Regulation of Cytochrome P450 During Hepatic Stress Responses

by

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This thesis is dedicated to my parents,
Olive and Frederick Bryden.

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ABSTRACT

Cytochrome P450 isoforms and related biotransformations are depressed by agents independently characterized as triggers of systemic inflammation and the hepatic acute phase response (APR). At the time this thesis work was undertaken, the recognition of the role of interleukin-6 (IL-6) as a modulator of hepatic gene expression was in its primary stage of investigation. This work complemented research on interleukin-1 and tumor necrosis factor alpha (TNF), known triggers of the hepatic APR, and also produced by the administration of many agents known to produce P450 depression. Recombinant TNF produced a dose- and time-dependent depression of hepatic P450 and related catalysis when administered to mice. This was accompanied by depressed expression of albumin mRNA, an indicator of the phenotypic changes associated with an APR to inflammation. Interleukin-6 administration did not consistently depress P450 *in vivo*, however, primary murine hepatocytes treated with IL-6 exhibited a dose-dependent depression of total P450 and catalysis, demonstrating that P450 regulation is sensitive to the presence of this cytokine. These findings indicate that P450 expression is modulated by the inflammatory cytokines TNF and IL-6, and that this depression occurs concomitantly with the phenotypic changes in hepatic gene expression known as the acute phase response.

There is evidence that the hepatic APR serves a "priming" function for hepatocellular proliferation. As P450 depression is a feature of both the acute phase response and hepatic regeneration, it was of interest to determine if this depression occurs by a common mechanism. A protein deficiency (PD) model of hepatic regeneration has been shown to produce a G0 to G1 transition in hepatocytes (Mead *et al.*, 1990) and was used to assess this change in cell cycle status in relation to hepatic P450 levels. The advantage of this model for this work is its absence of superimposed tissue damage and inflammation. Protein deficiency resulted in depression of total hepatic and renal P450 and elevation of pulmonary P450. Hepatic P450-related catalysis was depressed in an isoform-specific manner, with significant depression of EROD but not constitutive N-demethylase or PNPH activities. Fasting and control-fed rats exhibited distinct profiles of total P450 and catalytic activity compared to PD animals. Microsomal heme and P4502C11 exhibited depression in both PD and fasting rats.

Systemic inflammation inhibits P450 induction in an isoform-specific manner. If inflammation serves to prime the hepatocyte for proliferation, therefore leading to P450 depression by a mechanism common with that seen in regeneration, it would be expected that the induction of P450 isoforms would be attenuated in a similar pattern for both inflammation and regeneration (protein deficiency). It was found that the transcriptional induction of P4504A1 by clofibrate was inhibited by Poly IC (PIC) treatment but not PD, the translational induction of P4502E1 by pyridine was attenuated by PIC but not PD, while the protein stabilising effect of acetone on P4502E1 produced significant induction in PD rats but not PIC-treated animals. These findings indicate that P450 modulation by inflammation and regeneration occurs by distinct mechanisms.

The role played by P450 in drug metabolism and carcinogenesis as well as in the metabolism of endogenous mediators including steroid hormones and eicosanoids makes elucidation of P450 regulation an important goal. Modulation of P450-related catalysis by infectious illnesses, malignancy, or rheumatological conditions, or by protein deficient conditions including alcoholism, inflammatory bowel disease and malabsorption syndromes, or poor nutrition in both Western and third world countries, requires further study to ensure the safe use of therapeutic agents in these patient populations.

ABBREVIATIONS AND SYMBOLS

APR	acute phase response
BALB/c	strain of mice
BNF	β-naphthoflavone
C57BL/6	strain of mice
CFPM	calcium-free perfusion medium
Ci	Curie
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin-o-deethylase assay
IFN	interferon
IL-1	interleukin-1
IL-6	interleukin-6
i.p.	intraperitoneal
i.v.	intravenous
Kb	kilobase
Kd	kilodalton
LAH	lauric acid hydroxylase assay
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
NADH	diphosphopyridine nucleotide, reduced form
NADPH	triphosphopyridine nucleotide, reduced form
PBS	phosphate buffered saline
PD	protein deficient
PNPH	paranitrophenol hydroxylase assay
poly IC	polyriboinosinic·polyribocytidylic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
SSPE	sodium chloride/sodium phosphate (monbasic)/EDTA
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TLC	thin layer chromatography
TNF	tumor necrosis factor
U	units
UV	ultraviolet

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A mind that is stretched to a new idea never returns to its original dimension.

-Oliver Wendell Holmes

INTRODUCTION

A. The Cytochrome P450 Superfamily of Genes

1. Historical Perspective

In 1958, Klingenberg and Garfinkle independently described a reduced hepatic pigment which exhibited a characteristic maximal absorption of light at a wavelength of 450 nanometers. The term "pigment" 450, or "P450" was ascribed to the cytochrome when Omura and Sato characterised it as a P450 hemoprotein in 1961. As researchers focused more effort on purifying the hepatic "carbon–monoxide–binding pigment", it became evident that multiple forms of P450 coexist in the mammalian liver, although the first definitive characterisation of multiple P450 isoforms, determined via enzyme fractionation techniques by Haugen, van der Hoeven and Coon, did not occur until 1975 (Haugen *et al.*, 1975).

Implicit in the multiple forms and structures of these enzymes is the existence of broad and overlapping catalytic specificities. The use of reconstituted enzyme systems in which purified P450s were reconstituted with phospholipid and NADPH–cytochrome P450–oxidoreductase demonstrated catalysis that was both substrate– and stereo–specific to varying degrees, depending on the isoform examined (Lu and Coon, 1968; Haugen and Coon, 1976; Guengerich, 1987). Gonzalez (1989) describes the nature of P450 substrate specificity using testosterone metabolism as an example. Different P450 isoforms exhibit varying K_m's for testosterone, and so many enzymes contribute to the overall metabolism of the hormone to varying degrees. There may be

only one or two forms which contribute significantly to the pool of metabolites, nevertheless, the sum total of minor contributions can be significant when taken as a whole. In addition, the P450s are capable of metabolising the hormone in a stereo-specific manner, with a single enzyme producing a spectrum of stereoisomers which can be quantitatively and qualitatively different from the mix generated from another form of P450 (Waxman, 1988). The mechanisms by which individual P450 enzymes can accommodate multiple substrates and/or incorporate oxygen into them in different ways is a subject of intense study.

Cytochrome P450s catalyse the incorporation of one atom of molecular oxygen into a substrate, producing the oxygenated product, while the second oxygen atom is reduced by two electrons to give water. There are several steps to this reaction which is electrochemically complex and incompletely understood. Figure 1 depicts the classical conceptualization of the reaction cycle. Much of what is known about the details of each step has been determined from work done with cytochrome $P450_{cam}$ (P450CI), a bacterial P450 derived from *Pseudomonas putida* (Ortiz de Montellano, 1986). This oxygen- and NADPH-dependent cycle is used to catalyse the majority of P450 reactions. Classically, P450s are said to catalyse reactions such as epoxidations, N-dealkylations, O-dealklylations, S-oxidations, and hydroxylations of aliphatic and aromatic compounds (Hollenberg, 1992), with greater than ten reaction types known (Ortiz de Montellano, 1986; Guengerich, 1991; Porter and Coon, 1991; Black, 1992). The insertion of a hydroxyl group into the substrate constitutes the initial reaction for all substrates (White and Coon, 1980). The specificity of the reaction lies in the nature

FIGURE 1.

The Cytochrome P450 Redox Cycle. Drugs bind to cytochrome P450 in its oxidized (3+) form. Reducing equivalents supplied by NADPH reduce the P450 heme prothesis to its 2+ form. Activation of molecular oxygen occurs, forming a complex with the drug/P450 complex and a second electron from NADPH or NADH. This complex then splits in turn to yield oxidized drug, water and P450 in its 3+ form. After White and Coon, 1980. (RH = substrate; ROH = corresponding product; e– = electron).

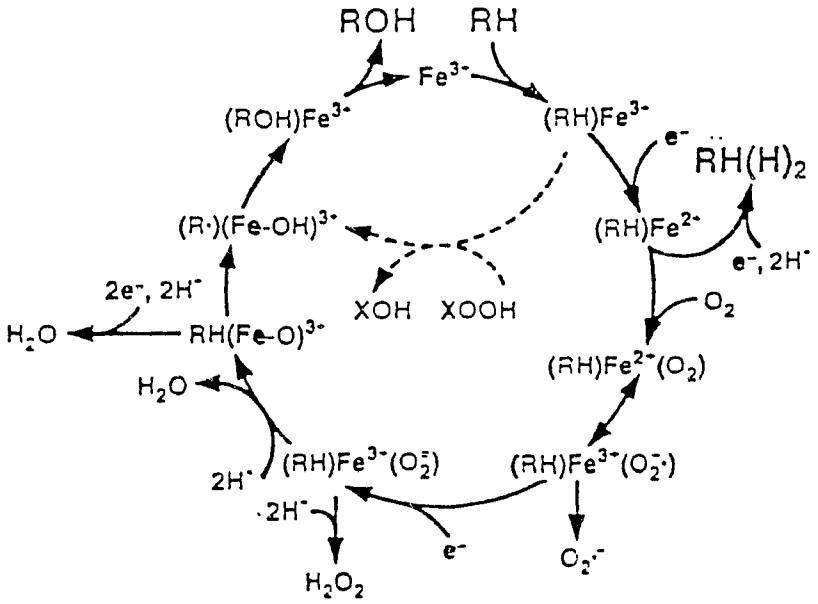

ROH
RH
Fe3+
(ROH)Fe3+
(RH)Fe3+
e-
RH(H)2
(R·)(Fe-OH)3+
(RH)Fe2+
e-, 2H+
2e-, 2H+
XOH
XOOH
O2
H2O
RH(Fe-O)3+
(RH)Fe2+(O2)
H2O
2H+
(RH)Fe3+(O2-)
(RH)Fe3+(O2-·)
2H+
e-
O2·-
H2O2

FIGURE 1.

of the substrate and the stability of the reactive intermediate formed via the particular P450 isoform (White and Coon, 1980; Kraut, 1988).

The physiological significance of P450 catalysis is suggested by the evolutionary conservation of P450 genes. Bacterial P450s constitute the phylogenetically oldest forms of the protein, and exhibit the same, highly conserved domains that are the hallmark of all P450 proteins (Poulos and Raag, 1992). Cytochrome P450 catalyses a number of endogenous anabolic and catabolic reactions primarily in pathways involving the metabolism of eicosanoids and other fatty acids (Kupffer, 1980), cholesterol derivatives such as bile acids and steroid hormones (Andersson *et al.*, 1989; Picardo–Leonard and Miller, 1987; Zanger *et al.*, 1992), and ketones (Koop and Coon, 1986). The precise, specific orchestration of P450 gene expression by developmental signals (Gonzalez *et al.*, 1986; Omiecinski *et al.*, 1990), by gender–specific hormonal changes (Gustafsson *et al.*, 1983), and by exposure to various xenobiotics (Nebert and Gonzalez, 1984; Adesnik and Atchison, 1986; Gonzalez *et al.*, 1984) suggests that an appropriately tailored P450 profile optimizes survival of the organism. The biological necessity of P450s is obscured, however, by the multitude of genetic mutations exhibited within the superfamily of P450 genes (Gonzalez, 1989). Genes which are absolutely essential for life normally do not exhibit high rates of mutation. Indeed, the multiplicity of P450 genes is derived from the propensity of P450 genes to mutate (Gonzalez, 1989).

The function of P450 in the regulation of the synthesis of endogenous biomolecules was only recognised some time after the enzymes had been studied in

association with their drug metabolic capacity. The numerous substrate specificities afforded by the multiplicity of the enzymes, each with perhaps several catalytic capabilities, provides mammalian organisms with the capacity to metabolise a huge number of chemical compounds found in the environment. These "chemicals" are estimated to number over 250 000 (Adesnik and Atchison, 1986), and are commonly referred to as pollutants, pesticides, drugs, vitamins, and "natural" ingredients, among others. The functional motif underlying the catalysis of these compounds is the conversion of relatively lipophilic substances to relatively hydrophilic ones that may be excreted from the body by way of the urine or bile. In this way, P450s perform the important function of eliminating foreign substances from the body.

While xenobiotic metabolism is correctly considered a "defensive" function of P450s in many instances, P450 oxidation has also been shown to result in the production of toxic, high–energy intermediates (*egs*, epoxides, free radicals) which are capable of damaging macromolecules critical to cellular functioning with the potential, therefore, to produce cellular death, dysfunction, or transformation. Thus, the role of P450 in the generation of mammalian cancers has been a topic for debate. Scientists have tried to determine the net effect of a process which is protective in its removal of carcinogens from the body, while at the same time toxic in the by–products it generates as a result of this metabolism.

2. Nomenclature

The broad catalytic profile of hepatic microsomes was recognized early and is

reflected in the terms used to denote the enzyme system. For instance, in 1957 Mason implied the variable usage of the oxygen molecule in the reactions with his term, "mixed function oxidase", while in the succeeding 5 years, Hayaishi (1962) recognized the fact that only a single oxygen atom is incorporated into the substrate by employing the term, "monooxygenase". Indeed, the nomenclature used to denote the various forms of P450 evolved as the understanding of the system unfolded.

When it was first recognised that the mixed function oxidase system of the liver was comprised not of one or a few enzymes, but of many, researchers could not have predicted the enormous multiplicity of P450 enzymes that would be found in the tissues and species examined. Researchers assigned names based on such parameters as the tissue/species source of the enzyme, spectral properties, electrophoretic mobility, substrate specificity or response to inducers. Nebert describes a typical example (Nebert *et al.*, 1989) for the names assigned to P450IA1, a form which metabolises planar, aromatic hydrocarbons: AHH, P–450c, P–450 form 6, P_1–450, CYP1, CYP2, P(I)450, P450DX, Cyp–1, P450C1, P450C1A1, and CYPIA1. Coon points out in his recent review (Coon *et al.*, 1992) that the standard approach to enzyme nomenclature, generally, was to name according to catalytic specificity. This proved impossible in the case of P450s, however, as various laboratories studied different substrates or different metabolites often with the same P450 enzyme.

The past few years have witnessed a tremendous expansion of the knowledge of P450 amino acid, cDNA, and gene sequences, prompting the development of a P450 classification system based on primary sequence alignment data (Nebert *et al.*,

1987; Gonzalez, 1989; Nebert *et al.*, 1991; Coon *et al.*, 1992; Nelson *et al.*, 1993). Briefly, the P450 superfamily of genes is divided into families, which are further grouped into sub-families, according to the degree of sequence homology of their genes. The limits for homology are entirely arbitrary and were determined in an examination of the 65 P450 protein sequences which were available at the end of 1986 with updates planned for every two year period (Gonzalez, 1989). A P450 in one gene family is less than or equal to 40% similar to P450s in other families, while P450s in a particular subfamily will share over 55% homology with each other (Nebert *et al.*, 1989). Accurate assignment of P450 genes to a family and subfamily can usually be accomplished only if its full amino acid coding sequence is known. The enzyme itself is referred to as cytochrome P450 or simply, "P450". The formerly used hyphen in "P-450" was dropped when systemic nomenclature was first introduced. The family is denoted by an arabic number, the subfamily by a capital letter, with the individual genes assigned an arabic number according to the chronological order in which they were described. To date, some 221 P450 forms have been recorded (Nelson *et al.*, 1993) with sequence homologies varying from 10 to 97% (Coon *et al.*, 1992). Cytochrome P450s have been recovered from microorganisms, plants and animals, from nearly all mammalian tissues examined, and from various organelles, predominantly the endoplasmic reticulum and the mitochondria.

It should be noted that the terminology ascribed to P450 proteins is distinct from that used to name the genes themselves (Nebert, 1989). Based on suggestions from the committees for Human Gene Mapping and for Standardized Genetic

Nomenclature for Mice, it was decided to denote P450 genes by the root symbol "CYP" followed by an arabic numeral for the family, a capital letter for subfamily and arabic number denoting the specific gene (Nebert, 1989). This convention is consistent with the nomenclature for other gene families including the human collagen genes, mouse homeobox genes, and yeast cytochrome genes, among others (Nebert, 1989). The systematic nomenclature will be used throughout this thesis when referring to specific P450 genes and proteins.

3. Levels of P450 Regulation

The cytochrome P450 superfamily exhibits multiplicity at the level of gene sequence, protein structure and function, substrate specificity, and tissue and species specificity. It is not surprising, therefore, to discover that these genes are under the control of a wide range of regulatory processes, with examples of nearly every known regulatory mechanism found within the set of P450 genes. The mix of regulatory influences for a given species of P450 ranges from simple to complex.

a. Present Understanding of Protein Synthesis and Degradation

The turn-over of any protein rests in the balance between the zero-order process of protein synthesis and the first-order process of protein degradation (Waterlow *et al.*, 1978). Following from this, then, it may be suggested that the rate constant of degradation frequently is the sole determinant of the steady-state concentration of individual proteins as they fluctuate from the basal to the induced or

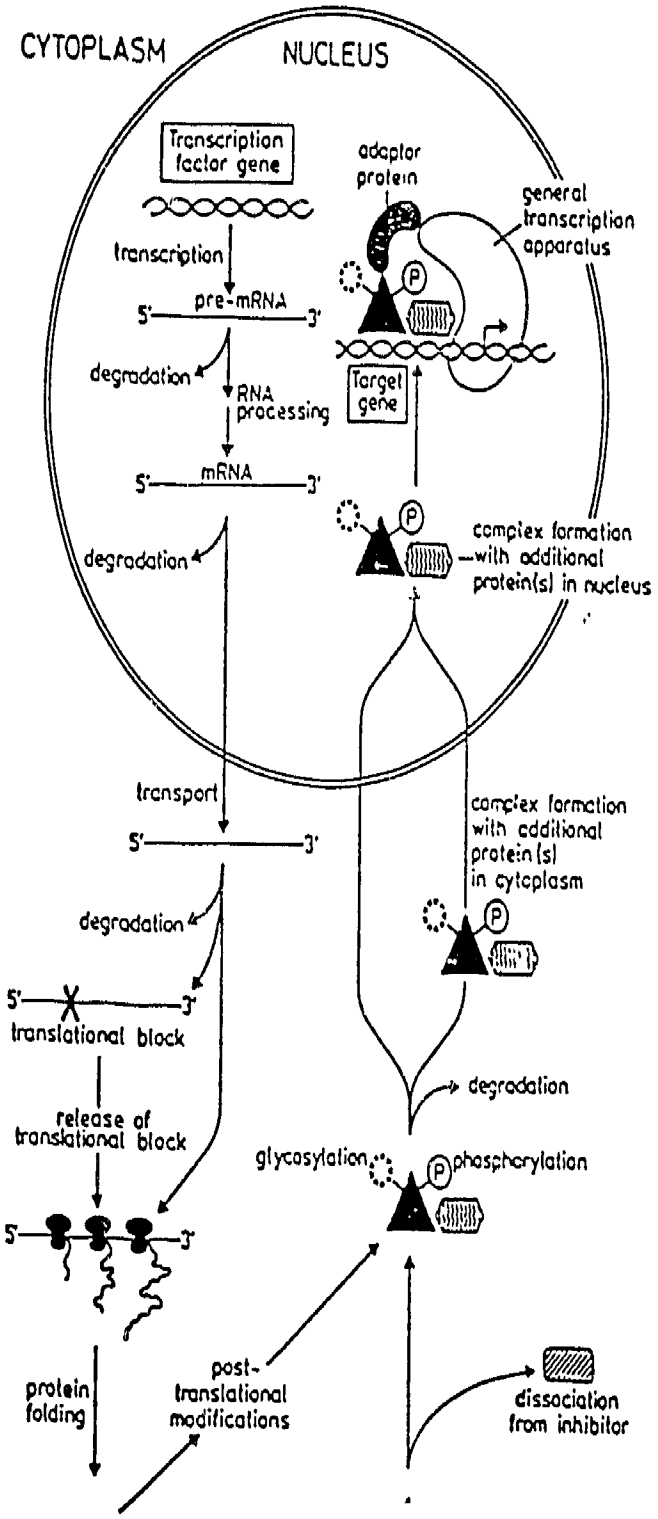
repressed state of expression (Correia, 1991). The understanding of net changes in protein expression requires an assessment of both the synthetic and degradative aspects of protein expression.

In recent years, advances in molecular biology techniques have allowed researchers to come closer than ever to a more complete understanding of the mechanisms governing expression of the prokaryotic and eukaryotic genome. These mechanisms act to up- or down-regulate protein expression from the moment of initial transcription of DNA in the nucleus to the time of protein degradation in the cytosol. Figure 2 depicts the sequential levels at which protein expression may be controlled.

Starting in the nucleus, the degree of gene transcription is known to be determined by the presence of several positive or negative transcription factors which serve to enhance or depress the functioning of the DNA transcription machinery. The pattern of factors binding to a particular gene depends on the presence of "promoter" and "enhancer" DNA sequences which are specific for different transcription factors. While relatively few specific transcription factors have been conclusively identified at this time, it is believed that the transcription of most mammalian genes is likely regulated by a host of such peptides. Indeed, the mechanisms governing the regulation of these factors themselves has been a subject of intense investigation in recent years (Mitchell and Tijian, 1989; Falvey and Schibler, 1991). The term, "*cis*-acting" is given to elements which are comprised of specific regulatory sequences contained within the gene itself. They may reside in the immediate vicinity of the start-site ("promoters"),

FIGURE 2.

Potential control points in the sequence of events leading to the synthesis of proteins. The figure depicts the regulation of gene transcription by transcription factors (black triangles), followed by pre–mRNA processing and degradation, mRNA transport to the nucleus, translation of transcripts, protein folding and post–translational modifications. It is feasible that gene expression may be regulated at only one or a few of these points, with variable patterns dominating under differing regulatory influences (taken from Falvey and Schibler, 1991).


