralized semicarbazide HCl, 2 µmoles aminopyrine and an NADPH generating system consisting of 0.6 mg NADP, 2 9 mg glucose-6-phosphate and 0.5 units glucose-6-phosphate dehydrogenase. The reaction mixture was made up to a total volume of 2 ml with phosphate buffer (0.1 M, pH 7 5). The reaction mixture was then incubated for 15 minutes at 37°C using a Dubnoff shaking water bath. The reaction was terminated and mixture deproteinized by adding 1 ml of zinc sulphate (50 g/l) followed by 1 ml of barium hydroxide (50 g/l). The mixture was thoroughly agitated on a vortex mixer after each addition and then centrifuged at 2000 x g for 5 minutes at room temperature. Formaldehyde was assayed in 2 ml of the clear supernatant by adding 1 ml Nash reagent (containing 150 gm ammonium acetate, 1 ml acetylacetone, 1.5 ml glacial acetic acid dissolved to give 500 ml solution with distilled water) agitating and incubating for 20 minutes at 60°C in a water bath. The absorbance of the cooled clear samples was determined at 412 nm using a Turner Model 350 spectrophotometer. In all experiments, reaction mixtures incubated without substrate, were subtracted from the experimental incubation mixtures

to correct for any formaldehyde or other materials reacting with Nash reagent which may be formed from a non-substrate source. Absorbance of blank reaction mixtures varied between 0.025 to 0.045. The reactions remained linear with respect to time utilizing these reaction conditions. Results were expressed as nmoles HCHO formed/mg protein/hr.

K. DETERMINATION OF MICROSOMAL BENZO(A)PYRENE HYDROXYLATION:

The hydroxylation of benzo(a)pyrene in microsomes was determined by measuring the formation of polar fluorescent products (the 1-,3-, 6-,7- and 9-hydroxy derivatives) by a modified method of Nebert and Gelboin (1968).

Throughout this assay all procedures were carried out in the dark. Incubation mixture contained 10 µl benzo(a)pyrene (5 mg/ml in acetone), 0.1 ml NADPH (10 mg/ml in potassium phosphate buffer, pH 7.5) and 0.5 ml diluted microsomes (1:5 in 1.15% KCl for hepatic microsomes; extra-hepatic microsomes were not diluted). Cold acetone (0.5 ml) was added to the blanks and samples were incubated at 37°C in the shaking water bath for 15 minutes. The reaction was terminated with the addition of 0.5 ml acetone and 2 ml of cold petroleum ether (including the blank). The tubes were then capped, agitated for 20 seconds each and centrifuged at 1000 x g for one minute. Approximately 2 ml of organic phase was removed and mixed with 2 ml of

cold NaOH (1.N). The tubes were then capped, agitated for 15 sec each and centrifuged at 1000 x g for one minute. The fluorescence of the aqueous phase was determined in a fluorometer (excitation wavelength = 396 nm and emission wavelength = 522 nm). 3-hydroxy benzo(a)pyrene was used to standardize the assay procedure. Because of the difficulties in obtaining, using, and storing authentic 3-hydroxy benzo(a)pyrene as a standard to calibrate the instrument, quinine hydrochloride (100 µg/ml) which was standardized against the 3-hydroxy benzo(a)pyrene was routinely used on a day-to-day basis. Quinine hydrochloride fluoresces strongly at the peak excitation and emission wavelength of 3-hydroxy benzo(a)pyrene. Results were expressed as nmoles of benzo(a)pyrene hydroxylated metabolites formed/mg protein/hr.

L. DETERMINATION OF MICROSOMAL ETHOXYRESORUFIN O-DE-ETHYLATION:

The O-de-ethylation of ethoxyresorufin was determined by the method of Burk et al., (1977), using the difference in fluorescent properties of ethoxyresorufin (excitation wavelength = 455 nm, emission wavelength = 560 nm) and the product, resorufin (excitation wavelength = 510 nm, emission wavelength = 586 nm).

A 2 ml aliquot of potassium phosphate buffer (0.1 M pH 7.4) was placed in a fluorometer cuvette followed by 50 µl

of undiluted microsomes. Subsequently 10 µl ethoxyresorufin (88.7 µM in methanol) was added. The cuvette was then transferred to the shaking water bath and incubated at 37°C for 3 minutes. After incubation, the cuvette was placed in the Perkin-Elmer LS-5 fluorometer, using an excitation wavelength of 510 nm and an emission wavelength of 586 nm.

Immediately after a steady baseline was established, a 10 µl aliquot of NADPH (24 mM) was introduced into the cuvette to initiate the reaction. The production of resorufin with respect to time was monitored as the increase in fluorescence at 586 nm. The fluorimeter was calibrated with 10 µl of resorufin standard (14.2 µM in methanol) in the buffer and microsomal assay system. Using the slope of the reaction recorded (increased in fluorescence with time), the results were expressed as nmoles resorufin formed/mg protein/min.

M. DETERMINATION OF MICROSOMAL LAURIC ACID HYDROXYLATION:

The hydroxylation of lauric acid was determined by the radiometric method of Parker and Orton (1980) for the detection of the hydroxylauric acid metabolites (11- and 12-hydroxylauric acid).

Each sample was prepared in a 15 ml screw cap tube containing 1 ml microsomes (2 mg/ml), 0.2 ml "cold" lauric acid (1 mM), 10 µl ^{14}C-lauric acid (0.1 Ci/µl) and 0.75 ml

distilled water to give a total volume of 1.96 ml. The tubes were incubated for 5 min at 37°C in the shaking water bath prior to the addition of 40 µl NADPH (40 mM) to initiate the reaction. Control microsomes were subsequently incubated for 15 minutes at 37°C and microsomes from clofibrate treated animals were incubated for 5 min. The reaction was terminated by the addition of 0.2 ml HCl (3 M). Petroleum ether (10 ml) was added to the reaction mixture which was then shaken for 10 min. A few minutes later (minimum of 5 min to clarify the phases), 7 ml of the upper ether layer was transferred to a test tube and evaporated to dryness under a stream of nitrogen.

The dried petroleum ether extracts were reconstituted in 60 µl methanol and 25 µl spotted onto silica gel GF t.l.c. plates (Merck brand). [redacted]lates were developed in hexane: diethylether: acetic a[redacted] solution (140:56:3 by volume). The developed plates were labelled with a ^{14}C-lauric acid bromophenol blue mixture at the origin and at approximately 10 cm height. X-ray film (XAR [redacted]) was sandwiched within the plate using an X-ray exposure holder (20.3 x 25.4 cm) and exposed in the dark for 3 days. Using the developed x-ray film, the radioactive area corresponding to unmetabolized lauric acid and 11- and 12-hydroxy lauric acid (lower RF values) were localized on the tlc plates. Equal sized areas enclosing substrate and product spots and corresponding areas in microsome blanks

were scraped from the plate, suspended in 10 ml Biofluor and radioactivity determined using a Beckman liquid scintillation counter.

The rates of hydroxylation were calculated from the fractional conversion of substrate to total hydroxy products (11- and 12-hydroxy lauric acid) making the estimation of recoveries in various extraction steps unnecessary. Results were expressed as nmol hydroxy laurate/mg protein/min.

N. DETERMINATION OF MICROSOMAL NADPH CYTOCHROME C REDUCTASE:

The activity of NADPH cytochrome c reductase was determined by following the absorbance change at 550 nm, reflecting the appearance of reduced cytochrome c after addition of NADPH, in microsomes as described by Williams and Kamin (1962).

Reaction mixtures, containing 1 ml cytochrome c (0.1 mM dissolved in 50 mM potassium phosphate buffer pH 7.4) and 0.3 ml microsomal suspension (0.3 mg protein/ml), were placed in both sample and reference cuvette and were incubated at 37°C for 1 min. The cuvettes were then transferred to the Pye Unicam SP-800 spectrophotometer. The reaction was initiated by adding 10 µl NADPH (25 µM) in the sample cuvette and 10 µl of phosphate buffer (50 µM) to the reference cuvette. The change in absorbance at 550 nm

was recorded over the first minute of reaction and the specific activity of NADPH cytochrome c reductase was determined from the tangent to the curve representing the rapid phase of reduction.

Using an extinction coefficient of 21 nM^{-1} cm^{-1} (Williams and Kamin, 1962), the activity of cytochrome c reductase was calculated and expressed as nmoles cytochrome c reduced/mg protein/minute.

O. DETERMINATION OF MICROSOMAL TOTAL HEME:

Total microsomal heme content was measured by the pyridine hemochromogen method described by Falk (1964).

One ml microsomes (2 mg/ml) were mixed with 0.5 ml of 1 N NaOH and 0.5 ml 20% pyridine. The mixture was divided equally into two cuvettes and a pinch of sodium dithionite was added to one cuvette. The cuvettes were then scanned from 600-500 nm in the Pye Unicam SP8-200 spectrophotometer.

The results were calculated from the difference spectrum at 558 nm and expressed as nmol of heme/mg protein using extinction coefficient of 31 cm^{-1} mM^{-1}.

P. DETERMINATION OF HEME OXYGENASE:

The activity of heme oxygenase in hepatic microsomes was determined spectrophotometrically by following the rate of formation of bilirubin from hemin at 468 nm (Schacter et

al., 1978). The initial product formed from heme oxygenase, biliverdin, was converted to bilirubin by NADPH-dependent biliverdin reductase obtained from the 125,000 x g cytosolic fraction.

The reaction mixture contained 1 ml microsome (undiluted), 3.6 ml potassium phosphate buffer (0.1 M pH 7.4), 0.1 ml biliverdin reductase (crude source of enzyme obtained from the 125,000 x g cytosolic fraction of untreated hamsters), 0.1 ml magnesium chloride (0.1 M) and 25 µl hemin (17 µM). Hemin (equine) Type III was prepared as follows: 13 mg hemin and 12 mg Tris base were dissolved in 2.5 ml of 0.1 N NaOH and the pH of the solution was adjusted to 7.4. The hemin solution was then made up to 5 ml with 2% serum albumin. Total volume of this preparation was 7.5 ml. The reaction mixture was incubated for 5 min at 37°C in a shaking water bath. The incubation mixture was gently agitated to mix the contents and then was equally divided (2.5 ml each) in two cuvettes (sample and reference). 0.1 ml of NADPH generating system, consisting of 1 ml cofactor N (containing 358 mg D-glucose-6-phosphate, 76 mg NADP and 10 ml of 1.15% KCl) and 5 µl of glucose-6-phosphate was added to the sample cuvette. Corresponding volume of 1.15% KCl was added to the reference cuvette. Both the sample and blank cuvette containing the reaction mixture were incubated for 10 min at 37°C in a shaking water bath. The difference spectrum

from 410 to 550 nm was recorded in a double beam spectrophotometer.

Enzyme activity in microsomes was calculated using the molar extinction coefficient of 0.057 cm^{-1} nm^{-1}. Specific activity was expressed as nmoles of bilirubin formed/mg protein/min.

Q. DETERMINATION OF CYTOSOLIC XANTHINE DEHYDROGENASE (TYPE D) AND XANTHINE OXIDASE (TYPE O) ACTIVITY:

The activity of xanthine dehydrogenase (type D) was assayed by measuring spectrophotometrically the amount of NADH formed at 350 nm in the presence of NAD and hypoxanthine. Xanthine oxidase (type O) activity was measured by the amount of uric acid formed from hypoxanthine in the presence of oxygen (Della, Corte and Stirpe, 1972). Supernatant from the 125,000 x g fraction of the liver homogenate was dialysed overnight (approximately 16 hrs) against potassium phosphate buffer (0.1 M pH 7.4) and was used in the following assays.

When measuring xanthine oxidase activity, both sample and reference cuvettes contained in a final volume of 1 ml the following: 0.1 mM potassium phosphate buffer and 0.1 mM EDTA which have been stabilized at 25°C (pH 8.1) and saturated with oxygen. Sample cuvette, in addition to the above reagents, contained 0.1 mM hypoxanthine. Reaction was initiated when 70 µl of supernatant was added to both

the sample and reference cuvette. Formation of uric acid was measured at 292 nm in a Pye-Unicam spectrophotometer. The slope of the reaction recorded (increase in absorbance with time) at 292 nm and the extinction coefficient of 7.6 x 10^{-3} cm^{-1} nM^{-1} were used to determine the activity of xanthine oxidase (nmol uric acid formed/mg protein/minute).

When measuring xanthine dehydrogenase activity, both sample and reference cuvette contained in a final volume of 1 ml the following: 0.1 mM potassium phosphate buffer, 0.1 mM EDTA and 0.1 mM NAD^+ which have been stabilized at 25°C (pH 8.1) in anaerobic condition. Sample cuvette contained in addition to above reagents 0.1 mM hypoxanthine. Reaction was initiated when 70 µl of supernatant was added to both cuvette. Formation of NADH was measured at 360 nm in a Pye Unicam spectrophotometer. The spectrophotometer was calibrated with 22 nM NADH standard contained in the buffer and supernatant assay system. Using the slope of the reaction recorded (increase in absorbance with time), the amount of NADH formed was determined (nmol NADH formed/mg protein/min).

R. DISCONTINUOUS SODIUM DODECYL SULPHATE POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS:

The method of Laemli (1970) was used to analyse hepatic microsomal proteins. The proteins were first rendered monomeric by solubilization with sodium dodecyl

sulphate (SDS) in the presence of 2-mercaptoethanol and then separated on 7% polyacrylamide gels according to molecular weights using a discontinuous buffer system. Stock solutions used in the procedure are as follows.

(a) Acrylamide - BIS solution:

2.1 g acrylamide

0.8 g N'N' BIS methylene acrylamide

Made up to 100 ml with distilled water

(b) Separating gel buffer:

Tris base (1.5 M pH 8.8) 18.15 g

distilled water 50 ml

Adjusted to pH 8.8 with 1 N HCl and then made up to 100 ml with distilled water

(c) Stacking gel buffer:

Tris base (0.5 M pH 6.8) 3.0 g

distilled water 50 ml

Adjusted to pH 6.8 with 1 N HCl and then made up to 100 ml with distilled water

(d) Electrode buffer:

Tris base (pH 8.3) 18.0 g

glycine 86.4 g

SDS 6.0 g

and then made up to 6 litre with distilled water

(e) Sample buffer:

Distilled water 2.0 ml

0.5 M Tris base (pH 6.8) 0.5 ml

99% glycerol	4.0 ml
10% (w/V) SDS	0.8 ml
2-mercaptoethanol	0.2 ml
0.05% (w/V) bromophenol blue	0.1 ml

(f) Ammonium persulphate 10% (w/v)

(g) N-N-tetramethylethylenediamine (TEMED)

Electrophoresis was carried out at room temperature using a vertical slab gel electrophoresis (BIO-RAD protean cell (R)) dual vertical slab gel electrophoresis cell). The gel was contained in glass plates of dimension 18 cm x 16 cm. The glass plates were washed in detergent, tap water, distilled water and ethanol, prior to use. The slab gels were cast outside the protean cell using the separate casting stand. A 3 mm spacer was used to determine the gel thickness. Sandwich clamps made from polycarbonate with nylon screws attached to it, was used to make a leak proof seal and were held vertical by a casting stand. The separating (lower) gel was prepared by mixing 33 ml distilled water, 20 ml separating gel buffer, 0.8 mls 10% (w/v) SDS and 20 ml acrylamide-bis solution. The mixture was then deaerated under vacuum for 30 min. Polymerization was initiated by the addition of 0.2 ml freshly prepared ammonium persulphate and 0.4 ml TEMED. The solution was poured into the sandwich to a height of 120 mm and a layer of distilled water was introduced above the gel mixture to

give a smooth interface after polymerization. When polymerization was complete (1 hr to 24 hrs) the water layer was removed. The stacking (upper) gel was prepared by mixing 12.8 ml distilled water, 5 ml stacking gel buffer, 0.2 ml 10% (w/v) SDS and 2 ml acrylamide-bis solution. The mixture was then deaerated under vacuum for 15 min. Ammonium persulphate (0.1 ml) and TEMED (10 µl) was added to the mixture to initiate polymerization. A 10 track teflon comb was introduced into the stacking gel before polymerization to form the sample wells. After polymerization (approximately 45 min), the sample comb was removed and the gel slab was immediately placed into the electrophoresis tank (protean cell).

Microsomal samples were diluted with sample buffer to give a final protein concentration of 1 mg/ml and were placed in a boiling water bath for 5 minutes. After cooling at room temperature 100 µl (100 µg) aliquots of the sample were applied to the stacking gel sample wells using a Finn pipette. A constant current of 40 mA was applied to the gel until the bromophenol blue indicator dye had moved to within 1 cm of the bottom of the gel. The power was then switched off, the sandwich removed, the glass plates separated and the stacking gel removed. The lower gel was retained for staining.

Electrophoretic bands were detected on the gel using coomassie blue as the protein stain.

Reagents

(a)	Staining solution	- coomassie blue	0.04%
		distilled water	325 ml
		isopropanol	125 ml
		acetic acid	50 ml
	Destaining solution	- acetic acid	75 ml
		methanol	40 ml

distilled water was added until total volume was 1 litre

Staining of protein bands was achieved by placing the gel in staining solution for 2 hr at 37°C. The gel background was destained with successive volumes of destaining solution on a gently rocking water bath until gel background had cleared. The gel was then placed in water and photographed.

Standard protein molecular weight markers used in SDS polyacrylamide gel electrophoresis

The following proteins, obtained from Sigma Co. (MW-SDS), of known molecular weight were used routinely to calibrate the polyacrylamide gels.

Proteins	Approximate Molecular Weight
Bovine Albumin	66000
Egg Albumin	45000
Glyceraldehyde-3 Phosphate Dehydrogenase	36000
Carbonic Anhydrase	29000
Trypsinogen	24000

Trypsin Inhibitor	20100
β-Lactalbumin	14200

A stock solution was prepared by dissolving 3.5 mg of the proteins in 1 ml of the sample buffer and later stored at -20°C. Before use, an aliquot was diluted (1:10) and heated in boiling water for 5 min. Once cooled, 100 µl was applied to the appropriate sample wells and the electophoresis carried out as previously described.

To determine the relative mobility (R_f) of a protein, the following formula was used.

Rf = distance of protein migration from top of the separating gel to the center of the protein divided by the distance of the bromophenol blue tracking dye from the top of the separating gel.

R_f values were calculated directly from the gel or from photographs. The R_f values (Abscissa) were plotted against the known molecular weights (ordinate) on semi-logarithmic paper. Identification of cytochrome P-450 apoprotein was determined from this calibration curve.

S. THE TURNOVER OF HEPATIC PROTEINS USING THE INCORPORATION OF LABELLED AMINO ACIDS INTO SUBCELLULAR FRACTIONS

i) Incorporation of a labelled amino acid into various subcellular fractions

[^{35}S]-methionine (specific activity 1054.4 Ci/mmole) was injected intraperitoneally (i p) into hamsters (50μ Ci/hamster) one hour before they were killed. The livers were excised, homogenized and radioactivity from different fractions determined as described later in this section.

(a) Determination of relative rates of synthesis of total hepatic microsomal protein and cytochrome P-450

The effect of IFN-αCON$_1$, (24 hr treatment period), on the relative rate of synthesis of microsomal proteins was determined by an adaptation of the double-isotope technique of Dehlinger and Schimke (1972). A saline treated animal was given 100 μCi of [^{14}C]-leucine intraperitoneally and 225 μCi [^{3}H]-leucine was simultaneously administered to an experimental animal treated with IFN-αCON$_1$, (1 x 10^6 units/hamster) 20 hrs previously (Figure 6). After 4 hrs both animals were killed and liver of equal weight each, was combined and homogenized in buffered KCl solution prior to microsomal preparation. Control experiments combining ^{3}H- and ^{14}C-labelled livers from two saline treated animals established the limits of experimental error. All experiments were repeated 3 times. Liver portions from interferon and saline treated hamsters were removed prior to combining them, for cytochrome P 450 assay. Determination of the ratio of ^{3}H/^{14}C for various subcellular fractions is described below. The four hour period between isotope injection and killing allowed for

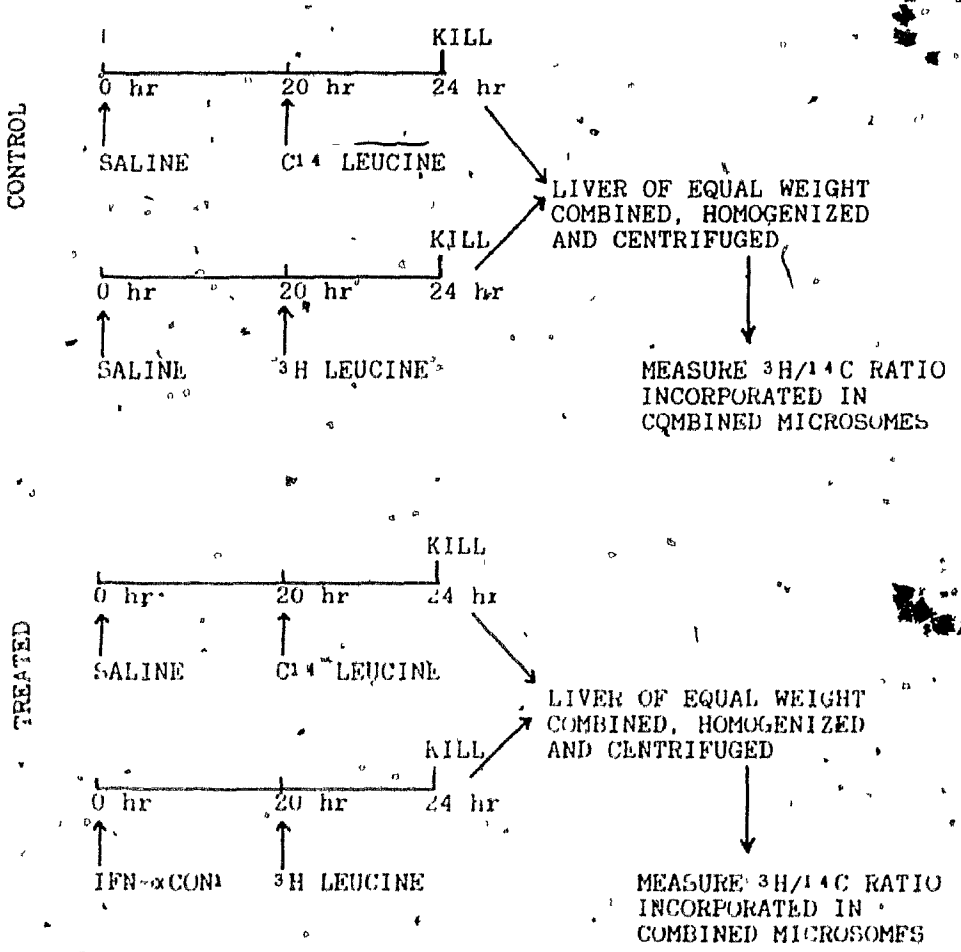


Figure 6.

Relative rates of protein synthesis: Double isotope technique.

the passage of the rapidly labelled serum proteins through the endoplasmic reticulum before isolation of the microsomal fraction (Arias et al., 1969).

ii) Determination of relative degradation rate of hepatic total microsomal protein and cytochrome P-450

The method of Arias et al (1969) was employed in which each interferon (10^6 units/hamster) or saline treated hamster, received 100 µCi [^{14}C]-leucine i.p. and 48 hr later received 225 µCi [^{3}H]-leucine i.p. They were then killed 4 hr later. Radioactivity was determined in various subcellular fractions from individual liver homogenates of equal weight, from interferon and saline treated hamsters (Figure 7). Liver portions were removed for cytochrome P-450 assay. All experiments were performed 3 times. Determination of the ratio of ^{3}H/^{14}C for various subcellular fractions is described below.

iii) Detection of labelled amino acids in various subcellular fractions

Aliquots (0.1 ml) of whole liver homogenates from either the single or dual labelled experiments (see above) were assessed for radioactivity using 10 ml Bioflour as scintillation fluid. The homogenates were centrifuged at 10,000 x g and the radioactivity in the supernatant (0.1 ml) was determined. Microsomal pellets were prepared as described earlier and the radioactivity in the cytosolic fraction (0.1 ml) was determined. The pellets were then

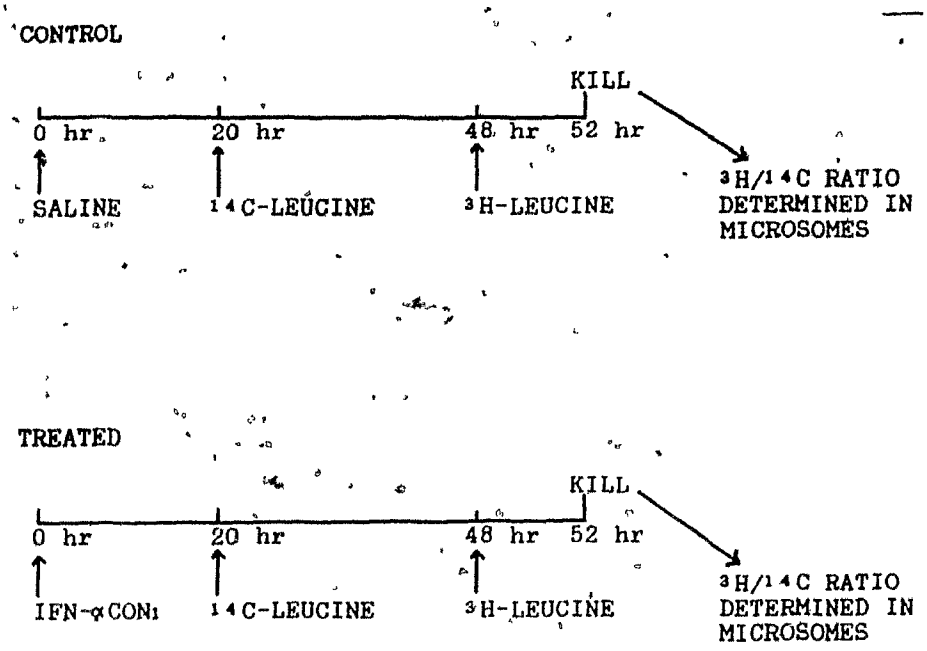


Figure 7

Relative rates of protein degradation: Double isotope technique.

washed twice with 8 ml cold 1.15% KCl and re-centrifuged for 40 min at 125,000 x g. The final pellet was suspended in the buffer as described earlier to yield a 50% microsomal suspension. Radioactivity in 0.1 ml of the resuspended microsomes was determined using 10 ml of Biofluor. One ml of the resuspended microsomes was then treated with ice-cold TCA (12 5%) and centrifuged at 5,000 x g for 10 min. The pellets were washed once with TCA and then solubilized in IN NaOH. An aliquot of the mixture (0 1 ml) was added to 10 mls of Biofluor to determine radioactivity in the TCA precipitable fraction. Total microsomal protein content was determined as described earlier.

The washed microsomes were also subjected to gel electrophoresis and subsequently stained and fixed as described earlier. The gels were sliced horizontally into consecutive slices (3 mm for single labelled and 2 mm for dual labelled experiment) from origin to solvent front. The gel slices were placed in scintillation vials and solubilized with 30% hydrogen peroxide (1 5 ml) at 60°C for approximately 13 hrs or more. Ten ml of Aquasol was placed in each vial and radioactivity determined.

In the [^{35}S]-methionine labelling experiment, radioactivity was counted in a Mark III Searle Analytic Inc. Model 6880 liquid scintillation system, programmed to measure ^{14}C-labelled compounds (since ^{14}C has a similar

liquid scintillation spectrum as ^{35}S). Results were expressed as total counts (cpm), cpm/mg protein and cpm/gel slice. In the dual labelled experiments, radioactivity (^{3}H and ^{14}C) was determined with a Beckman LS5801 liquid scintillation counter, programmed for dual-labelled counting. The program performed individual quench correction and automatically compensated for ^{14}C spillover into the ^{3}H channel. To account for unequal initial incorporation of the two isotopes (^{3}H versus ^{14}C), a correction factor was determined. This correction factor involved the administration of both isotopes simultaneously into an animal with subsequent isolation of various subcellular fractionation after 4 hrs Determination of the ratio of $^{3}H/^{14}C$ for various subcellular fractions and gel slices is described below The ratio of $^{3}H/^{14}C$ was divided by the correction factor in order to standardize each experiment. Results for the dual labelled experiments were expressed as $^{3}H/^{14}C$ ratio in the various fractions and gel slices. Autoradiography (as described later) was carried out with gels obtained from the SDS electrophoresis of microsomes (1 mg/ml) derived from [^{35}S]-methionine treated animals.

The methods used in the following three sections which deal with RNA extraction, cell-free protein translation and analysis of <u>in vitro</u> translated proteins are summarized in Figure 8.

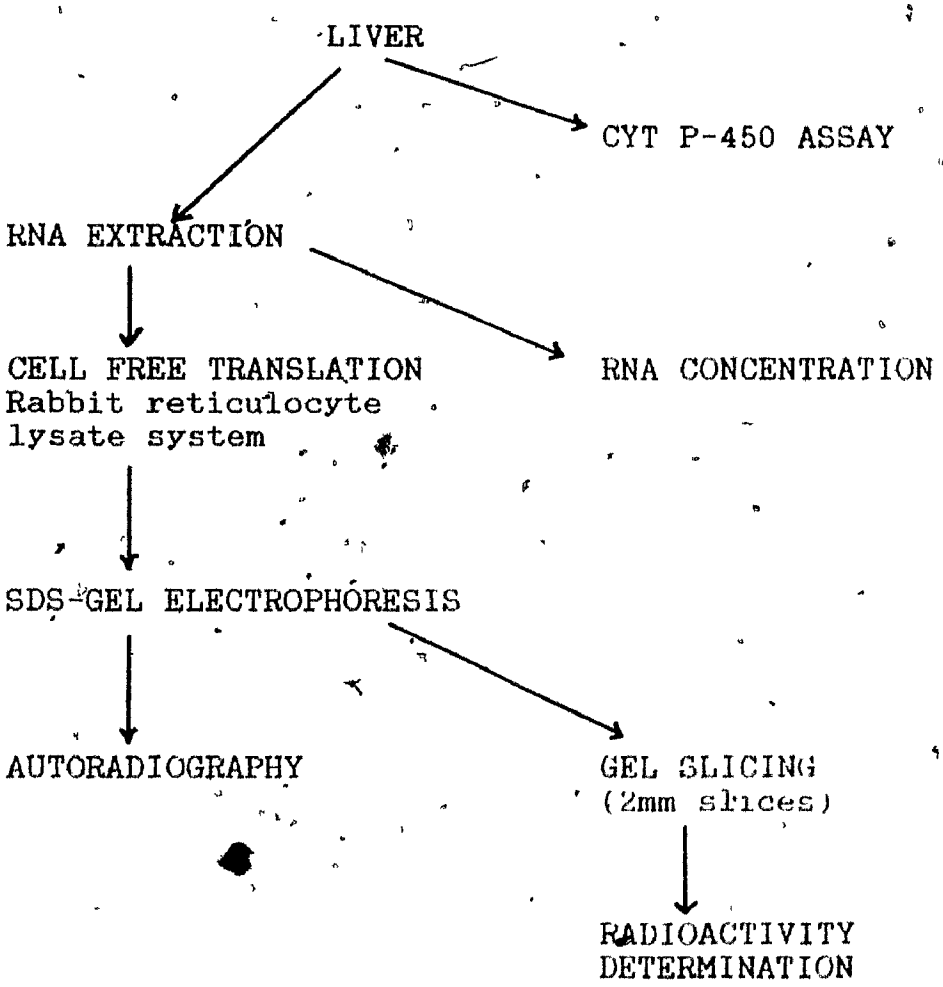


Figure 8

In vitro synthesis of proteins in a cell free system.

T. ISOLATION OF TOTAL CELLULAR RNA:

Total RNA was extracted from the liver using a modification of the procedure of Chirgwin et al., (1979). The use of a strong denaturant, guanidine hydrochloride together with reducing agents such as 2-mercaptoethanol and dithioerythritol ensured that the nucleolytic degradation of RNA by endogeneous ribonuclease was totally inhibited.

Approximately 2 g liver was rapidly removed from the hamster and then homogenized in a guanidine hydrochloride buffer (20 ml/g liver). This buffer contained guanidinium HCl (7.5 M pH 7.0), citric acid (25 mM), 2-mercaptoethanol (10 mM), dithioerythritol (5 mM) and triton X-100 (0.1% v/v). The homogenate was centrifuged at 10,000 x g for 20 min using a Beckman (J2-21) refrigerated centrifuge. The supernatant was decanted into a sterile test-tube and the RNA precipitated by the addition of 0.025 volumes of 1 M acetic acid and 0.75 volume of ethanol. The sample was thoroughly mixed and stored at -25°C overnight to allow precipitation of RNA. The next day the RNA precipitate was collected by centrifugation at 2,500 x g for 10 min in a Beckman benchtop centrifuge. The resultant RNA pellet was again dissolved in guanidinium HCl buffer. A small amount of the buffer was used initially to dissolve the pellet before making up the mixture to a total volume of 15 ml. RNA was reprecipitated by adding 0.025 volume of 1 M acetic

acid and 0.5 volume ethanol. The solution was allowed to stand at -20°C for at least four hours (usually left overnight). The latter step was repeated two more times, this time using 20 ml of buffer to dissolve the RNA. After a final precipitation, the RNA pellet was dissolved in sterile water (1 ml) and 3 M sodium acetate (pH 5.5) was added to give a final concentration of 0.3 M acetate. RNA was then precipitated by the addition of 2.5 volumes of ethanol and stored for at least 4 hr at -20°C. The RNA was pelleted by centrifugation and solubilization and precipitation steps repeated. The ethanol was then removed by drying the pellet in a freeze drier for at least 4 hr to ensure complete evaporation of ethanol. The RNA pellet was dissolved in a small volume of sterile water (100 µl) and stored in 20 µl aliquots at -80°C.

Concentration of RNA in the aliquots was measured spectrophotometrically by adding 10 µl of the RNA preparation to 990 µl of sterile water. The RNA obtained from the above extraction procedure routinely gave an absorbance ratio (E 260 nm/ E 280 nm) of above 1.8. The concentration of RNA was determined spectrophotometrically at 260 nm assuming that an optical density of one corresponded to 40 µg RNA/ml (Avadhari and Buetow, 1972).

U CELL-FREE PROTEIN SYNTHESIS ASSAY

This assay was carried out in rabbit reticulocyte lysate *in vitro* translation system, which actively translates exogenously supplied mRNA into proteins. The lysate system provided in a kit from Bethesda Research Laboratories (BRL), permitted the choice to select the amino acid and isotope for labelling the *in vitro* synthesized protein. The procedure for translation was followed as described in the kit supplied. All procedures were carried out using rubber gloves to prevent contamination from ribonuclease from skin. The components for the assay consisted of the following:

a) Rabbit recticulocyte lysate

Solution containing 3.5 mM $MgCl_2$; 0.05 mM EDTA; 0.05 mM EDTA; 25 mM KCl; 0.5 mM dithioerythritol; 25 µM hemin; 50 µg/ml creatine kinase; 1 mM $CaCl_2$; 2 mM EGTA and 70 mM NaCl.

b) Protein biosynthesis reaction mixture

Solution containing 250 mM HEPES; 400 mM KCl; 100 mM creatine phosphate and 500 µM each of 19 amino acids.

c) Potassium acetate solution (2.0 M at pH 7.2).

d) [^{35}S]-methionine (0.2 µCi/µl)

e) Sterile water

Initially all of the above components were thawed in an ice bath, and were mixed in the following order: a) 30 µl reaction mixture; 2) 7 µl sterile water; 3) 13 µl

potassium acetate; 4) 50 µl [^{35}S]-methionine and 5) 100 µl rabbit reticulocyte lysate. Each of the components was placed in a clean sterile polypropylene microtube on ice and was gently mixed in a Beckman microfuge. Addition of the last component (lysate) brought the total volume of the so called master mix to 200 µl. Twenty µl of master mix were then pipetted into each microtube This was followed by 8 µl of sterile water and 2 µl of RNA (from the 20 µl aliquots). Blank incubation mixtures contained 10 µl of sterile water with the master mix without the addition of RNA. The tubes were swirled gently after each addition and were then incubated for 60 minutes at 30°C. Reaction was stopped by placing the microtubes in ice.

The extent of incorporation of [^{35}S]-methionine into total protein was determined by measuring the amount of radioactivity incorporated into acid-precipitable protein. Six µl of the reaction mixture were added to 1 ml water followed by 0.5 ml of the following solution (1N NaOH, 0.5 M H_2O_2 and 1 mg/ml "cold" methionine) to decolorize the sample and to hydrolyze the t-RNA. This mixture was incubated at 60°C for 15 min. Then 2 ml cold 25% TCA was added and the tubes were placed on ice for at least 1 hr. 100 µl carrier protein (BSA 50 µg/ml) was added to facilitate precipitation. The precipitate was collected by vacuum filtration (Millipore) on glass fibre discs (Whatman GF/C) and washed with approximately 6 mls of 5% cold TCA.

The filters were counted in 5 ml Aquasol using the Beckman LS counter.

By subtracting endogenous incorporation (blank tube) from the observed incorporation, net radioactive amino acid incorporation due to the presence of exogenously added RNA could be measured. Results were expressed as (^{35}S incorporated) cpm per µg RNA or (^{35}S incorporated) cpm/g liver. Equal amounts of radioactivity in TCA precipitable protein translated from saline and IFN treated animals were then subjected to gel electrophoresis.

ELECTOPHORESIS

V. ANALYSIS OF IN VITRO TRANSLATED PROTEINS

Analysis of the translated products of *in vitro* protein synthesis was achieved by fluorographic analysis of the gel which allowed visual detection and fractionation to allow quantitation of radioactive incorporation in various proteins.

i) Fluorography

The purpose of fluorography was to reduce the X-ray film exposure time necessary for visualization. The following procedure was also used in the analysis of [^{35}S]-methionine incorporated in microsomes.

After the translated products (equal amounts of radioactivity in the sample buffer/well) were resolved on gel fixed, stained and destained; the destained gel was

impregnated with Enhance (R) (NEN) in a glass tray for 2 hr with gentle agitation. The Enhance was discarded and excess cold water was added to the tray containing the gel. After 1 hr under gentle agitation, the gel was placed in a solution containing 1% glycerol and 10% acetic acid for 30 min. The consolidated gel was then dried with a filter paper under heat (between 80°-82°C) and vacuum on a slab gel dryer (Biorad Model 224) for 2-3 hr. To facilitate alignment of the stained, dried gel and the developed x-ray film, radioactive coomasie blue dye was spotted on the filter paper of the gel prior to autoradiography. The stained dried gel with radioactive marker spots or filter paper was then placed in direct contact with the X-ray film (Kodak O-Mat AR (R) film), clamped in a radiographic cassette and then wrapped in a plastic bag. The film was exposed at -70°C for 3 days.

(ii) Gel fractionation and counting

Fractionation of the gel and the determination of radioactivity in the solubilized fractions were to quantitate labelled protein components following gel electrophoresis.

Gels were fractionated first by cutting the gel into individual tracks using a long blade which enables cutting the length of the track in a single movement. The gel tract was then sliced transversely (2 mm/slice) using a manual gel slicer (Bio-Rad Model 190). The slicer

consisted of a series of razor blades separated by 2 mm thick metal spacers Each slice was placed in a scintillation vial and solubilized for 13 hr or more with 1 ml 30% H_2O_2 at 60°C. The vials were then allowed to cool to room temperature before opening Ten ml of aquasol was added to each vial and radioactivity counted in a Beckman LS counter. Net incorporation of ^{35}S-labelled protein in various slices was determined by subtracting the corresponding slice of incubation mixture which did not contain RNA from the corresponding slice of gel containing radiolabelled proteins synthesized from exogenously added RNA. Results were expressed as cpm/gel slice.

It must be noted that before fluorography and gel fractionation were performed, a calibration curve was obtained based on the molecular weight standards (as described earlier). This facilitated identification of molecular weights of various proteins. As the molecular weight of cytochrome P-450 apoproteins are within the region of 66,000 D to 45,000 D, gel fractionations were restricted to this region.

W ELECTRON MICROSCOPY:

Right and left liver lobe sections (approximately 1. mm^3) were fixed for 4 hr in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. The sections were washed in the same buffer with sucrose added, post

fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 hr. The sections were dehydrated through graded alcohols and embedded in TAAB embedding resin. For light microscopy, 0.5 μm sections of liver were stained with Toluidine Blue (Trump et al., 1961). For electron microscopy, 60 nm section were stained wtih uranyl acetate and lead citrate and examined on a Phillips 300 electron microscope.

X. STATISTICAL METHODS:

The students t-test for unpaired data was utilized in this thesis to determine statistical significance of the differences between two means. Analysis of variance and Student-Newman-Keuls was used to determine statistical differences when multiple groups were compared. Significance throughout these studies is defined at the 5% level, i.e. $p < 0.05$.

RESULTS

A. The effect of interferon on hepatic microsomal mixed function oxidase in hamsters

The effect of a single intraperitoneal (i.p.) injection of three different doses of IFN-αCON1 on hepatic mixed function oxidase activity, 24 hr after administration are shown in Table 4. IFN-αCON1 caused a dose-dependent decrease in cytochrome P-450 content, but the cytochrome b_5 content remained unchanged following the acute interferon treatment. The loss of cytochrome P-450 at each dose of IFN-αCON1 paralleled the loss of aminopyrine N-demethylase activities. The loss in activity of benzo(a)pyrene hydroxylase with various doses of IFN-αCON1 did not parallel the loss of cytochrome P-450 content.

A single dose of buffy coat interferon (a naturally occurring interferon derived from human leukocytes) caused a comparable depression in cytochrome P-450, aminopyrine N-demethylase and benzo(a)pyrene activities (Table 4) 24 hrs later. In contrast, buffy coat interferon (Singh et.al., 1982) and IFN-αCON1 (Table 5) has no effect on mouse hepatic mixed function oxidase activities.

In the multiple dose study, IFN-αCON1, injected once daily (1×10^6 units, i.p.) for 3 days (Table 6), caused no greater reduction in cytochrome P-450 content or cytochrome P-450 dependent catalytic activity (as clearly seen in subsequent study) compared to a single dose (Table 4). No

TABLE 4

The depression of hepatic cytochrome P-450-dependent drug oxidation by interferon preparations in the hamster liver.

Treatment	Cyt P-450	Cyt b_5	Aminopyrine N-demethylase	Benzo(a) pyrene Hydroxylase
IFN-αCon_1 (5×10^4 units)	90.8 ± 11.3	108.5 ± 7.4	102.9 ± 0.9	133.0 ± 2.5
IFN-αCon_1 (1×10^6 units)	75.1 ± 2.7*	97.3 ± 10.2	80.7 ± 0.9*	32.9 ± 10.3*
IFN-αCon_1 (2×10^7 units)	50.9 ± 9.8*	114.7 ± 15.4	36.2 ± 3.6*	69.2 ± 5.6*
Buffy coat (1×10^6 units)	58.0 ± 2.3*	97.5 ± 3.1	61.9 ± 3.5*	78.2 ± 5.4*
Poly IC (10 mg/kg)	68.2 ± 2.1*	108.6 ± 9.2	55.3 ± 2.1*	77.5 ± 3.6*

*Significantly different from corresponding control, $p < 0.05$.

Values are the mean ± S.E. of the percentage of control values obtained for each treatment group (n=4). Animals were treated i.p. with various doses of interferon which are indicated in parenthesis. After 24 hours, animals were killed and hepatic microsomes prepared. Control values were: cytochrome P-450 = 0.832 ± 0.058 nmoles/mg protein; cytochrome b_5 = 0.461 ± 0.103 nmoles/mg protein; aminopyrine N-demethylase = 424 ± 7 nmoles HCHO/mg protein/hour; benzo(a)pyrene hydroxylase = 9.44 ± 1.27 nmoles 3 OHBP/mg protein/hr.

TABLE 5

The effect of interferon and interferon inducer on cytochrome P-450 levels in mouse liver.

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)
Control	0.758 ± 0.014	0.252 ± 0.015	338 ± 11	7.07 ± 0.11
IFN-αCon_1 (1×10^6 units)	0.788 ± 0.036	0.248 ± 0.15	336 ± 25	6.49 ± 0.32
Control	0.747 ± 0.054	0.306 ± 0.02	329 ± 11	7.26 ± 0.21
Poly IC (10 mg/kg)	0.465 ± 0.064*	0.258 ± 0.01	221 ± 8*	6.59 ± 0.24*

*Significantly different from corresponding control, $p < 0.05$, n=4.

Animals were treated with a single dose of interferon or poly IC, i.p. and killed 24 hours later. Control animals received a corresponding volume of phosphate buffered saline (pH 7.4) at the same time.

TABLE 6

The effect of chronic interferon treatment on cytochrome P-450 levels in hamster hepatic microsomes.

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)
Control	0.819 ± 0.027	0.453 ± 0.057	552 ± 9	8.02 ± 1.24
IFN-αCon$_1$ (1×10^6 units)	0.403 ± 0.059*	0.376 ± 0.065	368 ± 31*	4.61 ± 0.70*

*Significantly different from corresponding control, $p < 0.05$, n=4.

Animals were treated with 3 daily doses of interferon and killed 24 hours after the final dose. Control animals received a corresponding volume of sterile phosphate buffered saline at the same time.

TABLE 7

Mixed function oxidase activity in hamster hepatic microsomes incubated <u>in vitro</u> with interferon or poly IC

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Ethoxyresorufin O-de-ethylase (nmoles resorufin formed/mg protein/min)
Control	0.811 ± 0.003	0.31$_2$ ± 0.006	476 ± 3	0.12 ± 0.02
IFN-αCon$_1$	0.813 ± 0.002	0.314 ± 0.001	478 ± 4	0.14 ± 0.01
Control	0.796 ± 0.002	0.302 ± 0.003	462 ± 3	0.13 ± 0.01
Poly IC	0.790 ± 0.001	0.300 ± 0.002	460 ± 4	0.11 ± 0.03

Poly IC (1 mg) or IFN-αCON$_1$ (10^6 units) was added to microsomal suspension 15 min prior to assay. Control mirosomal suspensions received equivalent volumes of vehicle. Each value represents means and standard error of three replicates.

significant reduction in cytochrome b5 content was observed with chronic IFN-αCON1 treatment.

The treatment of hamsters with poly IC, an interferon inducer (10 mg/kg, i.p., 24 hrs), also produced a significant depression in cytochrome P-450, aminopyrene N-demethylase and benzo(a)pyrene hydroxylase (Table 4). These results are similar to those reported by Renton and Mannering (1976) in rats and Singh and Renton (1981) in mice.

IFN-αCON1 and Poly IC had no effect on the hepatic mixed function oxidase when incubated with hepatic microsomes in vitro (Table 7), demonstrating that interferon has no direct effect on cytochrome P-450

B. Temporal changes in hepatic microsomal mixed function oxidase in hamsters following a single dose of interferon or poly IC

The effects of poly IC (10 mg/kg i.p.) on hepatic cytochrome P-450, aminopyrine N-demethylase and benzo (a)pyrene hydroxylase activities in microsomes prepared from hamsters, at various times after treatment are shown in Figure 9. After 6 hr of poly IC treatment, cytochrome P-450 content was significantly elevated to 123% of control, but the content was depressed significantly to 68% of control by 24 hours. The content of cytochrome P-450 returned to within 90% of control within 120 hrs of

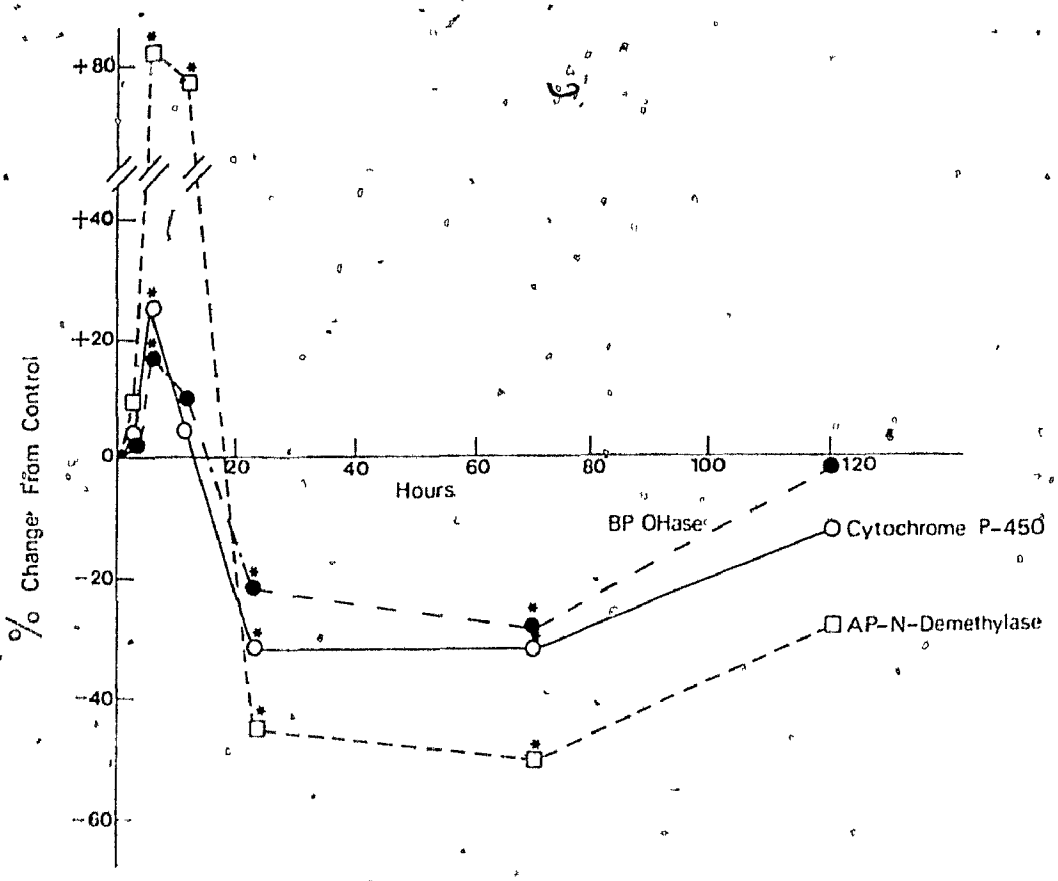


Figure 9

Hepatic Mixed Function Oxidase in the Liver of Hamsters Treated with Poly IC for Various Times

Control values at zero times were: cytochrome P-450 = 0.73 ± 0.01 nmoles/mg protein; aminopyrine N-demethylase = 566 ± 45 nmoles HCHO formed/mg protein/hr; benzo(a)pyrene hydroxylase = 9.99 ± 0.12 ^{3}OH-benzo(a)pyrene formed/mg protein/hr. Each value is the mean of 4 individual animals at each time period. *Significantly different from control, $p < 0.05$.

treatment. Aminopyrine N-demethylase and benzo(a)pyrene hydroxylase activity paralleled the changes in cytochrome P-450 content throughout the treatment time course. The biphasic change in activity of aminopyrine N-demethylase fluctuated between 187% of control at 6 hr after poly IC treatment to 50% of control at 24 hr. On the other hand, benzo(a)pyrene hydroxylase activity was elevated to only 116% of control at 6 hr and dropped to 77% of control after 24 hr, before recovering to control value by 120 hr. There was no significant change in cytochrome b_5 content in the hepatic microsomes throughout the time course except at 6 hr and 72 hrs after poly IC treatment, where the levels were elevated to 125% and 150% respectively (control cytochrome b_5 level = 0.22 ± 0.01, n = 4). Poly IC had no effect on body weight or liver weight throughout the time period studied but microsomal protein content was significantly elevated at 6 hrs. There was no difference in microsomal protein content subsequent to the 6 hr treatment period. Round and Stebbing (1981) have shown that a single dose of poly IC in hamster induces serum interferon level which peaks at 3-4 hrs. No interferon levels were detected following 24 hr poly IC treatment period in their experiments. It seems that the appearance of interferon in serum correlates with the stimulation of hepatic mixed function oxidase activity and that the

depression of mixed function oxidase activity is not due to the direct interaction of interferon with cytochrome P-450.

The biphasic pattern of stimulation and the depression of hamster hepatic mixed function oxidase activity with time following a single dose of poly IC, was similarly observed with a single dose of IFN-α CON1 (Figure 10). Cytochrome P-450 content was maximally stimulated 3 hrs after IFN-αCON1 treatment ($1x10^6$ units, i.p.). The activity of aminopyrine N-demethylase and benzo(a)pyrene hydroxylase were also stimulated to 149% and 110% of control respectively after 3hr IFN-αCON1 treatment. The first significant depression of cytochrome P-450 occurred between 12 to 24 hr after IFN-αCON1 administration; at 24 hr the cytochrome P-450 content was 79% of control. Biphasic changes in the activities of aminopyrine N-demethylase and benzo(a)pyrene hydroxylase were also observed after IFN-αCON1 and these changes paralleled the cytochrome P-450 content in the microsomes. Depression of aminopyrine N-demethylase activity occurred between 6 to 12 hr after IFN-αCON1 administration; at 12 hr, the activity of demethylase was 80% of control. The first significant depression of benzo(a)pyrene hydroxylase activity was observed between 12 to 24 hrs after IFN-αCON1 treatment. By 170 hr of IFN-αCON1 treatment, cytochrome P-450 content and aminopyrine N-demethylase activity had returned to

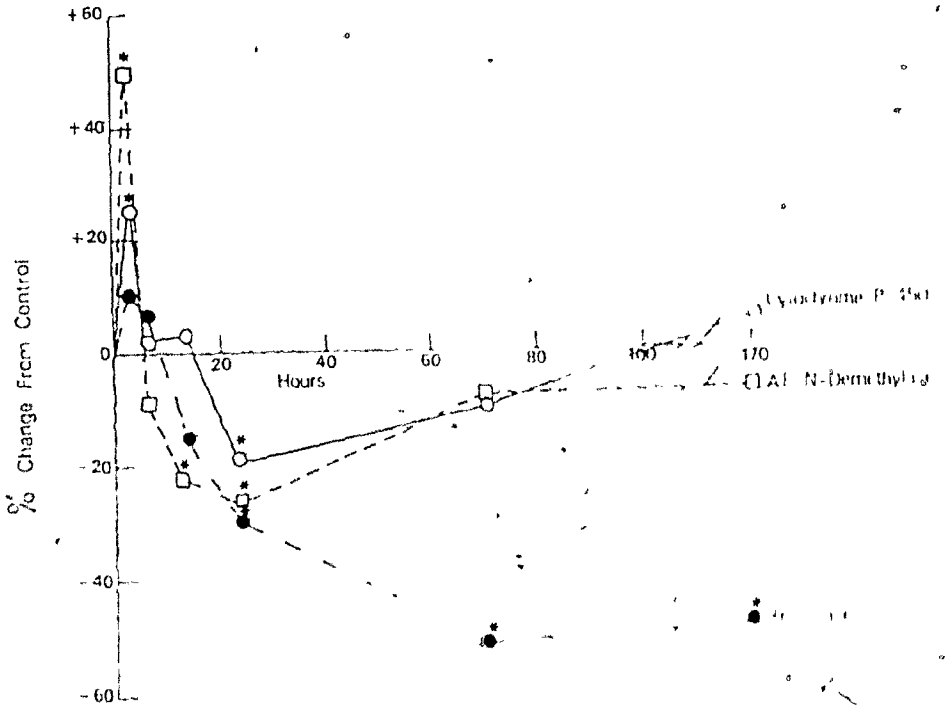


Figure 10

Hepatic Mixed Function Oxidases in the Liver of Hamsters Treated with IFN αCON1 for Various Times

Control values at zero time were: cytochrome P 450 0 75 ± 0 02 nmoles/mg protein, aminopyrine n demethylase 420 ± 13 nmoles/mg protein/hour; benzo(a)pyrene hydroxylase 11 7 ± 1.5 nmoles ^{3}OH benzo(a)pyrene/mg protein/hour Each value is the mean of 4 individual animals at each time period. *Significantly different from control, p < 0.05

control levels, however benzo(a)pyrene hydroxylase activity remained depressed even at this time. Cytochrome b5 content was not altered throughout the time period studied Also, there was no effect on body weight or liver weight during the time course studied. Microsomal protein content was significantly higher at 3 hrs (119% of control) and 6 hr (125%) but returned to control values after 12 hr of IFN treatment.

In a separate study, a single dose of IFN-αCON1 (1×10^6 units/hamster i.p.) for 3 hrs stimulated cytochrome P-450 content, aminopyrine N-demethylase and benzo(a)pyrene activities to 156%, 158% and 145% of control respectively. In this study the substrates, lauric acid and ethoxyresorufin, were used as markers for specific cytochrome P-450 isoenzymes. Lauric acid hydroxylase and ethoxyresorufin O-de-ethylase activities were increased to 117% and 187% of control after 3hr of IFN-αCON1 treatment. After 24 hr of IFN-αCON1 treatment, cytochrome P-450 content and the activities of aminopyrine N-demethylase, benzo(a)pyrene hydroxylase, ethoxyresorufin O-de-ethylase and lauric acid hydroxylase were depressed to 65%, 64%, 79%, 60% and 63% of control respectively. There was no change in cytochrome b5 level at either 3 hr or 24 hr of after IFN-αCON1 treatment (Table 8).

TABLE 8

The effect of interferon treatment for 3 hours and 24 hours on hepatic microsomal mixed function oxidase activity of hamsters

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)	Ethoxyresorufin O-de-ethylase (nmoles/mg protein/min)	Lauric Acid Hydroxylation (11 + 12 OH laurate/mg protein/min
Control	0.77 ± 0.05	0.26 ± 0.03	408 ± 10	8.91 ± 0.60	0.104 ± 0.012	2.21 ± 0.28
IFN-αCon_1 (3 hr)	1.20 ± 0.08*	0.34 ± 0.03	645 ± 60*	12.88 ± 0.13*	0.194 ± 0.009*	*2.58 ± 0.22
Control	0.80 ± 0.01	0.34 ± 0.02	415 ± 16	11.94 ± 0.42	0.12 ± 0.02	2.13 ± 0.12
IFN-αCon_1 (24 hr)	0.52 ± 0.01*	0.33 ± 0.02	265 ± 18*	9.39 ± 0.43*	0.072 ± 0.04*	1.34 ± 0.15*

*Significantly different from corresponding control, $p < 0.05$, n=4.
Animals were treated with a single dose of IFN-αCON_1 (1×10^6 units) and killed 3 and 24 hours later.
Control animals received a corresponding volume of sterile phosphate buffered saline at the same times.

C. The effect of poly IC and interferon on NADPH cytochrome c reductase activity

The comparison in the response of cytochrome P-450 content and NADPH cytochrome c reductase activity to a single dose of IFN-αCON1 (1 x 10^6 units, i.p.) for 3 hrs and 24 hrs, and 24 hrs after poly IC treatment is shown in Table 9 and Table 10, respectively. The response of cytochrome P-450 content and NADPH cytochrome c reductase activity after 3 daily doses of IFN-αCON1 (1 x 10^6 units i.p.) is shown in Table 11. Although microsomal cytochrome P-450 content was elevated following 3 hrs and depressed after 24 hrs by IFN-αCON1 treatment, NADPH cytochrome c reductase activity was not significantly changed at either times. Similarly, NADPH cytochrome c reductase activity did not significantly change after either 24 hr treatment period with poly IC or 24 hr after 3 daily doses of IFN-αCON1, although cytochrome P-450 content was depressed It therefore appears that the effect of poly IC and IFN-αCON1 is restricted to the cytochrome P-450 component of the hepatic mixed function oxidase system.

The effect of interferon on total microsomal heme content and heme oxygenase activity in hepatic microsomes

The effect of IFN-αCON1 (1 x 10^6 units, i.p.) on total microsomal heme content and heme oxygenase activity after a single dose for 3 hr or 24 hrs and 24 hr after three daily doses of IFN-αCON1 is shown in Table 12. Total heme

TABLE 9

The effect of interferon treatment for 3 hours on NADPH-cytochrome c reductase and cytochrome P-450 level in hepatic microsomes in hamsters

Treatment	NADPH-cytochrome c reductase nmole/min/mg protein	Cytochrome P-450 nmole/mg protein
Control	171 ± 8.3	0.811 ± 0.06
IFN-αCon_1 (1×10^6 units)	193 ± 11.3	1.294 ± 0.06*

Animals were treated with interferon (IFN-αCon_1, 1×10^6 units i.p.) for 3 hours. Each value is the mean of 4 individual animals.

*Significantly different from corresponding control, $P < 0.05$.

TABLE 10

The effect of interferon treatment for 24 hours on NADPH-cytochrome c reductase and cytochrome P-450 level in hepatic microsomes in hamsters

Treatment	NADPH-cytochrome c reductase nmole/min/mg protein	Cytochrome P-450 nmole/mg protein
Control	163.1 ± 6.1	0.812 ± 0.08
IFN-αCon_1 (1 x 10^6 units)	176.6 ± 6.3	0.576 ± 0.12*
Control	170.1 ± 5.2	0.876 ± 0.12
Poly IC (10 mg/kg)	166.2 ± 7.1	0.510 ± 0.16*

Animals were treated with interferon (IFN-αCon_1, 1 x 10^6 units) and interferon inducer (poly rI.rC. 10 mg/kg) for 24 hours. Each value is the mean of 4 individual animals.

*Significantly different from corresponding control, $P < 0.05$.

TABLE 11

The effect of 3 daily doses of interferon treatment on NADPH-cytochrome c reductase and cytochrome P-450 level in hepatic microsomes in hamsters

Treatment	NADPH-cytochrome c reductase nmole/min/mg protein	Cytochrome P-450 nmole/mg protein
Control	166 ± 6	0.819 ± 0.027
IFN-αCon_1 (1×10^6 units)	141 ± 7	0.403 ± 0.059*

Animals were treated with 3 daily doses of interferon (IFN-αCon_1, 1×10^6 units i.p.) and killed 24 hours after the final dose. Each value is the mean of 4 individual animals.

*Significantly different from corresponding control, $P < 0.05$.

TABLE 12

The effect of interferon on heme oxygenase and total heme content in hepatic microsomes in hamsters

Treatment	Total Heme nmole/mg protein	Heme Oxygenase nmole bilirubin/ mg protein/hour
Control	1.00 ± 0.08	0.06 ± 0.01
IFN-αCon_1 (3 hr)	1.30 ± 0.10	0.07 ± 0.02
Control	0.72 ± 0.02	0.05 ± 0.01
IFN-αCon_1 (24 hr)	0.53 ± 0.02*	0.10 ± 0.01*
Control	0.71 ± 0.02	0.053 ± 0.01
IFN-αCon_1 (3 daily doses)	0.54 ± 0.03*	0.111 ± 0.02*

*Significantly different from corresponding control, $p < 0.05$, n=4. Animals were treated with a single dose of interferon (IFN-αCON_1, 1×10^6 units i.p.) and killed 3 hrs or 24 hrs later, or 3 daily doses of interferon and killed 24 hrs later. Control animals received corresponding volume of sterile phosphate saline at the same times.

content was significantly depressed after a single dose of IFN-αCON1, 24 hrs and after 3 daily doses of IFN-αCON1 by 73% and 75% of control respectively. The depression of total microsomal heme content corresponded to an increase in oxygenase activity to 224% and 209% of control respectively after a single and 3 daily doses of IFN-αCON1. There was no change in total microsomal heme content and heme oxygenase activity after 3 hr IFN-αCON1

D. The effect of poly IC and interferon on extrahepatic cytochrome P-450 and benzo(a)pyrene hydroxylase activity

The effect of a single dose of IFN-αCON1 (1 x 10^6 units i.p.) for 3 hr and 24 hr of treatment; and poly IC (10 mg/kg i.p.) for 24 hr treatment, on extrahepatic microsomal cytochrome P-450 content and benzo(a)pyrene hydroxylase activity is illustrated in Tables 13. 14 and 15.

Following a 3 hr treatment period with IFN-αCON1, cytochrome P-450 in the lung, spleen and adrenals increased to 240%, 160% and 119% of control respectively. Benzo(a)pyrene hydroxylase activity in the corresponding tissues was increased to 190%, 119% and 118% of control respectively. In contrast, the cytochrome P-450 content and benzo(a)pyrene hydroxylase activity in the kidney, however were depressed to 56% and 94% of control respectively.

TABLE 13

The effect of interferon treatment for 3 hours on cytochrome P-450 and benzo(a)pyrene hydroxylase activity in extrahepatic microsomes

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control	0.135	2.60
	IFN-αCON$_1$	0.324	4.93
Kidney	Control	0.185	5.35
	IFN-αCON$_1$	0.103	5.01
Spleen	Control	0.131	0.75
	IFN-αCON$_1$	0.210	0.89
Adrenals	Control	0.236	2.03
	IFN-αCON$_1$	0.280	2.39

Animals were treated with a single dose of IFN-αCON$_1$ (1 x 10^6 units i.p.) and killed 3 hours later. Control animals received a corresponding volume of sterile phosphate buffered saline at the same times. Results are mean of two sets of experiments from microsomes pooled from a group of 4 animals in each set.

TABLE 14

The effect of interferon treatment for 24 hours on cytochrome P-450 and benzo(a)pyrene hydroxylase activity in extrahepatic microsomes

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control	0.138	2.00
	IFN-αCON_1	0.061	1.34
Kidney	Control	0.154	5.70
	IFN-αCON_1	0.046	3.14
Spleen	Control	0.121	0.80
	IFN-αCON_1	0.085	0.64
Adrenals	Control	0.220	2.81
	IFN-αCON_1	0.130	1.96

Animals were treated with a single dose of IFN-αCON_1 (1×10^6 units i.p.) and killed 24 hours later. Control animals received a corresponding volume of sterile phosphate buffered saline at the same times. Results are mean of two sets of experiments from microsomes pooled from a group of 4 animals in each set.

TABLE 15

The effect of poly IC treatment for 24 hours on cytochrome P-450 and benzo(a)pyrene hydroxylase activity in extrahepatic microsomes

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control	0.160	1.91
	Poly IC	0.076	1.24
Kidney	Control	0.092	5.51
	Poly IC	0.029	2.92
Spleen	Control	0.121	0.71
	Poly IC	0.082	0.57
Adrenals	Control	0.220	1.88
	Poly IC	0.132	1.35

Animals were treated with a single dose of poly IC (10 mg/kg, i.p.) and killed 24 hours later. Control animals received a corresponding volume of sterile phosphate buffered saline at the same times. Values represent results from one set of experiment of microsomes pooled from a group of 4 animals of each treatment.

After a 24 hr treatment period with IFN-αCON1 and poly IC, both cytochrome P-450 and benzo(a)-pyrene hydroxylase were depressed in all of the extrahepatic tissues, however the degree of depression of extrahepatic mixed function oxidase differed. Whereas the kidney cytochrome P-450 content and benzo(a)pyrene hydroxylase activity were depressed by 30% and 55% of control respectively by IFN-αCON1 treatment, spleen cytochrome P-450 content and benzo(a)pyrene hydroxylase activity were only depressed to 60% and 70% of control respectively. Lung and adrenal cytochrome P-450 content were depressed to 48% and 60% of control respectively with concomitant depression of benzo(a)pyrene hydroxylase activities to 67% and 70% of control respectively. Poly IC treatment after 24 hrs caused comparable depression of cytochrome P-450 content and benzo(a)pyrene hydroxylase activity in these tissues.