CYTOPLASM
NUCLEUS
Transcription factor gene
adaptor protein
general transcription apparatus
transcription
pre-mRNA
5'
3'
degradation
RNA processing
Target gene
mRNA
complex formation with additional protein(s) in nucleus
transport
complex formation with additional protein(s) in cytoplasm
degradation
translational block
release of translational block
degradation
glycosylation
phosphorylation
protein folding
post-translational modifications
dissociation from inhibitor

or be distributed up–stream or down–stream of the transcription site ("enhancers") (McClure, 1985; Maniatis *et al.*, 1987; Jones *et al.*, 1988; Saltzman and Weinmann, 1989). Enhancers are capable of regulating promoter function from a distance (1 to 30 kilobases), in an orientation–independent fashion (Ptashne, 1986). The factors which effect regulation of gene expression by binding to the *cis*–acting elements are the "*trans*–acting factors", and usually consist of a set of highly regulated nuclear proteins specific to each gene and regulatory context.

The nascent RNA transcript, or "pre–mRNA" is prepared for entry into the cytoplasm by processes such as "splicing" in which the transcript is shortened by the removal of intervening sequences called "introns" leaving the "exon" sequences to generate a polypeptide by way of translation. It is known that the expression of some proteins is regulated by altering this processing of the pre–mRNA molecule such that it does not leave the nucleus, or perhaps exhibits altered susceptibility to degradation upon its entry into the cytosol (Brawerman, 1989; Falvey and Schibler, 1991).

It is now recognized that mRNA decay is an important control point in the regulation of gene expression, and that the stability of different mRNA species varies widely. For example, transcripts for transiently expressed genes such as the proto–oncogenes c–fos and c–myc have half–lives as low as 15 minutes, while beta–globin mRNAs appear to be fully stable (Brawerman, 1987). It is probable that mRNA degradation is a regulated rather than a random process. If decay were mediated by random endonucleolytic attack, variations due to differences in transcript length and perhaps secondary structure would be observed. Experiments in prokaryotic systems

have shown that there is no relation between transcript length and its susceptibility to decay (Belasco *et al.*, 1986).

A common regulatory motif is manifested by the presence of secondary structures in the 3' and 5' non-coding regions of the mRNA molecule. Poly(A) sequences in association with proteins are known to confer resistance to 3' exonucleolytic degradation (Bergman and Brawerman, 1977; Huez *et al.*, 1981), while transcripts lacking the 3' poly(A) terminus have been shown instead to contain sequences with the capacity to form stem-loop structures potentially resistant to 3' exonuclease (Georgiev and Birnstiel, 1985). Similarly, sequences in the 5' non-coding regions of certain bacterial transcripts also have a stabilising influence on the transcript (Belasco *et al.*, 1986). Conversely, examples of destabilising sequences in both the 3' and 5' non-coding regions of eukaryotic genes have been revealed (Albrecht *et al.*, 1984; Piechaczyk, 1985; Rabbitts *et al.*, 1985; Morris *et al.*, 1986; Shaw and Kamen, 1986), including such secondary structures as 3' stem-loops (Mullner and Kuhn, 1988) and 3' AU-rich sequences (Brawerman, 1989).

Ribosomes are an important component of the translation machinery in eukaryotic cells, and there is evidence for their involvement in yet another control point of gene expression. Some workers provide evidence of enhanced mRNA stability following the assembly of translation machinery (Morse and Yanofsky, 1969; Belasco *et al.*, 1985), although more recent experiments suggest that in some systems protection by ribosomes may not be a significant factor in reducing mRNA decay (Stanssens *et al.*, 1986). It has also been found that for certain eukaryotic transcripts,

mRNA decay does not occur unless the transcript has been translated to a certain degree. One interpretation of this phenomenon is that decay is mediated by a ribosome–bound nuclease that degrades the 3' terminus of the mRNA once brought into close proximity via continued translation of the transcript (Graves *et al.*, 1987; Yen *et al.*, 1988). Clearly, the assembly of translation machinery along with the process of translation itself, constitute important points for differential regulation of protein synthesis in eukaryotic cells.

There is comparably little information available as to the nature of the regulation that is effected at the level of the endo– and exonucleases that are directly responsible for the differential metabolism of mRNA species. Site–specific endo–nucleases responsible for mRNA degradation in prokaryotic systems have been identified, however much less is known about the control of such processes in higher organisms (Brawerman, 1989).

The elucidation of the mechanisms of cellular protein degradation has lagged behind our understanding of protein synthetic mechanisms. The reason for this, in part, is due to the large number of pathways responsible for protein catabolism in the cell. In a review of the subject, Stadtman (1990) notes that for many proteins, no one pathway is responsible, with the possible involvement of ATP–dependent and –independent, lysosomal and nonlysosomal, and ubiquitin–dependent and –independent pathways for any given species under differing conditions. Within this complexity, a few basic patterns of protein degradation have begun to emerge.

Of the multiple ATP–dependent proteolytic pathways discovered in eukaryotic

cells including reticulocytes, yeast, liver, and muscle (Goldberg and St. John, 1976; Beynon and Bond, 1986; Ciechanover, 1987; Seglen, 1987), the reticulocyte ubiquitin-dependent system has been the most extensively characterized (Ciechanover, 1987; Seglen, 1987; Hershko, 1988; Hough, 1988; Hershko, 1991). Ubiquitin, a small protein found in all eukaryotic cells (Ciechanover *et al.*, 1984; Finley and Varshavsky, 1985), serves as a marker for proteins targeted for destruction, including abnormal and short-lived species. Regulation of the ubiquitin pathway may occur at several sites including activation of ubiquitin by ubiquitin-activating enzymes, the conjugation of proteins by ubiquitin-conjugating enzymes, lysis of these conjugates by isopeptidases, ATP-dependent proteolysis of the target protein by a multisubunit protease (Hough *et al.*, 1986; Waxman *et al.*, 1987) or by ubiquitin itself (Fried *et al.*, 1987), the latter of which can be attenuated by intracellular inhibitors (Murakami and Etlinger, 1986). Ubiquitin-dependent pathways appear to be responsible for the degradation of several short-lived proteins, heme-stripped apohemoproteins, and structurally damaged or aberrant proteins (Beynon and Bond, 1986; Ciechanover, A., 1987; Seglen, 1987; Hershko, A., 1988; Hershko, A., 1991).

Two calcium-dependent pathways have been described which utilize the "calpain" proteases found in a wide variety of cell types (Ohno *et al.*, 1984; Pontremoli and Melloni, 1986). Intracellular inhibitors and activators have also been described for the calpains, allowing for another level of regulation in this pathway (Finley *et al.*, 1987). Lysosomal pathways of degradation have classically been ascribed to the catabolism of membrane proteins (Libby and Goldberg, 1981; Masaki

et al., 1987) and long–lived cytosolic proteins (McElligott *et al.*, 1985). Other pathways which have been less completely described include ATP–dependent cytosolic pathways which are ubiquitin–independent (Waxman *et al.*, 1987); ATP– and ubiquitin–independent cytosolic pathways (Fagan *et al.*, 1986); pathways within mitochondria and chloroplasts (Mayer and Doherty, 1986); and pathways within the cisternae of the endoplasmic reticulum and/or the Golgi apparatus that degrade a proportion of secreted or membrane proteins before they reach their final destination (Mayer and Doherty, 1986; Minami *et al.*, 1987).

Sorting of targeted proteins into a particular degradative pathway is dependent on protein modification. Some proteins have been found to contain particular peptide sequences which destine them to their degradative fate (Chiang and Dice, 1993; Dice *et al.*, 1993). Others may be labelled by any of many covalent modifications which have been found to confer susceptibility to proteolytic degradation. These modifications include acetylation, carbamylation, deamidation, several oxidative processes, and phosphorylation (reviewed by Rivett, 1986; Stadtman, 1990).

The hormonal, nutritional and growth status of the cell establishes the balance of metabolic controls on protein degradative pathways. Alterations in parameters such as cAMP, catalase, oxygen, ATP, and the net cellular redox state will confer important influences on protein stability and the reactions which mark proteins for degradation. In addition, abnormal or partially denatured proteins will be produced under conditions of cellular stress including high temperatures and the presence of oxidizing agents. Protein unfolding, oxidative damage, and exposure of the amino–terminal residue

determine conjugation with ubiquitin. The resultant modified proteins will then be subject to the various proteolytic pathways which no doubt, are themselves subject to modulation by the factors cited above.

The preceeding paragraphs present a brief summary of the current understanding of the factors controlling the level of specific cellular proteins, based on the information provided in several current reviews. The degree of mRNA synthesis is determined by the efficiency of the transcription apparatus, consisting of various transcription factors and RNA polymerase II (Mitchell and Tijian, 1989). The presence of mRNA transcripts in the cytosol is also a function of pre–mRNA processing coupled with degradative mechanisms imposed and modified by a host of factors such as the secondary structure of the transcript, the efficiency of the translation machinery and the presence of endo– and exonucleases. The specific direction of proteins into one of several degradative pathways is incompletely understood, and the subject of intense investigation. This process is believed due to the targeting of proteins by inherent peptide sequences or covalent modification, allowing them to be selectively degraded by a variety of proteolytic mechanisms.

b. Present Understanding of P450 Regulation

The tremendous complexity in terms of the number of genes as well as the number of regulatory influences has made an understanding of precise regulatory mechanisms a difficult goal in the field of cytochrome P450 research. Much of the recent experimental progress has focused on the isolation of genes, the elucidation of

their sequences (genomic and/or cDNA) and the expression of these sequences in transfected cells to determine their catalytic specificities (Nebert *et al.*, 1987; Gonzalez, 1989; Nebert *et al.*, 1989; Nebert *et al.*, 1991). When P450 regulation is considered in the context of Figure 2, many more questions than answers are to be found for each level of expression. A sufficient amount of information is now available to allow researchers to embark on a more detailed investigation of the mechanisms leading to changes in P450 gene expression.

The binding of transcription factors to the appropriate DNA promoter and enhancer sequences is one of the initial events leading ultimately to the production of new proteins. The CYP1A1 gene codes for a P450 which metabolizes and is strongly induced by planar aromatic hydrocarbons. CYP1A1 is one of the best-understood P450 genes with regard to the control of transcription by the specific interaction of protein factors with DNA (Fujisawa-Sehara *et al.*, 1985; Jones *et al.*, 1986; Fujisawa-Sehara *et al.*, 1987; Fujisawa-Sehara *et al.*, 1988). Aspects of both *cis*- and *trans*-acting control mechanisms have been determined for CYP1A1/2. The transient transfection of hepatoma and other cell lines with chimeric genes coding for the 5' region of CYP1A1 with chloramphenicol acetyl-transferase (CAT) as the reporter gene allowed researchers to elucidate the sequences of two regulatory DNA sequences which are required for maximal induction of the CYP1A1 gene (Gonzalez and Nebert, 1985; Jones *et al.*, 1985; Fujisawa-Sehara *et al.*, 1986; Fujisawa-Sehara *et al.*, 1987; Yanagida *et al.*, 1990). The "xenobiotic responsive element" (XRE) is a *cis*-acting control element distributed 5 times in the 5' flanking sequence of the gene, and binds

the activated (ligand–bound) aryl–hydrocarbon (Ah) receptor, presumably initiating increased gene transcription (Poland *et al.*, 1976; Denison *et al.*, 1986; Cutchill *et al.*, 1987; Denison *et al.*, 1988; Fujisawa–Sehara *et al.*, 1988; Hapgood *et al.*, 1989; Neuhold *et al.*, 1989). The second *cis*–acting element is believed to be responsible for complete response to inducers but also to control the basal expression of the gene (Fujii–Kuriyama *et al.*, 1992). This "basal transcription element" (BTE) is located at –43 bp, just upstream from the TATA initiation sequence (Yanagida *et al.*, 1990), and has been found in the region proximal to the TATA sequence in several genes, most commonly in P450 genes, including CYP2B1/2 (phenobarbital–inducible), CYP2E1 (ethanol–inducible), and the steroidogenic forms CYP11A1 and CYP21A2 (Yanagida *et al.*, 1990). The latter gene is also responsive to altered levels of cAMP, and recent work (Kagawa and Waterman, 1990) has revealed the presence of a 5' cAMP responsive element which is distinct from the consensus cAMP response elements found in other cAMP responsive genes (Roesler *et al.*, 1988).

Other work has focused on the elucidation of the *trans*–acting factors themselves. Gel retardation and DNA foot–printing assays led to the identification of a phenobarbital– and heme–induced transcription factor in rat liver which was correlated with the transcriptional activation of CYP2B1/2 (Rangarajan and Padmanaban, 1989). Later studies demonstrated that dexamethasone reduces phenobarbitone–mediated increases in the binding of this 85 kilodalton protein to the upstream region of CYP2B1/2 as evidenced by gel retardation and Southwestern blot analysis (Rao *et al.*, 1990). Work done with the genes encoding the steroidogenic P450s 27A1 and 21A1

are illustrative examples of the diverse regulatory processes which can affect even genes which are coordinately regulated. The presence of ACTH serves to coordinately upregulate both CYP17A and CYP21A. This results from the binding of a putative adrenal–specific transcription factor in the case of CYP21A, whereas the binding of a ubiquitous protein apparently active only in steroidogenic cells mediates the response by CYP17A (Zangar *et al.*, 1992). Thus the coordinated regulation of these functionally related genes employs biochemically distinct mechanisms, underscoring the complexity of regulatory mechanisms to be found within the P450 superfamily of genes. Taken together with the present knowledge of *cis*–acting regulatory sequences found within P450 genes, these findings provide a starting point for a more comprehensive view of how P450 induction is mediated at the level of gene transcription.

Although the precise mechanisms governing P450 turnover are as yet poorly understood, degradation of the cytochromes P450 is felt to be a highly selective event. The large degree of variation in the half–lives of P450 isoforms supports this hypothesis (Watkins *et al.*, 1987; Correia, 1991). Studies of hepatic intracellular loci and proteolytic systems have focused on the turn–over of membrane–bound proteins, including the P450s (Watkins *et al.*, 1987). The T–cell antigen receptor, 3–hydroxy–3–methylglutaryl coenzyme A reductase and the asialoglycoprotein receptor are integral proteins of the endoplasmic reticulum which are apparently degraded within or at the endoplasmic reticulum (Lippincott–Schwarz *et al.*, 1988; Amara *et al.*, 1989; Chun *et al.*, 1990). In contrast, outer mitochondrial membrane proteins (Evans and

Mayer, 1984) and the endoplasmic reticulum-bound P450s 2B1 and 2E1 and NADPH-P450 reductase are believed to undergo autophagic degradation in rat liver (Masaki *et al.*, 1987; Ronis and Ingelman-Sundberg, 1989a; Ronis and Ingelman-Sundberg, 1989b; Ronis *et al.*, 1991). Degradation of endoplasmic reticulum proteins is generally believed to occur via the autophagic/lysosomal pathway of protein catabolism (Khairallah *et al.*, 1985; Henell and Glaumann, 1984; Ahlberg *et al.*, 1985; Bolander and Weibel, 1973), however, the lack of specificity for this "bulk-flow" process (Omura, 1982) resulted initially in scepticism for its involvement in P450 turn over. Nevertheless, experimental evidence has shown that certain P450s (*eg*, P4502B1) are, in fact, degraded by this mechanism (Toshiro *et al.*, 1987; Masaki *et al.*, 1987; Shiraki and Guengerich, 1984; Ronis *et al.*, 1991; Masaki *et al.*, 1989). The ubiquitin pathway has also been implicated in the turn over of the P450s. A 33-kDa ubiquitin-immunoreactive protein appeared following the loss of hepatic CCl_4-inactivated 2E1 (Tierney *et al.*, 1990; Tierney and Koop, 1990; Tierney *et al.*, 1992), and the enhanced affinity of CCl_4-inactivated 2E1 for reticulocyte lysate fraction II-dependent ubiquitination (Koop *et al.*, 1991) is believed to support the existence of ubiquitin-dependent proteolysis of CCl_4-inactivated 2E1 in rats.

Targeting of P450s for degradation is believed to occur by a number of mechanisms including oxidation of P450 amino acid residues by oxygen radicals (Stadtman, 1990; Davies, 1987; Salo *et al.*, 1990; Schaefer *et al.*, 1985), phosphorylation (Eliasson *et al.*, 1990; Bartlomowicz *et al.*, 1989), heme modification by N-alkylation or other reactions (Ortiz de Montellano and Mathews, 1981), or loss

of the prosthetic heme (Watkins *et al.*, 1986). So-called "mechanism-based", "suicide" inhibition by the catalysis of certain substrates is a well-studied process in which destruction of the heme and/or active site occurs as a result of substrate catalysis. The specific destructive mechanisms differ according to P450 isoform and substrate (Coffman *et al.*, 1982; De Matteis *et al.*, 1983; Tephly *et al.*, 1985; Correia *et al.*, 1987; Sugiyama *et al.*, 1989).

Understanding the mechanisms of P450 degradation is particularly challenging as both heme and apoprotein kinetics must be taken into account. The turn-over of the prosthetic heme occurs at a more rapid rate than that of the apoproteins (Correia, 1991), and debate continues as to the role of heme stripping in the regulation of P450 turn over. An assessment of the kinetics of the hepatic heme pool is a necessary prerequisite to the analysis of P450 turn-over in the liver. It is beyond the scope of this work to discuss the details of heme regulation in the liver, however, an understanding of the mechanisms of P450 induction and depression is dependent on an understanding of this important component of P450 regulation.

Genes in the first, second, third and fourth P450 gene families are known to respond to exogenous inducers. While all four families include genes which are induced by way of increased rates of transcription, there are many examples of mRNA stabilization, as well as alterations in translation and protein degradative influences. The mix of regulatory mechanisms is exceedingly complex, and there are few common motifs to serve as a framework for organization. Indeed, individual genes can exhibit altered inductive regulation depending on dosing schedules, species, gender, tissue

type, or the presence of other xenobiotic compounds (reviewed by Gonzalez (1989)).

In addition to influences which serve to increase P450 expression, several factors have been shown to lead to a down-regulation of P450 isoforms. Among these are the sex-specific suppression of P4501A2 in female rats (Gonzalez *et al.*, 1986) and P4502A1 in males (Matsunaga *et al.*, 1988; Nagata *et al.*, 1987). The modulation of P450 seen following the administration of agents such as endotoxin, viruses or the "interferon-inducer", Poly IC has been a topic of interest since it was first documented (Kato *et al.*, 1963). Depression of P450 and catalytic activities has also been noted following partial hepatectomy in the rat (Jean-Marie *et al.*, 1988).

The earliest observations of modulation of cytochrome P450 expression during inflammation were described in the 1960's by Kato (1963) who noted alterations in phenobarbital induction in mice infected with murine hepatitis virus, and Strenger (1969) who found pre-treatment of animals with carbon particles modulated the subsequent hepatotoxicity of carbon tetrachloride. In the years leading to the present state of knowledge it became evident that a wide variety of immune stimulants and inflammatory agents exhibit the propensity to cause P450 depression. Table 1 summarizes these observations which have been made following the administration of parasites, bacteria, bacterial cell wall components, stimulants of the reticuloendothelial system, viruses, vaccines, interferon inducers, and cytokines to rodents and humans.

Suggestions were made early that this depression resulted from the release of soluble mediators from inflammatory cells. Direct incubation of hepatic microsomes with high concentrations of *Salmonella* glycolipids (Egawa and Kasai, 1979), zymosan

TABLE 1. Infectious, immunomodulating and inflammatory agents known to depress cytochrome P450.

AGENT	REFERENCE
PARASITES	
Schistosoma mansoni	Cha and Edwards, 1976; Cha *et al.*, 1980
Trypanosoma brucei	Shertzer *et al.*, 1981
Plasmodium berghei	McCarthy *et al.*, 1970
Fasciola hepatica	Maffei Facino *et al.*, 1981
BACTERIA	
Bacillus Calmette–Guerin (BCG)	Farquhar *et al.*, 1976; Sonnenfield *et al.*, 1980
Mycobacterium butyricium (Freund's adjuvant)	Morton and Chatfield, 1970; Dipasquale *et al.*, 1974; Cawwthorne *et al.*, 1976
Corynebacterium parvum	Sokya *et al.*, 1976; Farquhar *et al.*, 1983
Bordetella pertussis	Renton and Mannering, 1976b; Williams and Szentivanyi, 1977
Listeria monocytogenes	Azri and Renton, 1987
CELL WALL COMPONENTS	
Endotoxin	Renton and Mannering, 1976b; Gorodischer *et al.*, 1976; Stanley *et al.*, 1988
N–Acetylmuramyl–1–analyl–D–isoglutamine (MDP)	Williams and Szentivanyi, 1983
Lipid A	Egawa and Kasai, 1979
Nocardia rubra cell wall skeleton	Iwasaki and Noguchi, 1982
PARTICULATE AND SOLUBLE RES STIMULANTS	
Maleic anhydride–divinyl ether copolymer (pyran copolymer)	Barnes *et al.*, 1979; Morahan *et al.*, 1972
Peptidoglycan monomer (PGM)	Trescec *et al.*, 1983
Methyl Palmitate	Wooles and Munson, 1971

TABLE 1. (Continued)

AGENT	REFERENCE
Zymosan	Wooles and Munson, 1971; Al-Tuwaijri *et al.*, 1982
Dextran Sulfate	Renton, 1981b; Peterson and Renton, 1984
Latex	Renton, 1981b; Peterson and Renton, 1986a
Colloidal Carbon	Strenger *et al.*, 1969; Leterrier *et al.*, 1973
VIRUSES	
Murine hepatitis virus	Kato *et al.*, 1963; Budillon, *et al.*, 1972
Mengo virus	Renton and Mannering, 1976b
Encephalomyocarditis virus	Renton, 1981a
Newcastle disease virus	Singh and Renton, 1981; Deloria *et al.*, 1985
Duck hepatitis virus	Ragland *et al.*, 1971
Sindbis virus	Moore *et al.*, 1978
VACCINES	
Influenza vaccine	Corbett and Nettesheim, 1973; Renton *et al.*, 1980
Bordetella vaccine	Renton, 1979
INTERFERON INDUCERS	
Poly IC	Renton and Mannering, 1976b; El Azhary and Mannering, 1979; El Azhary *et al.*, 1980; Singh and Renton, 1981, 1984; Singh *et al.*, 1982; Ghezzi *et al.*, 1985; Deloria *et al.*, 1985; Mannering *et al.*, 1988.
Tilorone	Renton and Mannering, 1976a, 1976b; Leeson *et al.*, 1976; El Azhary and Mannering, 1979; El Azhary *et al.*, 1980
Quinacrine	Renton and Mannering, 1976b
Statolon	Renton and Mannering, 1976b
CP 20,961	Renton and Mannering, 1976b
OK-432	Hojo and Hashimoto, 1977

TABLE 1. (Continued)

AGENT	REFERENCE
CYTOKINES	
α–Interferon (crude)	Singh *et al.*, 1982
α–Interferon (recombinant)	Singh *et al.*, 1982; Parkinson *et al.*, 1982; Renton *et al.*, 1984; Taylor *et al.*, 1985; Franklin and Finkle, 1985; Bertini *et al.*, 1988; Moochala and Renton, 1991a, 1991b
β–Interferon (crude)	Singh *et al.*, 1982
γ–Interferon (crude)	Sonnenfield *et al.*, 1980; Smith *et al.*, 1983
γ–Interferon (recombinant)	Franklin and Finkle, 1985; Mannering *et al.*, 1988; Bertini *et al.*, 1988
Interleukin–1	Bertini *et al.*, 1988
Tumor necrosis factor	Ghezzi *et al.*, 1986; Bertini *et al.*, 1988
Lymphotoxin	Bertini *et al.*, 1988

After Mannering and Deloria (1986).