E. Analysis of microsomal proteins by gel electrophoresis after interferon treatment

The SDS-polyacrylamide gel electrophoretic pattern of protein staining bands from hepatic microsomal preparation, was used as a crude determination of changes in concentration of particular forms of cytochrome P-450 polypeptide. Chiang and Steggles (1983) have assigned electrophoretic bands of polypeptides at 48,500, 50,000 and 53,500 as subunits of cytochrome P-450 apoproteins based on

induction studies in hamsters with Aroclor 1256, phenobarbital and 3-methylcholanthrene. We have noted changes in the microsomal proteins at the above mentioned molecular weight regions. The effect of administration of IFN-αCON1 (1 x 10^6 units i.p.) for 3 hr, 24 hr and 24 hr after 3-daily doses to hamsters, on the electrophoretic pattern of 100 μg of microsomal proteins is shown in Figures 11, 12 and 13.

After 3 hr of IFN-αCON1 treatment, the intensity of a protein band with the mobility in the region associated with cytochrome P-450 of Mr = 48,000 was increased compared to the same protein band obtained for saline treated hamsters. No significant change in intensity in other polypeptides was observed. Proteins with mobilities at Mr = 48,000 and 50,000 appeared to be present in reduced concentration at 24 hrs after a single dose and 24 hr after 3 daily doses of IFN-αCON1. No appreciable change in intensity of other bands were noted with either treatment protocol. It was concluded that the changes of certain bands assigned as cytochrome P-450 apoprotein were correlated with the content of cytochrome P-450, determined in hepatic microsomes, after a single dose for 3 hr and 24 hr and 24 hr after 3 daily doses of IFN-αCON1. It must be noted that the overall different intensity of protein bands in the different photographs were probably due to

Figure 11

SDS-polyacrylamide gel electrophoresis of microsomal proteins from hamsters treated with IFN-αCON1 for 3 hr A 3 mm slab gel was used as described in Materials and Methods. Samples analyzed were: Wells (a) and (b) contained liver microsomes (100 μg protein) from saline and interferon treated hamsters respectively. Molecular weight standards used were: Bovine albumin (66,000), Egg Albumin (45,000), Glyceraldehyde-3-phosphate Dehydrogenase (36,000), Carbonic Anhydrase (29,000) and Trypsinogen (24,000).

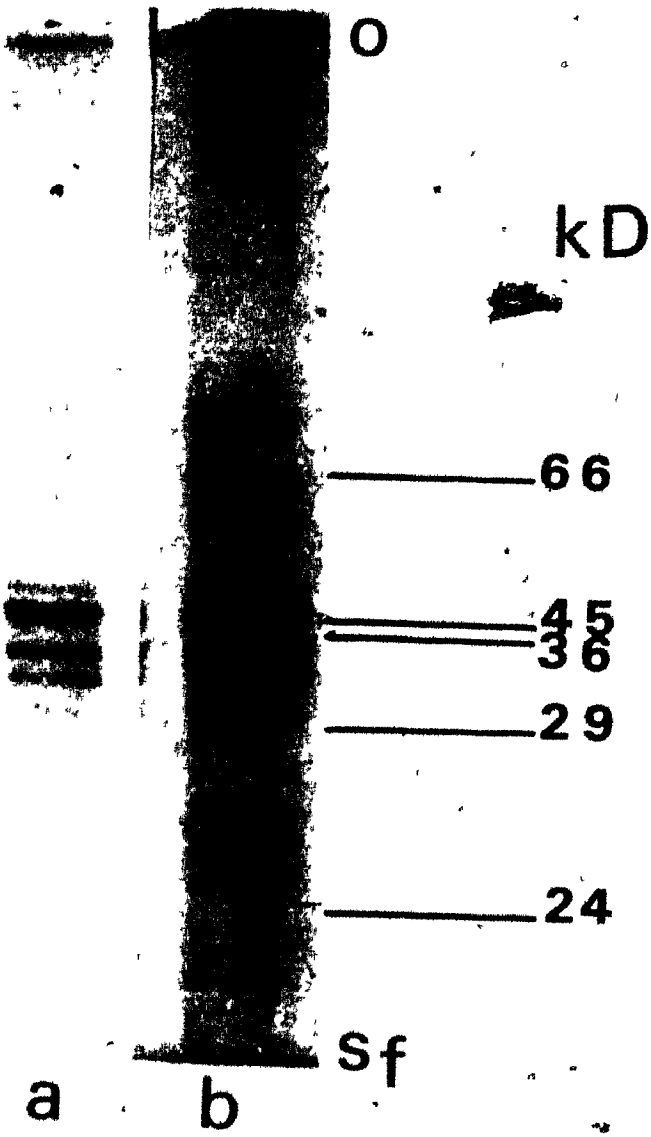

o
kD
66
45
36
29
24
sf
a
b

Figure 12

SDS-polyacrylamide gel electrophoresis of microsomal proteins from hamsters treated with IFN-αCON1 for 24 hr A 3 mm slab gel was used as described in Materials and Methods. Samples analyzed were: Wells (a) and (b) contained liver microsomes (100 µg protein) from saline and interferon treated hamsters respectively. Molecular weight standards used were: Bovine albumin (66,000), Egg Albumin (45,000), Glyceraldehyde-3-phosphate Dehydrogenase (36,000), Carbonic Anhydrase (29,000) and Trypsinogen (24,000), and Trypsin Inhibitor (20,000).

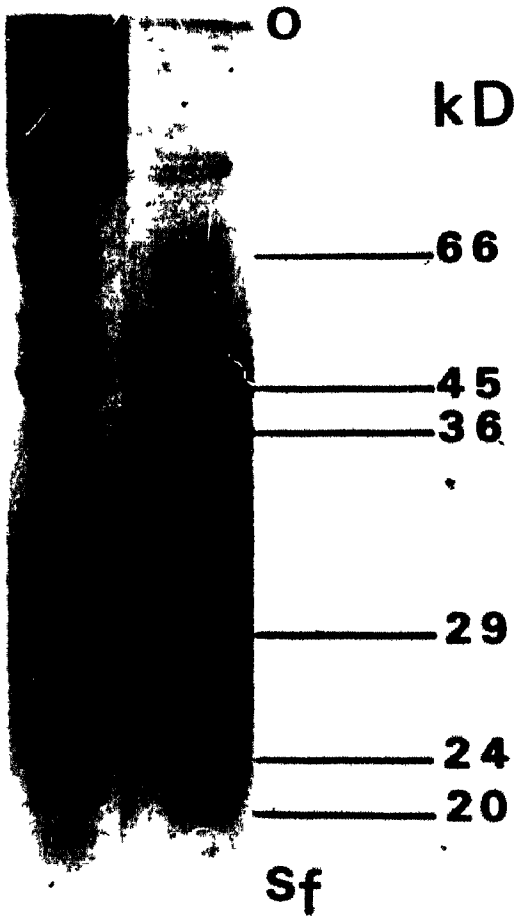
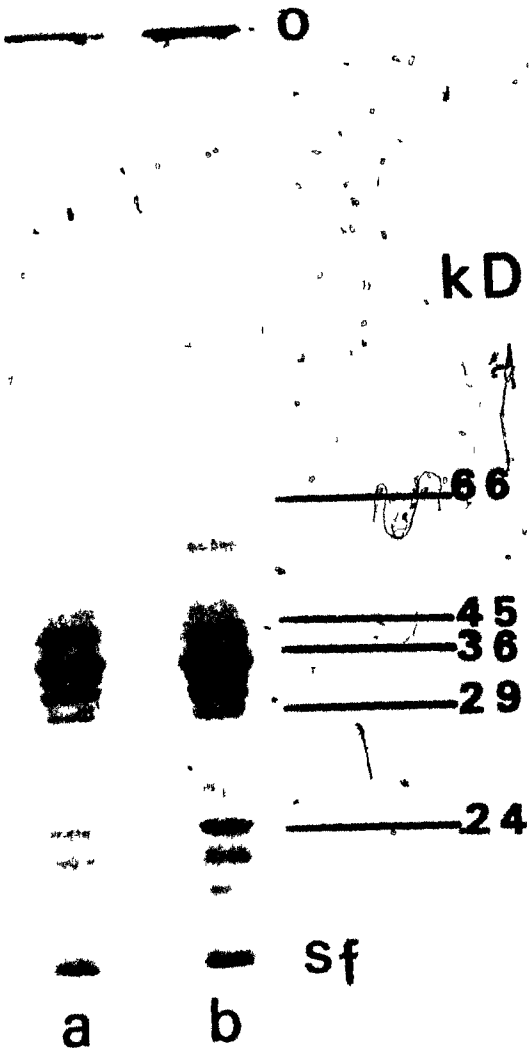

O
kD
66
45
36
29
24
20
Sf
a
b

Figure 13

SDS-polyacrylamide gel electrophoresis of microsomal proteins from hamsters treated with IFN-αCON1 daily for 3 days. A 3 mm slab gel was used as described in Materials and Methods. Samples analyzed were: Wells (a) and (b) contained liver microsomes (100 µg protein) from saline and interferon treated hamsters respectively. Molecular weight standards used were: Bovine albumin (66,000), Egg Albumin (45,000), Glyceraldehyde-3-phosphate Dehydrogenase (36,000), Carbonic Anhydrase (29,000) and Trypsinogen (24,000).


o
kD
66
45
36
29
24
sf
a
b

differences in timing of destaining and photographing the gel.

F. The effect of interferon on xenobiotic-induced cytochrome P-450

This study was undertaken to ascertain whether the depression of hepatic mixed function oxidase activity by IFN-αCON1 after 24 hr treatment period was the result of various cytochrome P-450 hemoproteins being equally or selectively depressed. The effect of IFN-αCON1 (1×10^6 units, i.p.) for 24 hrs, on hepatic cytochrome P-450 content and mixed function oxidase activities were determined in animals induced by phenobarbital, β-napthoflavone and clofibrate (Table 16). Administration of IFN-αCON1 by itself significantly depressed cytochrome P-450, aminopyrine N-demethylase and benzo(a)pyrene hydroxylase activity to 66%, 65% and 81% of control respectively. Lauric acid hydroxylation was significantly depressed to 63% of control by IFN-αCON1 administration (control = 2.20 ± 0.09, IFN-αCON1 = 1.39 ± 0.06 11+12 OH lauric acid/mg protein/min).

The respective 141% and 363% increases in cytochrome P-450 content and aminopyrine N-demethylase activity induced by phenobarbital were lowered by interferon by 23% and 45% respectively from the induced level. The 143%

TABLE 16

Effects of interferon treatmnt on hepatic mixed function oxidase of hamsters induced with different xenobiotics

Treatment	Inducer	Cyt P-450 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/ mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP/ mg protein/hr)
Saline	None [12]	0.93 ± 0.04	471 ± 20	12.7 ± 0.7
IFN-αCON$_1$	None [12]	0.62 ± 0.03$^+$	306 ± 19$^+$	10.3 ± 0.3
Saline	Phenobarbital [4]	1.31 ± 0.01*	1709 ± 32*	18.2 ± 0.5*
IFN-αCON$_1$	Phenobarbital [4]	1.01 ± 0.04$^+$	935 ± 42$^+$	16.6 ± 0.5
Saline	β-naphthoflavone [4]	2.21 ± 0.03*	410 ± 16	26.3 ± 1.1*
IFN-αCON$_1$	β-naphthoflavone [4]	0.98 ± 0.03$^+$	314 ± 9$^+$	21.6 ± 0.8
Saline	Clofibrate [4]	0.90 ± 0.04	650 ± 27	16.3 ± 0.8
IFN-αCON$_1$	Clofibrate [4]	0.42 ± 0.09$^+$	506 ± 27$^+$	15.8 ± 0.6

* Significantly different from saline control. $P < 0.05$
$^+$ Significantly different from corresponding inducer treatment. $P < 0.05$
Animals, pretreated with phenobarbital, β-naphthoflavone and clofibrate (see Method section for treatment protocol), received a single dose of interferon (IFN-αCON$_1$, 1 x 10^6 units i.p.) and killed 24 hours later. Control animals received corresponding volume of vehicles and interferon. Numbers in parenthesis show the number of animals.

increase in benzo(a)pyrene hydroxylase activity by phenobarbital was decreased by 12% by IFN-αCON1

The 238% and 207% increase in cytochrome P-450 and benzo(a)pyrene hydroxylase activity respectively, induced by β-napthoflavone were lowered by 56% and 18% respectively from the induced level. Aminopyrine N-demethylase activity was not induced by β-napthoflavone However, IFN-αCON1 decreased the activity of aminopyrine N-demethylase of β-napthoflavone treated animal by 23%

Clofibrate, a known inducer of a cytochrome P-452, did not significantly change total cytochrome P-450 content, aminopyrine N-demethylase and benzo(a)pyrene hydroxylase activity. The activity of lauric acid hydroxylase was increased to 760% of saline control (control = 2.20 ± 0.09, clofibrate = 16.71 ± 1 00 11+12 OH lauric acid/mg protein/min). The cytochrome P-450 content and lauric acid hydroxylase activity were lowered by 54% and 31% respectively from the induced level by IFN-αCON1 .

It was concluded that hepatic aminopyrine N- demethylase and lauric acid hydroxylase, both substrates for diffent cytochrome P-450 isozymes were depressed to a greater extent than benzo(a)pyrene hydroxylase activity, by IFN-αCON1 treatment in hamsters.

The effect of IFN-αCON1 for 24 hr on lung cytochrome P-450 content and benzo(a)pyrene hydroxylase activity

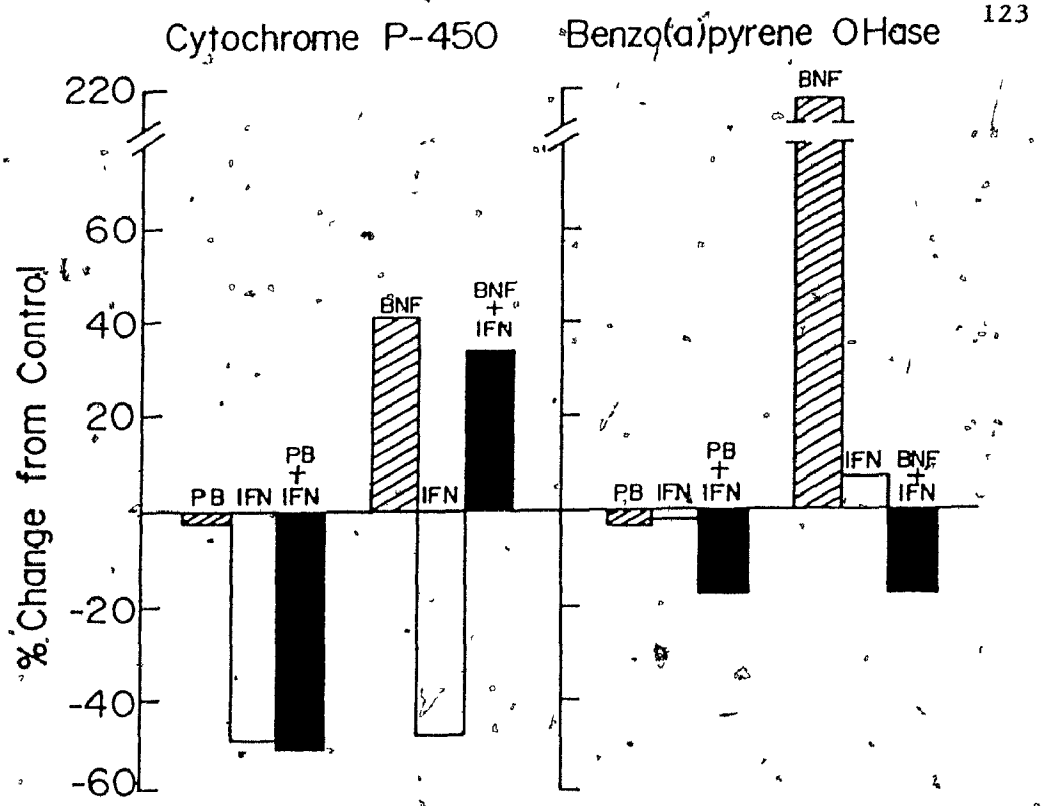


Figure 14

Animals pretreated with phenobarbital or β-napthoflavone (see Method section for treatment protocol), received a single dose of interferon (IFN-αCON1, 1 x 10⁶ units, i.p.) and were killed 24 hours later. Control animals received corresponding volumes of vehicles and interferon. Enzymatic assay was performed from one set of experiments of microsomes from a group of 4 animals of each treatment. Control value for phenobarbital experiment: cytochrome P-450 = 0.15 nmoles/mg protein; benzo(a)pyrene hydroxylase = 0.42 nmoles ^{3}OH BP/mg protein/hr. Control value for β-napthoflavone experiment: cytochrome P-450 = 0.147 nmoles/mg protein; benzo(a)pyrene hydroxylase = 0.50 nmoles ^{3}OH BP/mg protein/hr.

induced by phenobarbital and β-napthoflavone as shown in Figure 14. Administration of IFN-α CON1 itself appreciably depressed cytochrome P-450 content, but had no effect on benzo(a)pyrene hydroxylase activity. Administration of phenobarbital itself, had no effect on lung cytochrome P-450 content or benzo(a)pyrene hydroxylase activity. IFN-αCON1 caused an appreciable depression in cytochrome P-450 content and benzo(a)pyrene hydroxylase activity of 49% and 82% respectively in phenobarbital treated animal. Administration of β-napthoflavone caused an appreciable increase in cytochrome P-450 content and benzo(a)pyrene hydroxylase activity to 140% and 317% of control respectively. The corresponding increase in cytochrome P-450 content and benzo(a)pyrene activity was depressed by 7% and 338% respectively after IFN-αCON1 treatment. It appeared that the depression of benzo(a)pyrene activity by IFN-αCON1 in β-napthoflavone treated hamsters was greater in the lung than in the liver.

G The effects of an inhibitor of protein synthesis on interferon-induced stimulation and depression of mixed function oxidase activity

The aim of these experiments was to determine whether the stimulation and depression of mixed function oxidase activity, at 3 hr and 24 hr after administration of IFN-αCON1 respectively, involved the synthesis of an intermediate protein. The effects of puromycin

TABLE 17

The effect of puromycin on interferon induced stimulation of hepatic mixed function oxidase activity in hamsters

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)	Ethoxyresorufin O-de-ethylase (nmoles resorufin/ mg protein/min)
Control	0.77 ± 0.05	0.26 ± 0.03	408 ± 10	8.91 ± 0.60	0.104 ± 0.012
IFN-αCon_1	1.20 ± 0.08*	0.34 ± 0.03	645 ± 60*	12.88 ± 0.13*	0.194 ± 0.004*
Puromycin	0.83 ± 0.02	0.26 ± 0.02	405 ± 32	8.71 ± 0.84	0.132 ± 0.013
IFN-αCon_1 plus puromycin	0.67 ± 0.04	0.27 ± 0.02	350 ± 31	9.78 ± 0.89	0.138 ± 0.014

*Significantly different from corresponding control, $p < 0.05$, n=4.
Animals were treatd with a single dose of interferon (IFN-αCON_1, 1 x 10^6 units i.p.) at 0 hour and 4 doses of puromycin (0.272 mg in 0.17 mls of PO_4 buffered saline) at -2, 0, 1, 2 hours and killed 3 hours after interferon treatment. Control and interferon only treated animals received a corresponding volume of PBS at the same times.

TABLE 18

The effect of puromycin on interferon induced depression of hepatic mixed function oxidase activity in hamsters.

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)
Control	0.80 ± 0.01	0.34 ± 0.02	442 ± 14	11.94 ± 0.42
IFN-αCon_1	0.52 ± 0.01*	0.33 ± 0.02	332 ± 11*	9.39 ± 0.43*
Pyromycin	0.77 ± 0.02	0.35 ± 0.02	452 ± 6	12.15 ± 0.45
IFN-αCon_1 plus puromycin	0.74 ± 0.02	0.33 ± 0.01	453 ± 12	12.1 ± 0.55

*Significantly different from corresponding control, $p < 0.05$, n=4.

Animals were treated with a single dose of interferon (IFN-αCon_1 1 x 10^6 units i.p.) at 0 hour and 6 doses of puromycin (2.72 mg in 0.17 ml of 0.15 M NaCl-0.04 M phosphate buffer i.p.) at -2, 0, 1½, 3, 4 and 5 hours and killed 24 hours after interferon treatment. Control and interferon only treated animals received corresponding volume of phosphate buffered saline at the same times.

TABLE 19

The effect of puromycin on interferon induced stimulation of extrahepatic mixed function oxidase activity in hamsters

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control (C)	0.136	2.53
	Puromycin (P)	0.097	3.14
	IFN-αCON_1 (IFN)	0.293	4.79
	P + IFN	0.101	2.67
Kidney	Control (C)	0.183	4.98
	Puromycin (P)	0.172	4.75
	IFN-αCON_1 (IFN)	0.113	4.58
	P + IFN	0.106	4.63
Spleen	Control (C)	0.123	0.53
	Puromycin (P)	0.077	0.51
	IFN-αCON_1 (IFN)	0.178	0.60
	P + IFN	0.079	0.49
Adrenals	Control (C)	0.203	1.93
	Puromycin (P)	0.171	1.73
	IFN-αCON_1 (IFN)	0.253	2.33
	P + IFN	0.208	1.94

Animals were treated with a single dose of interferon (IFN-αCON_1, 1×10^6 units i.p.) at 0 hour and 4 doses of puromycin (0.272 mg in 0.17 ml of PO4 buffered saline) at -2, 0, 1 and 2 hours and killed 3 hours after interferon treatment. Control and interferon only treated animals received a corresponding volume of PBS at the same times. Values represent results from one set of experiments of microsomes from a group of 4 animals of each treatment.

TABLE 20

The effect of puromycin on interferon induced depression of extrahepatic mixed function oxidase activity in hamsters

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control (C)	0.128	2.32
	Puromycin (P)	0.120	2.30
	IFN-αCON_1 (IFN)	0.053	1.36
	P + IFN	0.115	2.20
Kidney	Control (C)	0.163	5.41
	Puromycin (P)	0.154	5.31
	IFN-αCON_1 (IFN)	0.056	3.48
	P + IFN	0.156	4.98
Spleen	Control (C)	0.119	0.63
	Puromycin (P)	0.108	0.59
	IFN-αCON_1 (IFN)	0.083	0.53
	P + IFN	0.106	0.59
Adrenals	Control (C)	0.216	2.63
	Puromycin (P)	0.208	2.54
	IFN-αCON_1 (IFN)	0.128	1.91
	P + IFN	0.199	2.36

Animals were treated with a single dose of interferon (IFN-αCON_1, 1×10^6 units i.p.) at 0 hour and 6 doses of puromycin (P) (2.272 mg in 0.17 mls of 0.15 NaCL - 0.04 M phosphate buffer) at -2, 0, 1½, 3, 4 and 5 hours and killed 3 hours after interferon treatment. Control (C) and interferon (IFN) only treated animals received a corresponding volume of phosphate buffered saline at the same times. Values represent results from one set of experiments of microsomes from a group of 4 animals of each treatment.

pretreatment on hepatic mixed function oxidase activities in microsomes isolated from animals given IFN-αCON1 treatment for 3 hr and 24 hr respectively is shown in Tables 17 and 18.

Following a 3 hr treatment period with IFN-αCON1 , the content and activity of cytochrome P-450, aminopyrine N-demethylase, benzo(a)pyrene hydroxylase and ethoxyresorufin O-de-ethylase were increased to 156%, 158%, 145% and 187% of control respectively. After 24 hr of IFN-αCON1 treatment, the content and activity of cytochrome P-450, aminopyrine N-demethylase, and benzo(a)pyrene hydroxylase were decreased to 65%, 75% and 79% of control respectively. The biphasic effect of IFN-αCON1 on cytochrome P-450 content and cytochrome P-450 mediated enzyme activity was prevented by the concurrent administration of puromycin (Tables 17 and 18). Puromycin itself had no effect on basal cytochrome P-450 content or enzyme activity. Similarly, the time dependent biphasic effects of IFN-αCON1 treatment on extrahepatic mixed function oxidase activity were essentially prevented by pretreatment with puromycin (Tables 19 and 20). It was noted that IFN-αCON1 induced depression of kidney cytochrome P-450 content was not prevented by puromycin, but the changes in benzo(a)pyrene hydroxylase activity were however prevented. This probably represents an exception to a general trend.

H. The effect of an inhibitor of RNA synthesis on interferon induced stimulation and depression of mixed function oxidase activity

The data in Tables 21 and 22 illustrate the effect of the RNA synthesis inhibitor, actinomycin D on IFN-αCON1 (1 x 10^6 units, i.p.) induced stimulation and depression of hepatic mixed function oxidase. Following 3 hr treatment period with IFN-αCON1, the content and activity of cytochrome P-450, aminopyrine N-demethylase, benzo(a)pyrene hydroxylase, and ethoxyresorufin O-de-ethylase was increased to 159%, 162%, 129% and 181% respectively After 24 hr treatment period with IFN-αCON1, the content and activity of cytochrome P-450, aminopyrine N-demethylase, benzo(a)pyrene hydroxylase and ethoxyresorufin O-de-ethylase were decreased to 67%, 63%, 83% and 74% of control respectively. The time dependent biphasic effect of IFN αCON1 on the hepatic mixed function oxidase activity was prevented by the concurrent administration of actinomycin D (Tables 23 and 24). Actinomycin D administered alone had no significant effect on basal cytochrome P 450 content and enzyme activity. Cytochrome b5 content was not changed after administration of either IFN-αCON1, actinomycin D or both.

The time dependent biphasic effect of IFN αCON1 administration on cytochrome P 450 content and benzo(a)pyrene hydroxylase activity in extrahepatic tissues

TABLE 21

The effect of actinomycin D on interferon induced stimulation of hepatic mixed function oxidase activity in hamsters

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)	Ethoxyresorufin O-demethylase (nmoles resorufin/ mg protein/min)
Control	0.81 ± 0.09	0.32 ± 0.04	392 ± 15	9.32 ± 0.53	0.110 ± 0.016
Actinomycin D	0.76 ± 0.06	0.28 ± 0.09	401 ± 4	9.41 ± 0.32	0.103 ± 0.010
IFN-αCon_1	1.29 ± 0.04*	0.29 ± 0.03	636 ± 10*	12.03 ± 0.81*	0.199 ± 0.011*
Actinomycin D plus IFN-αCON_1	0.70 ± 0.12	0.27 ± 0.06	381 ± 28	8.98 ± 0.93	0.103 ± 0.019

*Significantly different from corresponding control, $p < 0.05$, n=4.
Animals were treated with a single dose of interferon (IFN-αCON_1, 1 x 10^6 units i.p.) at 0 hour and 3 doses of actinomycin D (19.8 µg in 0.17 mls of 0.15 M NaCl - 0.04 M sodium phosphate buffer) at -2, 0, and 1 hours and killed 3 hours after the interferon (IFN) treatment. Control and interferon only treated animals received a corresponding volume of phosphate buffered saline at the same times.

TABLE 22

The effect of actinomycin D on interferon induced depression of hepatic mixed function oxidase activity

Treatment	Cyt P-450 (nmoles/mg protein)	Cyt b_5 (nmoles/mg protein)	Aminopyrine N-demethylase (nmoles HCHO/mg protein/hr)	Benzo(a) pyrene Hydroxylase (nmoles 3-OHBP mg protein/hr)	Ethoxyresorufin O-de-ethylase (nmoles resorufin/ mg protein/min)
Control	0.82 ± 0.01	0.38 ± 0.04	498 ± 10	12.11 ± 0.52	0.108 ± 0.011
IFN-αCon_1	0.55 ± 0.02*	0.37 ± 0.06	313 ± 15*	10.05 ± 0.61*	0.080 ± 0.003*
Actinomycin D	0.79 ± 0.05	0.31 ± 0.06	473 ± 15	12.03 ± 0.55	0.111 ± 0.012
IFN-αCON_1 plus Actinomycin D	0.76 ± 0.03	0.37 ± 0.03	443 ± 14	12.35 ± 0.63	0.106 ± 0.006

*Significantly different from corresponding control, $p < 0.05$, n=4.
Animals were treated with a single dose of interferon (IFN-αCON_1, 1×10^6 units i.p.) at 0 hour and 3 doses of actinomycin D (19.8 µg in 0.17 mls of 0.15 M NaCl - 0.04 M sodium phosphate buffer) at -2, 0, and 4 hours and killed 24 hours after the interferon treatment. Control and interferon only treated animals received a corresponding volume of phosphate buffered saline at the same times.

TABLE 23

The effect of actinomycin D on interferon induced stimulation of extrahepatic mixed function oxidase activity

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control (C)	0.130	2.51
	Actinomycin D	0.121	2.13
	IFN-αCON_1	0.300	3.98
	Act D + IFN-αCON_1	0.168	2.53
Kidney	Control (C)	0.179	5.12
	Actinomycin D	0.141	5.71
	IFN-αCON_1	0.104	4.57
	Act D + IFN-αCON_1	0.132	4.21
Spleen	Control (C)	0.128	0.68
	Actinomycin D	0.098	0.58
	IFN-αCON_1	0.202	0.98
	Act D + IFN-αCON_1	0.131	0.63
Adrenals	Control (C)	0.232	2.01
	Actinomycin D	0.212	2.01
	IFN-αCON_1	0.278	2.43
	Act D + IFN-αCON_1	0.220	2.28

Animals were treated with a single dose of interferon (IFN-αCON_1, 1×10^6 units i.p.) at 0 hour and 6 doses of actinomycin D (2.272 mg in 0.17 mls of 0.15 NaCL - 0.04 M phosphate buffer) at -2, 0, 1½, 3, 4 and 5 hours and killed 3 hours after interferon treatment. Control (C) and interferon (IFN) only treated animals received a corresponding volume of phosphate buffered saline at the same times. Values represent results from one set of experiments of microsomes from a group of 4 animals of each treatment.

TABLE 24

The effect of actinomycin D on interferon induced depression of extrahepatic mixed function oxidase activity

Organ	Treatments	Cytochrome P-450 (nmoles/mg protein)	Benzo(a)pyrene Hydroxylase (nmoles 3-OHBP mg protein//hr)
Lung	Control (C)	0.129	1.96
	Actinomycin D	0.109	1.90
	IFN-αCON_1	0.052	1.34
	Act D + IFN-αCON_1	0.093	1.83
Kidney	Control (C)	0.148	5.78
	Actinomycin D	0.110	5.00
	IFN-αCON_1	0.046	3.71
	Act D + IFN-αCON_1	0.083	4.96
Spleen	Control (C)	0.128	0.71
	Actinomycin D	0.098	0.65
	IFN-αCON_1	0.086	0.60
	Act D + IFN-αCON_1	0.091	0.06
Adrenals	Control (C)	0.218	2.86
	Actinomycin D	0.213	2.54
	IFN-αCON_1	0.132	1.98
	Act D + IFN-αCON_1	0.168	2.48

Animals were treated with a single dose of interferon (IFN-αCON_1, 1 x 10^6 units i.p.) at 0 hour and 6 doses of actinomycin D (2.272 mg in 0.17 mls of 0.15 NaCL - 0.04 M phosphate buffer) at -2, 0, 1½, 3, 4 and 5 hours and killed 24 hours after interferon treatment. Control (C) and interferon (IFN) only treated animals received a corresponding volume of phosphate buffered saline at the same times. Values represent results from one set of experiments of microsomes from a group of 4 animals of each treatment.

was similar to that seen previously (Table 23 and 24). In all tissues examined, actinomycin D prevented the biphasic effect of IFN-αCON1 on the mixed function oxidase activity Although there was no effect of actinomycin D administration alone on the hepatic mixed function oxidase activity, this antibiotic did depress extrahepatic cytochrome P-450 content and benzo(a)pyrene activity in certain other organs. This might reflect the ability of the inhibitor to reach its site of action in adequate concentration.

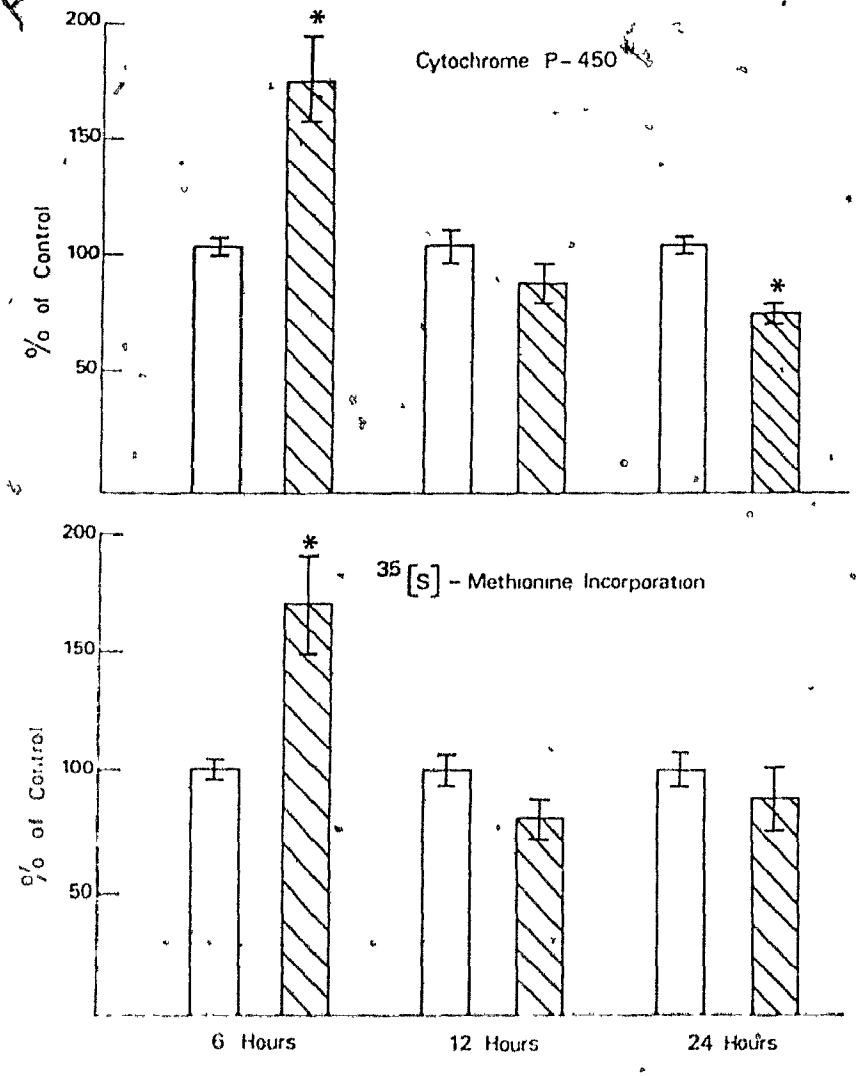
I. Incorporation of [^{35}S]-labelled amino acid in various subcellular fractions

Hamsters were treated with poly IC (10mg/kg, i.p.) for various time intervals and 1 hr before sacrifice, [^{35}S]-methionine was administered i.p. Whole liver homogenates and various subcellular fractions were prepared as described in the method section. The amount of label incorporated in the whole homogenate and subcellular fractions was determined.

Administration of poly IC (10 mg/kg, i.p.) into hamster for 6 hr, 12 hr and 24 hr had no significant effect on total [^{35}S]-radioactive incorporation in the whole homogenate, 10,000 x g supernatent and cytosolic fraction compared to saline treated hamsters. Radioactive incorporation in the microsomal fraction (Figure 15) after

Figure 15

Incorporation of [^{35}S]-amino acid in hepatic microsomes following poly IC treatment for various time intervals. Open bar represents percentage change in cytochrome P-450 content and radiolabelled amino acid incorporation of saline treated animals at various times and represents mean ± SE of 4 individual hamsters (Cytochrome P-450 content and [^{35}S]-amino acid incorporation respectively, at 6 hrs: 0.81 nmoles/mg protein, 9833 cpm/mg protein; at 12 hr: 0.84 nmoles/mg protein, 9390 cpm/mg protein; at 24 hr: 0.78 nmoles/mg protein, 9532 cpm/mg protein) Shaded bars represent poly IC treated animals. *Significantly different from control, $p < 0.05$.


Cytochrome P-450
% of Control
200
150
100
50
*
*
35 [S] - Methionine Incorporation
% of Control
200
150
100
50
*
6 Hours
12 Hours
24 Hours

a 6 hr treatment with poly IC was significantly increased to 172% of control (saline treated = 9833 ± 1101 cpm/mg protein; poly IC treated = 16914 ± 1083 cpm/mg protein). Incorporation of the labelled amino acid into TCA precipitable microsomal fraction was similarly increased to 168% of control by poly IC after 6 hr After a 12 hr or 24 hr poly IC treatment the incorporation of the radiolabelled amino acid into microsomal fraction was not different from control value. Incorporation of radiolabelled amino acid in TCA-precipitable microsomal fraction after a 12 or 24 hr of poly IC treatment period was unchanged from control Microsomal cytochrome P-450 content was also measured at these time courses. Cytochrome P-450 content was significantly increased to 171% of control after 6 hr treatment with poly IC, whereas cytochrome P-450 content after 12 hr and 24 hr treatment were not changed or depressed to 77% of control respectively (Figure 15). It appears that the increase in total radiolabeled incorporation of amino acid into microsomes at 6 hr after poly IC treatment was associated with increase in protein synthesis which was then associated with the increase of cytochrome P-450 content. The depression of cytochrome P-450 content seen after 24 hr treatment period with poly IC was without any concomitant depression in incorporation of the labelled amino acid into the microsomal protein.

J. Incorporation of [^{35}S]-labelled amino acid in various constituents of hepatic microsomal proteins

To examine the extent of incorporation of labelled amino acid into hepatic microsomal cytochrome P-450, saline or poly IC treated (10 mg/kg i p.) hamsters were injected i. p. with 50 µCi [^{35}S]-methionine per hamster and killed 1 hr later. The livers were removed, microsomes were prepared and proteins were separated by gel electrophoresis as described in the methods. The gels were either subjected to fluorography or sliced into 3 mm sections. The gel slices were then solubilized in H_2O_2 and assayed for radioactivity.

Fluorographs in Figures 16 and 18 show the effect of poly IC (6 hr and 24 hr treatment respectively) on the extent of ^{35}S-incorporation in hepatic microsomal protein. The corresponding profile of the distribution of ^{35}S radioactivity in microsomal proteins after gel slicing is shown in Figures 17 and 19. It was evident that in saline treated hamsters an appreciable amount of radioactivity was incorporated into the cytochrome P-450 region proteins (between Mr = 66,000 to 45,000), than in other regions. Following 6 hr treatment with poly IC, there was a marked increase in the amount of radioactivity incorporated in the cytochrome P-450 region, especially Mr = 47,500 and 50,500 (Figure 16 and 17). It was also noted in Figure 17 that

Figure 16

Fluorographed SDS-polyacrylamide gel electrophoresis of microsomal proteins incorporated with [^{35}S]-amino acid and of hamsters treated with poly IC for 6 hr. Hamsters were injected i.p. with [^{35}S]-methionine and killed 1 hr later. Hepatic microsomes were prepared, subjected to electrophoresis and fluorographed as described in Materials and Methods. Representative profiles of microsomes from single hamsters are shown; microsomes from 3 hamsters each have yielded similar results Samples analysed were: wells (a) and (b) contained liver microsomes (100 µg protein) from saline and poly IC treated hamsters respectively. Molecular weight standards used are shown on the right hand side. The arrows mark the molecular weight region of the cytochrome P-450


kD
66
45
36
29
a
b

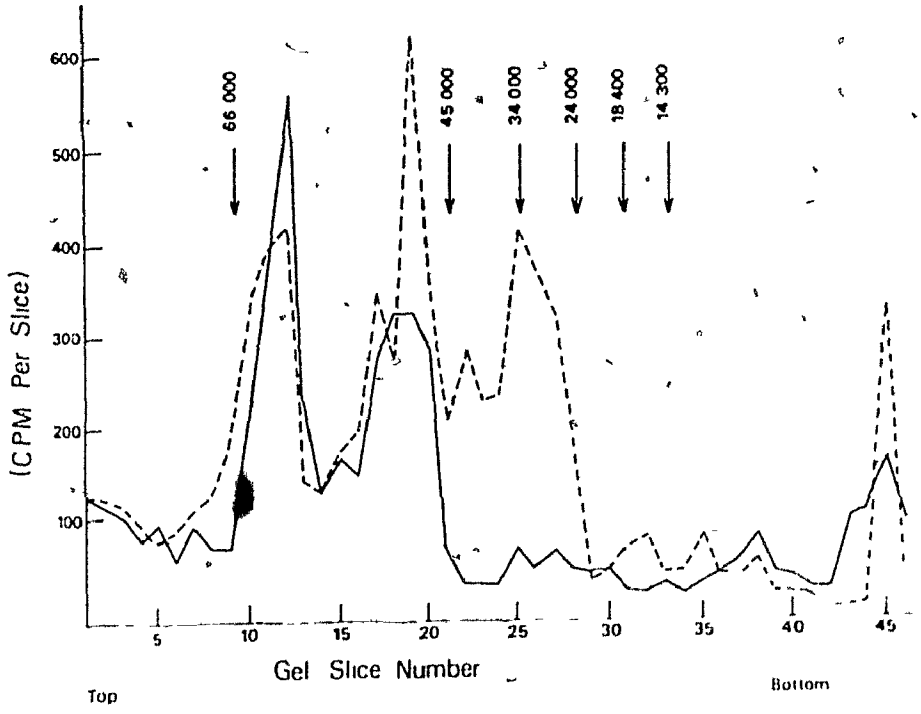


Figure 17

Distribution profile of [^{35}S]-amino acid incorporation in microsomal proteins after gel slicing of hamsters treated with poly IC for 6 hr. Hamsters were injected i.p. with [^{35}S]-methionine and killed 1 hr later. Hepatic microsomes were prepared, subjected to electrophoresis and assayed for radioactivity as described in Materials and Methods. Representative profiles of microsomes from single hamsters are shown; microsomes from 3 hamsters each have yielded similar results. The arrows mark the molecular weight standards used. Solid line, liver from hamsters treated with saline; dotted line, liver from hamster treated with poly IC for 6 hr.

Figure 18

Fluorographed SDS-polyacrylamide gel electrophoresis of microsomal proteins incorporated with [^{35}S]-amino acid and of hamsters treated with poly IC for 24 hr. Hamsters were injected i.p. with [^{35}S]-methionine and killed 1 hr later. Hepatic microsomes were prepared, subjected to electrophoresis and fluorographed as described in Materials and Methods. Representative profiles of microsomes from single hamsters are shown; microsomes from 3 hamsters each have yielded similar results. Samples analysed were: wells (a) and (b) contained liver microsomes (100 µg protein) from saline and poly IC treated hamsters respectively. Molecular weight standards used are shown on the right hand side. The arrows mark the molecular weight region of the cytochrome P-450.

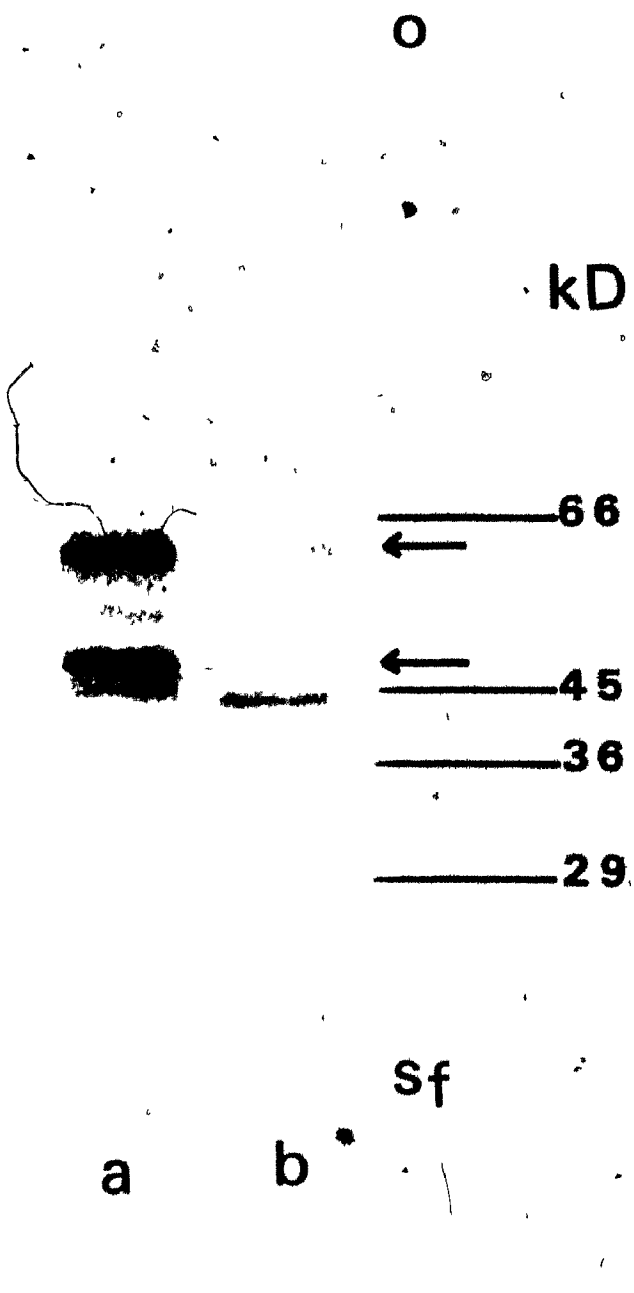
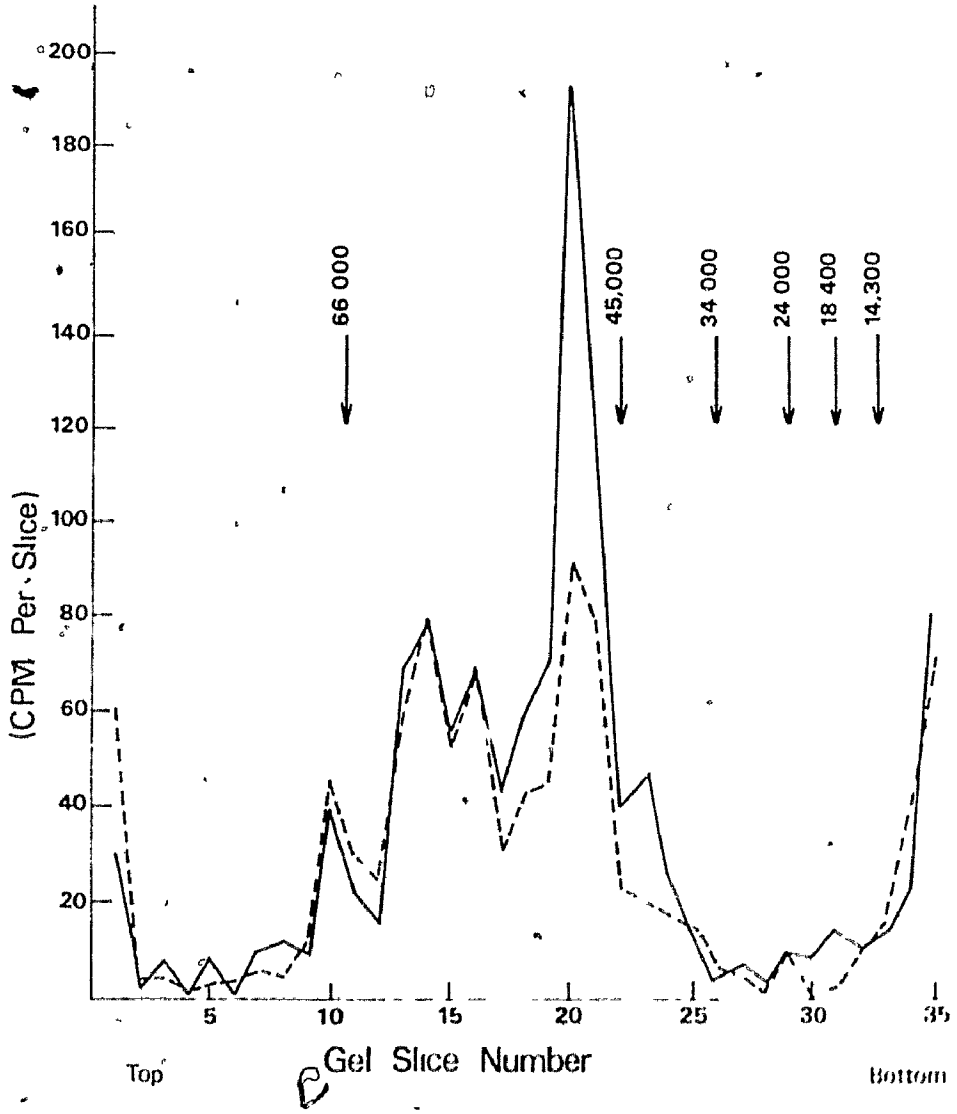

o
kD
66
45
36
29
sf
a
b

Figure 19

Distribution profile of [^{35}S]-amino acid incorporation in microsomal proteins after gel slicing of hamsters treated with poly IC for 24 hr. Hamsters were injected i.p. with [^{35}S]-methionine and killed 1 hr later. Hepatic microsomes were prepared, subjected to electrophoresis and assayed for radioactivity as described in Materials and Methods. Representative profiles of microsomes from single hamsters are shown; microsomes from 3 hamsters each have yielded similar results. The arrows mark the molecular weight standards used. Solid line, liver from hamsters treated with saline; dotted line, liver from hamster treated with poly IC for 24 hr.


66 000
45,000
34 000
24 000
18 400
14,300
(CPM Per Slice)
200
180
160
140
120
100
80
60
40
20
5
10
15
20
25
30
35
Top
Gel Slice Number
Bottom

regions other than between Mr = 66,000 and 45,000 had increase in radioactive incorporation. This effect may be due to an overall increase in microsomal protein synthesis by poly IC. The increase in radioactive incorporation in various gel fractions is in accordance to overall radioactive incorporation in microsomal fraction and increase in cytochrome P-450 content (Figure 17).

The profile of [^{35}S]-radioactive incorporation in microsomes of animals treated with poly IC for 24 hr is shown in Figure 18 and 19. The apocytochrome P-450 region (Mr=48000) in both the fluorograph (Figure 18) and the gel fractionation profile (Figure 19) shows that the amount of radioactivity incorporated into these proteins was reduced. The reduction in radioactive incorporation in the apocytochrome P-450 region is correlated with the reduction in cytochrome P-450 content in the hepatic microsomes, although the total incorporation of radiolabel amino acid in microsomes was not different from control.

K. <u>The effect of interferon on relative rates of hepatic microsomal protein and apocytochrome P-450 synthesis</u>

The effect of IFN-αCON$_1$ (1 x 10^6 units, i.p.) for a 24 hr treatment, on the synthesis of microsomal proteins was studied by comparing the ratio of incorporation of [^{3}H] leucine into microsomal proteins of hamsters treated with IFN αCON$_1$ with the incorporation of [^{14}C]-leucine into

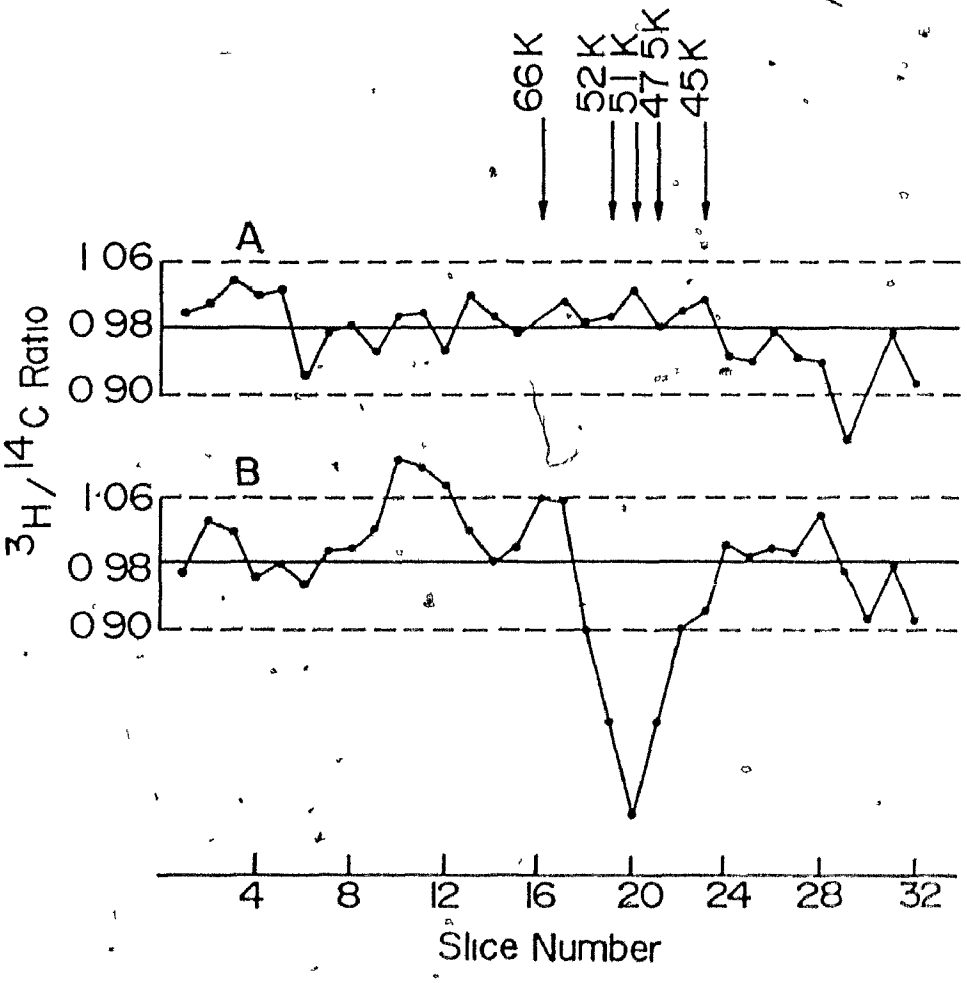
microsomal proteins of hamsters treated with saline. The microsomal proteins were then fractionated on SDS polyacrylamide gels.

In order to establish limits of variation inherent in this method, control experiments were executed whereby both [H^3]-leucine and [^{14}C]-leucine were independently administered (see method section) to saline treated animals. The hepatic microsomes were then prepared and examined by electrophoresis The upper panel in Figure 20 shows the distribution pattern of $^3H/^{14}C$ ratio in a control experiment. As seen, no point lies outside the 95% confidence limits (indicated by the dotted lines) from the mean microsomal $^3H/^{14}C$ ratio of individual slices. Thus experimental ratios (i.e treatment with IFN-αCON1) that are outside the 95% confidence limits (CL) of the control ratio (mean = 0.98, lower and upper CL = 0.90 and 1.06 respectively) are considered to be indicative of decreased and increased rates of protein synthesis respectively.

When this method was applied to an analysis of the effect of IFN-αCON1 after 24 hr treatment period (Figure 20, lower panel) a marked deviation from control limits were seen in some areas of the polyacrylamide gel. In particular, the $^3H/^{14}C$ ratio of apoproteins in the molecular weight region of cytochrome P-450, Mr 47,500, 51,000 and 52,000 (gel slice no = 21, 20 and 19 respectively), was decreased indicating that the relative

Figure 20

Relative rate of synthesis of microsomal proteins. Panel A, control hamster given [^{3}H]-leucine compared with control hamster given [^{14}C]-leucine; Panel B, hamster treated with IFN-αCON$_1$ (1 x 10^6 units, i.p., 24 hr) given [^{3}H]-leucine compared with control hamster given [^{14}C]-leucine. Microsomes were prepared from each group, electrophoresed, and fractionated as described in Materials and Methods. Dotted horizontal lines represent ± 2 standard deviation from means microsomal ^{3}H/^{14}C ratio (95% confidence limits) in control experiment. The two panels are representative profile; microsomes from 3 hamsters each have yielded similar results. The arrows 52K, 51K and 47.5K mark the molecular weight regions of the cytochrome P-450.


66K
52K
51K
47.5K
45K
A
B
1·06
0·98
0·90
$^{3}H/^{14}C$ Ratio
4
8
12
16
20
24
28
32
Slice Number

rate of synthesis of these species of apocytochrome P-450 was decreased. The $^{3}H/^{14}C$ ratio of gel slices in this region was consistently below the confidence limits in two further experiments. It was also noted that the $^{3}H/^{14}C$ ratio of a number of higher molecular protein bands in the IFN-αCON1 treated hamsters was increased particularly in the molecular weight region greater than Mr = 66,000. This indicates that synthesis of other microsomal proteins is stimulated by IFN-αCON1.

The effect of IFN-αCON1 treatment for 24 hrs on the relative rate of protein synthesis in the above mentioned molecular weight regions in three separate experiments are summarized in Table 25. The individual ratios were obtained by pooling ^{3}H and ^{14}C counts (dpm) in the respective gel region of individual experiments. The mean $^{3}H/^{14}C$ ratio for the individual gel slice was then divided by the correction factor (see method section). The $^{3}H/^{14}C$ ratio for the individual slices corresponding to the cytochrome P-450 region, Mr = 47,500, 51,000 and 52,000, were depressed by 66%, 64% and 80% of control respectively. Cytochrome P-450 content (as determined spectrophotometrically) in microsomes from IFN-αCON1 treated animals was significantly depressed by 65% compared to control value. It was concluded that the depression of apocytochrome P-450 content after 24 hr IFN-αCON1

TABLE 25

The effect of interferon on relative rates of synthesis of putative apocytochrome P-450

Treatment	Mr=47500 $^3H/^{14}C$	Mr=51000 $^3H/^{14}C$	Mr=52000 $^3H/^{14}C$	Cyt P-450 (nmoles/mg protein)
Control	1.00	1.00	0.99	0.85 ± 0.05
IFN-αCON$_1$ (1×10^6 units)	0.66	0.64	0.79	0.55 ± 0.09*

* Significantly different from saline control, $p < 0.05$, n=3.

The respective molecular weight regions represent putative apocytochrome P-450 as assessed by gel electrophoresis. The individual ratios were obtained by pooling ^3H and ^{14}C counts of hepatic microsomes of three individual sets of experiments including figure 20 (see result section). Cytochrome P-450 content was determined from liver microsomes of individual animals (n=3).

treatment is due to decrease in apocytochrome P-450 synthesis.

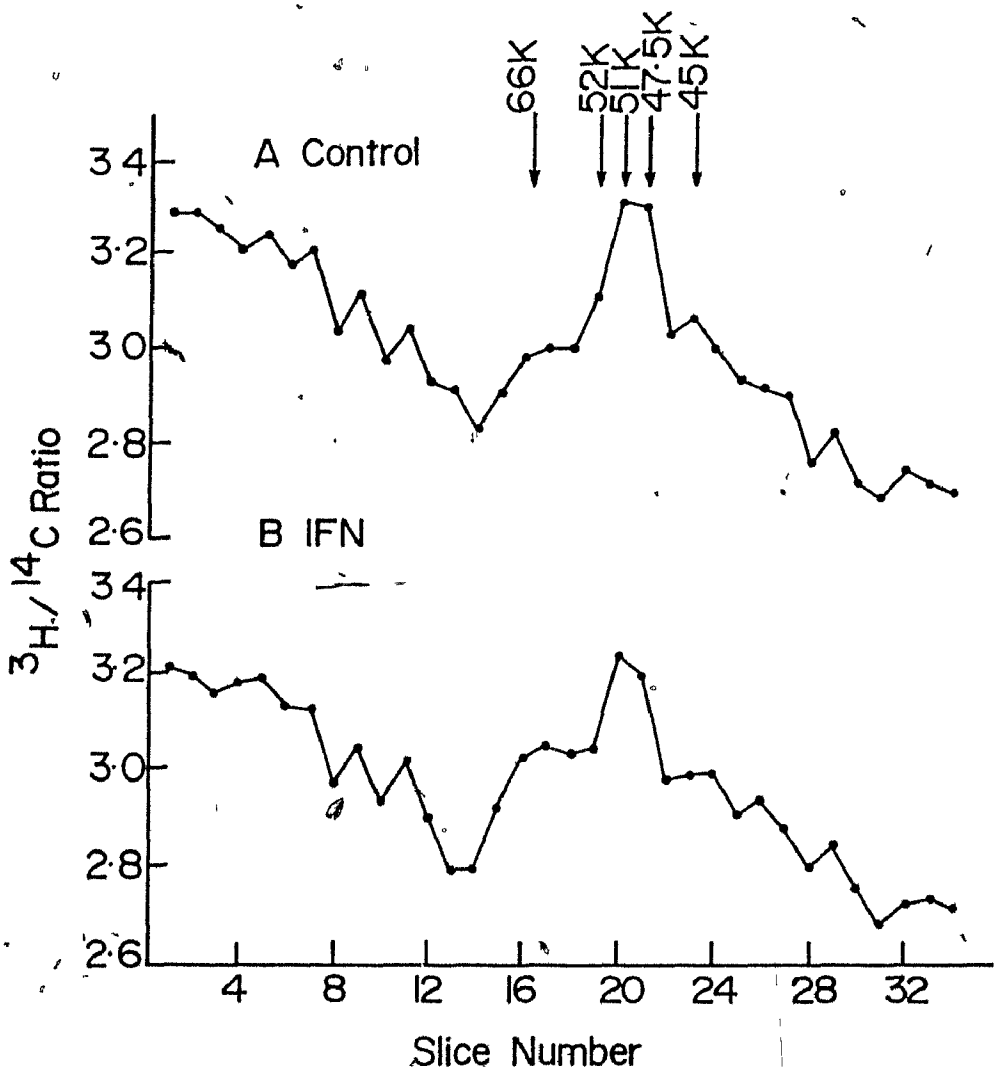
L. The effect of interferon on relative rate of hepatic microsomal protein and cytochrome P-450 degradation

In this experiment, one isotopic form of an amino acid, [^{14}C]-leucine, was administered in hamsters initially and was allowed to be eliminated for 48 hrs. Then, a second isotopic form of the same amino acid [^{3}H]-leucine was given, and the animal was killed 4 hrs later. The ^{3}H counts represented the initial time point on the labelled protein degradation curve of the microsomal proteins which have incorporated this label while the ^{14}C counts represented the amount of labelled protein remaining in the microsomal proteins after 52 hr Hepatic microsomes were then prepared and electrophoresis carried out. The ^{3}H/^{14}C ratio in gel fractions of microsomes from IFN-αCON$_1$ treated hamsters was compared to corresponding fraction of microsomes from saline treated hamsters. Changes in ratios in the same molecular weight region indicates changes in relative rates of protein degradation.

The pattern of distribution of ^{3}H/^{14}C ratio of individual proteins that were fractionated in the polyacrylamide gel is shown in Figure 21. The profile of the differential ratio across the different molecular weight regions indicates heterogeneity of relative rate of

Figure 21

Relative rate of degradation of microsomal proteins. Panel A, control hamster given [^{14}C]-leucine followed 48 hr later by [^{3}H]-leucine. Panel B, IFN-αCON$_1$ treated (1 x 10^6 units, i.p., 24 hr) hamster given [^{14}C]-leucine followed 48 hr later by [^{3}H]-leucine. Microsomes were prepared from each group, electrophoresed and fractionated as described in Materials and Methods. The ^{3}H/^{14}C ratio for each gel slice was divided by the correction factor (see Materials and Methods). The two panels are representative profile: microsomes from 3 hamster each have yielded similar results. The arrows 52K, 51K and 47.5K mark the molecular weight regions of the cytochrome P-450.


66K
52K
51K
47·5K
45K
A Control
B IFN
3·4
3·2
3·0
2·8
2·6
$^{3}H/^{14}C$ Ratio
4
8
12
16
20
24
28
32
Slice Number

degradation of constituent microsomal proteins. As seen in the Figure 21 higher molecular weight proteins were degraded more rapidly than smaller polypeptides (as indicated by higher $^{3}H/^{14}C$ ratio). A marked exception was seen with the proteins between Mr = 66,000 and 45,000. According to Dehlinger and Schimke (1972), this region, which is associated with cytochrome P-450 region, have higher $^{3}H/^{14}C$ ratio and therefore proteins in this region turn over more rapidly. Following IFN-αCON1 treatment (Figure 21), heterogeneity of degradation rate of proteins were still apparent, however there was reduction in the relative rate of degradation of cytochome P-450 apoproteins, Mr = 47,500, 51,500 and 52,000 (gel slice no. 21, 20 and 19 respectively). The effect of IFN-αCON1 after 24 hr treatment period on the relative rate of protein degradation in gel slices, at the molecular weight region of Mr = 47,500, 51,500 and 52,500, obtained from 3 separate experiments, is summarized in Table 26. The individual ratios were obtained by pooling ^{3}H and ^{14}C counts of individual sets of experiments in the respective gel regions. The mean $^{3}H/^{14}C$ ratio for the individual molecular weight region was then divided by the correction factor (see methods section). As seen, the $^{3}H/^{14}C$ ratio in the respective protein regions, i.e. Mr = 47,500, 51,000 and 52,000, was depressed by approximately 10% from control after IFN-αCON1 treatment. In other words, the relative

TABLE 26

The effect of interferon on relative rates of degradation of putative apocytochrome P-450

Treatment	Mr=48000 a/b	Mr=51000 a/b	Mr=52000 a/b	Cyt P-450 (nmoles/mg protein)
Control	2.96	2.86	2.82	0.88 ± 0.06
IFN-αCON$_1$ (1×10^6 units)	2.67	2.67	2.65	0.61 ± 0.09*

* Significantly different from corresponding control, $p < 0.05$, n=3.

The respective molecular weight regions represent putative apocytochrome P-450 as assessed by gel electrophoresis. The individual ratios were obtained by pooling ^3H and ^{14}C counts of hepatic microsomes of three individual sets of experiments including figure 21 (see result section). Cytochrome P-450 content was determined from liver microsomes of individual animals (n=3).

rate of degradation of the putative apocytochrome P-450 was depressed after IFN-αCON1 treatment in hamsters. Cytochrome P-450 content (as measured spectrophotometrically) in the microsomes was also depressed to 69% of control.

For the determination of the apparent rate of degradation and half-lives from these ratio ($^3H/^{14}C$), the following formula of Bock et al (1971) was used.

$$\text{Apparent rate of degradation, } Kd = \frac{2.303}{t_2 - t_1} \log R$$

$$\text{Apparent half-lives } t_{1/2} = \frac{0.693}{Kd}$$

where $t_2 - t_1$ denotes the time interval in hours between administration of the two isotopic forms of leucine (in this experiment $t_2 - t_1 = 48$ hr), and R corresponds to the "corrected" dpm ratio of ^3H to ^{14}C radioactivity after sequential isotope administation (i.e. $^3H/^{14}C$ divided by correction factor). The apparent rate of degradation (Kd) and half-life (t 1/2) of the putative apocytochrome P 450 in gel slices corresponding to molecular weight regions of 47,500, 51,500 and 52,500 (gel slice no 21, 20 and 19 respectively) Table 27. The half lives of the putative apocytochrome P-450 from microsomes of saline-treated hamsters were approximately similar to those reported by others for the rat (Parkinson et al., 1983; Sadano and Omura, 1983). After 24 hr of IFN αCON1 treatment period, the average half-lives of the putative apocytochrome P 450

TABLE 27

Estimated rate of degradation and half lives of putative apocytochrome P-450 of hamsters treated with interferon

Treatment	Approximate Mr of Gel Slice	K_d (hr^{-1})	$t_{\frac{1}{2}}$ (hr)
Control	47,500	0.023	30.1
	51,500	0.022	31.5
	52,500	0.022	31.5
IFN-αCON$_1$ (1×10^6 units)	47,500	0.021	33.0
	51,500	0.021	33.0
	52,500	0.020	34.7

Values for K_d and $t_{\frac{1}{2}}$ were obtained by pooling radiolabelled incorporation in three sets of experiments (three sets of microsomes each from control and treated animals).

was increased from 31 hr in saline treated hamsters to approximately 34 hr in the IFN-αCON1 treated hamsters.

This suggests that the reduction of cytochrome P-450 content (as measured spectrophotometrically) to 69% of control (Table 26), 24 hr after IFN-αCON1 treatment was not due to an increase in degradation rate of apocytochrome P-450. In fact the slight reduction in the rate of degradation may contribute to a process of stabilization of the enzyme at the new steady state of protein turnover.