(Renton, 1983), dextran sulfate (Peterson and Renton, 1984) or latex beads (Peterson and Renton, 1986a) produced no effect on microsomal catalytic activities. Moreover, incubation of primary hepatocyte cultures with lipopolysaccharide (Williams and Szentivanyi, 1985; Ghezzi, 1986; Bertini, 1989), dextran sulfate (Peterson and Renton, 1984), or latex beads (Peterson and Renton, 1986a) also had no depressant effect on the P450 system. This led to interest in the role played by the non-parenchymal cell population of the liver, and in particular, the Kupffer cells of the reticuloendothelial system.

The reticuloendothelial system is composed primarily of monocytes and macrophages and the endothelial cells lining the reticulin network of the liver, lung, lymph nodes, spleen and bone marrow (Aschoff, 1924; Bjorneboe and Prytz, 1976). A role for such inflammatory cells and their mediators was implicated by experiments in which hepatocytes incubated with the conditioned medium from dextran sulfate-treated Kupffer cells exhibited significantly reduced levels of P450 catalysis (Peterson and Renton, 1984). Experiments which employed Marbrook vessels provided direct evidence for the existence of a soluble mediator released by Kupffer cells. The identity of this mediator remains unknown, however, it is less than 12kD, the pore size of the semi-permeable membrane employed in the experiments.

The role of the inflammatory cytokines as mediators of P450 depression has been the focus of recent research. Interleukin-1 (Dinarello, 1984), tumor necrosis factor (Ertel *et al.*, 1991) and interleukin-6 (Heinrich *et al.*, 1991; Ertel *et al.*, 1991) are released by macrophages following the administration of endotoxin to mice, and

have been shown to either directly or indirectly produce depression of cytochrome P450 in mouse models. Purified interleukin-1 is capable of directly depressing P450 metabolism *in vitro* when incubated with primary cultures of hepatocytes (Peterson and Renton, 1986b; Ghezzi *et al.*, 1986b). Furthermore, *in vivo* administration of human or murine recombinant interleukin-1-α or murine interleukin-1-β produced significant reductions in P450 catalysis in both C3H/HeN and C3H/HeJ mice (Ghezzi *et al.*, 1986b; Shedlofski *et al.*, 1987; Bertini *et al.*, 1988), demonstrating that both strains are sensitive to the effects of the cytokine. This is an important distinction as the C3H/HeJ strain has been shown to lack interleukin-1 (Rosenstreich *et al.*, 1978), tumor necrosis factor (Beulter *et al.*, 1986) and interferon (Apte *et al.*, 1977) release in response to endotoxin treatment, and to be resistant to ethoxycoumarin deethylase depression following the administration of endotoxin (Ghezzi, 1986b). The fact that the C3H/HeJ strain exhibits no P450 depression following LPS treatment, but does exhibit depression in response to IL-1 administration implies IL-1 as a potential mediator of P450 depression following treatment with LPS.

The capacity of tumor necrosis factor for direct P450 depression appears to be limited. Although the administration of human recombinant tumor necrosis factor was associated with depressed levels of P450 and ethoxycoumarin activities in mice (Ghezzi, 1986b), it was found that the *in vitro* incubation of hepatocytes with the cytokine had little effect on catalytic activity (Ghezzi, 1986a; Bertini *et al.*, 1988). Further experiments by Bertini (1989) demonstrated that conditioned medium from tumor necrosis factor-treated monocytes contained a substance capable of depressing

ethoxycoumarin activity in primary cultures of mouse hepatocytes, and that this depressant effect could be blocked by the presence of anti–interleukin–1 antibodies. This difference in the properties of interleukin–1 and tumor necrosis factor is in contrast to the usual spectrum of effects in that these two cytokines generally have similar biological actions (Dinarello, 1987).

The depressant action of interleukin–6 on the induction of P4502B1/2 in isolated rat hepatocytes has been demonstrated by Williams and co–workers (1991), however, *in vivo* adminstration of human recombinant interleukin–6 had no effect on P450 levels in rats 24 hours following treatment (Wright and Morgan, 1991). The role played by interleukin–6 and more importantly, the interplay of the various inflammatory cytokines on the regulation of P450 requires clarification.

4. Importance of P450 Research

It has long been recognized that the safe administration of drugs to humans can only be accomplished in light of a thorough understanding of the physiological fate of these compounds following their entry into the body. The risk for drug toxicity, and conversely, reduced therapeutic benefit, along with drug interactions can be substantially reduced when therapeutic regimens are designed with a knowledge of the P450 system in mind. The advances made in P450 research have made this task much easier.

The diversity of human P450 gene regulation is illustrated by studies revealing the wide variability of catalytic activities of certain isoforms among individuals.

Marked differences in the content of P450IA1 (Jaiswal *et al.*, 1985), P450IA2 (Wrighton *et al.*, 1986; Sesardic *et al.*, 1989), P4503 isoforms (Guengerich *et al.*, 1986; Gonzalez *et al.*, 1988; Waxman *et al.*, 1988; Yoo *et al.*, 1989), and P4502E1 (Wrighton *et al.*, 1986; Yoo *et al.*, 1989) have been observed in studies of human liver samples. These differences can be attributed in part to exposure to environmental or dietary chemicals. Patients should therefore be treated with caution until their unique P450 profile has been assessed through observation of the side effects and therapeutic efficacy of their medications.

A recognized source of diversity among human P450 genes is the propensity of certain gene families for mutations leading to the presence of mutant alleles. The presence of two or more forms of a P450 gene (or any gene) is termed a genetic "polymorphism". A well-studied P450 polymorphism is that leading to the presence of both "poor" and "extensive" metabolizers of the antihypertensive agent, debrisoquine (Idle and Smith, 1979; Eichelbaum, 1986; Idle, 1988). Recent research has determined that in poor metabolizers, the presence of a defective 2D gene leads to the production of a variant RNA transcript which cannot be properly spliced. In keeping with the variability of P450 gene structure and function, there are several types of mutant transcripts which were found to give rise to the poor metabolizer phenotype (Gonzalez *et al.*, 1988). Other polymorphisms have been identified for the metabolism of S-mephenytoin (Inaba *et al.*, 1984; Kupffer and Preisig, 1984; Ward *et al.*, 1987) and for the metabolism of nifedipine by P4503 isoforms (Kleinbloesem, *et al.*, 1984; Wrighton, *et al.*, 1989). An additional level of complexity is added by the association

of ethnic background and P450 polymorphisms: while the debrisoquine polymorphism seen in Caucasians is rare among the Japanese (Nakamura *et al.*, 1985), the S–mephenytoin defect is relatively prominent in Oriental populations, affecting up to 18% of subjects tested (Ward *et al.*, 1987).

Given the scope for P450 variability with regard to the multiplicity of isoforms, the potential for induction or inhibition, the multiplicity of substrate preferences, and individual genetic differences, it is not surprising that drug toxicity and drug interactions commonly arise. Cytochrome P450–dependent toxicity is commonly observed in particular for drugs with a narrow therapeutic window, including the anticoagulant coumadin compounds, antiepileptics such as phenytoin and carbamazepine and the respiratory stimulant, theophylline. The complexity of P450 regulation and function must be understood to avoid such iatrogenic morbidity. The depression of P450 genes and proteins in the presence of hepatic stress responses to inflammation and regeneration presents a further obstacle to the safe use of medications in the treatment of medical illness. While information is accumulating by way of animal studies, relatively little is known of how human P450s are altered by generalized acute or chronic inflammatory illness, or by the host of diseases which specifically affect the liver. In particular, the interaction of P450 depression with the induction by environmental and pharmaceutical chemicals requires clarification.

Another focus of questioning in P450 research is the potential for particular P450 profiles to confer either susceptibility or resistance to certain forms of cancer. Cytochromes P4501A1 and 1A2 are induced by cigarette smoking (Guengerich, 1989;

Guengerich and Shimada, 1991), and are well-known activators of carcinogens. However, the clearance of carcinogenic compounds is enhanced by the induced levels of enzyme, pointing to a potentially protective effect of induction. A link between cancer and elevation of P4502E1 has been suggested (Guengerich *et al.*, 1991). P4502E1 is readily induced by small polar compounds including ethanol (Koop and Coon, 1986), and industrial solvents such as benzene (Johansson and Ingelman-Sundberg, 1988), chloroform (Brady *et al.*, 1989), and trichloroethylene (Guengerich *et al.*, 1991). Sustained induction due to ethanol abuse is correlated with elevated risk for certain forms of cancer in alcoholism (Shikata *et al.*, 1976; Popper *et al.*, 1977; Brechot *et al.*, 1985; Farinati *et al.*, 1989). The propensity for P4502E1 to efficiently support hydroxyl radical-mediated reactions (Feierman *et al.*, 1985; Dicker and Cederbaum, 1987) provides a mechanistic link to the association of cancer and elevated levels of the enzyme. In keeping with this link, disulfiram, a mechanism-based inhibitor of P4502E1 (Guengerich *et al.*, 1991; Brady, *et al.*, 1987), inhibits the carcinogenicity of 1,2-dimethylhydrazine (Fiala, 1981). Indeed, cruciferous vegetables such as cabbage and brussel sprouts have been found to contain the 2E1 inhibitor, phenethyl isothiocyanate (Ishizaki *et al.*, 1990) suggesting a mechanism for the protective effect that consumption of these vegetables appears to have with regard to colon cancer (Wattenberg, 1983). Attempts have also been made to draw empirical links between debrisoquine phenotype and lung cancer and bladder cancer (Ayesh *et al.*, 1984), but the suggestion remains controversial. Continued study of human P450s will provide a basis for understanding individual susceptibilities to many types of

chemically induced cancer.

Apart from the tremendous clinical impact of P450 research, information gained on the mechanisms of genetic expression and regulation of these enzymes has contributed to the general understanding of gene regulation, mutation and evolution. The study of P450 genes offers potential for elucidating the mechanisms governing the processes of developmental gene regulation, as well as the processes of gene conversion, duplication and regulation by *cis*– and *trans*–acting factors.

Coon discusses an interesting biochemical application of P450 research in his recent review (Coon *et al.*, 1992), describing the scientific development of techniques by which P450s may be used in the synthesis of chemicals which require the stereo– or regio–specific insertion of oxygen atoms. These reactions can be very difficult to accomplish by the available methods of organic chemistry. Microsomal preparations are being used increasingly to screen pharmaceutical compounds for the production of toxic metabolites or other adverse effects (Coon *et al.*, 1992). In light of increasing environmental concerns, it appears that P450s may be able to play a role in the metabolism and detoxification of pollutants (Coon *et al.*, 1992). The heterologous expression of specific forms of P450 in bacteria would provide a useful tool for ridding the environment of unwanted chemicals, exploiting the mechanisms which evolved to clear the mammalian system of these same xenobiotics. Overall, the study of this diverse and versatile group of enzymes has generated important contributions to research in fields as diverse as molecular biology, evolutionary biology, clinical pharmacology, oncology, and endocrinology, among others. Continued research will

permit further understanding of how P450s contribute to a host of physiologic and pathologic processes in mammalian systems.

B. Hepatic Response to Physiological Stressors

1. The Hepatic Response to Nutritional Deficiency

a. General Concepts

To explore the impact of isocaloric protein deficiency on hepatic cytochrome P450 regulation, it is necessary to understand the physiology of amino acid and protein metabolism in individual tissues as well as in the body as a whole. Much of the foundation of knowledge in this field was built up in the late 1960's to late 1970's, drawing on a broad spectrum of disciplines including protein chemistry, endocrinology, nutritional chemistry and third world nutrition and healthcare.

Protein intake is considered nutritionally adequate when the nitrogen and amino acid requirements of the animal are satisfied. Alterations in the pattern of protein synthesis in both liver and muscle of rats can be seen when the protein proportion of daily intake is shifted above or below the optimum (Giovanetti and Stothers, 1975; Wannemacher and Allison, 1968; Howarth, 1972; Coward *et al.* 1977; Garlick *et al.*, 1975; Millward *et al.*, 1975). The liver possesses the unique ability to rapidly and continuously respond to changes in the nutritional status of the body by altering its patterns of protein expression. This is both practical and necessary attribute for an organ which sits at the junction of incoming nutrients and other substances with the body's circulation. The liver's primary function in protein metabolism is to act as a

buffer for the maintenance of appropriate levels of circulating amino acids and plasma proteins. The total mass of liver protein in the rat is only twice the total albumin mass, necessitating considerable economy in the utilization of hepatic protein stores (Waterlow *et al.*, 1978).

Following the ingestion of a protein/carbohydrate meal, the liver has been shown to convert up to 57% of the absorbed amino acids to urea, allowing only 23% to pass through unchanged. The remaining 20% are utilized for hepatic synthesis of fixed and plasma proteins (Elwyn, *et al.*, 1968; Elwyn, 1970). This anabolic response seen during the post–absorptive phase is followed by a catabolic state in which the liver maintains the circulating level of amino acids and plasma proteins at the expense of its own protein and of certain circulating proteins which are catabolized for their amino acids (Bloxam, 1972). The liver therefore removes a large fraction of the dietary influx of amino acids, and it modifies the fraction which is later released from the liver into the systemic circulation (Bloxam, 1972).

Figure 3 summarizes the fluctuations in hepatic protein content which occur according to the pattern of food intake in rats (Millward, *et al.*, 1974). Following a high protein meal, the protein content of the liver begins to rise, peaking at 125% of initial values by 12 hours post ingestion. The protein content then declines by 18 hours post ingestion. When given a protein free meal, the liver exhibits a steady decline in hepatic protein content.

The response of hepatic protein mass to isocaloric protein deficiency and starvation was first quantified in the 1930's by Addis. It was found that after 10 days

FIGURE 3.

Variation in liver protein content in meal-fed rats. In response to a protein-containing meal at the 24 hour time point, liver protein content increases by about 25%, falling again over the subsequent 18 hours. After a protein-free meal at the 48 hour time point, there is a steady fall in total liver protein. After Waterlow *et al.*, 1978; p.699.

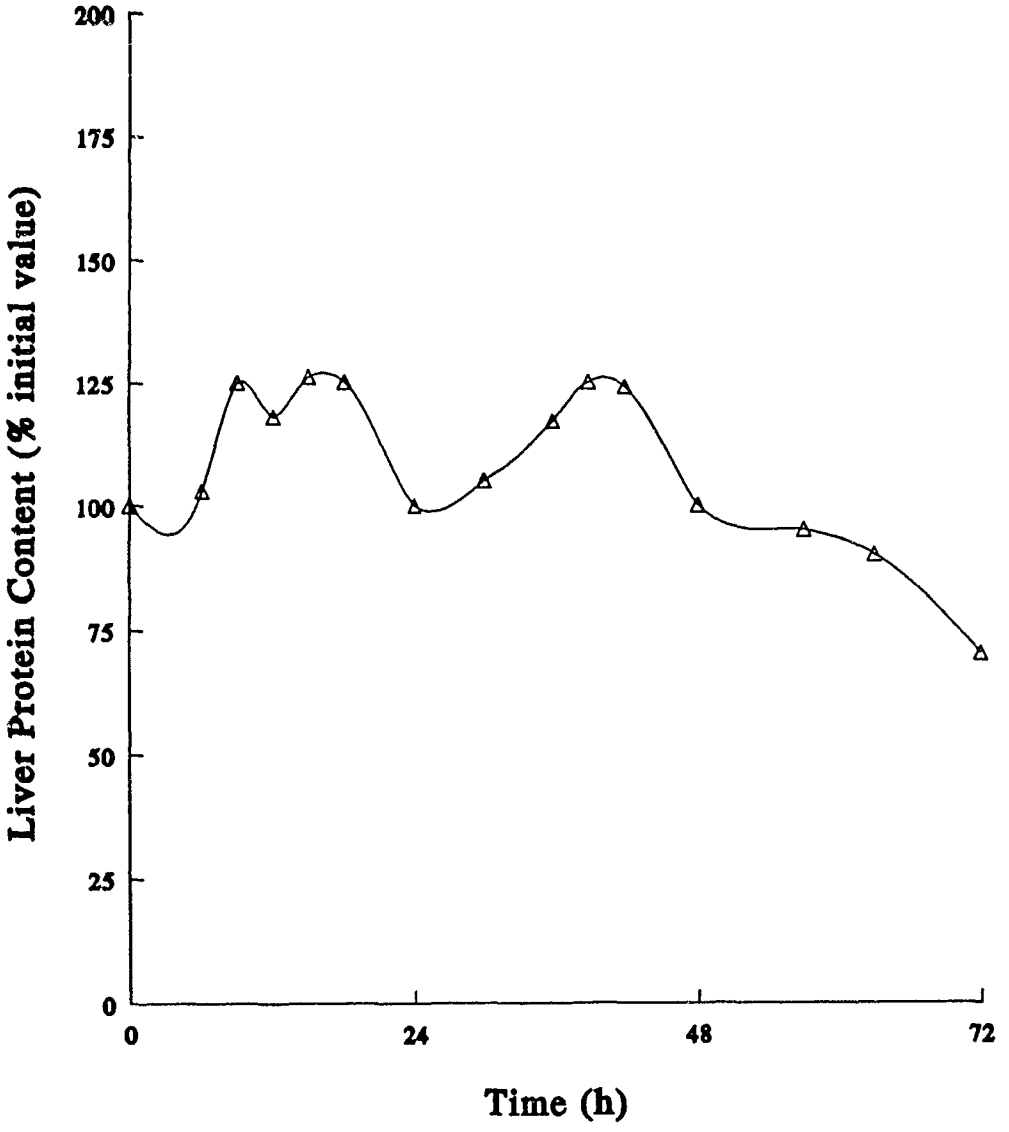

Liver Protein Content (% initial value)
200
175
150
125
100
75
50
25
0
0
24
48
72
Time (h)

FIGURE 3.

on a protein free diet, 28% of hepatic protein mass was lost, and that most of this loss took place in the first 2 days of nutritional deficiency (Addis *et al.*, 1936). It has subsequently been shown that even for periods of up to 4 weeks of protein deprivation, the maximum loss of hepatic protein is in the range of 20 to 30 percent. If the caloric supply is also curtailed, the loss is amplified to 60% or more, the maximum which can be tolerated (Coward *et al.*, 1977).

Early studies attempting to determine the mechanism of protein fluctuation in the liver focused on rates of total liver synthesis and breakdown. Following food intake, liver protein content increased at the same rate as the measured rate of synthesis suggesting that protein breakdown was negligible during this phase. However, as the animals entered a fasting state, a rapid loss of hepatic protein occurred despite well maintained protein synthesis, suggesting that protein breakdown had increased. The conclusion drawn is that while protein synthesis remains fixed independent of food intake, the rate of protein breakdown is adjusted up or down respectively depending on the relative scarcity or abundance of circulating amino acids (Millward, *et al.*, 1974; Garlick, *et al.*, 1973; Mortimore and Ward, 1976). The total synthesis of hepatic proteins was found to be well maintained in animals which were starved or protein deprived for periods of up to 48 hours (Garlick *et al.*, 1975). Upon re-feeding, previously malnourished rats show a dramatic increase in hepatic protein mass, exhibiting an increase in the synthesis of both fixed and secreted liver proteins (Conde and Scornik, 1976; Morgan and Peters, 1971; Kirsch, 1968).

The fractional synthetic rate of fixed liver proteins has been found to be well

maintained in isocaloric protein deficiency and total caloric restriction for periods of up to 3 weeks (Millward *et al.*, 1975; Garlick *et al.*, 1975). The synthesis of plasma proteins, however, is reduced (Waterlow and Stephen, 1968). The reduced synthesis of albumin during isocaloric protein deficiency has been well-studied (Haider and Tarver, 1969; Kirsch *et al.*, 1968; Jeejeebhoy *et al.*, 1973), as has that of transferrin (Jeejeebhoy *et al.*, 1973). Jeejeebhoy *et al.* (1973) also found that the synthesis of the circulating protein, fibrinogen, was increased during isocaloric protein deficiency. This pattern of depressed albumin synthesis and increased fibrinogen synthesis is analogous to other situations of hepatic stress including the acute phase response to inflammation.