M The effect of interferon on in vitro biosynthesis of proteins by translation of hamster liver messenger RNA

The aim of this experiment was to determine whether the biphasic change in catalytic activities of cytochrome P-450 mediated drug biotransformation at 3 hr (stimulation) and 24 hr (depression) after IFN-αCON1 treatment was due to changes in (a) amount of mRNA level and (b) efficiency of mRNA to translate into proteins, especially apocytochrome P 450. In this experiment total RNA was extracted from hamsters treated with IFN-αCON1 after a 3 hr or 24 hr treatment or in saline treated hamsters; and translated using [^{35}S] methionine incorporation in a rabbit reticulocyte lysate translation system. The proteins synthesized were then isolated by SDS polyacrylamide gel electrophoresis as described in the method section. The effects of IFN-αCON1 treatment (1 x 10^6 units i.p.), 3 hrs

and 24 hrs treatment, on total RNA per gram liver, specific activity of mRNA to translate and capacity of total liver to translate [^{35}S]-methionine into TCA-precipitable proteins is shown in Table 28. After 3 hr of IFN-αCON1 treatment, microsomal protein and cytochrome P-450 content (as measured spectrophotometrically) were increased to 150% and 152% of control respectively The amount of total RNA extracted, the specific activity of mRNA to translate and the capacity of total liver to translate into proteins were increased by 151%, 153% and 230% of control.

Following a 24 hr treatment period with IFN-αCON1 there was no change in microsomal protein content, however cytochrome P-450 content was depressed to 65% of control. The amount of total RNA extracted, the specific activity of mRNA to translate or capacity of total liver to translate were not affected by a 24 hr treatment period with IFN-CON1 .

Analysis of total translation products by SDS polyacrylamide gel electrophoresis following 3 hr and 24 hr treatment period with IFN-αCON1 are shown in Figures 22 and 24, respectively. In all these experiments, approximately equal amounts of TCA precipitable radioactive counts were resolved on the gel. The gels were either sliced or subjected to fluorography. Gels were sliced in the molecular weight region between Mr = 66,000 and 45,000

TABLE 28

The effect of interferon on hamster liver RNA content and the ability of the messenger RNA to translate *in vitro* into proteins

Treatment	Microsomal Protein (mg/ml)	Cyt P-450 (nmoles/mg protein)	µg RNA/ g liver	Incorporation of [^{35}S]-methionine/ µg RNA	Incorporation of [^{35}S]-methionine/ g liver
Saline (3 hr)	7.61 ± 0.21	0.81 ± 0.02	179.1 ± 12.1	4653 ± 578	837365 ± 152424
IFN-αCon$_1$ (3 hr) (1 x 10^6 units)	11.42 ± 0.56*	1.23 ± 0.04*	270.0 ± 13.0*	7106 ± 612*	1921845 ± 198647*
Saline (24 hr)	8.23 ± 0.41	0.85 ± 0.05	107.0 ± 50.0	3954 ± 1849	228206 ± 62937
IFN-αCon$_1$ (24 hr) (1 x 10^6 units)	8.11 ± 0.21	0.55 ± 0.03*	201.0 ± 45.0	1391 ± 196	284319 ± 66017

*Significantly different from control, $p < 0.05$, n=3.

Animals were treated with a single dose of interferon (IFN-αCON$_1$, 1 x 10^6 units, i.p.) and killed 3 and 24 hours later. Control animals received corresponding volume of sterile phosphate buffered saline. Livers were excised and RNA extracted from 2 g of liver. The rest of the liver was used to assay cytochrome P-450 content. Quantity of RNA extracted was determined spectrophotometrically (1 unit of optical density = 40 µg RNA/ml). Incorporation was measured as the amount of [^{35}S]-methionine incorporated in TCA precipitable fraction of the translation incubation mixture.

Figure 22

Fluorographed SDS-polyacrylamide gel electrophoresis of total translation products of hepatic RNA from hamsters treated with IFN-αCON1 for 3 hr. Aliquots (130,000 cpm) of translated TCA precipitable protein were resolved on SDS-PAGE and fluorographed. Wells (b) and (c) RNA from control and interferon treated hamster liver, respectively, (a) bands detected in the absence of exogenous RNA. Molecular weight standards are shown on the right; where cytochrome P-450 region lies between 66K and 45K. Fluorograph shown is a representative of 3 sets of experiments yielding similar results.

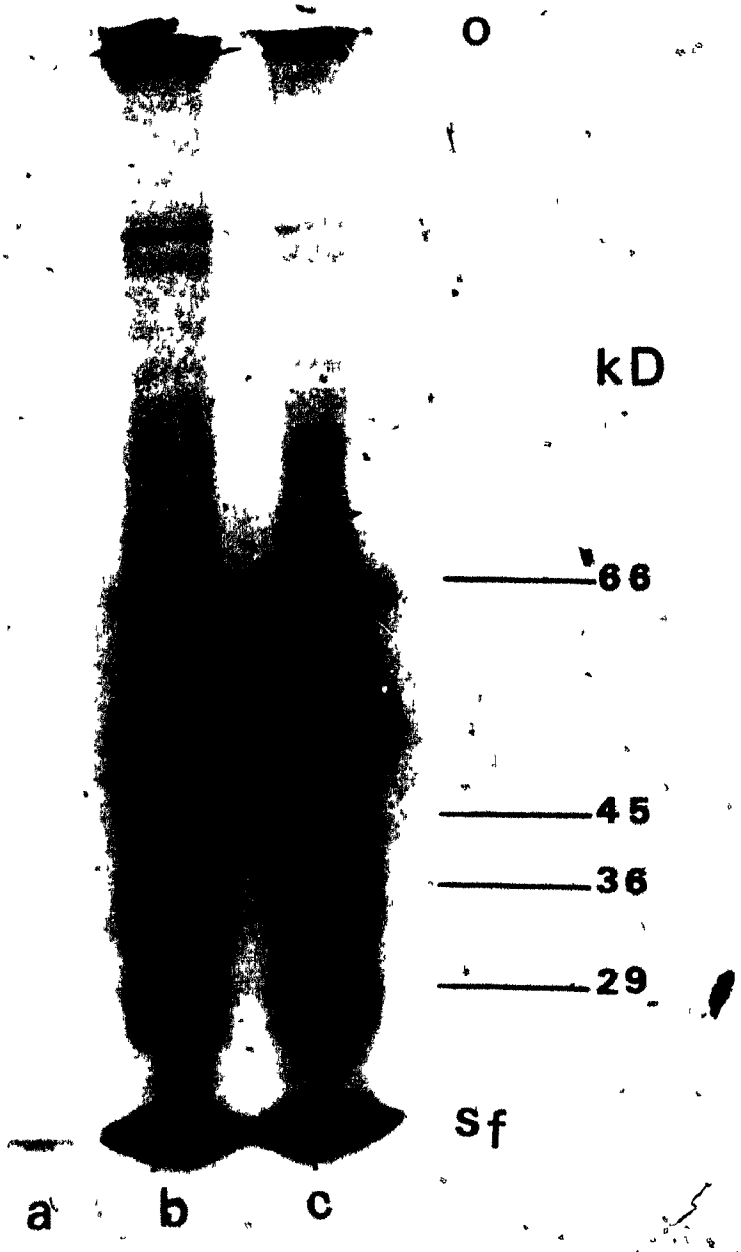

o
kD
66
45
36
29
sf
a
b
c

Figure 23

Fractionated SDS-polyacrylamide gel electrophoresis of total translation products of hepatic RNA from hamsters treated with IFN-αCON1 for 3 hr. Aliquots (130,000) of translated TCA precipitable proteins were resolved on SDS-PAGE and assayed for radioactivity after gel fractionation. Arrows mark the molecular weight standards, where cytochrome P-450 region lies between 66K and 45K. Distribution profile shown is a representative of 3 sets of experiments yielding similar results.

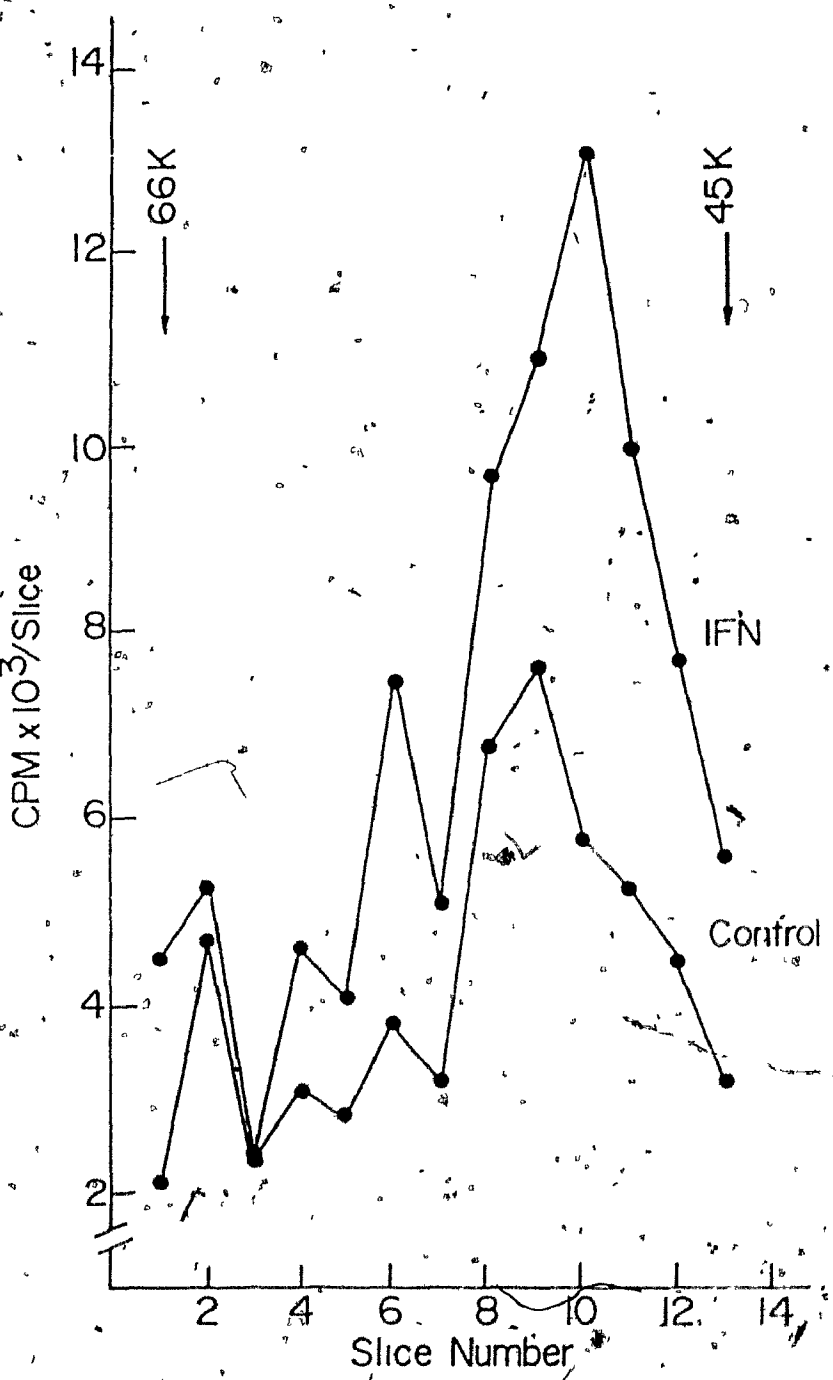

66K
45K
IFN
Control
CPM x 10³/Slice
Slice Number
2
4
6
8
10
12
14

Figure 24

Fluorographed SDS-polyacrylamide gel electrophoresis of total translation products of hepatic RNA from hamsters treated with IFN-αCON1 for 24 hr. Aliquots (110,000 cpm) of translated TCA precipitable protein were resolved on SDS-PAGE and fluorographed. Wells (b) and (c) RNA from control and interferon treated hamster liver, respectively, (a) bands detected in the absence of exogenous RNA. Molecular weight standards are shown on the right; where cytochrome P-450 region lies between 66K and 45K. Fluorograph shown is a representative of 3 sets of experiments yielding similar results.

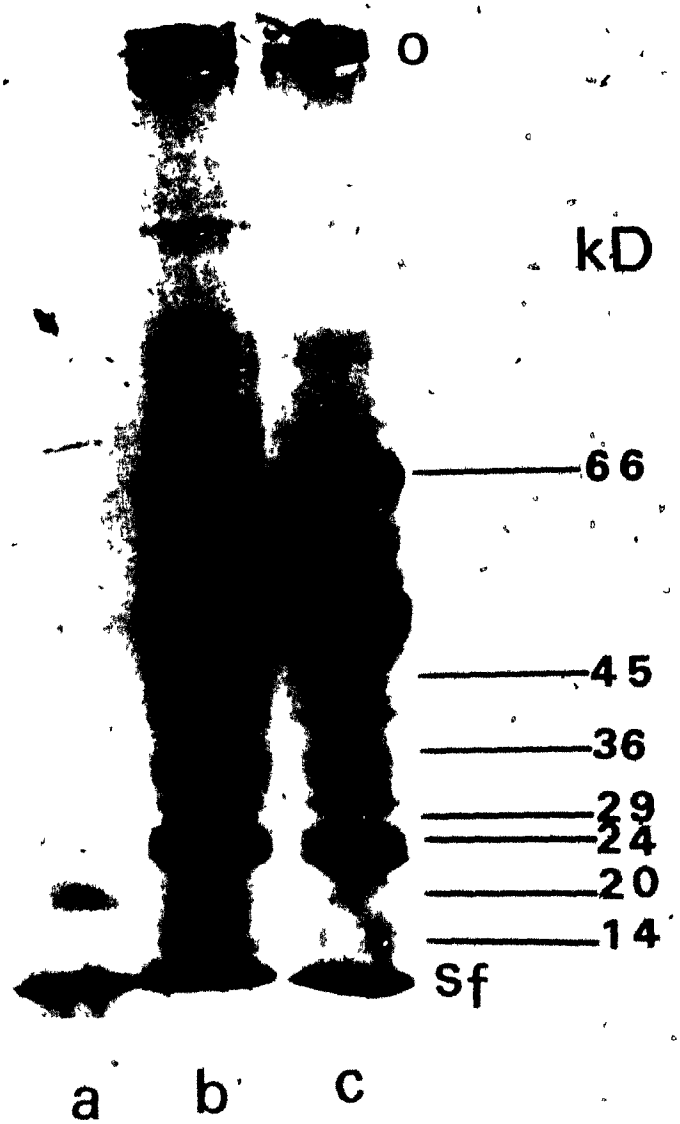
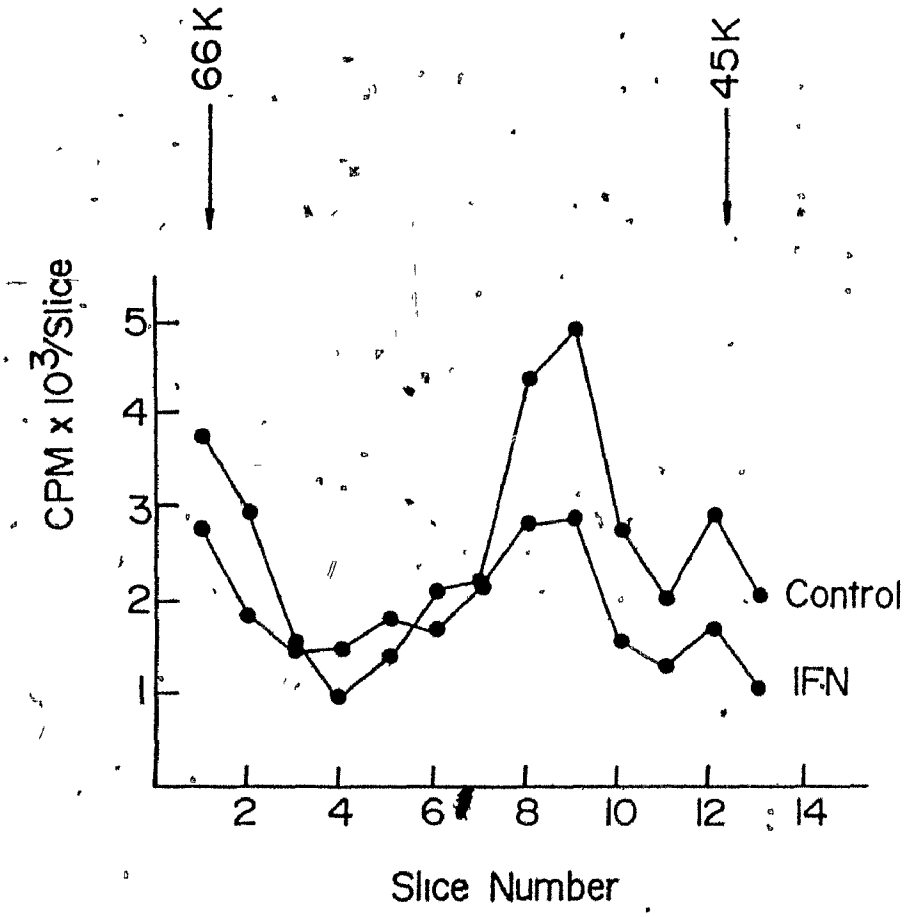

o
kD
66
45
36
29
24
20
14
Sf
a
b
c

Figure 25

Fractionated SDS-polyacrylamide gel electrophoresis of total translation products of hepatic RNA from hamsters treated with IFN-αCON1 for 24 hr. Aliquots (110,000) of translated TCA precipitable proteins were resolved on SDS-PAGE and assayed for radioactivity after gel fractionation. Arrows mark the molecular weight standards, where cytochrome P-450 region lies between 66K and 45K. Distribution profile shown is a representative of 3 sets of experiments yielding similar results


66K
45K
CPM x 10³/Slice
5
4
3
2
1
2
4
6
8
10
12
14
Slice Number
Control
IFN

(region corresponding to apocytochrome P-450 ; Chiang and Steggles, 1983)

After 3 hrs of IFN-αCON1 treatment, the intensity of the radioactive signals in the fluorogram between Mr = 47,000 and 54,000 appear to be greater than those of control (Figures 22 and 23). When the gel was subjected to fractionation (2 mm gel slice) and counting between the molecular weight region of 45,000 and 66,000, increase in radioactive counts in individual gel slices were noted in the translation products from RNA of IFN-αCON1 treated animals, especially at the molecular weight region between Mr = 47,000 and 54,000 (gel slice no 11, 10, 9, 8 and 7 respectively). After 24 hrs treatment period with IFN-αCON1 (Figures 24 and 25), the converse was seen. Between the region of Mr = 47,000 and 54,000, the intensity of the radioactive signals in certain bands in the fluorogram was reduced compared to those of control. In addition, a band around Mr = 65,000 was reduced in intensity compared to control. Counts from gel slices between Mr = 47,000 and 54,000 were reduced compared to control (slice no. 11, 10, 9, 8 and 7 respectively).

The effect of IFN-αCON1 treatment, after 3 hr and 24 hr on the efficiency of the RNA to translate into proteins from the livers of hamsters treated with IFN-αCON1 at the corresponding time periods in three experiments is summarized in Table 29 . The ratios in Table 29 were

TABLE 29

The effect of interferon after 3 hour and 24 hour treatment on the translatability of liver RNA into proteins

Experiment No.	Treatment	Time After Treatment	
		3 hr	24 hr
1	Saline	0.25	0.27
	IFN-αCON$_1$	0.40	0.21
	Ratio (IFN-CON$_1$/saline)	1.60	0.84
2	Saline	0.37	0.19
	IFN-αCON$_1$	0.62	0.08
	Ratio (IFN-CON$_1$/saline)	1.66	0.42
3	Saline	0.54	0.20
	IFN-αCON$_1$	0.81	0.12
	Ratio (IFN-CON$_1$/saline)	1.50	0.60

Table summarizes 3 experiments for the effect of single administration of IFN-αCON$_1$ (1×10^6 units, i.p.) on the efficiency of RNA, derived from the liver of treated animals, to translate into specific proteins. Control animals received corrsponding volume of sterile phosphate buffers saline. The translated protein of equal radioactive count were then subjected to SDS/PAGE, as described in the Methods section. The ratios were derived from areas of total counts in gel slices between Mr = 47,000 and 54,000 under "interferon treatment" curve to areas under the "saline treated" between the corresponding molecular weight region.

derived from areas of total counts in gel slices between Mr = 47,000 and 54,000 under IFN-αCON1 treatment curve to areas under the saline treated between the corresponding molecular weight region. After 3 hr and 24 hr of IFN-αCON1 treatment period, the ratio of total counts of treatment over control between 47,000 and 54,000, was 159% and 62% of control respectively which is almost identical to the changes seen in cytochrome P-450 content following the two interferon treatment periods.

In summary, this experiment demonstrated that after 3 hr treatment with IFN-αCON1 , there was increased amount of RNA and the efficiency of translation was increased especially in the cytochrome P-450 region. This correlated with the increase in microsomal cytochrome P-450 content (as measured spectrophotometrically) in this time period. After a 24 hr treatment period with IFN-αCON1 , the total translational capacity of the liver remained unchanged, however only the efficiency of translation was depressed in the apocytochrome P-450 region which is in keeping with the observed depression of microsomal cytochrome P-450 content (as measured spectrophotometrically).

N. The effect of interferon on hepatic xanthine oxidase.

It has been reported that xanthine oxidase activity in the liver was increased following the administration of interferon or various interferon inducers (e.g. poly IC,

tilorone and bacterial lipopolysaccharide) (Ghezzi et al., 1984). As it is known that the (O) from the xanthine oxides has the ability to generate reactive oxygen intermediates (superoxide and hydrogen peroxide) which may have a detrimental effect on the cytochrome P-450 system, we investigated the effect of IFN-αCON1 on two forms of xanthine oxidase, ((D) form and the (O) form). Xanthine oxidase was extracted from the whole liver homogenate as described in the method section.

The effect of IFN-αCON1 (1 x 10^6 units i.p.) for 3 hr or 24 hr on the (D) and (O) form of xanthine oxidase is shown in Table 30. Following a 3 hr treatment period with IFN-αCON1, the activity of the (D) form of xanthine oxidase was increased by 420% of control without any concomitant increase in the (O) form of xanthine oxidase. After a 24 hr of IFN-αCON1 treatment period, the activity of (O) form of xanthine oxidase was increased to 165% of control while the (D) form of xanthine oxidase decreased to a level of 175% of control. Neither IFN-αCON1 nor poly IC had any effect on either the (D) form or (O) form of xanthine oxidase in vitro (Table 31).

The effect of the RNA synthesis inhibitor, actinomycin D, on the IFN-αCON1 induced xanthine oxidase activity is shown in Figure 26. Actinomycin D prevented the induction of (D) form of xanthine oxidase activity while the (O) form remained at the normal level after 3 hr treatment period

TABLE 30

The effect of interferon after 3 hour and 24 hour treatment on hepatic xanthine oxidase activity

Treatment	Protein Concentration in Supernatant (mg/ml)	Xanthine Oxidase (O form) (nmoles uric acid/ mg protein/min)	Xanthine Oxidase (D form) (nmoles NADH/ mg protein/min)
Control	8.91 ± 0.93	2.35 ± 0.27	1.06 ± 0.10
IFN-αCON$_1$ (3 hr)	11.75 ± 0.75	2.40 ± 0.14	4.47 ± 0.87*
Control	8.19 ± 0.29	2.15 ± 0.09	2.63 ± 0.11
IFN-αCON$_1$ (24 hr)	8.61 ± 0.42	3.55 ± 0.06*	4.60 ± 0.39*

* Significantly different from corresponding control, $p < 0.05$ (n=4).

Animals were treated with a single dose of interferon (IFN-αCON$_1$, 1 x 10^6 units i.p.) and killed 3 hours and 24 hours later. Control animals received corresponding volume of sterile phosphate buffered saline at the same time.

TABLE 31

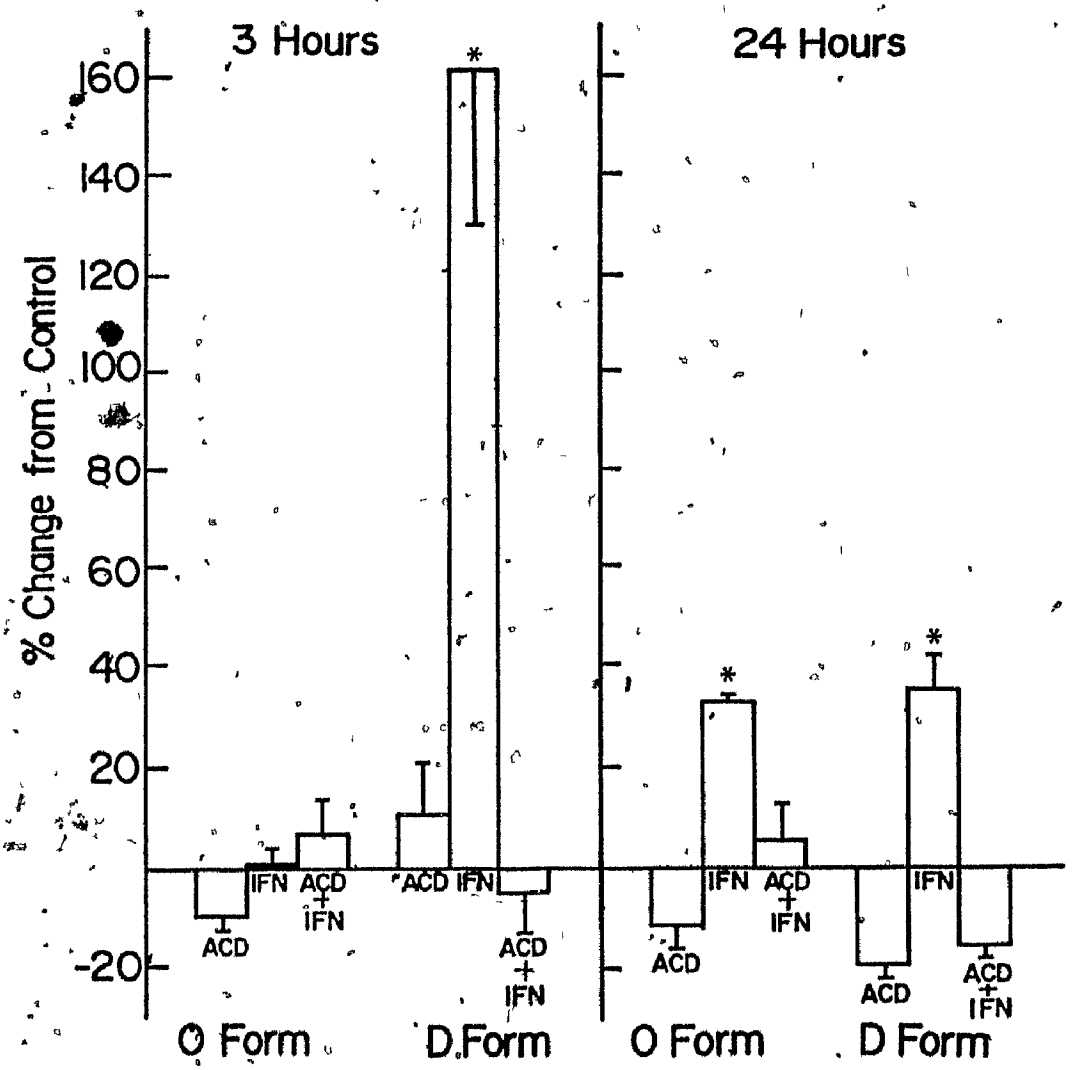
Xanthine oxidase activity (O form and D form) in hamster hepatic cytosolic fraction incubated in vitro with interferon or poly IC

Treatment	Protein Concentration in Supernatant (mg/ml)	Xanthine Oxidase (O Form) (nmoles uric acid/ mg protein/min)	Xanthine Oxidase (D Form) (nmoles NADH/ mg protein/min)
Control	21.32	1.26 ± 0.86	2.13 ± 0.11
IFN-αCON_1	20.01	2.93 ± 0.36	3.98 ± 0.13
Control	22.42	1.38 ± 0.34	2.07 ± 0.12
Poly IC	21.36	2.62 ± 0.18	4.18 ± 0.13

Poly IC (1 mg) or IFN-αCON_1 (10^6 units) was added to cytosolic fraction, 15 min prior to assay. Control microsomal suspension received equivalent volume of vehicle. Each value represents means and standard error of three replicates.

Figure 26

The effect of actinomycin D on interferon induced stimulation of xanthine oxidase. Control values for 3 hr experiment were: xanthine oxidase (O) form = 1.70 ± 0.29 nmoles uric acid/mg protein/min; xanthine oxidase (D) form = 1.06 ± 0.10 nmoles NADH/mg protein/min. Control values for 24 hr experiment: xanthine oxidase (O) form = 2.15 ± 0.09 nmoles uric acid/mg protein/min; xanthine oxidase (D) form = 2.63 ± 0.11 nmoles NADH/mg protein/min. Each value is the mean of 4 individual hamster. *Significantly different from control, $p < 0.05$.


3 Hours
24 Hours
% Change from Control
160
140
120
100
80
60
40
20
-20
*
ACD
IFN
ACD + IFN
O Form
D Form

with IFN-αCON1. In this experiment, actinomycin D by itself had no significant effect on the activity of either the (O) form or the (D) form of xanthine oxidase. Actinomycin D prevented the induction of both forms of xanthine oxidase which occurred 24 hrs after interferon treatment At this time, actinomycin D pretreatment by itself decreased the activity of both the (O) and the (D) form of xanthine oxidase Actinomycin D pretreatment, prevented both interferon induced stimulation of cytochrome P-450 after 3hr treatment period and interferon induced depression of cytochrome P-450 (Tables 21 and 22)

From these studies, two main conclusions can be drawn regarding the stimulation of xanthine oxidase activity by interferon in the liver Firstly, it appears that the increase in xanthine oxidase activity, particularly the (D) form, and cytochrome P-450 after 3 hr of interferon treatment requires protein synthesis denovo. Secondly, the depression of cytochrome P 450 after 24 hrs of interferon treatment is also dependent on the synthesis of an intermediate protein (e.g. O form of xanthine oxidase)

D Effects of xanthine oxidase inhibition or free radical scavenging on the interferon mediated depression of hepatic mixed function oxidase activity

Allopurinol, a xanthine oxidase inhibitor and α-tocopherol, a free radical scavenger, were used to investigate the role of free radicals in the depression of hepatic mixed function oxidase activity induced by the interferon inducer poly IC. Poly IC was used instead of IFN-αCON1 in these experiments because (a) poly IC stimulates xanthine oxidase activity more strongly than IFN-αCON1 and (b) the limited availability of IFN-αCON1.

The effect of allopurinol (7 mg/kg p.o. and 17 mg/kg p.o. respectively) on the depression of hepatic mixed function oxidase induced by a single dose of poly IC (10 mg/kg i.p.) 24 hrs later is shown in Tables 32 and 33. Cytochrome P-450 content and ethoxyresorufin O-de-ethylase activity were depressed by poly IC to 44% and 39% of control respectively. Concurrent administration of allopurinol 17 mg/kg p.o.) protected cytochrome P-450 from the depression induced by poly IC. Low doses of allopurinol (7 mg/kg, p.o.), partially protected cytochrome P 450. Allopurinol itself, at the corresponding doses had no effect either on cytochrome P-450 content or ethoxyresorufin O-de-ethylase activity in the hamsters. Previous studies (Ghezzi et al., 1985) have shown that

TABLE 32

The effect of allopurinol and poly IC on cytochrome P-450, (O) form of xanthine oxidase and (D) form of xanthine oxidase activity in the hamster liver

Treatment	Cyt P-450 (nmoles/mg protein)	Ethoxyresorufin O-de-ethylase (nmoles protein resorufin/mg protein/min)	Xanthine Oxidase (O form) (nmoles uric acid/ mg protein/min)	Xanthine Oxidase (D form) (nmoles NADH/ mg protein/min)
Control	1.00 ± 0.05	0.24 ± 0.03	1.06 ± 0.05	2.75 ± 0.09
Allopurinol	0.93 ± 0.07	0.23 ± 0.03	0.48 ± 0.04*	1.55 ± 0.03*
Poly IC	0.44 ± 0.02*	0.11 ± 0.01*	2.38 ± 0.04*	4.78 ± 0.05*
Allopurinol plus Poly IC	0.56 ± 0.02*	0.19 ± 0.02*	0.93 ± 0.11	1.90 ± 0.12

*Significantly different from corresponding control, $p < 0.05$ (n=4).

Animals were treated with a single dose of poly IC (10 mg/kg, i.p.) at 0 hour and two doses of allopurinol (7 mg/kg p.o.) at -1 and 6 hours and killed 24 hours after poly IC treatment. Control and poly IC only treated animals received corresponding volume of phosphate buffered saline at the same time.

TABLE 33

*** The effect of allopurinol and poly IC on cytochrome P-450, (O) form of xanthine oxidase and (D) form of xanthine oxidase activity in the hamster liver**

Treatment	Cyt P-450 (nmoles/mg protein)	Ethoxyresorufin (nmoles protein resorufin/mg/min)	Xanthine Oxidase (O form) (nmoles uric acid/ mg protein/min)	Xanthine Oxidase (D form) (nmoles NADH/ mg protein/min)
Control	1.207 ± 0.064	0.166 ± 0.017	0.67 ± 0.08	1.90 ± 0.35
Allopurinol	1.100 ± 0.096	0.143 ± 0.005	0.21 ± 0.05*	0.69 ± 0.32*
Poly IC	0.540 ± 0.046*	0.053 ± 0.019*	1.49 ± 0.12*	2.28 ± 0.46*
Allopurinol plus Poly IC	0.902 ± 0.058	0.107 ± 0.008	0.49 ± 0.09	1.48 ± 0.35

*Significantly different from corresponding control, $p < 0.05$ (n=4).

Animals were treated with a single dose of poly IC (10 mg/kg, i.p.) at 0 hour and two doses of allopurinol (17 mg/kg p.o.) at -1 and 6 hours and killed 24 hours after poly IC treatment. Control and poly IC only treated animals received corresponding volume of phosphate buffered saline at the same time.

allopurinol at 50 mg/kg p.o. can depress cytochrome P-450 in the mouse.

The effect of allopurinol (7 mg/kg p.o. and 17 mg/kg p.o respectively) on the induction of xanthine oxidase (O form and D form) 24 hr after a single dose of poly IC is shown in Tables 32 and 33. Poly IC treatment increased the activity of the (O) form and (D) form of xanthine oxidase to 223% and 145% of control respectively. Administration of allopurinol (7 mg/kg or 17 mg/kg p.o.) prevented poly IC induced increase in activity of both form of xanthine oxidase. Basal level of both forms of xanthine oxidase was also depressed significantly at both doses of allopurinol

Administration of the free radical scavenger, α tocopherol (416 mg/hamster i.p.) protected against the depression of cytochrome P-450 content and ethoxyresorufin O-de-ethylase activity caused by poly IC (Table 34). α Tocophenol by itself had no effect on xanthine oxidase activity, but caused a significant depression in cytochrome P-450 content and ethoxyresorufin O-de ethylase activity

These findings suggest that the generation of free radicals (probably via xanthine oxidase activity) may play an obligatory role in the depression of the hepatic mixed function oxidase system caused by interferon.

TABLE 34

The effect of α-tocopherol and poly IC on cytochrome P-450 (O) form of xanthine oxidase and (D) form of xanthine oxidase activity in the hamster liver

Treatment	Cyt P-450 (nmoles/mg protein)	Ethoxyresorufin (nmoles protein resorufin/mg/min)	Xanthine Oxidase (O form) (nmoles uric acid/ mg protein/min)	Xanthine Oxidase (D form) (nmoles NADH/ mg protein/min)
Control	0.798 ± 0.032	0.092 ± 0.013	1.04 ± 0.17	2.61 ± 0.1
α-tocopherol	0.525 ± 0.0541*	0.037 ± 0.001*	1.27 ± 0.11	2.73 ± 0.2
Poly IC (24 hr)	0.351 ± 0.036*+	0.010 ± 0.001*+	2.51 ± 0.19*	4.45 ± 0.1*
α-tocopherol plus Poly IC	0.786 ± 0.035	0.097 ± 0.02	2.69 ± 0.17*+	3.96 ± 0.24*+

*Significantly different from saline treated animals, $p < 0.05$.

+Significantly different from α-tocopherol treated animals, $p < 0.05$.

Animals were treated with a single dose of poly IC (10 mg/kg, i.p.) at 0 hour and one dose of α-tocopherol (419 mg/hamster i.p.) at 24 hours and killed 24 hours after poly IC treatment. Control and poly IC only treated animals received corresponding volume of phosphate buffered saline at the same time.

P Liver histology

Liver histology was carried out to assess if damage occurred in the endoplasmic reticulum (site of hepatic mixed function oxidase) during IFN-αCON1 treatment. Hamsters received a single dose of IFN-αCON1 (1 x 10^6 units i.p.) and were killed at 3 hr and 24 hr. Control animals received corresponding volumes of saline. The livers were removed, fixed with glutaraldehyde and osmic acid, embedded in TAAB embedding resin and stained with uranyl acetate and lead citrate.

Examination with electron microscopy (Figures 27, 28 and 29), the ultrastructures, including that of the endoplasmic reticulum, of the hepatocytes of the IFN αCON1 treated hamsters in these preparations appeared normal in all details compared to control. This demonstrates that decrease in cytochrome P-450 content after IFN-αCON1 treatment is not due to physical damage to the endoplasmic reticulum.

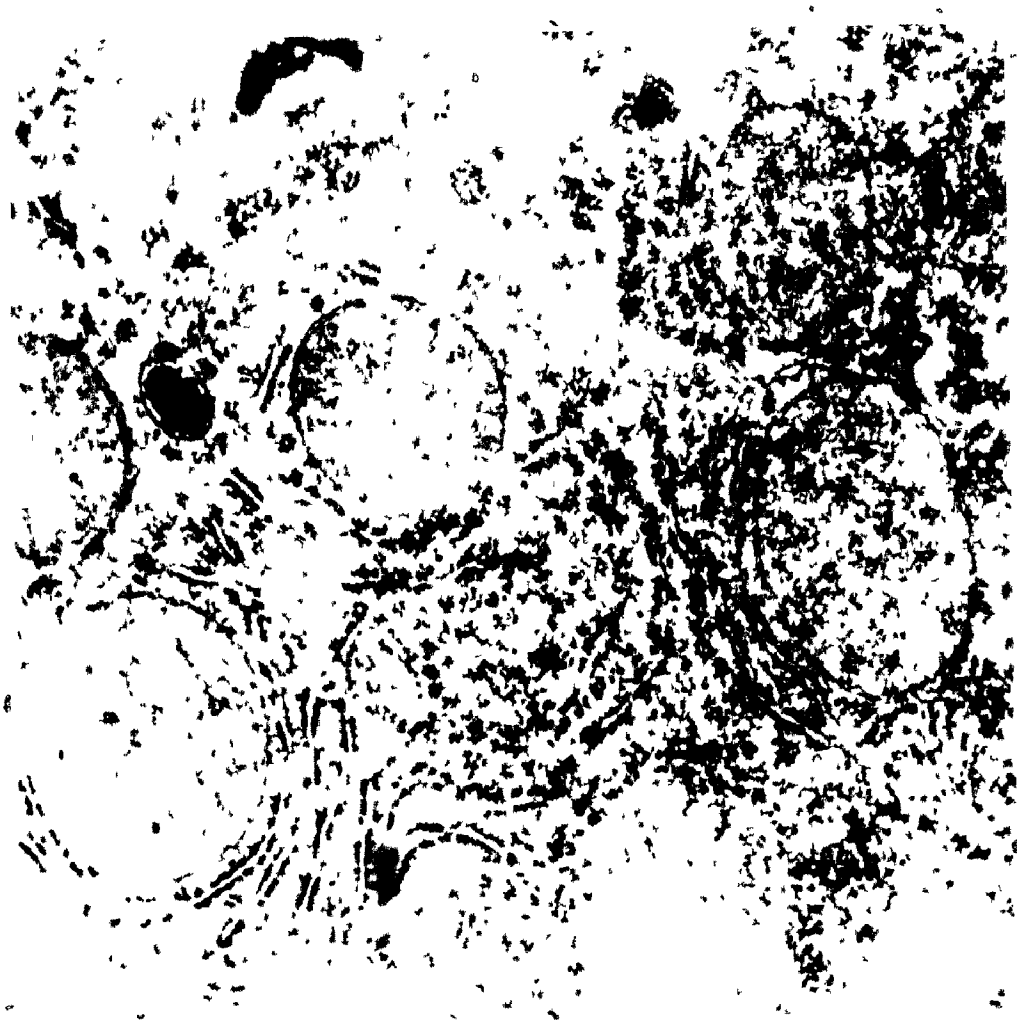


Figure 27

Electron micrograph of liver section from hamster treated with saline. Magnification = 30,600 x.

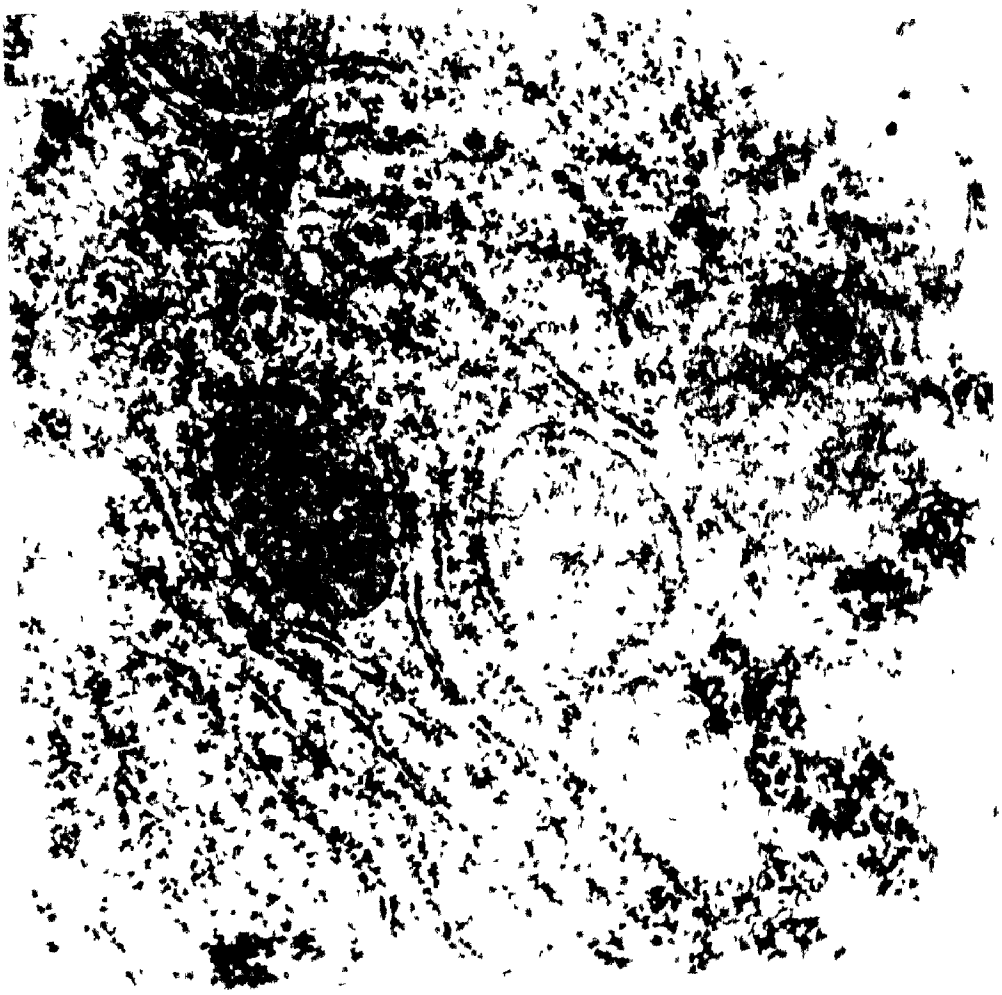


Figure 28

Electron micrograph of liver section from hamster treated with IFN-γCON$_1$ for 3 hr. Magnification 30,000x.

Figure 29

Electron micrograph of liver section from hamster treated with IFN-αCON$_1$ for 24 hr. Magnification - 30,600 x.

DISCUSSION

SECTION I

In 1976, two laboratories (Renton and Mannering, 1976; Leeson et al., 1976) demonstrated that interferon inducers depressed hepatic metabolism of drugs by depressing the content of cytochrome P-450. At this time, there was considerable speculation as to whether this depression was due to interferon or resulted from other actions common to these agents (e.g. immunomodulation). With the advent of large scale production of highly purified interferons, direct evidence for the involvement of interferon in depressing the cytochrome P-450 content was established (Singh et al., 1982; Parkinson et al., 1982). Recently, with the capacity to synthesize complete gene sequences, Amgen Inc. in California, conceived a novel analog human interferon called alpha consensus one (IFN αCON1) which, unlike other synthetic interferons, was derived by picking the most frequently observed amino acid at each position of the known IFN-α subtypes (Alton et al., 1983). The biological property that stimulated initial interest in IFN-αCON1 was with respect to its significant antiviral activities in human and hamster cell lines and in viral infections and experimental tumors in hamsters (Stebbing et al., 1986). In this thesis, we determined the extent by which IFN αCON1 could depress hepatic drug metabolism, an

effect of clinical concern, particularly when other drugs are used concomitantly.

Preliminary experiments of this study demonstrated that administration of IFN-αCON1 in hamsters caused an initial increase in hepatic cytochrome P-450 followed by a significant depression within 24 hrs This response in hamster with the consensus interferon (IFN-αCON1) was quite different from all previous work in which only depression of cytochrome P-450 was observed. The results described in this thesis demonstrate for the first time that interferon, in addition to causing depression of cytochrome P-450, could also stimulate this enzyme system.

Interferon induced stimulation of cytochrome P-450 system

During the "time course studies" of this investigation, it was noted that both poly IC and IFN-αCON1 stimulated the hamster hepatic mixed function oxidase prior to depression. Administration of IFN-αCON1 or poly IC caused an initial increase (3 hrs and 6 hrs after IFN-αCON1 and poly IC treatment respectively) in cytochrome P-450 levels followed by significant depression within 24 hrs. This early increase in cytochrome P-450 content and related enzyme activity was also associated with increases in total microsomal protein concentration. The response appears to be specific for the cytochrome P-450 apoprotein component of mixed function oxidase systems as 1) no increase in

NADPH cytochrome c reductase activity and cytochrome b5 content was observed, 2) putative cytochrome P-450 apoprotein of a molecular weight region of 48,000 was increased when the microsomes were analyzed by SDS-PAGE. This stimulatory effect of IFN-αCON1 on mixed function oxidase system was not restricted to the liver as lung, adrenals and spleen were also affected in a similar manner. In contrast, kidney cytochrome P-450 content and benzo(a)pyrene activities were depressed by 44% and 69% from control respectively.

The depression of kidney cytochrome P-450 content after 3 hrs of treatment with IFN-αCON1 could be due to a) differences in cytochrome P-450 isozymes in different tissues (Bend and Serabjit-Singh,1984), b) temporal factors in activation of this biological function, or c) the concentration of interferon reaching the tissues. With regards to the latter point, it was found that one third of a dose of a human α-interferon labelled with ^{125}I was found in the kidney of mice within 5 min of i.v. administration, whereas liver and stomach contained 5.5% and 1.4% of the dose respectively (Palleroni and Bohoslawec, 1984). Alternatively the different response in the kidney may be due to differences in biological systems. For example, El Ashary and Mannering (1979) reported that the cytochrome P 450 content and benzo(a)pyrene activity of rat intestines and adrenals and cytochrome P 450 content in the kidney

cortexes were not affected by 4 daily doses of poly IC however, the benzo(a)pyrene hydroxylase activity was lowered by 40%. It is also interesting to speculate that the effect of interferon could be related to the macrophage type cells present (Kupffer cells in the liver and alveolar macrophages in the lung) or absent (kidney) in a particular tissue.

Although the present study demonstrates that poly IC and IFN-αCON1 stimulates microsomal P-450 systems in vivo, it is still uncertain if the action is a property of a interferon per se, as preliminary studies in our laboratory have shown that IFN-αCON1 does not stimulate the cytochrome P-450 system in isolated hamster hepatocytes (Renton et al., 1984). It is possible that interferon-induced stimulation in vivo of microsomal cytochrome P-450 dependent catalytic activity may be due to (a) release of humoral factors (e.g. glucorticoid) that may stimulate cytochrome P-450 activity, or (b) through an intracellular component which stimulate cytochrome P-450 system and whose mechanism of action is "short lived" (Mannering and Deloria, 1986).

The paradoxical result (stimulation of mixed function oxidase as opposed to the depression normally reported) has also been observed by others in two other situations. Poly IC or crude mouse interferon preparations can increase cytochrome P-450 levels and related enzyme activity in

cultured mouse hepatocytes (Renton et al , 1978), and crude mouse interferon stimulates benzanthracene-induced aryl hydrocarbon hydroxylase activity in fetal mouse cell cultures (Nebert and Friedman, 1973) Other enzymes can also be enhanced by interferon or interferon inducers including (a) xanthine oxidase (Ghezzi et al., 1984, 1985; Deloria et al , 1985; and this study), b) 2'5 oligoadenylate synthetase (Krispin et al., 1984), c) creatine kinase (Fisher et al., 1983), d) tRNA methylase (Rozee et al , 1969), e) guanylate cyclase (Vesely and Cantell, 1980), f) indoleamine 2,3, dioxygenase (Yoshida and Hayaishi, 1978), g) tryptophan dioxygenase (El Azhary and Mannering, 1979), h) heme oxygenase (El Azhary and Mannering, 1979; Renton, 1981 and this study).

Interferon-induced depression of mixed function oxidase

The temporal aspects of hepatic cytochrome P-450 depression of hamsters following a single dose of IFN and poly IC were similar to those previously reported for human IFN-αAD (Bgl) and gamma interferon in mice (Singh et al , 1984; Parkinson et al., 1982; Franklin and Finkle, 1985) and poly IC in mice (Singh 1982b) and rats (El Azhary and Mannering, 1979). The depression of metabolism of typical substrates such as aminopyrine N-demethylase, benzo(a)pyrene hydroxylase, ethoxyresorufin O-deethylase and lauric acid hydroxylase paralleled the depression of

cytochrome P-450. The depression appears to be selective for cytochrome P-450 hemoprotein as cytochrome b_5 and NADPH cytochrome c reductase are not affected by interferon treatment.

At present, the pharmacokinetic elimination profile of IFN-αCON1 is unknown and it is therefore impossible to correlate the depression of cytochrome P-450 system with serum level of IFN-α CON1. Round and Stebbing (1983) have shown that a single dose of poly IC in hamsters induces serum interferon levels which peak at 3-4 hrs during which the hepatic mixed function oxidase activity is increased. No detectable level of interferon was observed after 24 hr after poly IC treatment during the time which hepatic mixed function oxidase activity was depressed. The lack of detectable serum interferon levels at the time of depression of cytochrome-P-450 systems was similarly observed by Parkinson et al. (1982). In contrast, Singh and Renton (1980) and Renton (1981) found that an increased serum interferon level correlated with depression of cytochrome P-450 system. Serum interferon levels however are not necessarily reliable indicators of the interferon content of the liver and other organs. This is because interferon can differentially be sequestered in different organs (Palleroni and Bohoslawee, 1984). Parkinson et al. (1982) suggested that the rapid disappearance of the interferon, IFN-α AD, after a single dose of this

interferon (i.p.) from blood serum relative to the rate of decrease in cytochrome P-450 content was not due to its direct interaction of interferon with cytochrome P-450. Our present study provides further evidence against a direct interaction between interferon and hepatic mixed function oxidase by showing a lack of inhibition of the cytochrome P-450 system in vitro by IFN-αCON1 or poly IC. No further reduction in the cytochrome P-450 system occurs after 3 daily doses (1 x 10^6 units i.p.) of IFN-αCON1 (chronic study) as compared to a single dose (1 x 10^6 units i.p.) suggesting that a new steady-state of cytochrome P-450 content is reached within 24 hrs of interferon treatment. A similar observation was noted by Mannering et al (1980) with interferon inducers poly IC and tilorone in rats and by Parkinson et al. (1982) with human interferon HuIFNr-AD in mice. When analyzed by SDS-PAGE, the concentration of hepatic microsomal proteins with molecular weights of approximately 48,000 and 50,000 daltons appeared to decrease 24 hrs after a single dose and multiple doses of IFN-αCON1. Microsomal proteins of these molecular weight ranges have been identified as cytochrome(s) P-450 (Chiang and Steggles, 1983). Previous workers using SDS PAGE, to separate microsomal proteins, reported that interferon inducers poly IC, tilorone or Freunds' adjuvant selectively depressed apocytochrome P-450 (Zerkle et al., 1980). During the course of this study, Balkwill et al.

(1984) reported that the concentration of selective apocytochrome P-450s in mice treated with purified mouse interferon was decreased. It can be concluded that interferon and interferon inducers depress the cytochrome P-450 content (as measured by its carbon monoxide-binding spectrum) by decreasing apocytochrome P-450 content

A single dose of IFN-αCON1 and poly IC for 24 hours also depressed cytochrome P-450 content and benzo(a)pyrene hydroxylase activity in lungs, kidney, spleen and adrenals. The extent of depression of cytochrome P-450 content and benzo(a)pyrene activity varied between different tissues. No correlation between the extent of depression of cytochrome P-450 content and benzo(a)pyrene activity occurred in these tissues Possible reasons for this would be: a) the depression of selective isozymes of cytochrome P-450 with/without a relatively high specific activity towards benzo(a)pyrene, b) regulatory mechanism for the maintenance of steady-state levels of cytochrome P-450 systems differ between tissues, c) the ability of the interferon to reach its site of action in adequate concentration. Kidney being the major site for metabolism of interferon is also the site where interferon had the greatest depressive effect.

In our experiments, the depression of cytochrome P-450 by either IFN-αCON1 or poly IC was not accompanied by any changes in body weight, liver weight or microsomal protein

concentration. In addition, no ultrastructural abnormalities were observed in tissues obtained from hamsters treated with single doses of IFN-αCON1 when examined using an electron microscope. These results demonstrate that the in vivo depression of cytochrome P 450 system by interferon is not accompanied by obvious toxic effects to the animal or ultra structural changes in the tissue.

The hypothesis that a direct relationship exists between antiviral and "anti-cytochrome P-450" properties of interferon (Singh et al., 1982) was also tested in this study IFN-αCON1 which has no antiviral activity against a mouse cell line (L929) when challenged with vesicular stomatitis virus (VSV), did not depress mouse hepatic cytochrome P-450 content or cytochrome P-450 dependent catalytic activity. On the other hand, poly IC depressed hepatic cytochrome P-450 system in the mouse in a similar extent to that reported by Singh and Renton (1980) [7] Human buffy coat interferon (HuIFN-α), derived from leukocytes, is antiviral against a hamster cell line (BHK) when challenged with VSV, depressed hamster hepatic cytochrome P-450 system. This interferon has virtually no antiviral activity in a mouse cell line and no depressive effect on mouse hepatic cytochrome P-450 (Singh et al., 1982). Although IFN-αCON1 has been shown to have antiviral activity against primate and human cell lines(Alton et

al.,1983), preliminary evidence from our own lab suggests that this interferon does not alter theophylline elimination kinetics in primates, possibly suggesting that the antiviral activity of this interferon is not necessarily accompanied by a depression in cytochrome P-450 system in all species (Renton et al, 1984). This would be an obvious advantage for the eventual use of this interferon in man.

In summary, this part of the study demonstrates that IFN-αCON1 caused a dose dependent and time dependent decrease in the cytochrome P-450 system in hamsters. Both hepatic and extrahepatic cytochrome P-450 content was depressed after 24 hrs interferon treatment period. The depression of the mixed function oxidase was restricted to the cytochrome P-450 component of the system as cytochrome b5 and NADPH cytochrome c reductase were not affected: IFN-αCON1 demonstrates a species specificity as mouse hepatic cytochrome P-450 system was not affected.

Effect of interferon on various isozymes of cytochrome P-450

The aim of this part of the study was to determine whether IFN-αCON1 had any suppressive effect on the levels of the cytochrome P-450s and specific mono-oxygenase activities induced by phenobarbital (PB), β-napthoflavone (BNF) or clofibrate (CF).

PB and BNF induced high concentrations of total cytochrome P-450 (as measured by carbon monoxide binding spectra) in hepatic microsomes. "Phenobarbital inducible" cytochrome P-450 has a high substrate specificity for aminopyrine, and "3-methylcholanthrene inducible" (or β-napthoflavone) cytochrome P-450 (cytochrome P-447) has a high substrate specificity for benzo(a)pyrene. On the other hand, "clofibrate inducible" cytochrome P-450 (cytochrome P-452) displayed specificity for the hydroxylation of lauric acid without inducing cytochrome total P-450 content (carbon-monoxide binding spectra) It has been shown recently that considerable proportion (greater than 50%) of the clofibrate-induced cytochrome P-450 isozyme is indeed cytochrome P-452 (Tamburini et al., 1984). Animals induced with various the inducers produce animals with microsomes which contain high levels of different isozymes of cytochrome P-450.

The results of these studies indicated that after a 24 hr treatment period IFN-αCON1 depressed cytochrome P 450 content and the activities of aminopyrine N-demethylase, benzo(a)pyrene hydroxylase, ethoxyresorufin o-dealkylase and lauric acid hydroxylase in non-induced hepatic microsomes to 66%, 65%, 81%, 63% and 63% of control respectively. When comparing the results to animals induced with PB, BNF and CF it becomes evident that the

forms of cytochrome P-450 induced are a determining factor in the extent of depression of P-450 hemoprotein.

PB-, BNF- and CF-induced cytochrome P-450 content, as measured by the amount of CO-binding pigment, were differentially decreased within 24 hrs of IFN-αCON1 treatment. Though this only indicates that total cytochrome P-450 is depressed by IFN-αCON1, it does not mean that specific isozyme of cytochrome P-450 are depress in the similar manner by interferon. However, different isozymes of cytochrome P-450, based on substrate specificity were depressed to varying degrees by IFN-αCON1 Cytochrome P-452, as measured by lauric acid hydroxylation, and cytochrome P-450 (PB inducible), as measured by aminopyrine N-demethylation were the isozymes most affected in both induced and non-induced microsomes by IFN-αCON1 treatment. On the other hand, cytochrome P-448 as measured by benzo(a)pyrene hydroxylation was least affected

It has been shown by Renton et al. (1979) that concurrent administration of poly IC or tilorone suppressed the induction of cytochrome P-450 and mono-oxygenase activity (ethylmorphine N-demethylase and benzo(a)pyrene hydroxylase) in PB and of 3-MC induced animals. However, induction of benzo(a)pyrene hydroxylase by 3-MC was lowered by only 40%. Recently, Crowe et al. (1986), as in the study by Renton et al (1979), showed that treatment of animals with poly IC induced with either PB or 3-MC

decreased the cytochrome P-450 content to the noninduced basal level within 24 hrs. They also showed that this effect was temporary in the 3-MC-treated animals since the cytochrome P-450 content returned to the pre-induced 3-MC level within 72 hrs of poly IC treatment. A similar recovery was not obtained in the comparable PB experiment

The effect of IFN-αCON1 on induced lung cytochrome P-450 content and benzo(a)pyrene hydroxylase activity was different from that seen in the liver In lung, IFN - CON1 depressed benzo(a)pyrene hydroxylase activity in the BNF induced animal accompanied by only a small effect on cytochrome P-450 systems. PB did not induce cytochrome P 450 content in hamster lung microsomes. This was not unusual as several reports have noted this phenomena in other species (Conney, 1971) However, in contrast to BNF treated hamsters, benzo(a)pyrene hydroxylase activity in the lung microsomes of the PB treated hamster was only depressed by 18%, compared to 338% in BNF treatment by IFN αCON1 . IFN-αCON1 treatment by itself only depressed benzo(a)-pyrene hydroxylase activity by 2% but, cytochrome P-450 content was depressed by 40%. It appears that IFN αCON1 , as in the liver, has little depressive benzo(a)pyrene hydroxylase activity in the lung, although there was unparalleled depression in the cytochrome P 450 content. In contrast, treatment with BNF alters the pattern of depression of benzo(a)pyrene hydroxylase

activity by IFN- CON1 "possibly" through a production of species of cytochrome P-450 isozyme that is susceptible to depression by interferon

The selective depression of enzyme activities (aminopyrine N-demethylase and lauric acid hydroxylase compared to benzo(a)pyrene hydroxylase) and cytochrome P-450 apoprotein content by administration of interferon may be due to effects on rapidly turning-over forms of cytochrome P-450, e g the rapid turnover of PB-inducible cytochrome P-450 (7-8 hr) relative to the 3-MC inducible cytochrome P-450 (46-48 hrs) (El Azhary et al., 1980). Turnover rate of clofibrate-inducible cytochrome P-450 has not been determined to date. Also this idea is supported by the observation that steroid induced tyrosine amino transferase or glutamine synthetase (both are thought to be rapidly turning over proteins) (Rossi, 1985) were depressed by interferon in rat and chicken cells.

SECTION II

Mechanisms involved in stimulation and depression of hepatic cytochrome P-450 caused by interferon

Enzyme induction as first defined in 1953 by Cohn et al., "is a relative increase in the rate of synthesis of a specific apoenzyme resulting from exposure to a chemical substance". In contrast enzyme repression was defined (Vogel, 1957) as "a relative decrease, resulting from the

exposure of cells to a given substance, in the rate of synthesis of a particular apoenzyme" The words "enzyme induction" and "enzyme repression" will be used henceforth to represent the increase and decrease of cytochrome P-450 following IFN treatment

In the first part of this thesis, I have demonstrated that interferon, IFN-αCON1 and the interferon inducer poly IC causes an initial increase in cytochrome P-450 content and related enzyme activities in the hamster which is then followed by a significant depression of the system within 24 hrs. It is remarkable that the increase and the loss of hepatic cytochrome P-450 caused by interferons or interferon inducers has rarely exceeded fifty percent of control. The finding that cytochrome P-450 loss does not exceed fifty percent of control even after repeated doses of interferon has been reported in this study as well as others (Parkinson et al., 1982; Singh, 1982). The early transient increase in cytochrome P-450 by interferon which is reported in this study is the first observation of such an effect.

The initial increase in cytochrome P-450 content by interferon is associated with increases in microsomal protein concentration and increases in apocytochrome P 450 content. The loss in cytochrome P 450 is, however, associated with loss in apocytochrome P-450 apoprotein content without an accompanying loss in total microsomal

protein concentration. Synthesis requires separate formation of the apoprotein and the heme moiety which are assembled to form the holoprotein (see Introduction). A perturbation in any process of holoprotein synthesis would result in a change in the steady-state level of the enzyme (Omura, 1980). Similarly alteration in the degradation rate would change the steady-state levels of hepatic cytochrome P-450 Several studies have been carried out to determine the mechanism of the effects of interferon and interferon inducers on cytochrome P-450.

Effects of interferon on hepatic microsomal cytochrome P-450 heme turnover

El Azhary and Mannering (1980) have suggested that interferon inducers depress cytochrome P-450 content by increasing heme degradation and thereby decreasing the availability of heme. These authors described the following sequence of events, which is based on the concept of heme synthesis being regulated by a heme pool (DeMatteis, 1978). They observed that interferon inducing agents caused an increase in the size of the regulatory heme pool. This could occur in two ways: (a) these agents can cause increased dissociation of heme from cytochrome P-450 or other hemoprotein; (b) or these agents depress the synthesis of apocytochrome P-450 and the apoproteins of other hemoproteins, thereby decreasing the demand for heme

from the heme pool. The increase in the size of the heme pool has been shown to depress δ-aminolevulinic acid synthetase (ALA-S), the rate-limiting enzyme in heme synthesis. The excess heme also induces heme oxygenase, the rate limiting enzyme in heme breakdown, which then relieves the inhibition of ALA-S by destroying heme (Bissell and Hammaker, 1976) In this study, heme oxygenase activity was increased 24 hrs following IFN-αCON1 treatment and corresponded to the time when the cytochrome P-450 content was decreased. A causal relationship between the increase in heme oxygenase activity and the depression of cytochrome P-450 has been described by others (Maines and Kappas, 1977; Jarvisalo et al., 1978; Bissell and Hammaker, 1976; El Azhary and Mannering, 1979; Renton, 1980) In 1980, El Azhary et al. suggested that interferon-inducing agents increased the degradation of heme in cytochrome P-450 but had no effect on heme synthesis. However in 1982, Singh (thesis) indicated that the results of El Azhary et al. (1980) cannot explain the increase in heme degradation as the mechanism by which interferon inducing agents depress cytochrome P-450. Singh's explanation, based on the measurement of rate constants of heme degradation calculated by El Azhary et al. (1980), allows only an 8% decrease in cytochrome P-450 by interferon inducers. A 40-50% total loss in hemoprotein is routinely observed. Subsequently, Singh (1982)

demonstrated that heme degradation as measured by the expiration of $^{14}CO_2$ from methene bridge carbon of the porphyrin ring was increased three fold in mice treated with interferon inducers. The area under the curve for $^{14}CO_2$ expiration was decreased in treated mice, suggesting that the synthesis of heme was also impaired. The magnitude of the changes measured in this way was much more compatible with the observed decrease in cytochrome P-450. Furthermore, Sonnefeld et al. (1982) noted that induction of gamma interferon in mice resulted in a transient decrease in serum iron levels. They proposed that translocation of iron from serum to liver could have resulted in a decrease in heme synthesis and increase in heme degradation, as proposed by others (Bissell and Hammaker, 1976). These authors attempted to block the translocation of iron by administering cysteine, which would complex with the metal via its sulphydryl groups. Administration of cysteine however did not block the decrease in cytochrome P-450 by the interferon, which suggested that the changes in heme components may play a secondary role in depression of cytochrome P-450 by interferon.

In regard to the early stimulation of cytochrome P-450 by IFN-αCON1 observed in the present experiments, it is interesting to note that heme content was also increased during this time period. Heme oxygenase activity however

was not affected during this treatment period. This is in keeping with the finding of El Azhary and Mannering (1979), that early stimulation of ALA-S occurs after treatment with an interferon inducer

Effects of interferon on hepatic apocytochrome P-450 content

Another mechanism which may be involved in altering the steady-state levels of cytochrome P-450 following interferon treatment would be to alter the content of the apocytochrome P-450. Indeed it has been suggested that, the rate limiting step involved in the formation of holocytochrome P-450 would be the availability of the apocytochrome (Correia and Meyer, 1975).

(i) Induction of apocytochrome P-450 by interferon

The results presented in this thesis have demonstrated for the first time that the amount of cytochrome(s) P-450 in hepatic and extrahepatic microsomes (except kidney microsomes) increased during the initial effects of IFN-αCON1 and poly IC (3 hrs and 6 hrs after IFN- CON1 and Poly IC treatment). The results of the [^{35}S]-methionine incorporation experiment (Hangen et al., 1976) shows that the amount of radioactivity incorporation per mg of microsomal protein in treated animals (6 hr treatment

period with poly IC) were higher than the control animals, indicating that an increase in the synthesis of microsomal proteins proceeded at a higher rate in interferon treated animals. When analyzed by SDS-PAGE (gel fractionation or fluorography), equally labelled microsomal proteins associated with the apocytochrome P-450 region from treated animals was increased. Renton and Singh (1984) previously demonstrated a significant increase in the incorporation of ^{3}H-labelled amino acids to the total protein of hepatic microsomes of animals at 3, 6, 9, 12 and 18 hrs after treatment with poly IC, however they did not associate this increase in amino acid incorporation with increases in specific or total apocytochrome P-450 during these time intervals.