The liver's key partner in the regulation of protein metabolism is skeletal muscle. Skeletal muscle has long been known to act as its own reservoir for glucose production via gluconeogenesis as well as for the provision of essential amino acids for the maintenance of essential glucose and protein synthesis for other tissues. The losses of body nitrogen incurred in the first few days of nutritional deficit are initially borne by the liver (Addis, 1936) and the gastro-intestinal tract (Ju and Nasset, 1959). Following this, however, the major part of the loss is incurred by skeletal muscle. In the rat, the skin also plays a substantial role in buffering lost body nitrogen as the proportion of skeletal muscle is smaller in these animals (Waterlow and Stephen, 1966).

In contrast to the liver's well-maintained level of protein synthesis in nutritional deficiency, in skeletal muscle, the rate of protein synthesis is markedly

reduced. This is the initial step in an adaptive response by muscle which leads to a decrease in the rate of muscle protein turnover generally. This response occurs very quickly, and is apparent after only 1 day of either isocaloric protein deficiency or fasting. Following this, there is a fall in the rate of breakdown so that over the initial 48 hours of deficiency, there is negligible net loss of protein from muscle. After 4 days of starvation, however, the fractional breakdown rate is nearly twice that found in the fed state (Waterlow *et al.*, 1978, pp. 628–637).

The patterns of protein synthesis and breakdown in liver and skeletal muscle resulting from nutritional deficits can be partially explained by known patterns of hormone flux which occur in response to food ingestion or deprivation. The effects of isocaloric protein deficiency and fasting on corticosteroid/insulin ratios in the rat are illustrated in Figures 4 and 5. It has been found that glucocorticoid concentrations are raised in starvation to a greater extent than in isocaloric protein deficiency in both rats (Coward *et al.*, 1977) and humans (Rao, 1968). The overall effect of corticosteroids is that of growth suppression and muscle wasting, inducing a negative nitrogen balance wherein protein is selectively lost from lean muscle tissue and gained by the liver (Long *et al.*, 1940; Munro, 1964). A corticosteroid/insulin ratio greater than 6 is considered the level beyond which further growth of muscle is prevented (Waterlow *et al.*, 1978, p.671). The activities of several hepatic gluconeogenic enzymes are increased by glucocorticoids, enhancing the liver's capacity to produce glucose from amino acids (Lin and Knox, 1957; Segal and Kim, 1963).

Insulin's primary action on skeletal muscle serves to promote the uptake of

FIGURE 4.

Changes in plasma insulin levels in adult rats fed low protein diets *ad libitum* or in restricted amounts. Open circles represent well–fed rats, open triangles represent isocaloric protein deficient rats, and open squares indicate fasted rats. Taken from Waterlow *et al.*, 1978, p. 672.

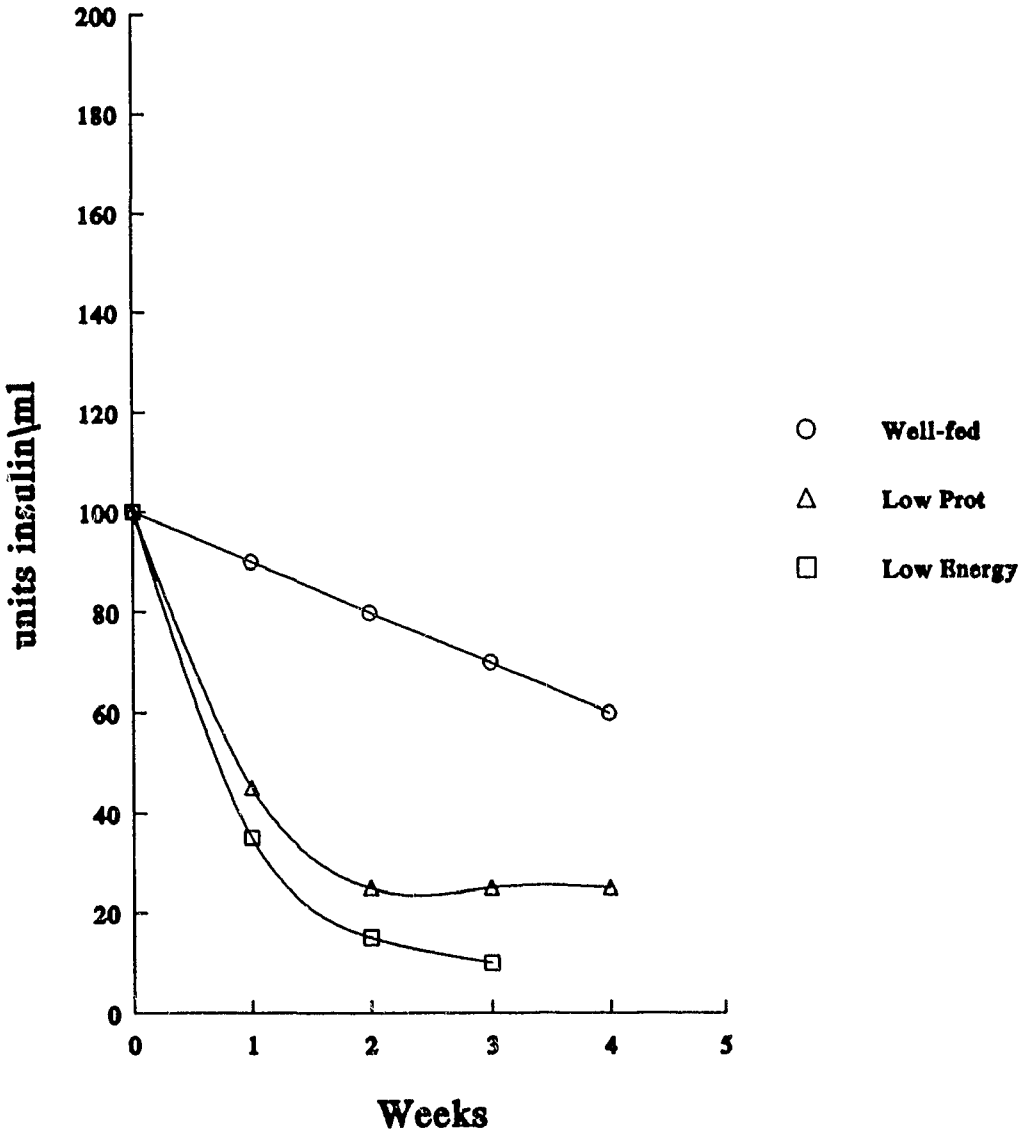

200
180
160
140
120
100
80
60
40
20
0
units insulin\ml
0
1
2
3
4
5
Weeks
Well-fed
Low Prot
Low Energy

FIGURE 4.

FIGURE 5.

Changes in plasma corticosterone levels in adult rats fed low protein diets *ad libitum* or in restricted amounts. Open circles represent well-fed rats, open triangles represent isocaloric protein deficient rats, and open squares indicate fasted rats. Taken from Waterlow *et al.*, 1978, p. 672.

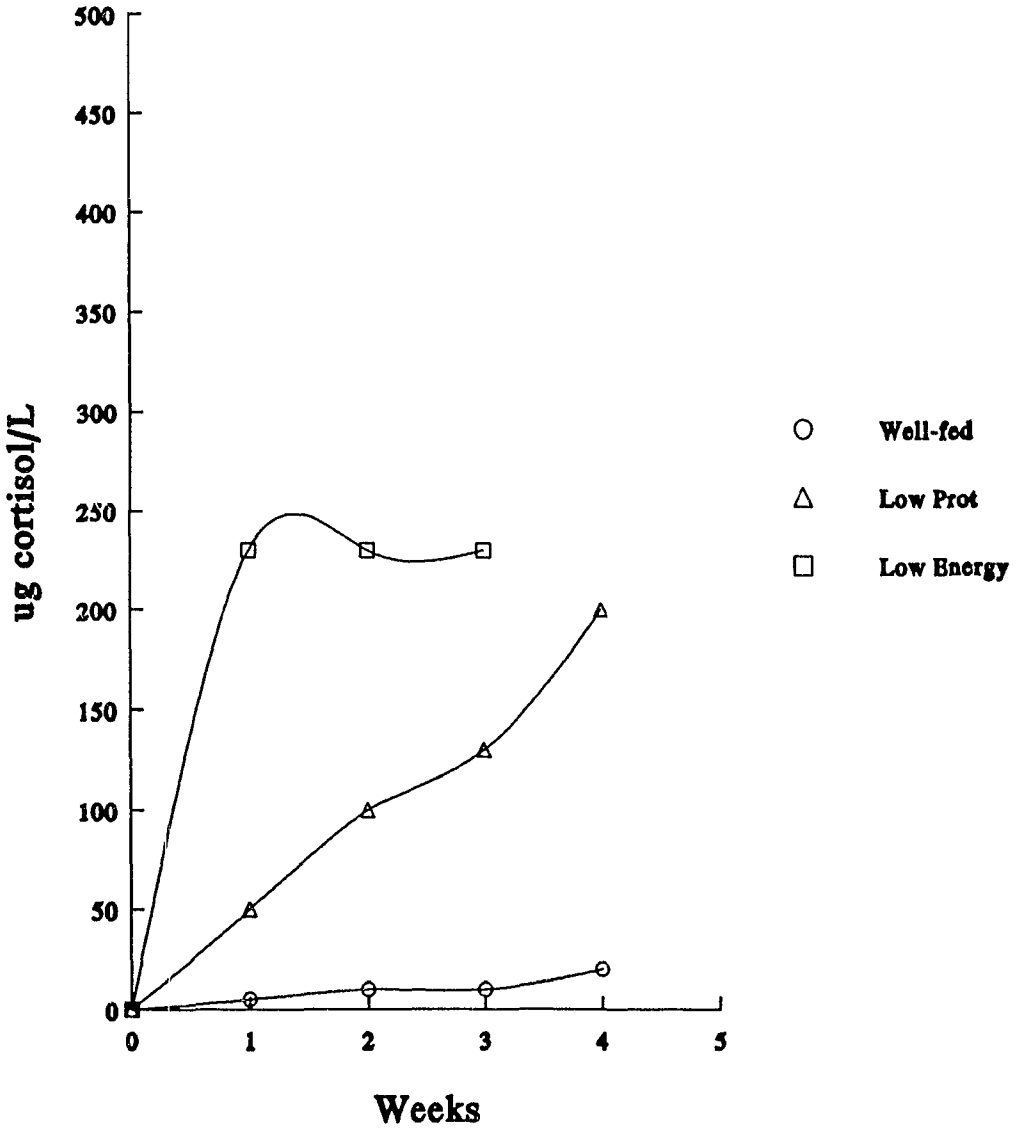

500
450
400
350
300
250
200
150
100
50
0
ug cortisol/L
0
1
2
3
4
5
Weeks
Well-fed
Low Prot
Low Energy

FIGURE 5.

FIGURE 6.

Changes in the cortisol to insulin ratio in adult rats fed low protein diets *ad libitum* or in restricted amounts. Open circles represent well–fed rats, open triangles represent isocaloric protein deficient rats, and open squares indicate fasted rats. Taken from Waterlow *et al.*, 1978, p. 672.

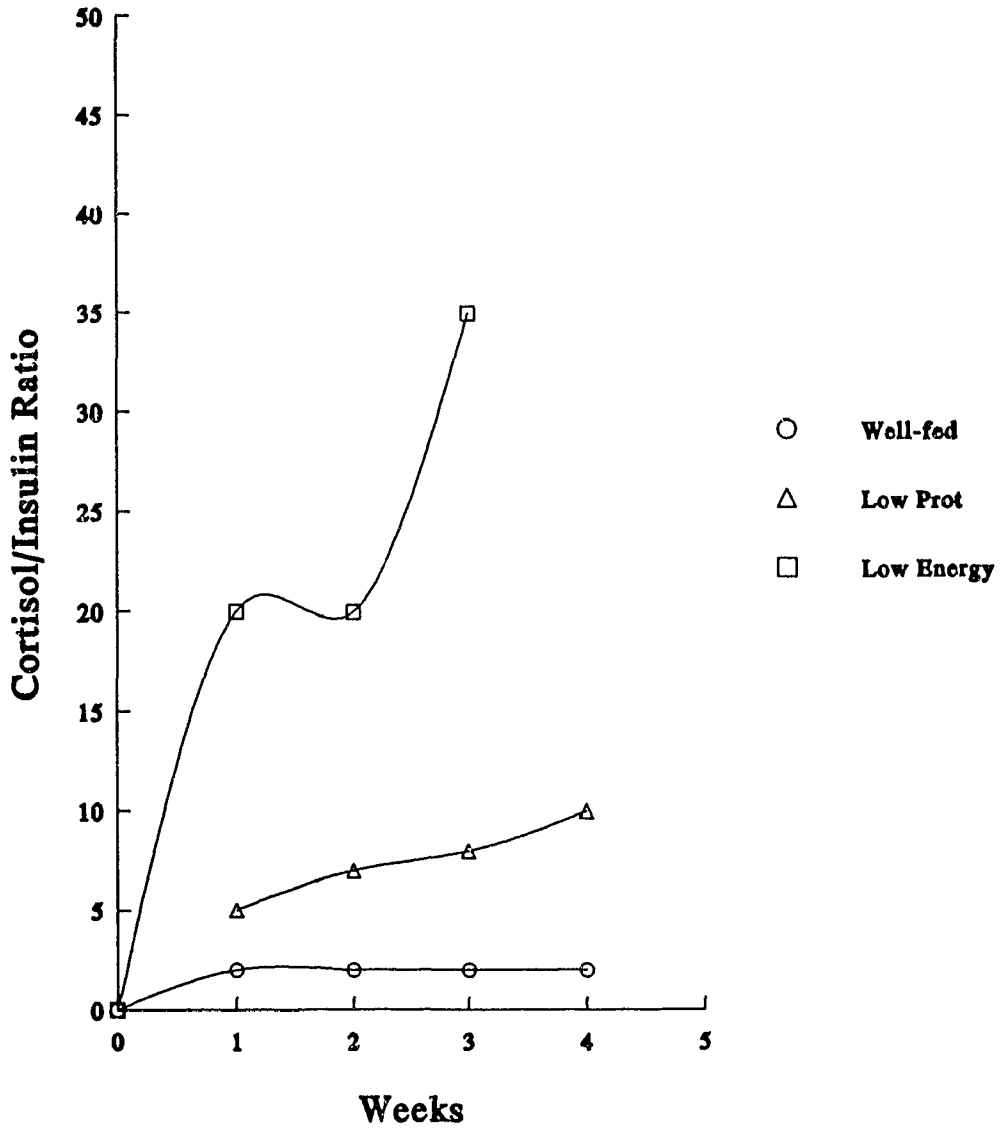

Cortisol/Insulin Ratio
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Weeks
Well-fed
Low Prot
Low Energy

FIGURE 6.

amino acids and synthesis of muscle protein (Hay and Waterlow, 1967; Kurihara and Wool, 1968; Sender and Garlick, 1973). The liver's response to insulin, found in depressed quantities in both isocaloric protein deficiency and starvation, is less clearcut. Early studies suggested that insulin promotes the marked suppression of protein breakdown, such as that seen in the fed state, with minimal effects on hepatic protein synthesis (Mortimore and Mondon, 1970; Woodside and Mortimore, 1972). More recent findings by McHardy, *et al.*, (1991), however, indicate that postprandial changes in hepatic protein metabolism occur independently of elevated postprandial insulin levels. Glucagon, found in high levels during the fasting state, acts primarily to stimulate proteolysis in the liver (Woodside *et al.*, 1974). The effects of growth hormone on liver and muscle protein synthesis are multiple and complex, and beyond the scope of this discussion. Briefly, the primary effect of growth hormone is to stimulate the uptake and oxidation of amino acids by the liver (Jefferson and Korner, 1967; Jefferson *et al.*, 1975), and to stimulate amino acid uptake and protein synthesis in skeletal muscle (Manchester, 1970; Young, 1970; Kostyo and Nutting, 1973; Kostyo and Reagan, 1976). It is notable that in both kwashiorkor (isocaloric protein deficiency) and marasmus (protein and caloric deficiency), the level of growth hormone is high, and falls to normal with refeeding (Pimstone, 1966; Pimstone, 1968; Gardner and Amacher, 1973). The levels of the somatomedins, believed to mediate many of the effects of growth hormone, conversely are low in nutritional deficiency, and rise during recovery (Pimstone *et al.*, 1973).

Several hormones including androgens, steroids, insulin, glucocorticoids, and

thyroxin have long been noted to influence P450 drug metabolism (Booth and Gillette, 1962; Conney and Klutch, 1963; Conney 1967). In particular, the high glucagon levels found in conditions of starvation and insulin-dependent diabetes are associated with decreased metabolism of aminopyrine, hexobarbital, chlorpromazine and codeine, and with increased aniline *p*-hydroxylation (Kato *et al.*, 1970; Dixon *et al.*, 1961; Aranda and Renton, 1975).

b. Isocaloric Protein Deficiency as a Model of Hepatic Competence to undergo Mitosis.

The liver is unique in its ability to regenerate lost tissue mass. Removal of up to 70% of the liver in rats can be fully compensated within one or two weeks. The process is not one of true "regeneration" *per se* in that the excised lobes do not regrow, but rather, the remaining lobes undergo one or two cycles of cell division and considerable hypertrophy to the point that the previous tissue mass is functionally restored (Higgins and Anderson, 1936). In addition, there is a significant increase in polyploidy of the hepatocytes (Goss, 1964).

Several models have been used to study the events of hepatic cell division. Hepatocyte proliferation occurs in response to viral hepatitis, cirrhosis and hepatotoxicity from agents such as carbon tetrachloride and dioxins (Smuckler and James, 1984; Zimmerman, 1982). In these instances, hepatocyte division occurs in the context of hepatic cell death, however, it is also possible to induce DNA synthesis and mitosis in normal hepatocytes with agents such as the organochlorine insecticides and

hexachlorobenzene, by carcinogenic agents such as 2–acetylaminofluorene, or by subjecting animals to an isocaloric protein deficient feeding regimen (Bucher, McGowan and Patel, 1978; Schulte–Hermann, 1974; Short *et al.*, 1972).

One of the difficulties of studying the effect of cell division on hepatic P450 expression is the concurrent influence of inflammation and physiological stress which is incurred by viral infections, hepatotoxic agents or by the process of partial hepatectomy itself. Agents such insecticides and carcinogens also have independent effects on P450 as they are generally substrates for P450 catalysis and are often P450 inducers. A nutritional model of hepatic competence for regeneration that is free of such confounding influences has been characterized by Mead *et al.* (1990). It was found that maintaining rats on an isocaloric protein deficient feed produced a "priming" effect on the liver such that hepatocytes prepared from the livers of isocaloric protein deficient animals underwent cell division at an earlier time point than hepatocytes prepared from control animals. In culture, hepatocytes generally require 48 to 72 hours to complete a mitotic cycle (Mead *et al.*, 1990), however, hepatocytes from isocaloric protein deficient rats had completed one mitotic cycle after only 24 to 36 hours in culture. Following partial hepatectomy, 24 to 30 hours is required for the completion of one mitotic division (Fausto and Mead, 1989).

In addition, liver from isocaloric protein deficient rats exhibited expression of the proto–oncogene "c–jun". The c–jun proto–oncogene is a cellular homologue of the avian sarcoma viral oncogene, "v–jun", and is expressed in cultured cells treated with agents such as epidermal growth factor, serum and phorbol esters. Its gene product is a

cis–acting transcription factor called "AP–1". While c–jun is not detectably expressed in normal liver, its expression was shown to increase 13–fold within 30 minutes of partial hepatectomy in mice (Alcorn *et al.*, 1990). The finding that this proto–oncogene is expressed both in the livers of partially hepatectomized animals and isocaloric protein deficient rats, further suggests that isocaloric protein deficient liver has been primed for entry into the cell cycle. These results support the conclusion of Mead *et al.* (1990) that isocaloric protein deficiency leads to a G0 to G1 transition in the hepatocellular cell cycle.

Additional experiments by the group showed that when isocaloric protein deficient rats are administered a hydrolysed casein meal (*i.e.*, a crude amino acid preparation) their livers exhibit an increase in the uptake of ^{3}H–thymidine, suggesting an increase in DNA synthesis. Histological exam revealed increased numbers of mitotic figures observed in the livers of isocaloric protein deficient animals, but not in the livers of control fed or fasting animals. This is taken as evidence that the hepatocyte has progressed from the G1 to the S (DNA synthetic) stage of the cell cycle.

This model is particularly useful for assessing the impact of priming for proliferative activity on the regulation of cytochrome P450. Isolating the replicative priming process from the confounding influences of the other models described above allowed a determination of the effect of alterations in cell cycle on the regulation of hepatic cytochrome P450. Indeed, the depressant effect of partial hepatectomy on hepatic P450 is well known. As part of the thesis studies, a total of sixteen partial

hepatectomies were performed on rats using sham operated animals for controls. The effect of P450 depression following partial hepatectomy was verified (data not included in thesis).

2. The Hepatic Response to Inflammation and Sepsis

"Inflammation" is the term used to denote the response of the body to essentially any type of tissue injury or infection. Perhaps the most familiar form of inflammation is the local inflammation that results from minor trauma, producing the classic signs of redness, heat and swelling. The physiological features of local tissue damage include stimulation of coagulation, generation of kinins, and phospholipid metabolism leading to vasodilatation and cellular migration. These are the direct result of the release of mediators such as histamine and prostaglandins, leading to vasodilatation (redness and heat), and increased vascular permeability (swelling) (Kushner, 1982). Where mild stimuli are involved, the reaction remains localized, however, if the inflammatory trigger is strong enough, the production of cytokines by accumulated granulocytes and mononuclear cells can reach systemic proportions, with cytokines spilling over into the circulation, triggering a cascade of events known collectively as the "acute-phase response".