Drug-induced increases in microsomal enzyme activity can be prevented by certain inhibitors of protein or deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) synthesis. Previous investigators have shown that inhibitors of protein synthesis such as puromycin (Gelboin and Blackburn, 1964) and actinomycin D (Gelboin and Blackburn, 1964) prevented the induction of microsomal enzymes (e.g. benzo(a)pyrene hydroxylase) by the inducer, 3-MC. The results presented here demonstrated that the interferon induced stimulation of both hepatic and extrahepatic cytochrome P-450 content and related enzyme activities could be prevented by both puromycin (protein

synthesis inhibitor) and actinomycin D (DNA-dependent RNA synthesis inhibitors) It has been shown that the response to interferon requires the generation of new RNA and protein synthesis In fact only two enzymes out of several IFN-induced proteins of unknown function (Sen, 1984) have been discovered that require de novo protein synthesis. This includes the 2'-5' oligosynthetase and protein kinase. We therefore conclude that interferon also induces cytochrome P-450 content and that this induction requires protein synthesis de novo.

An increase in the amount of extractable RNA and in vitro synthesis of RNA directed TCA precipitable protein isolated at 3 hrs following IFN-αCON1 administration coincides with the maximum appearance of microsomal protein concentrations. Because the translational systems used here were designed such that exogenous mRNA is the principle determinant for the rate of protein synthesis, these results indicate that induction with interferon increases not only the amount of mRNA but also the specific activity of mRNA (i.e. translated protein/μg RNA) Such an effect is not surprising as the antiviral action of IFN requires initial new mRNA and protein synthesis. Rubin and Gupta (1980) have reported that the synthesis of proteins in IFN-α-treated cells is tightly regulated. In IFN-treated human fibroblasts the induced proteins are not synthesized continuously and the rate of synthesis of some

of these proteins peak within a few hours of commencement of IFN-treatment. On analysis of total products by SDS-PAGE, we have shown that the intensity of radioactivity were greatest in the 47,000-54,000 dalton molecular weight region. It is in this region that apocytochrome P-450 would be expected to migrate. The 60% increase in the *in vitro* synthesis of apocytochrome P-450 observed at 3 hrs following interferon administration suggests (but does not prove) that an increase in the number of cytochrome P-450-specific mRNA sequences are involved in the induction process. Furthermore, it was found that the apocytochrome P-450 synthesized *in vitro* is indistinguishable in size from the apocytochrome isolated from hamster liver microsomes. Therefore, processing of interferon induced apocytochrome P-450 upon insertion into the endoplasmic reticulum may not occur. Similar observation was reported by Kumar and Padmanaban (1980) with phenobarbital as the inducer.

Depression of cytochrome P-450 by interferon

The results discussed in the previous section indicate that interferon induced depression of cytochrome P-450 is associated with a decrease in the steady-state level of apocytochrome P-450. This has also been suggested by several other workers (Zerkle and Wade, 1980; Mannering *et al.*, 1983; Balkwill *et al.*, 1984). This study however

provides for the first time evidence to directly support the hypothesis that the interferon induced depression of the apocytochrome P-450 content is due to a decrease in protein synthesis and is not influenced by changes in degradation of proteins This conclusion is based on the following results

The incorporation of [^{35}S]-methionine into total microsomal protein was identical in control and 24 hours after the treatment of poly IC. This indicates that the overall synthesis of total microsomal proteins is unchanged by an interferon inducer. When analyzed by SDS-PAGE, however, microsomal proteins of the molecular weight range of apocytochrome P-450s (47,000 dalton - 55,000 dalton) contained less radioactivity in animals treated with poly IC. A possible reason to explain the unchanged amount of amino acid incorporation in total microsomes could be that the rate of synthesis of some microsomal proteins in the liver was induced by poly IC treatment, while the synthesis of other proteins (e.g. cytochrome P-450) was depressed. A similar suggestion was made by Singh and Renton (1984) who demonstrated that the incorporation of amino acids into a cytochrome P-450 rich fraction of microsomes was depressed but the overall incorporation of amino acids into total microsomes was actually increased. These two studies which measured incorporation of amino acids into fractions of microsomes (Singh and Renton, 1984; this thesis) suggest

that interferon inducers inhibit apocytochrome P-450 synthesis. Conversely, Zerkle and Wade (1984) suggested that poly IC depresses cytochrome P-450 by increasing the rate of degradation of apocytochrome P-450. This conclusion was based on the fact that ^{14}C-leucine incorporation into a cytochrome P-450 enriched fraction of poly IC treated rats was increased, and therefore the depression of apocytochrome P-450 interferon was due to increases in degradation rate.

Unfortunately studies of protein synthesis which simply measure incorporation into a protein have interpretation problems. That is, depressed radiolabelled incorporation could be associated with increased rate of protein degradation rather than decreased synthesis Another approach, which could alleviate this problem, was taken in this study in order to define accurately the mechanism involved in the depressed steady-state level of apocytochrome P-450 after interferon treatment. The techniques of Aria et al (1969) and Dehlinger and Schimke (1970) enabled us to determine the effect of interferon on the relative rates of apocytochrome P-450 degradation and synthesis respectively. Through this study, we could pinpoint the mechanism by which interferon depresses apocytochrome P-450 content (i.e. rate of synthesis or degradation, or both). It was found that the relative rate of degradation of putative cytochrome P-450 was actually

decreased, rather than increased, following interferon treatment Three putative apocytochrome P-450 of approximately Mr = 47,500, 51,500 and 52,500 had mean half lives of 30 1, 31.5 and 31 5 hrs respectively in control animals. These half-lives of putative apocytochrome P-450s from microsomes of control hamsters were similar to those reported by others in the rat (Parkinson et al., 1983; Sadono and Omura, 1983). It is concluded that these 3 proteins turnover at similar rates The evidence that these proteins represent apocytochrome P-450 however is indirect and is based on the similarity of their molecular weight to purified cytochrome P-450 obtained from induced hamsters and their associated differential changes in monooxygenase activities as described by Chiang and Steggles (1983). Unequivocal identification of these proteins would require the use of multiple monospecific antibodies. The half-lives of these 3 proteins was increased by about 10% in animals treated with interferon for 24 hrs even though cytochrome P-450 content was depressed by 31%. The decrease in the rate of degradation of apocytochrome P-450s may contribute to a process of stabilization of the enzyme at the new steady state of protein turnover. In further experiments, the relative rate of synthesis of the above mentioned putative apocytochrome P-450s were decreased significantly in hamsters treated with interferon for 24 hrs. This clearly

indicates that the depressed steady-state level of cytochrome P-450 is caused by a loss in the capacity of the liver to synthesize the apocytochrome P-450 and that degradation of the apocytochrome P-450 plays no significant role in this process. It must be emphasized that a serious drawback in the accurate determination of degradation (Schimke, 1975) in these studies, is isotope reutilization (i e labelled proteins degraded to constituent labelled amino acid, can be reutilized for synthesizing the same proteins and therefore could underestimate degradation rate constantly). As reutilization of labelled amino acid into protein would be the same for both saline and interferon treated animals, this would therefore represent the actual effect of interferon on rate of synthesis and degradation of apocytochrome P-450.

To determine if the decrease in cytochrome P-450 synthesis was due to (a) decrease in mRNA, (b) decrease in translatability of mRNA, or (c) both, the effect of IFN on the in vitro synthesis of apocytochrome P-450 was measured in a cell free translation system. This idea was developed from reports that indicate that poly IC and interferon decrease the synthesis of certain other cellular proteins (other than viral proteins) via such a mechanism (Rossi et al., 1985). The results obtained in these experiments demonstrate that (a) total amount of RNA isolated from control and interferon treated animals was identical, (b)

after proteins were translated from isolated RNA, the total amount of label incorporated into TCA precipitable material was identical in both groups of animals The latter (b) indicates that the total translational capacity of the liver in interferon treated hamsters were identical to that of saline treated animals. This is perhaps not surprising as several reports indicate differential rates synthesis and degradation of several different proteins which occur after interferon treatment as stated in the review by Rossi, et al.(1985). On analysis of total translation products by SDS/PAGE, the amounts of apocytochrome P 450s translated was depressed. This indicates that the treatment of hamsters with interferon specifically affects the translatability of the mRNA coding for apocytochrome synthesis. Recent experiments in our laboratory using specific antibodies have indicated that the translation of specific forms of apocytochrome P-450 by interferon (apocytochrome P-452) was impaired (Renton et al., 1986). These experiments provide for the first time unequivocal proof that interferon is depressing cytochrome P 450 content in the liver by diminishing capacity of the liver to synthesise the apocytochrome.

During the course of this study the question arose "does interferon act directly on a mechanism of protein synthesis or does interferon require some form of intermediate?" This idea was developed from the

observations that interferon requires the synthesis of an "intermediate protein" to impart its antiviral action on a cell. Such a possibility had been discussed for many years and was in fact tested in 1984 by Gorce and Wade These authors, however, made a serious mistake by using protein synthesis inhibitors in combination with interferon inducer. As interferon itself is a protein which requires de novo protein synthesis (Mannering and Deloria, 1986) the conclusion that an intermediate protein is required as made by Gorce and Wade (1984) was totally unjustified. This type of experiment could only be carried out with pure interferon such as used in this thesis.

In experiments carried out with protein synthesis inhibitors, actinomycin D and puromycin prevented the interferon mediated depression of cytochrome P-450 and related enzyme activities by the purified interferon. This demonstrates conclusively for the first time that the depression of cytochrome P-450 caused by interferon requires the synthesis of an intermediate protein and does not act directly on the protein synthetic machinery. The protein(s) responsible for the actual depression of cytochrome P-450 steady-state level are probably enzymes which act on the protein synthetic machinery (e.g. mRNA, ribosomes, etc.) and as discussed in the next paragraph may be xanthine oxidase.

Role of reactive oxygen intermediates in the interferon-mediated depression of cytochrome P-450

Ghezzi et al. (1984) have proposed a novel mechanism to explain the depression of the cytochrome P-450 system caused by interferon. These authors reported that xanthine oxidase activity (O form) was enhanced in the liver and other tissues by several fold following interferon treatment. Without any direct evidence, they speculated that the production of superoxide by this enzyme could account for many biological roles of interferon including the loss of cytochrome P-450. Deloria et al. (1985), using the genetic model developed in our laboratory, demonstrated that cytochrome P-450 was lost only in strains of mice which produced interferon and high levels of liver xanthine oxidase (O form). Ghezzi et al. (1985) demonstrated the concomitant administration of allopurinol, a xanthine oxidase inhibitor or free radical scavenger (N-acetylcysteine) protected against the interferon-mediated depression of cytochrome P-450.

In the present study we have demonstrated that the (D) form of xanthine oxidase is induced in the first 3 hours following the administration of interferon and that the (O) form of the enzyme within the next 24 hours. The increase in the (D) form of xanthine oxidase by interferon treatment correlates with the times when the cytochrome P-450 is induced; and the increase in (O) form of xanthine oxidase

coincides with the time when the cytochrome P-450 is depressed. That the prevention of interferon induced (O) form of xanthine oxidase and depressionof cytochrome P-450, by actinomycin D, supports the hypothesis that interferon induces protein intermediate(s) (e g. xanthine oxidase) which are responsible for depression of cytochrome P-450 The (O) form of xanthine oxidase which is formed after interferon treatment, is likely to originate entirely from the (D) form of xanthine oxidase, as is suggested from the following: (a) (D) form of xanthine oxidase is the only form of the enzyme that exists normally in the liver (Wand and Rajagopalan, 1976), (b) only the (D) form was induced after 3 hrs of interferon treatment as shown in this study, (c) prevention of formation of (D) form of xanthine oxidase by actinomycin D prevented the formation of the (O) form of xanthine oxidase, 24 hrs later, as shown in this study, (d) decrease in the activity of the (D) form of xanthine oxidase is correlated with increases in the (O) form of xanthine oxidase 24 hrs after interferon treatment. It has been proposed that the conversion of the (D) form of xanthine oxidase can occur in two ways: (a) through oxidation of its thiol groups, which is reversible with dithioerythritol, or (b) by proteolysis, an irreversible process (Della Corte and Stirpe, 1972). Deloria et al (1985) demonstrated that the (O) forms of xanthine oxidase induced by poly IC and almost entirely converted to (D)

form of xanthine oxidase by dithioerythritol, thus showing that its conversion from (D) form of xanthine oxidase was oxidative rather than proteolytic.

The protective effect of allopurinol on poly IC mediated depression of liver cytochrome P-450 reported in this study supports the hypothesis that an increase in the generation of reactive oxygen intermediates resulting from the induction of (O) form of xanthine oxidase is responsible for the depression of cytochrome P-450. Ghezzi et al (1985) reported that allopurinol protected poly IC induced depression of cytochrome P-450 in mice. The dose of allopurinol used in their study also depressed the cytochrome P-450 In our study, the dose of allopurinol used per se did not depress cytochrome P-450 yet was able to depress xanthine oxidase activity. Further, the protective effect of α-tocopherol on the poly IC induced depression of cytochrome P-450 reported here supports the hypothesis that an increased generation of reactive oxygen intermediates, possibly resulting from induction of xanthine oxidase by interferon, is reponsible for the depression.

In summary, we speculate that the (O) form of xanthine oxidase formed via the induction of the (D) form of xanthine oxidase may be responsible for the depression of hepatic mixed function oxidase (via generation of active oxygen). Ghezzi et al. (1985) concluded that interferon

induced xanthine oxidase results in the destruction of cytochrome P-450 and drug biotransformation in the liver via generation of free radicals. While this hypothesis appears to be logical and much of our own data can support such an idea, several major problems with this conclusion are apparent.

i) The oxidative destruction of cytochrome P-450 is usually accompanied by severe membrane damage, but in our study, the membrane of the endoplasmic reticulum of the liver cells appears normal in interferon treated animals as examined in the electron microscope. Recently Koizumi et al (1986) proposed that the depression of cytochrome P-450 content might be caused by enhanced lipid peroxidation associated with increased activity of xanthine oxidase. On evaluating this data, the modest increase in lipid peroxidation caused by poly IC treatment could not possibly have caused the depression of cytochrome P-450. The destruction of cytochrome P-450 by such mechanism is usually accompanied by severe membrane damage, such as seen in carbon tetrachloride-induced hepatoxicity (Willis, 1980). Further, Finkle and Franklin (1985) demonstrated that the microsomal conjugating enzyme systems (Phase II) are not affected by interferon. In fact, administration of interferon inducers protected rats from hyperoxic pulmonary damage (Kikkawa et al., 1984). Hence, possible modest generation of reactive oxygen species (through the

induction of (O) form of xanthine oxidase) are unlikely to cause the destruction of cytochrome P-450

ii) This study and others (Renton et al., 1986; Gooderham and Mannering and Deloria, 1986), have clearly demonstrated in a number of different ways that it is the synthesis of the apocytochrome P-450 which is impaired and the rate of degradation of apocytochrome P-450 is not responsible for the loss of hemoprotein

iii) Very recently, Mannering and Deloria (personal communication, and 1986) have found that in mice in which xanthine oxidase activity is lowered to 10% of control by tungstate the loss of cytochrome P-450 can still be mediated by the interferon inducer, poly IC

What then is the role of xanthine oxidase in the loss of cytochrome P-450 following treatment with interferon?. Although at this stage of investigation it is pure speculation, two possibilities can be suggested.

i) The increase in xanthine oxidase following interferon is purely coincidental to the loss of cytochrome P-450. Interferon has many unrelated actions in a cell and these effects can be totally unrelated. The fact that XO can create reactive molecules which can potentially destroy cytochrome P-450, may be an unfortunate fact which may have led us and others in pursuit of an erroneous mechanism.

ii) The generation of free radicals or reactive oxygen species by xanthine oxidase may inhibit protein synthesis

rather than the more obvious mechanism of destroying cytochrome P-450 The free radicals formed in the cytoplasm of the cell might well destroy the mRNA involved in protein synthesis. Proteins which turnover with a faster rate will be affected to a greater extent. The fast turnover rates of the cytochrome P-450s might well make them preferential candidates for such an effect to account for their lower rate of synthesis and the decrease in cytochrome P-450 content.

CONCLUSIONS

The results summarized below represent the major original descriptions and contributions to new knowledge which are described in this thesis.

(1) Interferon and interferon inducers have a biphasic effect on hepatic and extrahepatic mixed function oxidase in hamsters. An initial increase in microsomal cytochrome P-450 is followed by a significant depression within 24 hours. The depression of mixed function oxidase by interferon appears to be selective for the most rapidly turning over forms of cytochrome P-450. IFN-αCON1, derived by selecting the most frequently observed amino acid at each position in the different human interferon α-subtypes, demonstrates species specificity in depressing cytochrome P-450.

(2) The mechanism of induction of apocytochrome P-450 requires synthesis of an intermediate protein which is mediated through production of mRNA (see Figure 30).

(3) The mechanism by which interferon represses apocytochrome P-450 content is due to a decrease in the rate of protein synthesis rather than degradation of the protein. This decrease in rate of synthesis of apocytochrome P-450 requires the synthesis of protein intermediate(s) through the production of mRNA (Figure 30). It appears that these protein intermediates

affect the translatability of apocytochrome P-450 mRNA.

(4) This study also demonstrates the induction of xanthine oxidase activity by interferon. This requires protein synthesis de novo. An initial increase in the (D) form of xanthine oxidase is followed by an increase in the (O) form of xanthine oxidase. The latter is responsible for the generation of reactive oxygen intermediates.

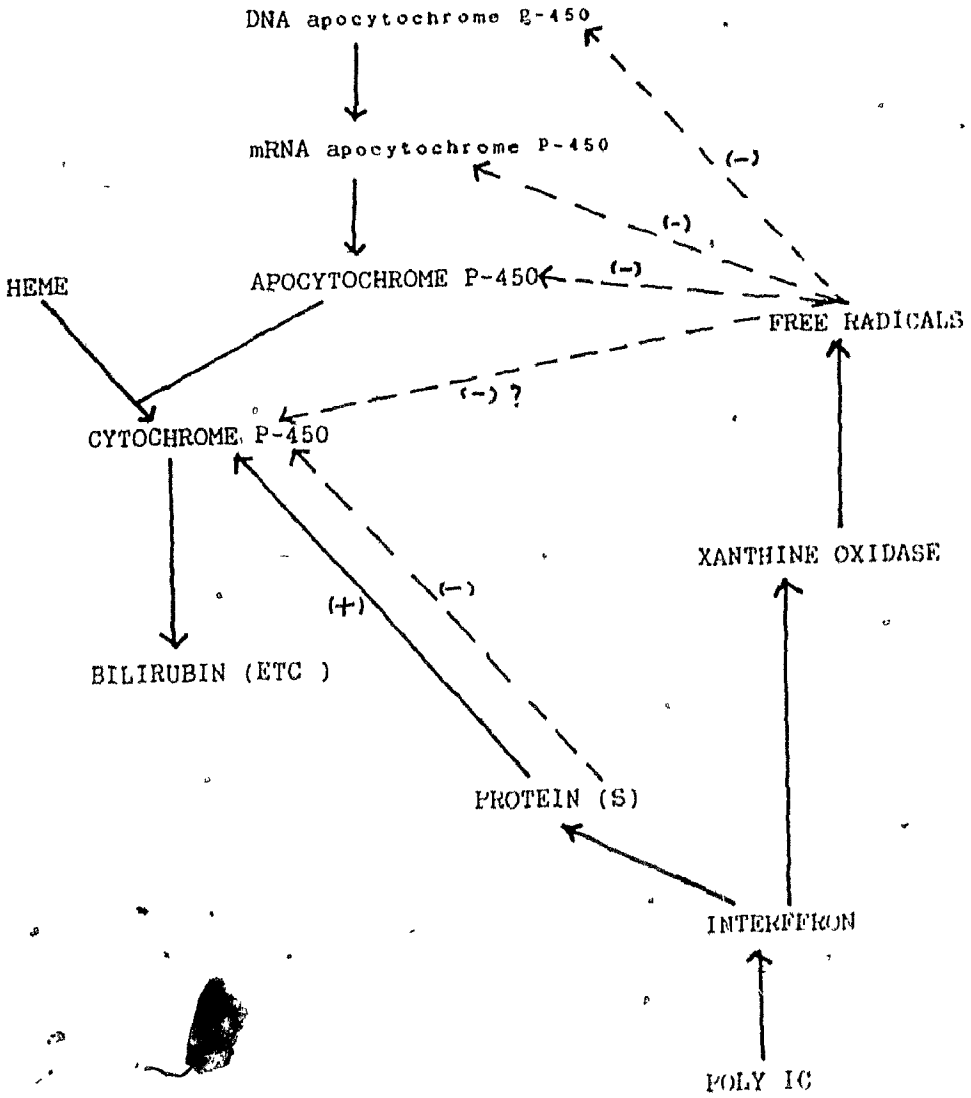


Figure 30

Proposed model for interferon induced stimulation and depression of mixed function oxidases.

PROPOSALS FOR FUTURE RESEARCH

(1) The turnover of messenger RNA and apocytochrome P-450 isozymes at different time periods following interferon treatment. This will enable us to (a) measure the effect of interferon on the rate of mRNA synthesis and degradation after interferon treatment of apocytochrome P-450 isozymes species. At the same time it will enable us to identify temporal relationship between turnover of mRNA and apoprotein synthesis before and after interferon treatment; and (b) to study the effect of interferon on the half-lives of mRNA and apoprotein of individual cytochrome P-450 isozymes. This will help us identify the mechanism of depression of certain isozymes of cytochrome P-450 by interferon.

The translation of apocytochrome P-450 mRNA can be quantified by translating fixed concentrations of mRNA isolated from liver from interferon treated animals using an in vitro cell free translation system. The translated products can then be immunoprecipitated and then analyzed on SDS/PAGE. Quantification of mRNA for specific apocytochrome P-450s can be analyzed with a cloned DNA probe. Measurement of the half-life of a specific apocytochrome P-450 can be determined in vivo by injecting $NaH^{14}CO_3$ into treated animals and using monoclonal antibodies to immunoprecipitate specific apocytochrome P-450 from microsomes. These methods have been used in other

laboratories to demonstrate the turnover of messenger RNA, apoprotein and heme of a particular cytochrome P-450 subtype following treatment of animals with phenobarbital (Ravishanker and Padmanaban, 1985). In addition, simultaneous measurement of 2'-5' oligoadenylate synthetase, protein kinase and xanthine oxidase (O and D form) can be measured to determine the temporal relationship of these enzymes to the turnover of the mRNA and apoprotein of cytochrome P-450. The synthetase and the kinase are shown to inhibit the synthesis of viral proteins and in some cases mammalian cell proteins at the translational stage (Rossi et al, 1985). Xanthine oxidase (O form) may have a role in providing reactive oxygen intermediates that may destroy nucleic acid

(2) To determine the effect of interferon on the cytochrome P-450 system in isolated hamster hepatocytes. Preliminary experiments in our laboratory have shown that IFN-αCON1 only depressed cytochrome P-450 content in isolated hamsters hepatocytes. The failure of this interferon to stimulate cytochrome P 450 in isolated hamster hepatocytes might be explained by humoral factors not present in hepatocytes but supplied by other cells. By method of elimination, either by systematic removal of organ (hypophysectomized or adrenalectomized animals) or by removal of different cell types in the liver (e.g. endothelial cells, Kupffer cells), we may be able to

determine if a humoral factor external to the hepatocytes is responsible for induction of cytochrome P-450 Interferon is known to (a) induce glucorticoid release from the adrenals and (b) have ACTH-like activities (Mannering and Deloria, 1986) - a possibility that interferon induces cytochrome P-450 through the action at these hormones.

REFERENCES

Ades, I Z. Biogenesis of mitochonrdrial proteins regulation maturation of δ-aminolevulinate synthetase by hemin. Biochem. Biophys. Res. Commun. 110: 42-47, 1983.

Alton, K., Stabinsky, Y., Richards, R., Ferguson, B., Goldstein, L., Altrock, B., Miller, L. and Stebbing, N. Production, characterization and biological effects of recombinant DNA- derived human IFN-α and IFN-Y analogs. In: The Biology of the Interferon System, ed. Deyaeyer, E and Schellkens, H., North Holland(1983).

Alvares, A.P and Mannering, G.J. Two-substrate kinetics of drug metabolizing enzyme systems of hepatic microsomes. Mol. Pharmac. 6. 206-212, 1970

Argyris, T.S and Heinemann, R. Ribosomes accumulation in 3-methylcholanthrene induced liver growth in adult male rats. Exp. Mol. Pathol. 22. 335-361, 1975

Arias, I.M., Doyle, D. and Schimke, R.T. Studies on the synthesis and degradation of proteins of the endoplasmic reticulum of rat liver J Biol Chem 244: 3303 3315, 1969.

Avadhani, N.G. and Buetow, D.E. Isolation of active polyribosomes from the cytoplasm, mitochondria and chloroplasts of Euglena gracilis. Biochem. J. 128: 353 365, 1972.

Badawy, A.A. and Evan, M. The regulation of rat liver tryptophan pyrvolase by its cofactor haem Biochem. J 150 511-520, 1975.

Baglioni, C., Minks, M.A. and Maroney, P.A Interferon action may be mediated by activation of a nuclease by PPPA2'p5'A2'p5'A. Nature 273: 684-687, 1978.

Balkwill, F.R., Mowshowitz, S., Seilman, S.S., Moodie, E.M., Griffin, D.E., Fantes, K.H. and Wolf, C.R. Positive interaction between interferon and chemotherapy due to direct tumor action rather than effects on host drug metabolizing enzymes. Cancer Res. 44 5249 5255, 1984

Barnes, D.W., Morahan, P.S., Loveless, S., and Munson, A.E. The effects of maleic anhydride divinyl ether (MVE) copolymers on hepatic microsomal mixed function oxidases and other biologic activities. J. Pharmacol. Exp Ther 208: 393-398, 1979.

Barnes, D.W. Alteration of murine hepatic microsomal proteins (MP) associated with the administration of the maleic anhydride-divinyl ether (MVE) copolymer, Divema. In: 6th International Symposium Microsomes and Drug Oxidation (Abstract) p. 30, 1984.

Barnun, S., Kriebich, G , Adesnick, M., Alterman, L., Negishi, M. and Sabatini; D.D. Synthesis and insertion of cytochrome P-450 into endoplasmic reticulum membranes Proc. Natl Acad: Sci. 77: 965-969, 1980.

Baron, S , Grossberg, S E , Klimpel, G.R and Brunell, P A Immune and interferon systems In: Antiviral Agents and Viral Diseases of Man, ed Galasso, G.J , Merigan, T C. and Merigan, R.A., Raven Press, 2nd ed. (1984).

Bend, J.R and Serabjit-Singh, C J Xenobiotic metabolism by extrahepatic tissues relationship to target organ and cell toxicity. In: Drug Metabolism and Drug Toxicity, ed Mitchell, J.R. and Horning, M.G., pp. 99-136, New York: Raven Press, 1984.

Bhat, K.B., Sardana, M.K. and Padmanaban, G. Role of heme in the synthesis and assembly of cytochrome P-450 Biochem. J. 164: 295-303, 1977.

Bissell, D.M. and Hammaker, L.E. Cytochrome P-450 heme and the regulation of δ-aminolevulinic acid synthetase in the liver. Arch Biochem. Biophys. 176: 103-112, 1976b.

Bissell, D.M. and Hammaker, L.E. Cytochrome P-450 heme and the regulation of hepatic heme oxygenase activity. Arch Biochem. Biophys. 176: 91-112, 1976a.

Bissell, D.M. and Hammaker, L.E. Effect of endotoxin on tryptophan pyrvolase and δ-aminolevulinate synthetase: Evidence for an endogenous regulatory haem fraction in the rat liver. Biochem. J. 166: 301, 1977.

Bock, K.W., Siekevitz, P. and Palade, G.E Localization and turnover studies of membrane nicotinamide adenine dinucleotide glycohydrolase in rat liver. J. Biol. Chem. 246: 188-195, 1971.

Bothello, L.H., Ryan, D.E., Yuan, P.M., Kutney, R., Shively, J.E. and Levin, W. Amino-terminal and carboxy-terminal sequence of hepatic microsomal P-450, a unique hemoprotein from rats treated with isosafrole. Biochemistry 21: 1152-1155, 1982.

Brenick, E., Brousseu, M , Levin, W , Reik, L., Ryan, D.E. and Thomas, P E. Administration of 3-methylcholanthrene to rats increases in specific hybridizable mRNA coding for cytochrome P-450 c Proc. Natl. Acad. Sci. 378: 4083-4087, 1981

Brenick, E., Foldes, R. and Hines, R N. Induction of cytochrome P-450 by xenobiotics. Pharmacological Reviews 36: 3602-3643, 1984

Brodie, B.B. Pathways of drug metabolism J. Pharm Pharmacol. 8: 1-22, 1956

Burke, D.M , Prough, R.A. and Mayer, R T. Characteristics of a microsomal cytochrome P-448-mediated reaction Ethoxyresorufin O-deethylation. Drug Metab. and Dispos 5. 1-8, 1977

Chany, C., Duc-Goivan, P., Robert-Galliot, B., Lebon, P. Study of human amniotic interferon. In: Interferons 4, ed , Merigan, T.C. and Friedman, R M., p. 241-248, 1982.

Cheng, K -C. and Sckenkman, J.B. Purification and characterization of two constitutive forms of rat liver microsomal cytochrome P-450. J. Biol Chem. 257: 2378-2385, 1982

Chiang, J.Y.L. and Steggles, A.w. Identification and partial purification of hamster microsomal cytochrome P-450 isoenzymes. Biochem. Pharmacol 32: 1389-1397, 1983.

Chirgwin, J.M., Przybyla, A.E., MacDonald R J. and Rutter, W.J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18 5294-5299, 1979.

Cohn, M., Monod, J., Pollock, M.R., Spiegelman, S. and Stanier, R.Y. Terminology of enzyme formation. Nature 172 1096, 1953.

Conney, A.H. Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev 19: 317-366, 1967

Conney, A.H. Environmental factors influencing drug metabolism In: Fundamentals of Drug Metabolism and Drug Disposition, ed. Ladu, B.N., Mandel, H.G. and Way, E.L., Baltimore: Williams & Wilkins, p. 253-278, 1971.

Cooper, D.Y., Levin, S., Narasimhulu, S., Rosenthal, O. and Estabrook, R.W. Photochemical action spectrum of the terminal oxidase systems. Science 147: 400-402, 1965.

Correia, M.A , and Meyer, U.A. Apocytochrome P-450. Reconstitution of functional cytochrome with hemin in vitro. Proc. Nat. Acad. Sci (Wash) 72: 400-404, 1975.

Crowe, D.O., Reiners, J.J., Nevland, D.E. and Sonnenfeld, G. Kinetics of depression and recovery of murine hepatic cytochrome P-450 levels after treatment with the interferon inducer polyriboinosinic-polyribocytidilic acid JNCI 76: 879-883, 1986.

Dannan, G.A., Guengerich, F.P., Kaminsky, L.S. and Hust, S.D. Regulation of cytochrome P-450 immunochemical quantitation of eight isozymes on liver of rats treated with poly brominated biphenyl congeners J. Biol. Chem. 258: 1282, 1983.

DeClerq, E. and DeSomer, P. Mechanism of the antiviral activity resulting from sequential administration of complementary hemopolyribonucleotides to cell cultures. J. Virol. 9: 721-731, 1972.

Dehlinger, P.J. and Schimke, R.T. Effects of phenobarbital, 3-methylcholanthrene, and hematin on the synthesis of protein components of rat liver microsomal membranes. J. Biol Chem 247: 1257-1264, 1972.

Della Corte, E. and Stirpe, F. The regulation of rat liver xanthine oxidase. Biochem. J. 126: 739-745, 1972

Deloria, L.B., Abbott, V., Gooderham, N., Mannering, G.J. Induction of xanthine oxidase and depression of cytochrome P-450 by interferon inducers: Genetic difference in the responses of mice Biochem. Biophys Res. Commun. 131: 109-116, 1985.

Deloria, L.B. and Mannering, G.J. Sequential administrations of polyriboinosinic acid and polyribocytidylic acid induce interferon and depress the hepatic cytochrome P-450 dependent monooxygenase system Biochem. Biophys. Res. Commun. 106: 947-952, 1982.

DeMatteis, F., and Gibbs, A. Stimulation of liver delta-aminolevulinic synthetase by drugs and its relevance to drug-induced accumulation of cytochrome P-450. Biochem. J. 126: 1149-1160, 1972.

DeMatteis, F. Loss of liver cytochrome P-450 caused by chemicals. In: Handbook of Experimental Pharmacology 44: 95-121, ed. DeMatteis, F. and Aldvidge, W.N., New York: Springer-Vorlag, 1978.

Diazani, F , Bucklèr, C.E. and Baron, S. Effect of cycloheximide on the antiviral action of interferon Proc Soc. Exp. Biol Med. 130. 519-523, 1969.

Eberhard-Muller, U and Vincent, S H Concepts of heme distribution within hepatocytes. Biochem. Pharmacol. 34. 719-725, 1985

Egawa, K., Yoshida, M., and Kasai, N. An endotoxin-induced serum factor that depresses hepatic δ-aminolevulinic acid synthetase activity and cytochrome P-450 levels in mice. Microbiol Immunol 25 1091, 1981.

El Defrawy el Masry, S and Mannering, G J. Sex-dependent differences in drug metabolism in the rat II. Qualitative changes produced by castration and administration of steroid hormone and phenobarbital. Drug Metab. and Disp 2. 279-284, 1974.

El Azhary, R., Renton, K W., and Mannering, G J. Effect of interferon inducing agents (polyriboinosinic acid polyribocytidylic acid and tilorone) on the heme turnover of hepatic cytochrome P-450 Mol. Pharmacol 17. 395-399, 1980.