This response is effected by a cascade of events which unfold in a predictable fashion following infection or other tissue injury. The intensity of the response can be semi-quantitated by the erythrocyte sedimentation rate (ESR) which is primarily determined by the ratio of albumin to globulins in the circulation (Schreiber *et al.*,

1989). This fact was recognized by Fahraeus in 1921 when he demonstrated the elevation of ESR in patients undergoing an inflammatory reaction. Tillett and Francis (1930) noted the consequences of the hepatic acute phase reaction in their description of the aggregation of pneumococcal C–polysaccharide by C–reactive protein in the circulation. C–reactive protein is one of the most responsive of the positive acute phase reactants, increasing several hundred fold in its expression during inflammation (Schreiber *et al.*, 1989). This rapid flux of plasma proteins was first recognized by Avery, who termed the phenomenon the "acute phase response" in 1941. The proteins affected are a heterogeneous group, differing in the kinetics and magnitude of their expression, their physicochemical properties and functional activities. The majority function to enhance immune function or control the non–specific damage of inflammation (for instance, anti–proteinases), although several have unknown functions despite extensive investigation.

The effects of systemic inflammation are far–reaching, encompassing nearly every organ system of the body. The broad range of influence is a result of the concerted action of a cascade of circulating cytokines, released not only from activated monocytes and macrophages, but also from endothelial cells and fibroblasts. The activation of these latter stromal cells affords the reaction its capacity to ensure all tissues of the body receive cytokine exposure.

The classic signs of systemic inflammation reflect the primary target organs of cytokines such as interleukin–1 (IL–1), interleukin–6 (IL–6), and tumor necrosis factor (TNF). These substances have pleiotropic, overlapping functions, and it is felt

that the particular mix of cytokines at the systemic or at the local tissue level serves to fine-tune the exact response of the target tissue (Gauldie, 1990; Koj, 1989). The bone marrow responds with an increased release of white blood cells, including the immature "band" forms characteristic of inflammatory leukocytosis (Kampschmidt, 1981; Elin *et al.*, 1982). In the central nervous system, IL-1 provokes the production of a sleep-inducing peptide (Kreuger *et al.*, 1984), while cytokine-induced PGE_2 production in the hypothalamus causes an elevation of the temperature set-point, resulting in fever (Dinarello and Wolff, 1982). Hypothalamic response also consists of increased corticotropin-releasing hormone, with the resultant increase in ACTH release by the anterior pituitary, leading to elevated levels of adrenocortical glucocorticoids in the circulation (Woloski *et al.*, 1985; Naitoh *et al.*, 1988; Jablons *et al.*, 1989). In addition, nutrient metabolism is altered, in the form of altered gluconeogenesis (Guillem *et al.*, 1982), fatty acid metabolism (Sganga *et al.*, 1985), and protein metabolism with resultant skeletal muscle wasting and negative nitrogen balance (Beisel, 1977; Ozawa *et al.*, 1983).

The liver is an important target of the inflammatory cytokines, and responds vigorously to their presence. The uptake of amino acids by the liver is vastly increased to allow for the increased synthesis of a group of serum proteins referred to as "acute-phase reactants" (Wannamacher *et al.*, 1975; Bereta *et al.*, 1989; Koj, 1989). These proteins comprise a heterogeneous group with differing electrophoretic mobilities and carbohydrate contents (Kushner, 1982). Their physiologic functions are poorly understood. The proteins are often grouped on the basis of the magnitude and direction

of their response to inflammation, with some serum proteins increasing to about 50% of control values, others increasing two- to four-fold, and still others increasing to several hundred-fold of control values. A fourth group consists of proteins which exhibit a decrease in the presence of inflammation (Fey and Fuller, 1987).

The marked commitment of the liver to the synthesis of these proteins suggests an important survival benefit to their presence during inflammation. Their survival benefit is further underscored by the conservation of acute phase reactants through evolution, with one of the phylogenetically oldest examples present in the horse-shoe crab (Atkins, 1984; Pepys and Baltz, 1983). Irving Kushner suggests in his review of the acute phase response that the systemic response to inflammation, and more specifically, the liver's response, promote survival for the same reasons as does the localized inflammatory response: that is, the containment or destruction of infectious agents, the removal of damaged tissue, and the repair of the affected tissues (Kushner, 1982). For example, elevated levels of fibrinogen along with other clotting factors enhance thrombus formation; the increased presence of anti-proteinases protects healthy tissue from proteases released by inflammatory cells or from the lysosomes of dead cells; and scavenger proteins such as hemopexin, which binds heme, and haptoglobin, which binds globin, serve to remove the components of lysed red blood cells.

Many of the hepatic proteins whose synthesis is increased during inflammation have been found to be immunomodulatory (Arora and Miller, 1978; Chisari, 1978; Mold *et al.*, 1981; Pizzo *et al.*, 1982; Baum *et al.*, 1983; Vetter *et al.*, 1983; Wangel

and Kontiainnen, 1983; Whistler *et al.*, 1983; Chisari *et al.*, 1985; Bumgardner *et al.*, 1989). The liver's involvement in both the initiation and modulation of several aspects of both the inflammatory and immune responses is further underscored by the work of Marshall.

The network of macrophages (Kupffer cells) and other inflammatory cells contained in the liver is part of the larger "reticuloendothelial system" (RES) of the body and serves to filter antigenic material entering the body via the portal vein (Marshall *et al.*, 1987). Altered phagocytic function (Hassner *et al.*, 1977) coupled with altered permeability of the gut wall (Howerton and Kolmen, 1972; Deitch *et al.*, 1987; Sori *et al.*, 1988) during critical illness have been associated with elevated levels of gut-derived bacterial products and the development of sepsis with the potential for mutiple organ failure (Saba and Jaffe, 1980; reviewed by Marshall *et al.*, 1993).

The hallmark of the acute phase response is its marked stereotypy. The components of the response are remarkably consistent despite the diversity of pathologic processes which induce it. A central event in the initiation of the cascade is the activation of the macrophage which responds with the production of two main cytokines, IL-1 and TNF (Koj, 1989). These cytokines lead in turn to the release of a third cytokine, IL-6, from a variety of cell types, most importantly, monocytes and macrophages (Aarden *et al.*, 1987; Kohase *et al.*, 1987; Bauer *et al.*, 1988; Horii *et al.*, 1988), fibroblasts (Sehgal and Sagar, 1980; Weissenbach *et al.*, 1980; Content *et al.*, 1982; Poupart *et al.*, 1984; Content *et al.*, 1985; Haegman *et al.*, 1986; Zilberstein *et al.*, 1986; Defilippi *et al.*, 1987; VanDamme *et al.*, 1987a; VanDamme *et al.*, 1987b;

Norioka *et al.*, 1988; VanDamme *et al.*, 1989), and endothelial cells (McAdam *et al.*, 1982; Jirik *et al.*, 1989; Sironi *et al.*, 1989).

Early reports of acute phase protein induction in mouse models by IL–1 (Dinarello, 1984), focused initial attention on the role of this cytokine as a primary mediator of the hepatic acute phase response (Perlmutter *et al.*, 1986). It was soon realized, however, that the full spectrum of acute–phase changes in hepatic gene regulation could not be attributed to this cytokine alone. It was found that TNF (Miura *et al.*, 1987) and IFN–γ (Andus *et al.*, 1988) were also able to independently regulate subsets of acute phase genes. Furthermore, experiments employing primary cultures of hepatocytes and recombinant preparations of cytokines demonstrated that IL–1 and TNF elicit only partial acute phase responses (Darlington *et al.*, 1986; Evans *et al.*, 1987; Gauldie *et al.*, 1987; Koj *et al.*, 1987; Castell *et al.*, 1989) compared with crude cytokine preparations from macrophages or tumor COLO–16 cells (Darlington *et al.*, 1986; Baumann *et al.*, 1987; Baumann *et al.*, 1989) or recombinant IL–6 preparations (Koj *et al.*, 1987; Magielska–Zero *et al.*, 1988; Bereta *et al.*, 1989; Castell *et al.*, 1989; Akira *et al.*, 1990). Interleukin–6 is now considered to be the final common mediator of the acute phase response (Evans *et al.*, 1987; Koj *et al.*, 1987; Mortensen *et al.*, 1988; Fey *et al.*, 1989; Koj, 1989; Kushner *et al.*, 1989; Schreiber *et al.*, 1989). Its production and release from inflammatory cells is an accepted sequela of the events which trigger the inflammatory cascade, and its effect on the liver is seen as central to the full expression of the hepatic acute phase response.

The role of other cytokines and hormones in modulating the primary response

to IL-6 should not be dismissed, however. Koj and coworkers found that additions of IFN-γ to IL-6 preparations attenuated IL-6-mediated stimulation of haptoglobin, α1-antichymotrypsin, and fibrinogen, but augmented the production of α2-macroglobulin (Koj, 1989). Similar interactions of IL-6 with other cytokines have been observed in studies where IL-1 or TNF prevented IL-6 stimulation of some proteins (Darlington *et al.*, 1986; Castell *et al.*, 1989), while IL-1, TNF, and glucocorticoids augmented IL-6 stimulation in an additive fashion for other acute phase reactants (Kilian *et al.*, 1986; Baumann *et al.*, 1988; Magielska-Zero *et al.*, 1988). These findings serve to emphasize the concept that the net effect of the inflammatory cytokine cascade results from a complex mix of signals with the final response determined by the pattern of cytokines present in the locale of each target organ.

Continued research has focused on the molecular mechanisms by which cytokines such as IL-6 effect their stimulation or depression of hepatic acute phase proteins. Specific, high-affinity receptors for IL-1 (Taga *et al.*, 1987; Sims *et al.*, 1988; Urdal *et al.*, 1988; Bomsztyk *et al.*, 1989), IL-6 (Yamasaki *et al.*, 1988; Hirata *et al.*, 1989; Taga *et al.*, 1989; Loetscher *et al.*, 1990), and TNF (Yonehara *et al.*, 1989; Schall *et al.*, 1990; Smith *et al.*, 1990) have been found on most tissue types. In addition, the IL-6 receptor has been found to associate with a 130 kilodalton membrane glycoprotein (gp130) via their extracellular domains following ligand binding to the receptor (Hirata *et al.*, 1989). A similar finding for the TNF receptor has demonstrated the involvement of an associated protein, "Fas" in the transducing

machinery of that receptor (Ganapathi *et al.*, 1989).

Events leading to the transduction of cytokine–receptor binding signals in a variety of cell types are slowly being elucidated. Interleukin–6 appears to have either no effect or an inhibitory effect on signalling pathways including cyclic adenosine monophosphate (cAMP), nitric oxide, eicosanoids and intracellular calcium. For example, IL–6–responsive rat thyroid FRTL–5 cells exhibited no increase in cAMP levels in response to IL–6 treatment (Nishiyama *et al.*, 1993), rat Kupffer cells exhibited no increase in nitric oxide production following IL–6 treatment (Gaillard *et al.*, 1992), fetal rat calvarial bone–forming cells exhibited no increase in phospholipase A2 activity in response to IL–6, but showed increased phospholipase A2 activity following treatment with IL–1 and TNF (Vadas *et al.*, 1991), IL–6–responsive gonadotropes demonstrated no increase in intracellular calcium levels when treated with IL–6 (Masumoto *et al.*, 1991), and in primary cultures of anterior pituitary cells, IL–6 exerted a modulatory effect on second messengers, reducing stimulated production of cAMP, inositol phosphate, and free intracellular calcium (Grimaldi *et al.*, 1992). In contrast, IL–6 was stimulatory for activation of the tyrosine kinase system in human trophoblasts (Neki *et al.*, 1993). Interleukin–1 has been reported to act through activation of tyrosine kinase in a T cell helper line (Munoz *et al.*, 1992) and to increase intracellular cAMP in primary cultures of rat adrenal cells (Tominaga *et al.*, 1991).

The identification of the second messenger pathways involved in the regulation of hepatic acute phase genes has not been clearly elucidated. The involvement of

protein kinase C in the induction of fibrinogen and alpha$_2$–macroglobulin was suggested by experiments employing the phorbol ester 12–*O*–tetradecanoyl–phorbol–13–acetate (TPA), and the diacylglycerol analogue, 1–oleoyl–2–acetylglycerol (OAG) (Gauldie *et al.*, 1987). However, other studies revealed an inhibitory effect of these compounds on the induction of C–reactive peptide, serum amyloid A and fibrinogen by IL–6 (Ciliberto *et al.*, 1987). Regions of the 5'–flanking DNA in the promoters of several acute phase genes have been demonstrated including those for human C–reactive peptide, alpha–1–acid glycoprotein, hemopexin, haptoglobin, and alpha–2–macroglobulin (Prowse and Baumann, 1988; Goldman *et al.*, 1989; Castell *et al.*, 1989; Oliviero and Cortese, 1989; Fey *et al.*, 1989). These regions are important for the binding of IL–6 responsive nuclear factors (Oliviero and Cortese, 1989).

The regulation of acute phase genes, while triggered by the same extracellular signals, is often determined by differing intracellular mechanisms which may be specific to the protein, species, or cell system under examination. For example, IL–6 alone is capable of induction of C–reactive peptide and serum amyloid A in the NPLC/PRF/5 cell line, but not in certain stocks of Hep3B cells which require instead, a combination of IL–6 and IL–1 for the induction of these proteins (Ganapathi *et al.*, 1988). Induction of alpha–1–antichymotrypsin occurs in response to IL–1 in some Hep3B stocks (Ganapathi *et al.*, 1988), but not in others (Helfgott *et al.*, 1989). The FAZA hepatoma line which lacks IL–1 receptors does not exhibit the expected increase in fibrinogen synthesis following treatment with TNF, hepatocyte stimulating factor or phorbol esters (Evans *et al.*, 1987). Similarly, the human hepatoma lines

Hep3B and HepG2 demonstrate differing patterns of acute phase gene expression following treatment with inflammatory agents (Ganter *et al.*, 1989; Prowse and Baumann, 1988).

It now well established that alterations in the synthesis of the acute phase proteins are due primarily to corresponding changes in transcription rates (Baumann *et al.*, 1987; Olivier *et al.*, 1987; Morrone *et al.*, 1988; Kushner *et al.*, 1989; Sehgal *et al.*, 1990). The *cis*–acting elements through which IL–6 alters transcription of several of the positive acute phase reactants have now been characterized. The elucidation of IL–6 responsive sequences in the 5' flanking regions of the genes for human C–reactive peptide, α_1–acid glycoprotein, hemopexin, haptoglobin, and α_2–macroglobulin (Evans *et al.*, 1987; Prowse and Baumann, 1988; Goldman *et al.*, 1989; Oliviero *et al.*, 1989; Poli *et al.*, 1989), has revealed the existence of a variety of discrete elements with little nucleotide sequence similarity (Prowse and Baumann, 1988). An apparently liver–specific *trans*–acting factor, "IL–6DBP", has been identified and characterized in the up–regulation of the promoters of the haptoglobin, hemopexin, and C–reactive peptide genes (Sori *et al.*, 1988). With the role of IL–6 now clearly established as a trigger to altered expression of the hepatic genome, much work remains to be done in elucidating the intracellular mechanisms by which these changes are effected.

RESEARCH PROPOSAL

Cytochrome P450 isoforms and related biotransformations have long been observed to be depressed by agents independently characterized as triggers of systemic inflammation and the hepatic acute phase response (summarized in Table 1). At the time this thesis work was undertaken, the recognition of the role of interleukin–6 as a paracrine and endocrine modulator of hepatic gene expression was in its primary stage of investigation (Gauldie *et al.*, 1987). This work complemented research on the better–known cytokines, interleukin–1 and tumor necrosis factor alpha, which had been found not only to provoke acute phase gene expression in the liver, but also to be produced following the administration of many of the agents also known to result in cytochrome P450 depression. To determine if administration of the cytokines tumor necrosis factor alpha and interleukin–6 is associated with P450 depression, a mouse model was employed for *in vivo* and *in vitro* experiments as mice require smaller doses of the cytokines which are expensive, and in addition, are responsive to human forms of the cytokines which were more readily available at the time the research was undertaken. Specifically, mice were administered tumor necrosis factor alpha or interleukin–6; *in vitro* experiments examined the effect of interleukin–6 on primary cultures of mouse hepatocytes.

There is evidence that the hepatic acute phase response to inflammation serves a "priming" function for hepatocellular proliferation (note Discussion). As P450 depression is a feature of both the acute phase response and hepatic regeneration, it was of interest to determine if this depression occurs by a common mechanism. The

partial hepatectomy model of hepatic regeneration does not consistently separate the process of inflammation from that of hepatic proliferation, making it impossible to determine if P450 depression is occurring in response to inflammation or to the proliferative state *per se*. An isocaloric protein deficiency model which has been shown to produce a G0 to G1 transition of the hepatocyte (Mead *et al.*, 1990) was used to assess this change in cell cycle status in relation to hepatic P450 levels. The advantage of this model for this work is its absence of superimposed tissue damage and inflammation.

It is important to realize that isocaloric protein deficiency is not viewed as a model of the hepatic acute phase response to inflammation. It is not expected that tissue damage and inflammatory phenomena such as fever, leukocytosis, and hepatic acute phase reactant production will occur as a result of isocaloric protein deficiency. Indeed, there are no such reports to indicate that this occurs. Instead, the hypothesis has been put forward that the G0 to G1 transition of isocaloric protein deficiency mimics an hypothetical G0 to G1 transition triggered in the liver as part of an hepatic acute phase response to inflammation, and that, by this common mechanism, P450 depression occurs in both settings.

The induction of various P450 isoforms has been well-studied, and has been shown to be attenuated by inflammation in an isoform-specific manner (Craig *et al.*, 1990; Renton and Knickle, 1990; Delaporte *et al.*, 1990). If inflammation serves to prime the hepatocyte for proliferation, and therefore leads to P450 depression by a mechanism common with that seen in regeneration, it would be expected that the

induction of P450 isoforms would be attenuated in a similar pattern for both inflammation and regeneration (or in this case, the priming for regeneration produced by isocaloric protein deficiency). The transcriptional induction of P4504A1 by clofibrate, the translational induction of P4502E1 by pyridine, and the protein stabilising effect of acetone on P4502E1 were compared in the contexts of isocaloric protein deficiency and systemic inflammation (Poly IC treatment) in order to determine if the two processes produce similar patterns of P450 depression. In addition, experiments which employed the transcriptional blocker, actinomycin D and the translational inhibitor, cycloheximide, allowed an indirect assessment of post-transcriptional and post-translational mechanisms in isocaloric protein deficiency.

The thesis contains 5 sets of distinct inter-related experiments. An itemized description of the underlying problem, hypothesis and experimental design for each is given below.

1. CYTOKINE EXPERIMENTS.

(Corresponding to sections A through C of "Results")

Problem: Cytochrome P450 depression that occurs following the administration of inflammatory agents to rodents had not been clearly linked to the hepatic acute phase response or its known triggers, the inflammatory cytokines.

Hypothesis: The inflammatory cytokines tumor necrosis factor and interleukin-6 will depress hepatic cytochrome P450 and evoke an hepatic acute phase response.

Experimental Design: Mice were treated *in vivo* with recombinant preparations of tumor necrosis factor and interleukin-6 and primary murine hepatocyte cultures were

treated with recombinant interleukin-6.

2. Determination of the effect of Isocaloric Protein Deficiency (G0 to G1 transition) on Cytochrome P450.

(Corresponding to section D of "Results")

Problem: Cytochrome P450 depression that occurs following partial hepatectomy in rodents could be linked either to the effects of inflammation and stress incurred by the surgical procedure itself, to the hepatic proliferative state, or to a combination of these factors. It was of interest to determine if G0 to G1 transition, in isolation from surgical stress and inflammation, would lead to P450 depression.

Hypothesis: Entry of hepatocytes into the cell cycle will be associated with P450 depression in the absence of superimposed inflammation.

Experimental Design: The isocaloric protein deficiency model of hepatocellular priming was employed (Mead *et al.*, 1990). Rats were maintained on isocaloric protein deficient, fasting or control-fed feeding regimens for variable periods of time prior to sacrifice. The fasting group serves as a control for sufficient caloric intake by the isocaloric protein deficient group which were maintained on a diet of 20% glucose. Insufficient intake of the 20% glucose solution would readily convert these rats from isocaloric protein deficiency to a state of fasting or hypocaloric protein deficiency. Where significant differences were observed between the fasting and isocaloric protein deficient groups, the isocaloric protein deficient groups were considered to be truly in a state of isocaloric protein deficiency.

3. Cytochrome P450 Induction during Isocaloric Protein Deficiency.

(Corresponding to sections E through G of "Results")

Problem: Given that isocaloric protein deficiency modulates the expression of constitutive cytochrome P450, is P450 *induction* also modulated by isocaloric protein deficiency.

Hypothesis: Isocaloric protein deficiency will modulate P450 induction in an isoform-specific manner.

Experimental Design: Induction that occurs by different mechanisms could potentially be influenced to different degrees by isocaloric protein deficiency. This may serve to indicate at which levels of regulation isocaloric protein deficiency interferes with P450 regulation. Accordingly, induction experiments were undertaken using the inducers clofibrate, pyridine and acetone, each with a distinct mechanism of induction. Clofibrate induces P4504A1 by increasing transcription, pyridine induces P4502E1 by enhancing translation, and acetone induces P4502E1 by inhibiting protein breakdown. Standard induction protocols were employed to compare the effect of isocaloric protein deficient, fasting and normal feeding on P450 induction.

4. Metabolic Inhibitor Experiments.

(Corresponding to sections H through K of "Results")

Problem: Given that isocaloric protein deficiency affects P450 induction, the level of regulation at which modulation occurs remains unclear.

Hypothesis: Isocaloric protein deficiency modulates P450 induction in a specific

manner.

Experimental Design: The metabolic inhibitors actinomycin D and cycloheximide were used to isolate the post-transcriptional and post-translational components of P450 turn-over in induction experiments. Experiments using actinomycin D allowed an indirect comparison of the post-transcriptional turn-over of P450 isoforms in isocaloric protein deficient versus control-fed rats. Experiments using cycloheximide allowed an indirect comparison of the post-translational turn-over of P450 isoforms in isocaloric protein deficient versus control-fed rats.

5. Cytochrome P450 Induction during Inflammation.

(Corresponding to sections L through N of "Results")

Problem: While the effect of inflammation on P450 induction has been well-studied, in order to allow a comparison between the effects of isocaloric protein deficiency on induction with the effect of inflammation on induction, it was necessary to repeat the induction protocols in the context of systemic inflammation produced by administration of Poly IC.

Hypothesis: The pattern of induction seen during inflammation will be the same as that seen during isocaloric protein deficiency.

Experimental Design: Induction protocols used in the third set of experiments with isocaloric protein deficiency, were repeated and well-fed rats were treated with the interferon inducer, Poly IC.

METHODS

A. Materials

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

acetylacetone

acrylamide

aminopyrine

bovine serum albumin

bromophenol blue

collagenase

clofibrate

dimethylsulfoxide

ethidium bromide

ethoxyresorufin

ethylene diamine tetra-acetic acid (EDTA)

Ficoll (type 400)

formamide

glucose

glucose-6-phosphate dehydrogenase

glycerol

glycine

guanidine hydrochloride

horseradish peroxidase conjugated antibody

lauric acid

lubrol PX

2–mercaptoethanol

molecular weight markers (Western blots)

N,N'–methylene–bis–acrylamide

N,N,N,N'–tetramethyl ethylenediamine (TEMED)

diphosphopyridine nucleotide (NAD)

diphosphopyridine nucleotide, reduced (NADH)

triphosphopyridine nucleotide (NADP)

triphosphopyridine nucleotide, reduced (NADPH)

4–nitrocatechol

o–phenylenediamine dihydrochloride (OPD)

paranitrophenol

polyvinylpyrrolidone (PVP–360)

poly IC

pyridine

resorufin

salmon sperm DNA

semicarbizide hydrochloride

sodium dithionite

sodium dodecyl sulphate

sodium potassium tartrate

sucrose

Tween 20

Reagents supplied by other suppliers:

Reagent	Supplier
acetone	BDH Chemicals, Dartmouth, Nova Scotia
agarose	Bio-Rad Laboratories, Mississauga,Ont.
anti-P4501A1	Oxygene™
anti-P4502C11	Dr. Stelvio Bandiera, University of British Columbia
anti-P4502E1	Oxygene™, Dr. Raymond Novak
barium hydroxide	Fisher Scientific, Toronto, Ontario
carbon monoxide	Union Carbide Canada Ltd., Halifax, N.S.
carbon dioxide	Union Carbide Canada Ltd., Halifax, N.S.
coomasie blue	Merck Chemical Company
copper sulphate	Fisher Scientific, Toronto, Ontario
crude [γ-^{32}P]ATP (~7000 Ci/mmol)	ICN Biomedicals Canada, Ltd., Montreal, Que.
crude [γ-^{32}P]ATP (~6000 Ci/mmol)	NEN Canada Ltd., Quebec
Enhance	NEN Canada Ltd., Quebec
formaldehyde	Fisher Scientific, Toronto, Ontario
fungizone	Flow Laboratories, Inc.

Reagent	Supplier
GeneScreen*Plus*™	DuPont Canada Inc. Dorval, Que.
interleukin-6	Genzyme®, R & D Sytems, Dr. Jack Gauldie, McMaster University
[1-^{14}C]lauric acid (10-30 mCi/mmol)	Amersham Canada, Ltd., Oakville, Ont.
magnesium chloride	BDH Chemicals, Dartmouth, Nova Scotia
oxygen	Union Carbide Canada Ltd., Halifax, N.S.
penicillin/streptomycin	Gibco Laboratories
phenol	BDH Chemicals, Dartmouth, Nova Scotia
phenol Folin-Ciocalteau reagent	Fisher Scientific, Toronto, Ontario
poly IC	Aldrich Chemical Co. Inc., Milwaukee, WI.
RNA ladder (0.24-9.5 kb)	Bethesda Research Laboratories
silica gel 60 TLC plastic sheets	BDH Chemicals, Dartmouth, Nova Scotia
spermidine trihydrochloride	Aldrich Chemical Co. Inc., Milwaukee, WI.
T4 polynucleotide kinase	Pharmacia Inc., Dorval, Que.
trichloroacetic acid	Fisher Scientific, Toronto, Ontario
tumor necrosis factor	Genzyme®
zinc sulphate	Fisher Scientific, Toronto, Ontario

B. Animals and Treatments

Male Sprague–Dawley rats (150–200g) were acquired from Canadian Hybrid Farms, Kentville, N.S. Rats were maintained according to the regimens described below, and were allowed at least 6 days to acclimatize to the facility before use in experiments. Rats were sacrificed by CO_2 asphyxiation, halothane overdose, or cervical dislocation.

Male C57BL/6 mice (25–30g), and male Swiss BALB/c strain mice (25–30g) were obtained from Charles River Laboratories, Montreal, Quebec. They were given free access to food and water and acclimatized for 6 days prior to use. Mice were sacrificed by cervical dislocation.

1. Nutritional Regimens

Experiments which tested the effect of altered cell cycle status on hepatic cytochrome P450 regulation employed the isocaloric protein deficiency model characterised by Mead *et al.* (1990). Isocaloric protein deficient rats were given access to 20% (w/v) glucose or 20% (w/v) sucrose as their only source of water and calories. This regimen was maintained for various periods from 12 to 96 hours, although most experiments involved a 36 hour regimen. There were two control groups. The first were maintained on the usual food and water *ad libitum*, while a second control group was denied food and received only water to determine the effects of global nutrient and caloric deficits relative to specific isocaloric protein deficiency. It should be noted that in characterising their model, Mead *et al.* demonstrated that rats maintained on 20% glucose exhibited the same pattern of effects as those which received a special protein–deficient feed.

In one experiment, the effect of amino acid ingestion following either isocaloric protein deficiency or fasting was assessed by the adminstration of hydrolysed casein to nutritionally deficient rats. Following 72 hours of nutritional deficiency, each rat received 5 ml of a 50% solution of hydrolysed casein by stomach tube. The procedure was carried out under light halothane anesthesia, and the rats were sacrificed 24 hours following.

2. Preparation of Primary Hepatocyte Cultures

Experiments using primary cultures of murine hepatocytes were performed in the laboratory of Dr. Gurmit Singh of McMaster University, Hamilton, Ontario. Mice were anaesthetized with thiopental and subjected to a retrograde perfusion technique in which the exposed heart was cannulated by way of the right atrium and the liver perfused via the superior vena cava. Fluids were drained freely from the liver by cutting the portal vein. The liver was initially flushed for 1 or 2 minutes with Seglen's calcium-free perfusion medium (CFPM) (Seglen, 1973) followed by a 5 minute perfusion with collagenase (50% (w/v) in CFPM). Collagenase was then flushed from the liver by 1 or 2 minutes' perfusion with calcium-free buffer. The digested liver was excised and gently broken into pieces, releasing the hepatocytes and non-parenchymal cells.

The preparation was enriched in hepatocytes via differential centrifugation at 500 rpm (Beckman TJ-6) for 1 minute. The supernatant, containing non-parenchymal cells, was discarded and the hepatocytes, collected at the bottom of the tube, resuspended in buffer. This cycle was repeated 3 or 4 times, after which the hepatocytes were assessed for viability (Trypan blue uptake) and cell number. Viability was generally 85% or higher, and cultures

were typically established at cell densities of 0.5–1.0 x 10^6 cells/ml media (Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin/streptomycin/fungisone). Cultures were allowed to "acclimatize" for 6 to 12 hours before the initiation of experimental protocols. In experiments in which cells were incubated with recombinant murine IL–6, control cultures received equal volumes of sterile saline containing 0.5% BSA (*i.e.*, the carrier protein in the IL–6 preparation).

3. Inflammatory Agents

a. Interleukin–6 (IL–6) (*in vivo*)

The effect of IL–6 on hepatic cytochrome P450 was determined following intravenous (tail vein) administration to mice. Controls received equivalent volumes of intravenous saline. Note that a preliminary experiment showed no effect of 0.5% BSA on P450 levels compared to saline controls. This is important as cytokine preparations are usually prepared and/or diluted with a solution containing 0.5% BSA as a "carrier protein". Several doses and preparations of IL–6 were employed including both human and murine recombinant preparations. Experiments were performed using either Swiss strain or C57/BALBc mice. Animals were sacrificed from 6 to 24 hours following injection in all experiments.

b. Interleukin–6 (IL–6) (*in vitro*)

In Vitro experiments in which cultures of primary murine hepatocytes (from C57/BALBc mice) were incubated with increasing concentrations of murine recombinant IL–6 or with saline were performed in collaboration with Dr. Gurmit Singh of McMaster University, Hamilton, Ontario. Cells were allowed to incubate for 18 hours in the presence of

50, 100, or 200 units of a recombinant murine IL-6 preparation supplied by Dr. Jack Gauldie of McMaster University. Hepatocytes were then solubilized in 4% (w/v) Lubrol and assessed for P450 content and N-Demethylase activity.

c. Tumor Necrosis Factor (TNF)

The effect of TNF on hepatic cytochrome P450 was assessed following intravenous injection of C57/BALBc mice with various doses of murine recombinant TNF. Controls received equivalent volumes of intravenous saline. Animals were sacrificed at 6, 12 or 24 hours following injection.

d. Polyinosinic Polycytidylic acid (Poly IC)

The interferon inducer, Poly IC, was administered to rats at a dose of 20 mg/kg (i.p.). This dose has been shown in previous experiments to produce a significant depression of total P450 and related catalytic activities.

4. P450 Inducing Agents

a. Clofibrate

Clofibrate is a peroxisome proliferator which has been shown to induce the P450 isoform responsible for the metabolism of ω and ω-1 fatty acids (P4504A1). Clofibrate was administered according to the induction protocol of Hardwick *et al.* (1987). This work showed that following the administration of clofibrate at a dose of 500 mg/kg (i.p.), 4A1 mRNA and proteins are significantly elevated. Furthermore, in the first 24 hours following administration, the increases in 4A1 mRNA can be entirely accounted for by elevated rates of gene transcription. Using this induction regimen, the effect of isocaloric protein deficiency and inflammation on the transcriptional induction of P4504A1 was evaluated. The experimental

TABLE 2. Clofibrate Induction Protocol.

Nutritional Regimen	Duration prior to induction	Induction Treatment	Duration prior to sacrifice
Control–Fed (n=8)	36h	Clofibrate or oil (each n=4)	24h
Fasting (n=8)	36h	Clofibrate or oil (each n=4)	24h
Protein Deficient (n=8)	36h	Clofibrate or oil (each n=4)	24h

TABLE 3. Pyridine Induction Protocol.

Nutritional Regimen	Duration prior to induction	Induction Treatment	Duration prior to sacrifice
Control–Fed (n=8)	36h	Pyridine or oil (each n=4)	12h
Fasting (n=8)	36h	Pyridine or oil (each n=4)	12h
Protein Deficient (n=8)	36h	Pyridine or oil (each n=4)	12h

TABLE 4. Acetone Induction Protocol.

Induction Treatment	Duration prior to Nutritional Regimen	Nutritional Regimen	Duration prior to Sacrifice
Acetone (n=12)	10 days	Control, Fast, or Protein Deficient (each n=4)	36h
no Acetone (n=12)	10 days	Control, Fast, or Protein Deficient (each n=4)	36h

design is summarized in Table 2.

b. Pyridine

In experiments designed to assess the effect of isocaloric protein deficiency or inflammation on the induction of P4502E1 by pyridine, animals were administered pyridine according to the induction regimen characterised by Kim and Novak (1990). Kim and Novak determined that the administration of 200 mg/kg (i.p.) pyridine produced an 2-fold induction of para-nitrophenol hydroxylation (PNPH) (a catalytic activity considered to be specific for P4502E1) at 12 hours following treatment. Furthermore, they determined that the induction likely occurs at the level of translation of the mRNA transcript as the induction was affected by the translational inhibitor, cycloheximide, but not by transcriptional interruption with actinomycin D. The experimental protocol is summarized in Table 3.

c. Acetone

In experiments designed to gain insight into the impact of isocaloric protein deficiency orinflammation on protein stability, induction of P450 was effected by the administration of acetone. Acetone was given in drinking water (5% (v/v)) according to the protocol established by Song *et al.* (1989) in which acetone administration was maintained for a period of 10 days before sacrifice. The experimental protocol is summarized in Table 4.

5. Metabolic Inhibitors

Experiments aimed at assessing post-translational kinetics of P450 isoforms employed the translational inhibitor, cycloheximide at a dose of 7.5 mg/kg (i.p.). This agent is very toxic and animals did not survive administration of a dose of 10 mg/kg used in initial experiments. Studies of the post-transcriptional kinetics of P450 isoforms were undertaken

TABLE 5. Protocol for Actinomycin D Treatment following Induction with Clofibrate.

Nutritional Regimen	Duration prior to induction	Inducer	Duration prior to AcD	Sacrifice
Control (n=12)	36h	Clofibrate	24h	0,3,6,12 hours post AcD (each n=3)
Protein Deficient (n=12)	36h	Clofibrate	24h	0,3,6,12 hours post AcD (each n=3)

TABLE 6. Protocol for Actinomycin D Treatment following Induction with Pyridine.

Nutritional Regimen	Duration prior to induction	Inducer	Duration prior to AcD	Sacrifice
Control (n=20)	36h	Pyridine	12h	0,3,6,10 hours post AcD (each n=5)
Protein Deficient (n=20)	36h	Pyridine	12h	0,3,6,10 hours post AcD (each n=5)

TABLE 7. Protocol for Cycloheximide Treatment following Induction with Pyridine.

Nutritional Regimen	Duration prior to induction	Inducer	Duration prior to CHX	Sacrifice
Control (n=12)	36h	Pyridine	12h	0,3,6,8 hours post CHX (each n=3)
Protein Deficient (n=12)	36h	Pyridine	12h	0,3,6,8 hours post CHX (each n=3)

TABLE 8. Protocol for Cycloheximide Treatment following Induction with Acetone.

Induction Treatment	Duration prior to Nutritional Treatment	Nutritional Regimen	Duration prior to CHX	Sacrifice
Acetone (n=12)	10 days	Control or Protein Deficient (each n=6)	22h	0,3,6,10 post CHX (each n=3)
no Acetone (n=12)	10 days	Control or Protein Deficient (each n=6)	22h	0,3,6,10 post CHX (each n=3)

using the transcriptional inhibitor, actinomycin D at a dose of 2 mg/kg (i.p.). This was the maximum tolerated dose, and animals did not survive past 12 hours following treatment. The experimental protocols are summarized in Tables 5 through 8.

C. Molecular Biological Techniques

1. Isolation of Total Cellular RNA

Hepatic tissue was excised immediately following sacrifice, fast–frozen in liquid nitrogen, and stored at −70°C. To extract total RNA, 0.20 to 0.30 g of tissue was measured and immediately homogenized in 1 ml of "solution D" (7.5 M guanidine thiocyanate, 175 μM sodium citrate, 0.79% sarcosyl, 0.72% 2–mercaptoethanol) usinga small glass homogenizer. When all samples were homogenized, 0.1 ml of 0.2 M sodium acetate was added to each tube and well mixed. Next, 1 ml of phenol was added to each and mixed followed by the addition of 0.2 ml of chloroform:isoamyl alcohol (49:1), and again well mixed. Samples were then left on ice for at least 15 minutes before proceeding.

Following centrifugation at 2000 x g for 30 minutes, the resultant aqueous phase from each tube was carefully transferred to clean test tubes, and diluted 1:1 with cold (−20°C) isopropanol. The RNA was allowed to precipitate at −20°C for at least 1 hour or overnight. Samples were centrifuged at 2000 x g for 2 minutes and the supernatant poured off. The RNA pellet was redissolved in 0.3 ml solution D, and upon the addition of 0.3 ml ice cold isopropanol (−20°C), was again allowed 1 hour or overnight to precipitate at −20°C. The precipitated samples underwent a washing procedure in which the samples were alternately pelleted by centrifugation, and resuspended in 1 ml 75% ice cold ethanol. This cycle was

repeated at least three times. Following the final centrifugation step, the pellet was allowed to air dry. When dried, the RNA was dissolved in DPC water (0.4–1.0 ml) and stored at –20°C.

Before RNA samples could be used for Northern or slot blotting, it was necessary to determine the concentration of each sample. This was accomplished by measuring the absorption of UV light at 260 nm. Concentration was calculated on the assumption that an optical density of 1 (through a 1 cm path) corresponds to a concentration of 40 μg RNA/ml (Maniatis *et al.*, 1982). In addition, RNA purity was assessed by measuring the ratio of absorbance at 260 nm versus 280 nm (Maniatis *et al.*, 1982) ensuring only samples with ratios of 1.6 or greater were used.

2. Gel Electrophoresis of RNA

A 1.3% agarose gel was used to separate RNA species under denaturing conditions (2.2 M formaldehyde). Briefly, 2g of high grade agarose was dissolved in 108 ml water by boiling When the solution had cooled to 65°C, 27 ml of 12.3 M formaldehyde, 15 ml of 10X MOPS (0.4 M 3(N–morpholino)propanesulfonic acid, 100 mM sodium acetate, 20 mM EDTA, pH 7.0) and 7.5 μl of ethidium bromide (10 mg/ml in water) were added. When cooled to 55°C, the mixture was poured onto a gel tray measuring 25 cm x 15 cm, and allowed to solidify for 45–60 minutes.

The RNA samples were prepared such that 20 μg RNA was contained in a volume of 4.5 μl of DPC water. A volume of 15.5 μl of FFM was added (deionized formamide:12.3 M formaldehyde:10X MOPS 5:1.75:1, v/v/v; note that formamide was deionized with AG 501–X8 ion exchange resin to neutral pH and stored in aliquots at –20°C). After incubating at

60°C for 15 minutes, the samples were quick-cooled on ice prior to the addition of 5 μl of loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol).

The gel was immersed in 1X MOPS in the electrophoresis chamber. Fifteen wells were available for sample loading but the 2 lateral-most wells were usually avoided. Samples were carefully pipetted into the wells with one well reserved for a kilobase standard (20 μg of lambda DNA *Hin*d III digestion fragments) prepared in a manner identical to the samples.

Upon completion of electrophoresis, the gel was visualized with an ultraviolet lamp to ensure the RNA was appropriately separated. The volume of material in each lane was also examined to confirm that equal volumes had been loaded in each lane.

3. "Northern" Blots

Following separation by gel electrophoresis, the gel was prepared for transfer by washing in 50 mM NaOH for 30 minutes followed by washing in 100 mM Tris-HCl, pH 7.0, for 30 minutes. RNA was transferred to nitrocellulose membrane. Transfer of RNA was carried out using a vacuum blotter (Bio-Rad, Richmond, CA.). Briefly, after washing, the gel was further prepared by filling the wells with molten agar, and allowed to solidify before proceeding. Meanwhile, 3MM filter paper and membrane were cut to measure 15 cm x 20 cm. The membrane was soaked in millipore water for 1 minute before being transferred to soak in 10X SSC (1.5 M NaCl, 0.15 M Na citrate) for 15 minutes. The 3MM filter paper was soaked for 1 minute in 10X SSC prior to use.

Filter paper, gel and membrane were arranged on the vacuum blotter according to the instructions given by Bio-Rad. The transfer was carried out with gel submerged in 10X SSC

with a vacuum of 5" Hg for 90 minutes at room temperature. The success of the transfer procedure was assessed by staining the gel with a solution of 1.0 μg/ml ethidium bromide for 30 minutes, then destaining with water for an additional 30 minutes before visualizing on an ultraviolet transilluminator. Upon transfer completion, the membrane was baked at 80°C for 2 hours in order to covalently link the nucleic acids to the membrane.

4. Albumin Oligonucleotide Probe

The albumin cDNA probe is a 20mer complementary to rat albumin mRNA transcripts along the region from bases 828 to 847 (Sargent *et al.*, 1981). The sequence was compared to other known sequences using a MicroGenie Sequence Analysis Program™ (Queen and Korn, 1984) in order to ensure its specificity. The sequence is also complementary to mouse albumin mRNA. The specificity of the probe was further ensured by Northern analysis which demonstrated that the oligomer binds to a single narrow band in the appropriate weight range for albumin message (2.0 kb). The albumin probe sequence chosen was 5'-d(CTGCCCTGTCATCCGCGCAT)-3', and was synthesized by the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta.

5. Probe Labelling

The oligomer was stored in Tris/EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) at a concentration of 10 pmol/μl. The 5'-OH end of the oligomer was labelled with ^{32}P using a T4 polynucleotide kinase 5' end-labelling kit obtained from Pharmacia. The reaction mixture contained 5 μl of 0.1 M $MgCl_2$/.05 M Tris HCl (pH 7.4), 25 mM spermidine, 60

pmol [γ-^{32}P]ATP (~6000 Ci/mmol or ~7000 Ci/mmol), 2 units of T4 Kinase, and 50 pmol of oligomer in a final reaction volume of 50 µl. The reaction was carried out at 37°C for 30 minutes and was terminated by the addition of 4.5 µl of 0.25 M EDTA. The labelled oligonucleotide was used for probing without further purification.