El Azhary, R. and Mannering, G J. Effects of interferon inducing agents (polyriboinosinic acid-polyribocytidylic acid, tilorone) on hepatic hemoproteins (cytochrome P-450, catalase, tryptophan 2, 3-dioxygenase, mitochondrial cytochromes, heme metabolism and cytochrome P-450-linked monopxygenase systems. Mol. Pharmacol. 15 698-707, 1979

El Azhary, R., Renton, K.W. and Mannering, G.J. Effect of interferon inducing agents (Polyriboinosinic acid and polyribocytoclylic acid and tilorone) on the heme turnover of hepatic cytochrome P-450. Mol. Pharmacol. 17: 395-399, 1980.

Estabrook, R.W., Cooper, D.Y. and Rosenthal, O. The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex Biochem. Z. 338: 741-755, 1963.

Evinger, M. Rubinstein, M., and Pestka, S. Antiproliferative and antiviral activities of human leukocyte interferons. Arch. Biochem. Biophys. 210: 319-329, 1981.

Falk, J.E. Porphyrins and metalloporphyrins. BBA Lib. 2: 232, 1964.

Farrell, G.c. and Correia, M.A. Structural and functional reconstitution of hepatic cytochrome P-450 in vivo J Biol. Chem. 255: 10128-10133, 1980.

Fisher, P B., Miranda, A.F , Bobiss, L , Pestka, S , and Weinstein, I.B. Opposing effects of interferon produced in bacteria and of tumor promoteus on myogenesis in human myoblast cultures. Proc. Natl. Acad. Sci. 80: 2961-2965, 1983.

Foidart, M., Eiseman, J., Engel, W.K., Adornato, B.T., Leim, H H. and Muller-Eberhard, U Effect of heme administration on hemopexin metabolism in the rhesus monkey. J Lab. Clin Med 100: 451-460, 1982

Franklin, M.R. and Finkle, B.S. Effect of murine gamma-interferon on the mouse liver and its drug-metabolizing enzymes: Comparison with human hybrid alpha-interferon. J. Interferon Rs 5: 265-272, 1985.

Friedman, R.M. Antiviral activity of interferons. Bact. Rev 41. 453-567, 1977.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K. and Muramatsu, M. Primary structure of a cytochrome P-450 coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. Proc. Nat'l Acad. Sci. USA 79: 2793-2797, 1982.

Fujii-Kuriyama, Y., Sogawa, K., Suwa, Y., Kawajiri, K and Gotoh, O. Multiplicity of cytochrome P-450 and its gene structure. In: Procedings of IUPHAR 9th International Congress of Pharmacology, London, 1984 (Paton, W., Mitchell, J. and Turner, P., eds) 3: 211-317, MacMillan, Basinstoke and London.

Furouno, K., Ishikawa, T., and Kato, K. Appearance of autolysosomes in rat liver after leupeptin treatment. J. Biochem. 91: 1485-1494, 1982.

Gander, J.E. and Mannering, G.J. Kinetics of hepatic cytochrome P-450 dependent monooxygenase systems. Pharmacol. and Therapeutics. Vol. 10, 2: 191-222, 1980.

Garfinkel, D. Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. Arch. Biochem. Biophys. 77: 493-509, 1958.

Gasser, R., Hauri, H.-P. and Meyer, U.A. The turnover of cytochrome P-450b. FEBS Lett. 147: 239-242, 1982.

Gelboin, H V. and Blackburn, N R The stimulatory effect of 3-methylcholanthrene on benzopyrene hydroxylase activity in several rat tissues. Inhibition by actinomycin D and puromycin. Cancer Res. 24: 356-360, 1964.

Ghezzi, P , Saccardo, B. and Bianchi, M. Recombinant tumor necrosis factor depresses cytochrome P-450 dependent microsomal drug metabolism in mice. Biochem. Biophys. Res. Commun. 136: 316-321, 1986

Ghezzi, P., Bianchi, M , Mantovani, A., Spreafico, F and Salmona, M. Enhanced xanthine oxidase activity in mice treated with interferon and interferon inducers Biochem. Biophys. Res. commun. 119: 144-149, 1984.

Ghezzi, P., Bianchi, M., Gianera, L , Landolfo, S , Salmona, M. Role of reactive oxygen intermediates in the interferon-mediated depression of hepatic drug metabolism and protective effect of N-acetylcysteine. Cancer Res. 45: 3444-3447, 1985

Gibson, G G. and Schenkman, J.B. Purification and properties of cytochrome P-450 obtained from liver microsomes of untreated rats by lauric acid affinity chromatography. J Biol Chem. 253: 5927-5963, 1978.

Gillette, J.R., Davis, D.C. and Sasame, H.A. Cytochrome P-450 and its role in drug metabolism. Ann. Rev. Pharmacol. 12: 57-82, 1972.

Gillete, J.R. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Adv. Pharmacol. 4: 219-261, 1966.

Goldstein, J.A Mechanism of induction of hepatic drug metabolizing enzymes: recent advances. T.I.P.S., 1984.

Gonzalez, F.J. and Nebert, D.W. Cytochrome P-450 gene expression and regulation. T.I.P.S. 6: 160-164, 1985.

Gooderham, N.J. and Mannering, G.J. Impaired cell-free translation of hepatic mRNA from mice treated with polyriboinosinic acid polyribocytidylic acid. A rationale for the depression of cytochrome P-450 by interferon inducers. In: Symposium for Prof. Beckett's Retiring Meeting (Submitted) (1986).

Gorce, P. and Wade, A.E. Inhibition of polyriboinosinic:polyribocytidylic acid-induced depression of mouse hepatic mixed function oxidases by atinomycin D or cycloheximide. Biochem. Biophys. Res. Comm. 121. 237-242, 1984.

Gram, T.E. The metabolism of xenobiotics by mammalian lung. In: extrahepatic metabolism of drugs and other foreign compounds. Ed., Gram, T.E., pp. 159-211, 1980).

Greim, H., Schenkevan, J.B., Klotzbucher, M. and Remner, H The influence of microsomal cyt b5 and cyt P-450 hemes in the rat. Biochem Biophys Acta 201: 20-25, 1970

Guengerich, F.P. Isolation and purification of cytochrome P-450 and the existence of multiple forms . Pharm. Ther 6: 99-21, 1979

Guengerich, F.P. Dannan, G.A., Wright, S.T., Martin, N V. and Kaminsky, L.S Purification and characterization of liver microsomal C-P-450: electrophoretic, spectral catalytic and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry 21: 6019, 1982.

Guzelian, P.S., Diegelmann, R.F., Lamb, R.G., Fallon, H.J. Effects of hormones on changes in cytochrome P-450, prolyl hydroxylase and glycerol phosphate acyltransferase in 10 monolayer cultures of parenchymal cells from adult liver . Yale. J. Biol. Med. 52: 52, 1979.

Haugen, D.A. and Coon, M.J. Properties of electrophoretically homogenous phenobarbital inducible and beta-napthoflavone inducible forms of liver microsomal cytochrome P-450. J. Biol. Chem. 251. 7929-7939, 1976.

Haugen, DA., Coon, M.J. and Nebert, D.W Induction of multiple forms of mouse liver cytochrome P-450. J. Biol. Chem. 351: 1817, 1976.

Henle, W., Henle, G. Interference of inactive virus with the propagation of virus of influenza. Science 98: 87-89, 1943.

Hopkinson, J., and Brenick, E. Stabilization of total and free ribosomes associated with 3-methylcholanthrene induced adult rat liver growth. Biochem. Pharmacol. 24: 435-437, 1975.

Huang, M.T., West, S.B., and Lu, A.Y H. Separation, purification and properties of multiple forms of c-P-450 from the liver microsomes of phenobarbital-treated mice. J Biol Chem 251: 4659-4665, 1976.

Isaacs, A. and Lindenmann, J. Virus interference. I. The interferon. Proc R Soc London, Sev B147 258-267, 1957.

Israel, D.I. and Whitlock, J P. Regulation of cytochrome P-450 gene transcription by 2,3,7,8-tetra-chlorodibenzo-p-dioxin in wild type and variant mouse hepatoma cells. J. Biol. Chem. 259: 5400-5402, 1984

Johnston, M.L., Torrence, P.F. The role of interferon-induced proteins, double-stranded RNA and 2',5'oligoadenylate in the interferon mediated inhibition of viral translation In; Interferon 3: 189-293, ed. Friedman, R.M., Amsterdam: Elsevier, 1983

Kato, R., Johdorfe, W.R , Loeb, L.A., Ben, T. and Gelboin, H.V. Studies on the mechanism of drug induced microsomal enzyme activities V. Phenobarbital stimulation of endogenous messenger RNA and polyuridylic acid directed 1-^{14}C-phenylalanine incorporation Mol. Pharmacol. 2 171-186, 1966.

Kikkawa, Y., Yano, S. and Skoza, L. Protective effect of interferon inducers against hyperoxic pulmonary damage Lab. Invest. 50: 62-71, 1984.

Klingenberg, M. Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75: 376-386, 1958.

Koizumi, A., Walford, R.L. and Imamura, T. Treatment with poly I.C. enhances lipid peroxidation and the activity of xanthine oxidase, and decreases hepatic P-450 content and activities in mice and rats. Biochem. Biophys. Res. Commun 134: 632-637, 1986.

Kramer, P. and McClain, C J. Depression of aminopyrine metabolism by influenza vaccination. N. Engl. J. Med. 305: 1262-1264, 1981.

Kramer, P., Tsurn, M. Cook, E., McClain, C.J. and Holtzman, J.L. Effect of influenza vaccine on warfarin anticoagulation. Clin. Pharmacol. Ther. 35: 416-418, 1984

Krispin, T.T., Parfenova, T.M., Itkes, A.V., Balandin, I.G., Kuznetsov, V.P. and Severin, E.S. Antiviral activity of α-β- and δ-interferons in the presence of theophylline involvement of 2'5'-oligo(a)synthetase. Biochem. Int. 8: 159-164, 1984.

Kumar, A. and Padmanaban, G. Studies on the synthesis of cytochrome P-450 and cytochrome P-448 in rat liver J. Biol. Chem. 255: 522-525, 1980.

Kuriyama, Y., Omura, T , Siekevitz, P and Palade, G.E. Effects of phenobarbital on the synthesis and degradation of the protein component of rat liver microsomal membranes J. Biol. Chem. 244: 2017-2026, 1969.

Kushnavyov, V.M., Sedmak, J J , Bendler, J.W. III and Grossberg, S.E. Ultrastructural localization of interferon receptors on the surface of cultured cells and erythrocytes. Infect. Immun. 36: 811-821, 1982.

Laemli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature 227: 680-685, 1970.

Lazarow, P.B. and DeDuve, C. The synthesis and turnover of rat liver perioxsomes. J. Cell. Biol. 59: 507-524, 1973.

Lebleu, B. and Content, J. Mechanism of interferon action: Biochemical and genetic approaches. In: Interferon 4. 47-94, Ed. Gresser, I., London: Academic press, 1982.

Leeson, G.A., Biedenback, S.A., Chan, K.Y., Gibson, J.B. and Wright, G.J. Decrease in the activity of the drug-metabolizing enzymes of rat liver following the administration of tilorone hydrochloride Drug Metab. Disp. 4: 232-238, 1976.

Levin, W., and Kuntzman, R. Studies on the incorporation of delta-aminolevulinic acid into microsomal hemoprotein: effect of 3-methylcholanthrene and phenobarbital. Life Sci. 8: 305-311, 1969b.

Lindenmann, J., Burke, D.C. and Isaacs, A. Studies on the production, mode of action and properties of interferon. Brit. J. Exp. Path. 38: L 551-562, 1957.

Lowry, O.H., Rosenbrough, N.J., Farr, N.J. and Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

Lu, A.Y.H. and Coon, M.J. Role of hemoprotein P-450 in fatty acid omega-hydroxylation in a soluble enzyme system from liver microsomes J. Biol. Chem. 243: 1331-1332, 1968

MacKinnon, M., Sutherland, E., and Simon, F.R. Effects of ethinylestradiol on hepatic microsomal proteins and the turnover of cytochrome P-450. J. Lab. Clin Med. 90: 1096-1106, 1977.

Maines, M.D. New developments in the regulation of heme metabolism and their implications CRC Crit Rev Toxicol. 12: 241-314, 1984.

Maines, M D and Kappas, A. Cobalt stimulation of heme degradation in the liver. Dissociation of microsomal oxidation of heme from cytochrome P-450. J Biol. Chem. 250. 4171-4177, 1975

Mannering, G.J. and Deloria, L.B. The pharmacology and toxicology of the interferons. An overview. Ann Rev. Pharmacol. Toxicol. 26: 455-515, 1986.

Mannering, G J., Renton, K.W., El Azhary, E and Deloria, L B. Effects of interferon-inducing agents on hepatic cytochrome P-450 drug metabolizing systems. Ann. N.Y. Acad. Sci. 350: 314-331, 1980

Mannering, G.J., Deloria, L.B., Kuwahara, S K Depression of allylisopropylacetamide induced hepatic ethylmorphine N-demethylase activity in mice by polyribinosinic acid, polyribocytidylic acid and recombinant hybrid human leukocyte interferon. Pharmacologist 25: 265 (Abstract), 1983.

Mannering, G.S., Renton, K.W., ElAzhary, R. and Deloria, L.B. Effects of interferon inducing agents on hepatic cytochrome P-450 drug metabolizing system. Ann. N.Y. Acad. Sci. 350: 314-331, 1980.

Marcus, P.I. Interferon induction by viruses: Double stranded ribonucleic acid as the proximal inducer. In Interferon 3: 113-175, ed. Friedman, R.M., 1983.

Masters, B.S.S., Parkhill, L.K., Yasukahi, Y., Okita, R.T., Theoharides, A.D. and Kupfer, D. Pig liver and kidney microsomal cytochromes P-450: Specificities for drugs, fatty acids and prostaglandins in reconstituted systems. In: Biochemistry, Biophysics and Regulation of Cytochrome P-450, Ed. Gustafsson, J.A., Carlstedt-Duke, J., Mode, A. and Rafter, J.: 101-104, 1980.

Meredith, C.G., Christian, C.D., Johnson, R.F., Toxell, P.A., Davis, G.L. and Scheuker, S. Effects of influenza virus vaccine on hepatic drug metabolism. Clin. Pharmacol. Ther. 37. 396-401, 1985

Mueller, G.C. and Miller, J.A. The reductive cleavage of 4-dimethyl-amino azobenzene by rat liver: The intracellular distribution of the enzyme system and its requirements for triphosphopyridine nucleotide. J. Biol Chem. 180. 1125-1136, 1949.

Morahan, P.S., Munson, A.E , Regelson, W., Commerford, S.L and Hamilton, L.D Antiviral activity and side effects of polyriboinosinic-cytidylic acid complexes as affected by molecular size. Proc. Natl. Acad Sci USA 69: 482-486, 1972

Mueller, G.C. and Miller, J.A. The metabolism of methylated amino-azo dyes. II. Oxidative demethylation by rat liver homogenates. J. Biol. Chem. 202. 579-587, 1953.

Nash, T. The calorimetric estimation of formaldehyde by means of the Hantzsch reaction. J. Biol. Chem. 55: 412-416, 1953.

Nebert, D.W. and Friedman, R.M. Stimulation of aryl hydrocarbon hydroxylase induction in cell cultures by interferon. J. Virol. 11: 193-197, 1973.

Nebert, D.W. and Gelboin, H.V. Substrate-inducible microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzymes. J. Biol Chem. 243: 6242-6245, 1968.

Nebert, D.W., Eisen, H.J., Negishi, M., Lang, M.A., Hjelmeland, L.M. and Okey, A.B. Genetic mechanisms of controlling the induction of polysubstrate monooxygenase (P-450) activities. Ann. Rev. Pharmacol. Toxicol. 21: 431-462, 1981.

Nebert, D.W. and Gonzalez, F.J. Cytochrome P-450 gene expression and regulation. T.I.P.S. 6: 160-164, 1985.

Negishi, M. and Kreibich, G. Coordinated polypeptide synthesis and insertion of protoheme in cytochrome P-450 during development of endoplasmic reticulum membranes. J. Biol. Chem. 253: 4791-4797, 1978.

Netter, K.J. Inhibition of oxidative drug metabolism in microsomes. Pharm. Ther. 10: 515-535, 1980.

Omura, T. and Sato, R. A new cytochrome in liver microsomes J Biol. Chem 237. 1375-1376, 1962.

Omura, T. and Sato, R. The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemoprotein nature. J Biol Chem. 239: 2370-2386, 1964.

Omura, T. Cytochrome P-450 linked mixed function oxidase turnover of microsomal components and effects of inducers on the turnover of phospholipids, proteins and specific enzymes. Pharmacol. Ther. 8: 489-499, 1980.

Paine, A.J. Excited states of oxygen in biology: their possible involvement in cytochrome P-450 linked oxidation as well as in the induction of P-450 by many diverse compounds. Biochem Pharmacol. 27: 1805-1813, 1978.

Palleroni, A.V. and Bohoslawee, O. Use of ^{125}I-interferons in pharmacokinetic and tissue distribution studies. J Interferon Res. 4. 493-498, 1984.

Parker, G.L. and Orton, T.C. Induction by oxisobutyrates of hepatic and kidney microsomal cytochrome P-450 with specificity towards hydroxylation of fatty acids. In. Biochemistry, Biophysics and Regulation of Cytochrome P 450, ed. Gustaffson, J.A., Carlsted, H., Duke, J., Mode, A and Rafter, J., 373-377, 1980.

Parkinson, A., Thomas, P.E., Ryan, D.E. and Levin, W. The in vivo turnover of rat liver microsomal epoxide hydrolase and both the apoprotein and heme moieties of specific cytochrome P-450 isozymes. Arch. Biochem Biophys. 225. 216-236, 1983.

Parkinson, A., Lasker, J., Kramer, M.J., Huang, M.T., Thomas, P.E., Ryan, D.E., Reik, L.M., Norman, R.L., Levin, W. and Conney, A.H. Effects of three recombinant human leukocyte interferons on drug metabolism in mice Drug Metab. Dispos. 10: 579-585, 1982.

Pestka, S. The human interferons from protein purification and sequence to cloning and expression in bacteria: before, between, and beyond. Arch. Biochem. Biophys. 221: 1 37, 1983.

Peterson, T.C. and Renton, K.W. The role of lymphocytes, macrophages and interferon in the depression of drug metabolism by dextran sulfate. Immunopharmacol. 11: 21 28, 1986b.

Peterson, T C. and Renton, K.W. Kupffer cell factor mediated depression of hepatic parenchymal cell cytochrome P-450. Biochem. Pharmacol 35: 1491-1497, 1986.

Peterson, T C. and Renton, K.W Depression of cytochrome P-450-dependent drug biotransformation in hepatocytes after activation of the reticuloendothelial system by dextran sulfate. J. Pharmacol. Exp. Ther. 229: 299-304, 1984.

Poland, A.P., Glover, E , Robinson, J.R and Nebert, D.W Genetic expression of aryl hydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P1-450 formation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Biol. Chem. 249: 5599-5606, 1974.

Powis, G., Lyon, L. and McKillop, D. NADH synergism of microsomal aniline metabolism in the presence of enhancing agents. Biochem. Pharmacol. 26: 137-141, 1977.

Rajamanickam, C., Satyanarayana-Rao, M.R and Padmanaban, G. On the sequence of events leading to cytochrome P-450 synthesis-effects of drugs. J. Biol. Chem 250: 2305-2310, 1975

Ravishankar, H. and Padmanaban, G. Turnover of messenger RNA, apoprotein and haem of cytochrome P-450b+e induced by phenobarbitone in the rat liver. Biochem J 229: 73-79, 1985.

Ravishankar, H. and Padmanaban, G. Effect of cobalt chloride and 3-amino-1,2,4-triazole on the induction of cytochrome P-450 synthesis by phenobarbitone in rat liver. Arch. Biochem. Biophysic. 225: 16-24, 1983

Ravishankar, H. and Padmanaban, G. Regulation of cytochrome P-450 gene expression. Studies with a cloned probe. J. Biol. Chem. 260: 1588-1592; 1985.

Renton, K.W and Moochhala, S M. The influence of highly purified interferon produced by recombinant DNA techniques on cytochrome P-450 dependent drug biotransformation. In: The 6th International Symposium on Microsomes and Drug Oxidation, Brighton 68 (Abstract).

Renton, K.W., Keyler, D.E. and Mannering, G.J. Suppression of the inductive effects of phenobarbital and 3-methylcholanthrene on ascorbic acid synthesis and hepatic cytochrome P-450-linked monoxygenase systems by the interferon inducers, poly r I.r.C. and tilorone. Biochem. Biophys. Res. Commun. 88: 1017-1023, 1979.

Renton, K.W. Relationships between the enzymes of detoxication and host defense mechanisms In: Biological Basis of Detoxication, ed Caldwell, J. and Jacoby, W.B. 307-324, New York: Academic, 1983

Renton, K.W., Gray, J.D., and Hall, R.I. Decreased elimination of theophylline after influenza vaccine. Can Med. Assoc. J. 123: 288-290, 1981.

Renton, K.W., Deloria, L.B. and Mannering, G.J. Effect of polyribornosinic acid-polyribocytidylic acid and mouse interferon preparation in cytochrome P-450 dependent monoxygenase systems in cultures of primary mouse hepatocytes. Mol. Pharmacol. 14: 672-681, 1978

Renton, K.W and Mannering, G.J. Depression of the hepatic cytochrome P-450 monooxygenase system by tilorone (2,7 bis[2-diethylamine ethoxy]fluovene-9-one dihydrochloride Drug Metab Dispos. 4: 223-231, 1976a.

Renton, K.W., Moochhala, S.M., Gibson, G.G. and Makowski, R. The interferon mediated loss of cytochrome P 450 In 10th European Drug Metabolism Workshop (Surrey) (Abstract) 37: 1986.

Renton, K.W., Gray, J.D. and Hung, O.R. The depression of theophylline elimination by erythromycin in man Clin. Pharm. Therap. In Press, 1981c.

Renton, K.W. The depression of hepatic cytochrome P 450 dependent drug biotransformation during infection with encephalomyocarditis virus in the mouse. Biochem. Pharmacol. 16: 2333-2336, 1981b.

Renton, K.W. Effects of interferon inducers and viral infection on the metabolism of drugs. Advances in Immunopharmacology, ed Haddon, J., et al., Pergamon Press, Oxford, pp. 17-24, 1981a.

Renton, K.W., Singh, G. and Stebbing, N. Relationship between the antiviral effects of interferons and their abilities to depress cytochrome P 450. Biochem. Pharmacol. 33: 3899 3902, 1984.

Renton, K.W. The deleterious effect of Bordetella pertussis vaccine and poly r (I.C.) on the metabolism and disposition of phenytoin. J. Pharmacol. Exp. Ther. 298: 267-270, 1979.

Renton, K.W. and Mannering, G.J. Depression of hepatic drug metabolizing systems with administered interferon inducing agents. Biochem. Biophys. Res. Comm. 73: 343-348, 1976b.

Revel, M. Molecular mechanisms involved in the antiviral effects of interferon. In: Interferon 1 101-163, ed, Gresser, London: Academic Press, 1979.

Rossi, G.B. Interferons and cell differentiation In. Interferon 6: 31-68, London, Academic Press, 1985.

Round, E.M. and Stebbing, N A putative interferon induced in hamsters by poly (I) . poly (C). J. Interferon Res. 1: 451-456, 1981

Rozee, K.R., Katz, L.J. and McFarlane, I S. Interferon stimulation of methylase activity in L-cells. Can. J Microbiol 15: 969-974, 1969.

Rubin, B.Y. and Gupta, S.L. Interferon-induced proteins in human fibroblasts and development of the antiviral state. J. Virol. 34 446-454, 1980.

Ryan, K.J. and Engel, L.L. Hydroxylation of steroids at carbon 21. J. Biol. Chem. 225: 103-114, 1957.

Sadano, H. and Omura, T. Incorporation of heme to microsomal cytochrome P-450 in the absence of protein biosynthesis. J. Biochem. 98: 1321-1331, 1985.

Sadano, H. and Omura, T. Turnover of two drug-inducible forms of microsomal cytochrome P-450 in rat liver. J. Biochem. 93: 1375-1383, 1983.

Samaras, S.C. and Dietz, N. Physiopathology of detoxification of phenobarbital sodium. Fed. Proc., Fed Amer. Soc. Exp. Biol. 12: 400, 1953.

Sassa, S., Kappas, S.E., Bernstein, S.E. and Alvares, A.P. Heme biosynthesis and drug metabolism in mice with hereditary hemolytic anemia. J. Biol. Chem. 254: 729-735, 1979.

Sato, R., Satako, H. and Imai, Y. Partial purification and some spectral properties of hepatic microsomal cytochrome P-450. Drug Metab. Dispos. 1: 6-13, 1973.

Schacter, B. Assay of microsomal heme oxygenase in liver and spleen. In: Methods of Enzymology 52: 367-368, ed Feicher, S. and Packer, L., 1978

Schimke, R.T. Methods for analysis of enzyme synthesis and degradation in Animal Tissues In: Methods of Enzymology 40, ed , O'Malley, B.W. and Hardman; J.G.: 241-301, 1975.

Schimke, R.T. Control of enzyme levels in mammalian tissues. Advanc. Enzymol. 37: 135-187, 1973.

Sen, G C Biochemical pathways in interferon action. Pharmac. Ther. 24: 235-257, 1984.

Sesardic, D , Boobis, A.R., McQuade, J., Baker, S., Lock, E.A., Elcombe, C.R., Robson, R.T., hayward, C. and Davies, D.S. Inter-relatedness of some isoenzyme of cytochrome P-450 from rat, rabbit and human, determined with monoclonal antibodies. Biochem. J. 236: 569-577, 1986.

Shiraki, H. and Guengerich, F.P. Turnover of membrane proteins: Kinetics of induction and degradation of seven forms of rat liver microsomal cytochrome P-450, NADPH cytochrome P-450 reductase, and epoxide hydroxylase Arch Biochem. Biophys 235: 86-96, 1984.

Singh, G. and Renton, K.W. Interferon-mediated depression of cytochrome P-450-dependent drug biotransformation. Mol. Pharmacol 20: 681-684, 1981

Singh, G., Renton, K.W. and Stebbing, N. Homogenous interferon from E coli depresses hepatic cytochrome P 450 and drug biotransformation. Biochem Biophys. Res. Commun. 106: 1256-1261, 1982.

Singh, G. and Renton, K.W. Inhibition of the synthesis of hepatic cytochrome P-450 by the interferon-inducing agent poly r I rC. Can J. Physiol. Pharmacol. 62: 379-383, 1984.

Singh, G. PhD thesis, 1982

Sladek, N.E. and Mannering, G.J. Induction of drug metabolism. II. Qualitative differences in the microsomal N-demethylating systems stimulated by polycyclic hydrocarbons and by phenobarbital. Mol. Pharmacol. 5: 186-199, 1969.

Sladek, N.E., and Mannering, G.J. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene-treated rats. Biochem. Biophys. Res. Commun. 24: 668-674,

1966.

Sladek, N.E. and Mannering, G.J. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun. 24: 688-674, 1966

Sladek, N E., Chaplin, M.D. and Mannering, G.J. I. Sex-dependent differences in drug metabolism in the rat II: Effect of morphine administration. Drug Met. and Disp. 2: 293-300, 1974.

Smith, S.S , Hill, R.N., Gleeson, R.A. and Vesell, E.S. Evidence for post-transcriptional stabilization of ribosomal precursor ribonucleic acid by phenobarbital. Mol. Pharmacol. 8: 691-700, 1972.

Smith, S.J., Hill, R.N., Gleeson, R.A. and Vessell, E.S. Evidence for post-transcriptional stabilization of ribosomal precursor ribonucleic acid by phenobarbital. Mol. Pharmacol. 8: 691-700, 1972.

Smith, A. and Morgan, W.T. Haem transport to the liver by haemopexin. Biochem. J. 182: 47-54, 1979

Sonnenfeld, G., Smith, P.K. and Nerland, D.E. Interferon and drug metabolism. In: Interferons, UCLA Symposium on Molecular Cell Biology 25: 329, ed. Merigan, T.C., Friedman, R.M., Fox, C.F., New York: Academic Press, 1982.

Sonnenfield, G., Harned, C.L., Thaniyavarn, S , Huff, T., Mandel, A D., and Nerland, D.E. Type II interferon induction and passive transfer depress the murine cytochrome P-450 drug metabolism system. Antimicrobiol. Agent. Chemother. 17: 969-972, 1980.

Soyka, L.F., Hunt, W.G , Knight, S.E. and Foster, R.S. Jr. Decreased liver and lung drug-metabolizing activity in mice treated with Corynebacterium parvum. Cancer Res. 36: 4425-4428, 1976.

Stebbing, N., Downing, M., Fagin, K.D., Altrock, B.W., Fish, E.N., Liggitt, H.D., Moochhala, S. and Renton, K.W. Experimental animal models for assessing clinical utility of human interferons: Studies in hamsters. In: The Biology of the Interferon System. Ed., Schellekens, H. and Stewart, W.E., Amsterdam: Elsevier, 1986.

Steward, W.E. II. The Interferon System. New York, Srpinger-Verlag 493: 2nd ed., 1981.

Swick, R.W. and Ip, M.M. Measurement of protein turnover in rat liver with [^{14}C]-carbonate J. Biol. Chem. 249: 6836-6841, 1974.

Tait, G.H. The biosynthesis and degradation of heme. In Handbook of Experimental Pharmacology, Vol. 44, eds DeMatteis, F. and Aldridge, W.N., Springer-Verlag, Berlin, Heidelberg and New York, pp 1-48, 1978.

Tamburini, P P., Masson, H.A., Bains, S.K., Makowski, M J., Morris, B. and Gibson, G.G., Multiple forms ofhepatic cytochrome P-450. Eur. J Biochem. 139: 235-246, 1984.

Taylor, J. Inhibition of interferon-action by actinomycin. Biochem. Biophys. Res. Commun. 14: 447-451, 1964.

Testa, B. and Jenner, P Inhibitors of cytochrome P-450 and their mechanism of action. Drug. Metab. REv. 12: 1-117 C19, 1981

Thomas, P.E., Lu, A.Y.H., Ryan, D.E. West, S.B., Kawalek, J. and Lewin, W. Multiple forms of rat liver cytochrome P-450: Immunochemical evidence with antibody against cytochrome P-450. J. Biol. Chem. 251: 1385-1391, 1976.

Tovey, M.G. Interferon and cyclic nucleotides. In: Interferon 4: 23-45, ed Gresser, London: Academic Press, 1982.

Trump, B.F., Smuckler, A.E. and Bendit, E.P. A method for staining epoxy sections for light microscopy. J. Ultrastruct. Res. 5: 343-348, 1961.

Vesely, D.L. and Cantell, K. Human interferon enhances guanylate cyclase activity. Biochem. Biophys. Res. Commun. 96: 574-579, 1980

Vogel, H.J. Repressed and induced enzyme formation: a unified hypothesis. Proc. Natl. Acad. Sci USA 43: 491-496, 1957.

Van Damme, J.V., De Ley, M., Opdenakker, G., Billiau, A., De Somer, P. and Van Becumen, J. Homogenous interferon-inducing 22k factor is related to endogenous pyrogen and interleukin-1. Nature 314: 266-268, 1985.

Waud, W.R. and Rajagopalan, K.V. The mechanism of conversion of rat liver xanthine dehydrogenase from an NAD+ dependent form (type D) to an O2-dependent form (type O). Arch. Biochem. Biophys. 172: 365-379, 1976.

Wang, P., Mason, P.S., and Guengerich, F.P. Purification of human liver c-P-450 and comparison to the enzyme isolated from rat liver. Arch. Biochem. Biophys. 199. 206-219, 1980.

Weck, P.K., Apperson, S., May, L. and Stebbing, N. Comparison of the antiviral activities of various cloned interferon-alpha subtypes in mammalian cell cultures. J. Gen. Virol. 57: 233-237, 1981

Whitlock, J.P. The regulation of cytochrome P-450 gene expression. Ann. Rev. Pharmacol toxicol 26. 333-369, 1986.

Williams, C.H. and Kamin, H. Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver. J. Biol. Chem.. 237: 587-595, 1962.

Willis, R.J. Possible role of endogenous toxigenic lipids in the carbon tetrachloride poisoned hepatocytes. Fed. Proc. 39: 3134-3137, 1980.

Wilton, A.F. and Aust, S.D. Multiplicity of cytochrome P-450 hemoprotein in rat liver microsomes. Biochem. Biophys Res. Commun. 56: 898-906, 1974.

Yoshida, R. and Hayaishi, O. Induction of pulmonary indoleamine 2,3,dioxygense by intraperitoneal injection of bacterial lipopolysaccharide. Proc. Natl. Acad. Sci. USA 75: 3998-4000, 1978.

Zerkle, T.B., Wade, A.E. and Rayland, W.E. Selective depression of hepatic cytochrome P-450 hemoprotein by interferon inducers. Biochem. Biophys. Res. Commun. 96: 121-127, 1980

Zerkle, T.D. and Wade, A.E. Depressant effects of the interferon inducer polyriboinosinic:polribocytidylic acid on cytochrome P-450 hemoproteins. Pharmacology 28: 1-11, 1984.

largely influenced by the geographical proximity of the group to the area in question. Basden and Dennis seem to have been familiar with the Onitsha - Awka area, and had little or no acquaintance with the central and eastern parts. In these parts, as the experience of Leonard clearly shows,[46] it would have been extremely difficult for them to miss the dominant presence of Abiriba and Nkwere smiths.

After years of familiarity with Igboland, Leonard came to a more realistic assessment of the Awka factor in parts of Igboland:

> The inhabitants of this district have been blacksmiths and travel all over that portion of the Ibo country which is contiguous to the Niger, as well as the Ijo, Oru and Brass territories, practically dividing the delta with the Nkwere, another Ibo clan of smiths, who take the eastern division.[47]

Leonard's view was shared by Jones, who was also very familiar with the southeastern Nigeria region in general and Igboland in particular.[48] The above caveats are not intended to detract from the undeniable evidence that Awka smiths did cast something of a colossal shadow on the landscape of metal technology in nineteenth century Igboland. What is being suggested is that the picture was less monolithic than the silence in the literature in respect of the other smithing centres might imply.

The first long distance travels by Agulu Awka smiths