6. Northern Analysis

Blots were pre-soaked in 2X SSPE (1X SSPE contains 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) for at least 5 minutes before being placed in prehybridization solution (6X SSPE, 1% SDS, 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone and 100 µg/ml sheared salmon sperm DNA; 12.5 ml for Northerns). Membranes were incubated at 42°C for at least 4 hours or left overnight, at which time 5µl of ^{32}P-labelled probe was added to the solution. The blot was incubated at 42°C for at least 4 hours or overnight. The blot was removed from the hybridization solution and washed at 48°C with 2X SSC for a total of 3 washes of approximately 30 to 40 minutes each. The wet blots were wrapped in plastic wrap and exposed to x-ray film (Kodak XAR-5) at -70°C with the aid of an intensifying screen (Cronex Lightning Plus) for 12 to 24 hours.

7. Gel Electrophoresis of Microsomal Proteins

A "mini-gel" protocol for the separation of hepatic microsomal proteins was employed. A 4% acrylamide stacking gel (a final volume of 5 ml contained 0.5 ml acrylamide stock solution (41% acrylamide (w/v), 1.4% bisacrylamide (w/v) in water), 2.5 ml buffer (0.25 M Tris base in 0.2% SDS, pH 6.8), with polymerization catalysed by the

addition of 50 μl 10% ammonium persulfate and 10 μl TEMED) was layered over a 10% acrylamide running gel (3.75 ml acrylamide stock, 3.75 ml buffer (1.5 M Tris Base in 0.4% SDS, pH 8.8) in a final volume of 15 ml, with polymerization catalysed by the addition of 75 μl 10% ammonium persulfate and 15 μl TEMED) to enhance the sharpness of band separation.

Microsomal samples containing 4.44 μg protein/μl were diluted 1:1 with Laemmli buffer (125 μM Tris HCl in 0.5% SDS, containing 20% glycerol (v/v) and 0.002% (w/v) bromophenol blue, pH 6.8) and boiled for 3 minutes. A volume of 4.5 μl of the denatured protein (10 μg) was added to each well. Each gel contained 10 wells. Eight wells were loaded with samples with the two outer-most lanes being reserved for molecular weight standards. The three molecular weight standards (glyceraldehyde-3-phosphate dehydrogenase (36 KDal), bovine serum albumin (66 KDal), egg albumin (45 KDal)) were prepared in a manner identical to the samples of microsomal proteins.

The gel was immersed in buffer (0.025 M Tris Base/0.2 M glycine in 0.1% SDS, pH 8.3) and run at 75V to run proteins through the 4% gel, "stacking" them against the border of the more resistant 10% gel. When proteins were sufficiently stacked, the voltage was increased to 125V to effect separation of the proteins. Stacking typically required about 20 or 30 minutes of 75 V electrophoresis, with protein separation requiring a duration of about 45 to 60 minutes of 125 V electrophoresis. Throughout electrophoresis, the progression of the proteins through the gel was monitored by observing the position of bromophenol blue in the gel.

8. Western Blots

Upon completion of protein separation, the "mini--gel" apparatus was disassembled. The stacking gel was gently scraped away and discarded, and the running gel containing the separated proteins, was placed on 3 pieces of buffer–soaked blotting paper situated on the cathodic chamber of a semi–dry electrophoretic transfer apparatus (Tyler Research Instruments). The gel was overlain with nitrocellulose membrane and 3 more pieces of blotting paper; both membrane and blotting paper had been soaked for at least 5 minutes in transfer buffer (25 mM Tris Base/150 mM Glycine in 20% Methanol (v/v)/0.05% SDS, pH 8.3). The anodic chamber of the apparatus was aligned and gently tightened in place, applying slight pressure to the stack. The transfer module was then connected to a power pack, and protein transfer from the gel to the nitrocellulose was carried out at a voltage of 25V for 15 to 20 minutes. Following transfer, the membrane was allowed to air–dry, and was stored dry at room temperature.

The extent of protein transfer was assessed in some instances by staining the gel with coomasie blue (0.2% (w/v) coomasie blue in 25% (v/v) methanol/10% (v/v) acetic acid). Subsequent destaining of the gel (10% (v/v) methanol/10% (v/v) acetic acid) removed background coomasie staining so that proteins could be visualized. In addition, it is possible to assess protein transfer by staining the blot itself with amido black. The lateral lanes containing the molecular weight standards were routinely stained with amido black in order to judge the molecular weights of the proteins detected in subsequent Western analysis.

9. Western Analysis

Microsomal proteins were identified with the use of specific antibodies. The membranes were blocked with a solution of 5% (w/v) skim milk powder in 2% (w/v) BSA in phosphate bufferred saline (PBS) (140 μM NaCl, 3 μM KCl, 10 μM Na_2HPO_4, 1.8 μM KH_2PO_4, pH 7.4) for about one hour at room temperature. Primary antibody (anti2C11 or anti2E1) at a concentration of 5 μg/ml in buffer (5% milk (w/v)/2% (w/v) BSA with 0.05% (v/v) Tween 20 in PBS) was incubated with membranes in a small plastic pouch, overnight at 4°C or for 4 hours at room temperature. Membranes were then washed three times for 10 minutes each with rocking, in 0.05% (v/v) Tween 20 in PBS. Following the washing procedure, membranes were incubated with horseradish peroxidase-linked secondary antibody diluted 1:5000 in buffer (5% milk (w/v)/2% (w/v) BSA with 0.05% (v/v) Tween 20 in PBS) for one hour at room temperature with rocking, after which the blots were washed as before. The colour reaction was carried out by equilibrating the blots in alkaline phosphatase reaction buffer (0.1 M Tris HCl/0.1 M NaCl/0.05 M MgCl, pH 9.0) for 5 minutes before replacing buffer with a mixture of 10 ml reaction buffer containing 66 μl 5% (w/v) nitro blue tetrazolium in 70% (v/v) dimethylformamide and 33 μl 5% (w/v) 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide. The reaction was allowed to proceed until sufficient colour had developed in the protein bands, usually 30 to 40 minutes, and was terminated by replacing the reaction mixture with "stop" buffer (0.02 M Tris HCl/5 mM EDTA, pH 8.0). Membranes were given a final rinse with distilled water and allowed to air-dry. Photographs were taken and densitometry performed as soon as possible before colour had faded. The comparison of band position to the position of the molecular weight markers

stained with amido black (described above) demonstrated that the antibodies were binding to proteins in the molecular weight range for P450 proteins.

10. Densitometry

Band intensities on Western blots were determined using a MacIntosh OneScanner™ and Ofoto™ and Scan Analysis™ scanning software. The data were expressed as optical density units.

D. Analysis of Microsomal Cytochrome P450

1. Preparation of Microsomes

Immediately following sacrifice, portions of liver (3–4 g for rats, 1–2 g for mice) were excised and placed in ice-cold 1.15% KCl. Using a glass homogenizer, livers were homogenized via 10 strokes with a tight pestle followed by 1 stroke with a loose pestle. Large cellular components such as nuclei and mitochondria were removed from the homogenate by an initial centrifugation at 10,000 x g for 10 minutes at 4°C. The resultant supernatant was centrifuged at 100,000 x g for 40 minutes at 4°C, with microsomal membranes forming a pellet. The pellet was then resuspended in a volume of glycerol buffer (50 mM KH_2PO_4, 50% glycerol, 0.4% KCl, pH 7.4; 4°C) sufficient to produce a 50% suspension by liver weight. Resuspension was performed using a glass homogenizer with 7 strokes of a tight-fitting pestle. Microsomes were stored in aliquots at −70°C.

2. Determination of Microsomal Protein Content

The concentration of microsomal protein was determined by the method of Lowry *et al.* (1951), with BSA (50 μg/ml) as standard. Briefly, microsomes were diluted 100-fold with water before a 1 ml volume was added to 5 ml of Reagent A (2% Na_2CO_3 in 0.1 N NaOH:1% $CuSO_4 \cdot 5H_2O$:2% NaKtartrate$\cdot 4H_2O$, 98:1:1, v/v/v). After 10 minutes' incubation at room temperature, 0.5 ml of 1 N phenol-Ciocalteau reagent was added and well mixed. A second 30 minute incubation allowed for colour reaction to occur, at which time the samples were read in a spectrophotometer for their absorbance at 700 nm. Absorbance readings were used to calculate microsomal protein concentration in units of mg/ml.

3. Determination of Cytosolic Protein Content

The concentration of cytosolic protein was determined by the method of Lowry *et al.* (1951) as described above, using the 100 000g supernatant obtained in the preparation of microsomes.

4. Determination of Microsomal P450 Content

The difference spectrum method (Omura and Sato, 1964) was employed for the quantitation of hepatic microsomal P450, expressed as nmol/mg microsomal protein. Approximately 1 ml of a mixture containing 0.5 ml of microsomal suspension, 0.25 ml of 1 M KH_2PO_4 (pH 7.4) and 1.25 ml of 1.15% KCl was placed in a cuvette. A few crystals of sodium dithionite were added and a baseline scan from 400-500 nm was determined in a single beam spectrophotometer (Beckman DU-70). The cuvette contents were bubbled with

carbon monoxide for 20 seconds and the 400–500 nm spectrum was determined. The concentration of cytochrome P450 was determined using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ at the difference spectrum peak (450–452 nm).

4. Ethoxyresorufin–O–Deethylase (EROD) Assay

Ethoxyresorufin–O–deethylase activity was determined by a modification of the methods of Prough, Burke and Mayer (1977), and of Burke (1985). A volume of hepatic microsomes containing 0.25 mg of microsomal protein was made up to a final volume of 2 ml with 0.1M KH_2PO_4, pH 7.4. Ethoxyresorufin substrate was prepared as a stock in DMSO; a working stock solution prepared from this in turn, contained about 10% DMSO, with an ethoxyresorufin concentration of 310 μM. Cuvettes contained 10 μl (in total volume of 2 ml) of a 31 μM dilution of the ethoxresorufin working stock. The samples were equilibrated at 37°C for 5 minutes. A baseline absorbance was then obtained in a Perkin–Elmer fluorescence spectrophotometer set at an excitation wavelength of 510 nm and an emission wavelength of 586 nm. The reaction was initiated by the addition of 10 μl of 25 mM NADPH (prepared in buffer). The reaction was allowed to proceed for approximately 3 minutes. When all samples had been analysed, the fluorescence of a resorufin standard (10 μM in water) was obtained and utilized to calculate rate of formation of the product. Specifically, the rate of formation was obtained by measuring the rate of rise (in millimetres) of absorbance per unit time, and adjusted according to the fixed scale setting on the fluorimeter. The use of the resorufin standard allowed a determination of how many nanomoles of resorufin product are required to produce a 1 millimetre rise in the absorbance.

6. Aminopyrine N–Demethylase Assay

The aminopyrine N–demethylase assay was performed after the method of Cochin and Axelrod (1959). Microsomes containing 1.2 mg microsomal protein in a volume of 0.4 ml were added to tubes containing 0.4 ml 5 mM aminopyrine, with blanks substituting 1.15% KCl for aminopyrine. A volume of 1.15 ml semicarbizide (13 mM in 100 μM KH_2PO_4/100 μM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) was added to each tube and well mixed. Tubes were equilibrated at 37°C for 5 minutes prior to initiation of the reaction through the addition of 50 μl of 40 mM NADPH. The reaction was allowed to proceed for 10 minutes at 37°C with rocking before being terminated by the addition of 1 ml of 5% (saturated) BaOH followed by the addition of 1 ml 5% $ZnSO_4$. The samples were left on ice for 15 minutes before centrifugation for 10 minutes at 2000 rpm in a benchtop centrifuge (Beckman TJ–6). A volume of 2 ml of supernatant was transferred to a clean test tube to which 1 ml of Nash's reagent (200 μM ammonium acetate prepared in aqueous solution containing 0.2% (v/v) acetylacetone and 0.3% (v/v) glacial acetic acid) had been added. The samples were then incubated for 20 minutes at 60°C. Samples were cooled to room temperature before reading absorbance at 412 nm in a Beckman DU–70 spectrophotometer. Absorbance readings were converted to nmol formaldehyde (*i.e.*, product) formed from a standard curve obtained by recording the absorbance from a formaldehyde dilution series.

A radiometric method (Poland and Nebert, 1973) was employed to determine aminopyrine N–demethylase activity in sonicated primary mouse hepatocytes. Methyl–^{14}C aminopyrine is employed as the substrate. The substrate is chloroform–soluble, while the end–product, ^{14}C–formaldehyde is extracted into the aqueous phase. The radioactivity

contained in the aqueous phase therefore provides a measure of N-demethylase activity.

7. Lauric Acid Hydroxylase Assay

A modification of the radiometric method of Parker and Orton (1980) was employed in the determination of microsomal lauric acid hydroxylation. Microsomes were diluted to 0.5 mg protein/ml with 1.15% KCl. A stock preparation of [1-^{14}C]lauric acid (10-30 mCi/mmol) was diluted 1:10 with methanol. The incubation mixture contained 1 ml of appropriately diluted microsomes (0.5 mg/ml), 0.2 ml of 1 mM lauric acid/1 mM Tris base (pH 7.4), 0.01 ml of diluted [1-^{14}C] lauric acid (1-3 mCi/mmol) and 0.75 ml of water. Samples were equilibrated for 5 minutes at 37°C prior to initiation of the reaction by the addition of 40 µl of 40 mM NADPH. The reaction proceeded for 5 minutes and was terminated by the addition of 0.2 ml of 3 M HCl. Lauric acid and its metabolites were extracted with diethylether (10 ml) with 10 minutes' shaking. The upper ether phase (7 ml) was pipetted into fresh tubes and dried under nitrogen gas or allowed to evaporate at room temperature in a fume hood. The dried samples were redissolved in methanol (60 µl) and then spotted onto a thin layer chromatography (TLC) plastic sheet (silica gel 60) in a 25 µl volume. The TLC plate was developed with hexane:diethylether:acetic acid (140:56:3, v/v/v). The dried plastic sheet was exposed to x-ray film (Kodak XAR-5) for 3-4 days at -70°C with the aid of an intensifying screen (Cronex Lightning Plus). The radioactive areas on the TLC sheet corresponding to the unmetabolized lauric acid and its ω-1 and ω hydroxylation products, 11- and 12-hydroxylauric acid respectively, were located and cut from the plastic sheet. Each piece was placed in 10 ml of scintillation fluid (Biofluor) and its radioactivity determined by

scintillation counting. The quantity of 11- and 12-hydroxylauric acid formed/mg microsomal protein/minute was calculated from the fractional production of 11- and 12-hydroxylauric acid from lauric acid.

8. Para-Nitrophenol Hydroxylase (PNPH) Assay

Microsomal para-nitrophenol hydroxylase (PNPH) activity was determined according to the method described by Kim (1988). A final reaction volume of 1 ml contained 1 mg microsomal protein, 10 μl of 25 mM para-nitrophenol prepared in dimethylsulfoxide (DMSO), 0.1 ml 10 mM ascorbic acid prepared in buffer (0.1M KH_2PO_4, pH 6.8), and 0.1 ml 10 mM NADPH prepared in buffer. Samples were allowed to equilibrate at 37°C for 5 minutes before the addition of NADPH. The reaction proceeded for 5 minutes at 37°C with rocking and was terminated by the addition of 0.5 ml 0.6 N perchloric acid. Samples were centrifuged for 10 minutes at 2000 rpm in a Beckman TJ-6 centrifuge. Supernatant was removed in a volume of 1 ml and added to clean test tubes containing 0.1 ml 10 N NaOH. Tubes were well mixed and read for absorbance at 546 nm in a Beckman DU-70 spectrophotometer.

A standard curve of 4-nitrocatechol absorption was prepared in the range of 0.5 to 2.5 μM by substituting 4-nitrocatechol (25 mM stock in DMSO) for para-nitrophenol in the initial reaction mixture, and proceeding via the usual procedure. Absorbance readings were plotted against 4-nitrocatechol concentration and a linear equation was calculated from the least squares regression line.

9. Enzyme-linked immunosorbent Assay (ELISA)

An ELISA was established after the method of Goksöyr (1991). The concentration of microsomal protein which, when added to wells, gave absorbance readings in the linear range was determined along with the appropriate concentration of primary antibody to produce readings in the linear range. These prerequisites were determined by an initial assay in which 8 microsomal protein concentrations from 0.80 μg to 100 μg (2-fold serial dilutions with 1.15% KCl starting with 100 μg) contained in a volume of 100 μl were added to wells (each of 8 rows of 12 wells had a different protein concentration). A dilution series of the primary antibody was prepared in 5% dry milk in buffer (Tris buffered saline (TBS); 0.02 M Tris HCL, 0.50 M NaCl, pH 7.5) giving concentrations of 0, 0.063, 0.125, 0.250, 0.500, and 1.00 μg antibody in a volume of 100 μl. Each row with its particular protein concentration contained 12 wells and therefore 2 wells were available for each of the 6 antibody concentrations. This produced a plate (96 wells) with a gradient of protein concentration according to row (8 rows) and a gradient of antibody concentration according to column (12 columns).

The plate was incubated with primary antibody (100 μl/well) for 12 to 16 hours at 4°C or for 2 hours at 37°C. The plate was washed with rocking 3 times for one minute each with buffer (0.05% (v/v) Tween 20 in 0.02 M Tris HCl/0.50 M NaCl, pH 7.5). A horseradish peroxidase conjugate was employed as the secondary antibody, and was diluted 1:10 000 in 5% (w/v) skim milk powder in TBS. Secondary antibody was incubated for 2 hours at 37°C (200 μl/well), and washed with 5 one minute washes with rocking (0.05% (v/v) Tween 20 in TBS). Horseradish peroxidase substrate consisted of 0.08% (w/v) o-phenylenediamine

dihydrochloride (OPD) in 0.15 M $NaPO_4$/0.05 M Na Citrate, pH 5.7, with hydrogen peroxide added to a concentration of 0.012% (v/v) just prior to use. A volume of 100 μl was added to each well. The colour was allowed to develop for 10 to 20 minutes and the reaction was terminated with the addition of 50 μl of 4 N H_2SO_4 to each well. Absorbance was read at 496nm in a Titertek ELISA plate reader.

The initial assay conducted to determine appropriate protein and antibody concentrations was performed for antibodies against P4502C11. Using these concentrations, the experimental assays were performed in a manner identical to that described above. Each sample was assayed in triplicate utilizing 3 serial dilutions for each replicate. A standard sample of microsomes was set aside from a control rat, and used on every plate in order to ensure that assay conditions were comparable for every plate. In addition, the standard microsomes were used as blanks on each plate to ascertain baseline absorbance. The specificity of the antibody was previously determined via Western analysis in this and other laboratories.

E. Statistical Analysis

Statistical analysis of the data was performed with the guidance of Dr. Brian Eastwood, a statistician in the Department of Community Health and Epidemiology at Dalhousie University. The Student's t–test (Statquik Parametric Stats, Lundon Software, Inc.) was used in experiments in which mice were administered tumor necrosis factor or interleukin–6. Isocaloric protein deficiency experiments were analysed via two–way analysis of variance (SYSTAT® software) in order to ascertain differences due to duration of

nutritional regimen or to interactions between nutrition and other variables such as duration of treatment (time) or the administration of inducing agents. One-way analysis of variance was used to determine differences between the nutritional groups at individual time points. Experiments which examined the effect of isocaloric protein deficiency on P450 induction were analysed by one-way analysis of variance to analyze differences between the induced nutritional groups or between the uninduced nutritional groups. Experiments which examined the effect of Poly IC treatment on P450 induction were analysed by one-way analysis of variance and by Student's t-test. Data were summarised as means ± standard error, and differences were considered significant where p values less than 0.05 were obtained.

RESULTS

A. Effect of recombinant murine TNFα on hepatic cytochrome P450 content and activities in mice.

The inflammatory cytokine, tumor necrosis factor α (TNFα) has been shown to cause changes directly and indirectly on hepatic phenotype which are reminiscent of the hepatic acute-phase response. It has been found that TNFα is elicited by many agents which produce a state of systemic inflammation in mammalian systems, as well as P450 down-regulation in the liver. It was therefore of interest to determine if TNFα administration in mice (C57BL/6) would lead to down-regulation of hepatic cytochrome P450, and to provide evidence that this down-regulation was occurring in parallel with an accepted marker for the acute-phase phenotype (depressed albumin expression).

1. Cytochrome P450 Content and EROD Activity

The effect of TNFα administration (1 μg/mouse (i.v.), Genzyme®) on total hepatic cytochrome P450 in microsomes prepared at 12 or 24 hours following treatment is shown in Figure 6, demonstrating a significant loss of P450 by 12 and 24 hours. The effect of TNFα on EROD activity was similar (Figure 6) with the finding that EROD activity was significantly reduced after 12 and 24 hours of cytokine treatment ($p<.05$).

FIGURE 7.

Murine hepatic P450 and EROD activity following TNF administration. Mice were treated with saline or 40 000 units recombinant murine TNF (i.v.) and sacrificed at 12 and 24 hours following treatment. Hepatic microsomes were prepared and assayed for total P450 content and EROD activity, expressed as percent control. The mean control level of P450 at 12 hours was .64±.04, and at 24 hours was .61±.01. The mean control level of EROD activity at 12 hours was .084±.007, and at 24 hours was .081±.01. Both P450 content and EROD activity were significantly depressed ($p<.05$) at 12 and 24 hours following TNF treatment.

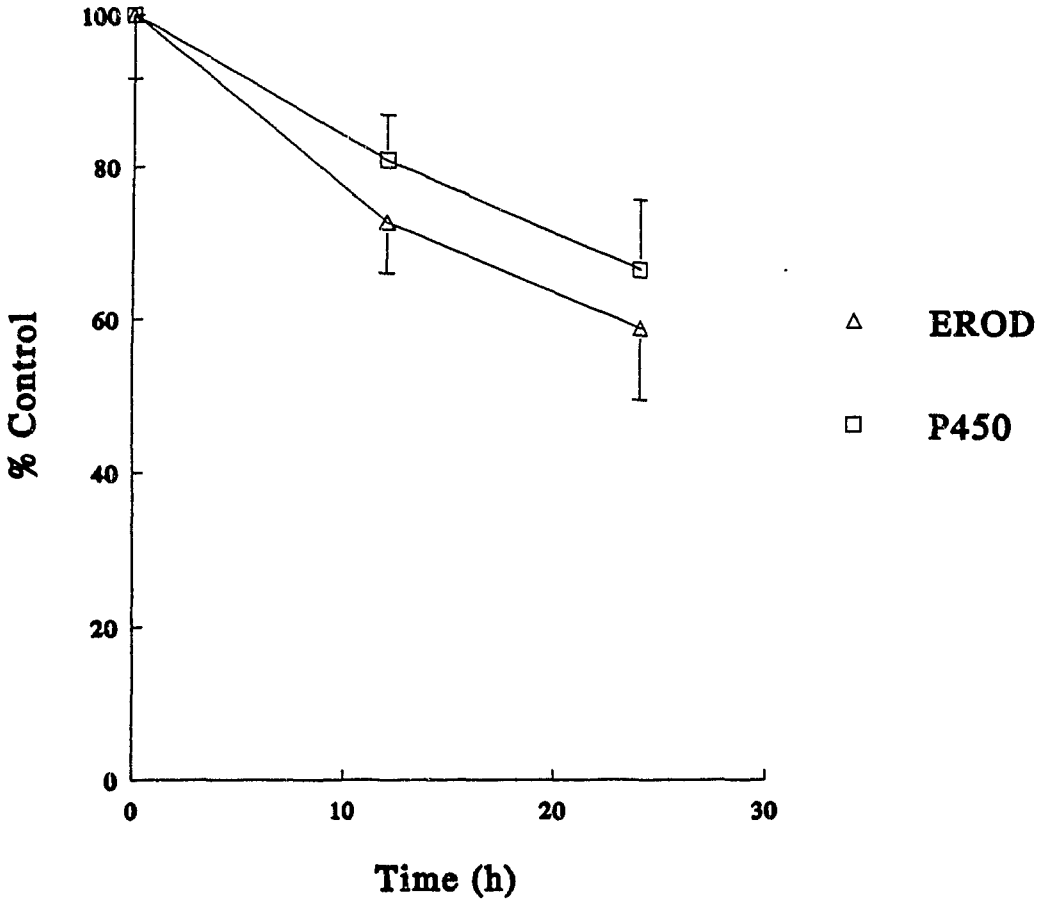


FIGURE 7.

2. Hepatic Albumin Expression

In another experiment the effect of TNFα administration on hepatic albumin RNA expression was determined, and is depicted in Figure 7. Mice (C57BL/6) were treated with 0.5 or 1.0 μg of the same TNFα preparation and sacrificed 24 hours following. Total hepatic RNA was extracted and separated by agarose gel electrophoresis. The RNA was transferred from the gel to a nitrocellulose membrane which was then hybridized with a cDNA oligomer specific for mouse and rat albumin mRNA. In Figure 7, lanes 9 to 12 contain RNA from saline-treated controls, lanes 5 to 8 from mice treated with 0.5 μg TNFα, and lanes 1 to 4 from mice treated with 1.0 μg TNFα. The RNA from mice receiving TNFα demonstrates appreciably lower levels of probe binding, indicating that the level of albumin mRNA is decreased following TNFα treatment. Lowered albumin mRNA indicates that the "acute-phase" phenotype has been initiated in the livers of mice which received TNFα.

B. Effect of recombinant murine IL-6 on hepatic cytochrome P450 content and activities in mice.

Experimental evidence suggests that the effect of TNFα on hepatic phenotype is evoked in part via interleukin-6 (IL-6) release from inflammatory cells such as monocytes and macrophages, fibroblasts and endothelial cells. In addition, IL-6 is believed to be a primary, direct regulator of many acute-phase genes, and so it was of interest to determine if IL-6 administration would produce P450 down-regulation in mice.

FIGURE 8.

Hepatic albumin mRNA expression following TNF administration. Mice were treated with saline (lanes 9–12), 20 000 units recombinant murine TNF (lanes 5–8), or 40 000 units recombinant murine TNF (lanes 1–4) and sacrificed 24 hours following treatment. Total hepatic RNA was extracted, separated by agarose gel electrophoresis, and transferred to a membrane. Equal loading of lanes with RNA material was confirmed by visualizing the gel with an ultraviolet lamp prior to transfer. A ^{32}P–labelled oligomer specific for rat and mouse albumin message was bound to a transcript approximately 2.1 kb in length.

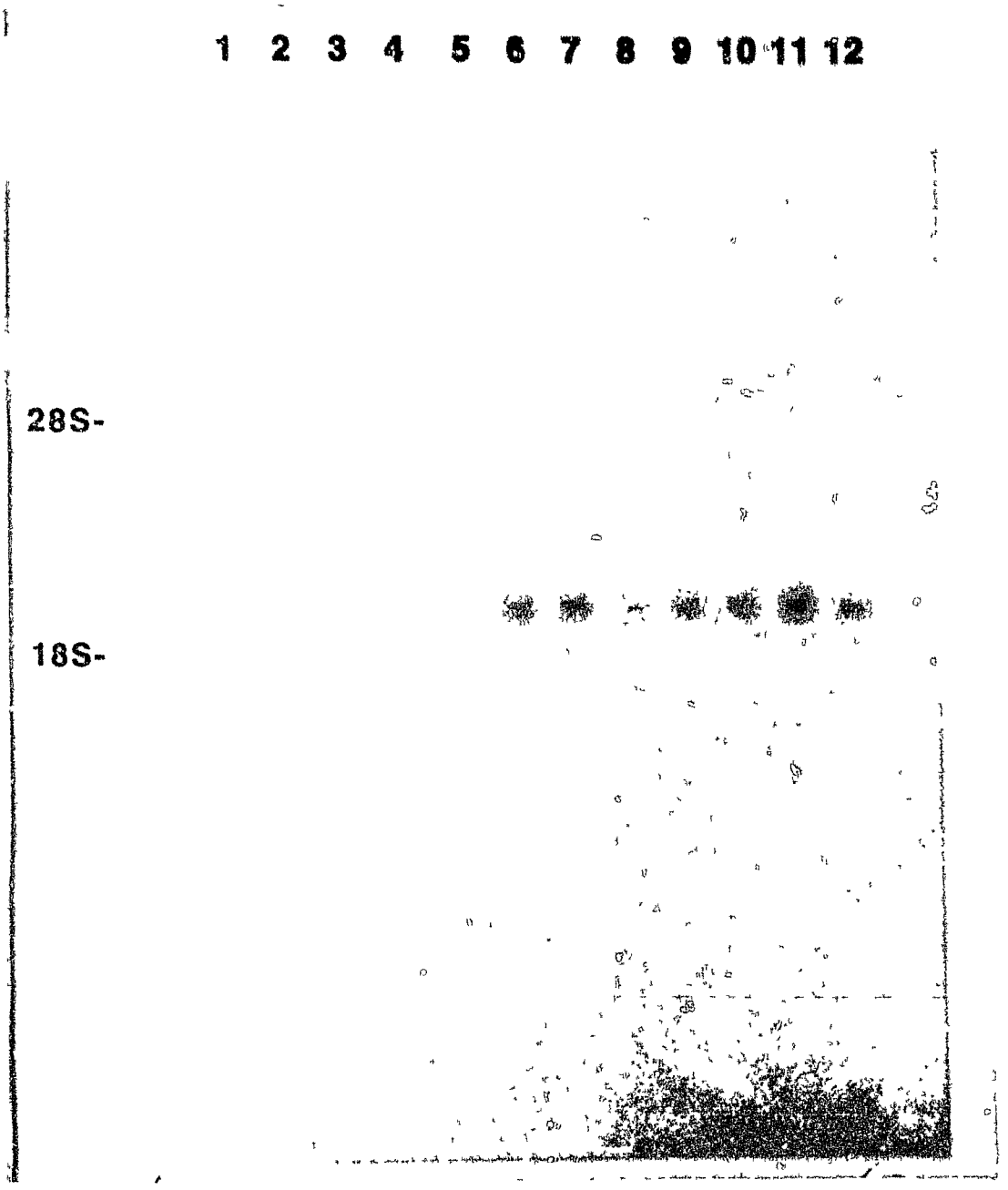


FIGURE 8.

TABLE 9. Summary of Interleukin-6 Experiments.

Source of IL-6	Strain of mouse	Dose	Duration of Treatment	Hepatic Microsomal P450	
				Ctrl	IL-6
Genzyme®, recombinant human	Swiss	30 000 U i.p.	12 hours	.94±.04	.71±.05*
Genzyme®, recombinant human	Swiss	30 000 U i.p.	12 hours	.53±.04	.57±.01
Genzyme®, recombinant human	Swiss	20 000 U i.v.	12 hours	.65±.02	.55±.02*
Genzyme®, recombinant human	Swiss	40 000 U i.v.	16 hours	.75±.12	.81±.07
Genzyme®, recombinant human	Swiss	50 000 U i.v. at 0 and 12 hours	24 hours	.48±.05	.42±.01
recombinant murine (gift from Dr. J. Gauldie, McMaster University)	C57BL/6	8 μg i.v.	24 hours	.55±.03	.52±.01

In an initial experiment, male Swiss BALB/c mice received 30 000 units of human recombinant IL–6 (Genzyme®) i.p., and were sacrificed 12 hours later. A significant down–regulation (25% loss) of total P450 was observed but could not be repeated in subsequent experiments. In attempts to confirm the initial results, experiments were conducted with doses of recombinant human IL–6 (Genzyme®) ranging from 20 000 units/mouse (i.v.) to 50 000 units/mouse. Treatments were given intraperitoneally as well as intravenously, for periods of 6, 12 or 24 hours following treatment before sacrifice. Significant losses of P450 were not consistently observed in any situation.

Experiments were also carried out using a recombinant murine IL–6 preparation generously supplied by Dr. Jack Gauldie of McMaster University, Hamilton, Ontario. These experiments involved the use of the C57BL/6 mouse strain used by Dr. Gauldie in his studies of the acute–phase response. This strain is known to exhibit an acute–phase response following treatment with the recombinant murine IL–6 preparation supplied by Dr. Gauldie. The animals were treated intravenously with doses known to evoke an acute–phase response (8 μg/mouse) and sacrificed 12 and 24 hours following treatment. No significant down–regulation of P450 was observed in any experiment, except in one case where a small (15%), statistically significant loss of total P450 was observed. It can be concluded from this section that *in vivo* IL–6 administration had no appreciable effect on hepatic cytochrome P450 in mice.

C. Effect of recombinant murine IL-6 on hepatocellular cytochrome P450 content and aminopyrine N-demethylase in primary hepatocyte cultures.

1. Cytochrome P450 Content and Aminopyrine N-Demethylase Activity

Experiments were undertaken to examine the effect of recombinant murine IL-6 on cytochrome P450 in primary hepatocyte cultures. Primary hepatocyte cultures were prepared from C57BL/6 mice and treated with 50, 100, or 200 units (per culture) of the recombinant murine IL-6 preparation provided by Dr. Jack Gauldie. After an 18 hour exposure to IL-6, the levels of cytochrome P450 and aminopyrine N-demethylase activity were determined in the hepatocytes. As illustrated in Figure 9, dose-dependent loss of P450 content and N-demethylase activity occurred in cultures incubated with IL-6. In contrast to the *in vivo* experiments described above, the *in vitro* study demonstrates that P450 regulation is sensitive to the presence of IL-6.

D. General effects of isocaloric protein deficiency (IPD) and effects on hepatic cytochrome P450 content and related activities.

Experiments designed to test the effect of altered cell cycle status on hepatic P450 regulation employed the isocaloric protein deficiency model characterized by Mead *et al.* (1990). The experiments described below employed three feeding regimens: control (food and water *ad libitum*), fasting (no food but water *ad libitum*), and isocaloric protein deficiency (*ad libitum* 20% glucose or 20% sucrose). The fasting group was included as a control group for the adequate consumption of calories by the isocaloric "protein

FIGURE 9.

Effect of interleukin-6 treatment on primary murine hepatocyte cultures. Primary cultures of hepatocytes were prepared and incubated with recombinant murine IL-6 (50, 100 or 200 units) for 18 hours following which the cells were assayed for microsomal P450 content and aminopyrine N-demethylase activity. Control values for microsomal P450 and N-demethylase activity were .53 nmol/mg protein and .22 nmol/mg protein/minute, respectively. Results are expressed as percent control.

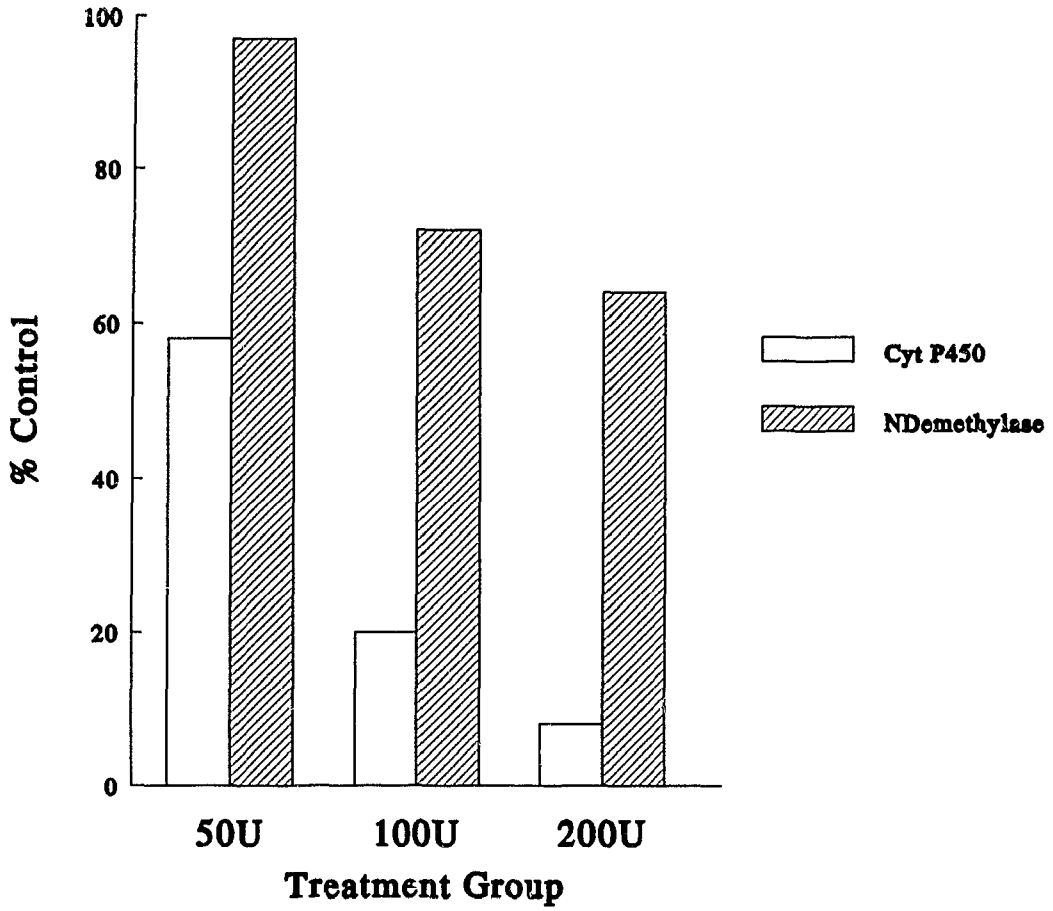

100
80
60
40
20
0
% Control
50U
100U
200U
Treatment Group
Cyt P450
NDemethylase

FIGURE 9.

deficient" group maintained on 20% glucose. A reduction in the intake of 20% glucose by this group could easily convert the animals from isocaloric protein deficiency to fasting protein deficiency, two quite different physiologic conditions. Where significant differences are exhibited between these two isocaloric protein deficient states, it can be safely concluded that the effects produced in the isocaloric protein deficient group are, in fact, specific to the isocaloric protein deficient state rather than the fasting condition.

The "isocaloric protein deficient" group was included as a model of the G0 to G1 cell cycle transition shown to occur in isocaloric protein deficiency but not the fasting or fed states (Mead *et al.*, 1990). To assess the effects of a G0 to G1 transition on hepatic cytochrome P450 levels, animals were sacrificed after 24, 48 or 72 hours of nutritional deficiency. In an additional experiment, the feeding regimens were maintained for 72 hours at which time rats received an amino acid "meal" by gavage, and were left for an additional 24 hours prior to sacrifice (96 hours in total). This tested the effect of a G_1 to S transition (Mead *et al.*, 1990) on P450 regulation. Effects on body weight and hepatic protein levels were assessed in each animal in addition to measurement of microsomal cytochrome P450 and related enzyme activities.

1. Total Body Weight and Organ Weights

This experiment was designed to assess the effect of isocaloric protein deficiency on total body weight and organ weight (Table 10) and on hepatic and extrahepatic levels of microsomal P450 (section D.4). Animals were maintained on

TABLE 10. Effect of Isocaloric protein deficiency on Total Body Weight and Organ Weights in Adult Male Rats.

Nutritional Regimen	Body Weight (g)	Liver (g)	Kidneys (g)	Spleen (g)	Lungs (g)
Control-Fed	287.0±8.4	13.9±0.93	2.84±0.06	0.90±0.27	1.40±0.12
Protein Deficient	228.4±7.7*	10.0±2.00*	2.12±0.19*	0.68±0.08	1.20±0.10

FIGURE 10.

Microsomal heme levels during isocaloric protein deficiency. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. Two-way analysis of variance determined significance for nutrition ($p<.00001$) and for time ($p<.00001$), with interaction between the variables ($p<.00001$). One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal heme expressed as nmol/mg microsomal protein.

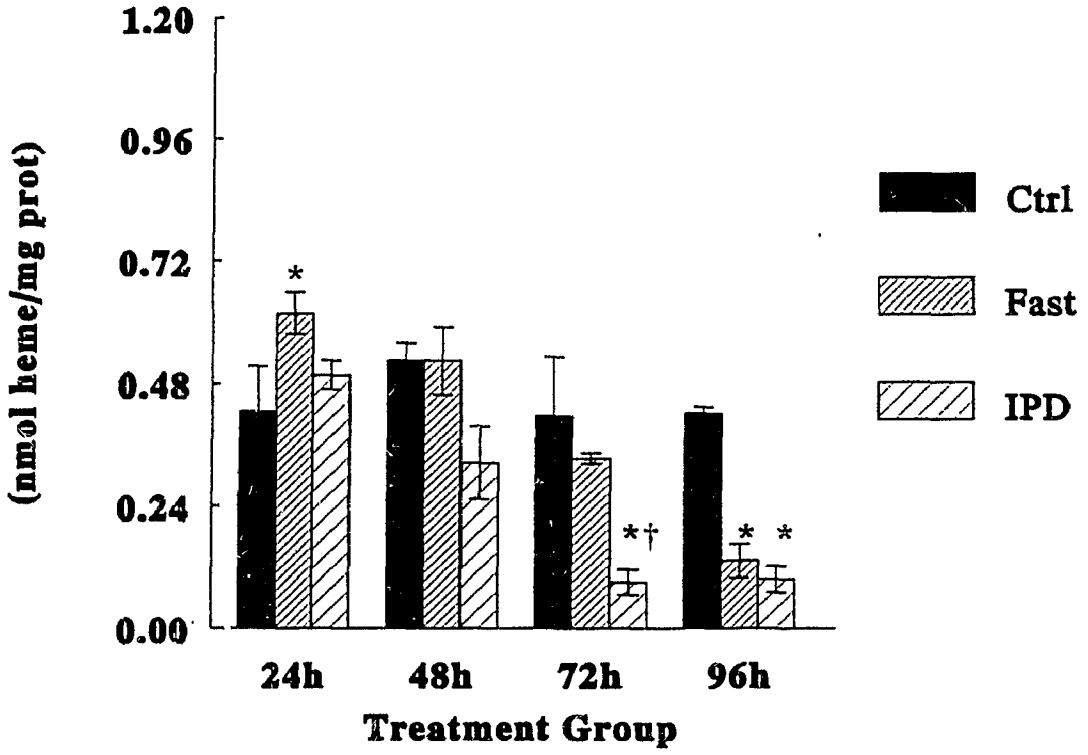

1.20
0.96
0.72
0.48
0.24
0.00
(nmol heme/mg prot)
*
*†
* *
24h
48h
72h
96h
Treatment Group
Ctrl
Fast
IPD

FIGURE 10.

20% glucose or normal feed for 60 hours prior to sacrifice. There was no fasting "control" group included in this experiment as it was performed at the end of the experimental series by which time it had been realised that the inclusion of a fasting "control" group for the verification of adequate caloric intake by the isocaloric protein deficient group was unnecessary. As shown in Table 10, isocaloric protein deficient animals exhibited a significant weight loss compared to control fed animals. This weight loss of approximately 20% is similar to the weight loss seen in the fasting group as noted in other experiments (data not shown). When various organ weights were compared (Table 10), it was found that the weights of both liver and kidney were significantly depressed in the isocaloric protein deficient group, but that there was no difference for spleen and lung weights. Organ weights were not recorded for fasting animals in any experiment.

2. Microsomal Heme Levels

The effect of fasting and isocaloric protein deficiency for 24, 48 and 72 hours on microsomal heme levels is illustrated in Figure 10. The effect of a protein meal administered by gavage at 72 hours to control, fasting and isocaloric protein deficient animals is also illustrated (*i.e.*, 96 hour time point). Both fasting and isocaloric protein deficient rats displayed a steady decline in heme levels with increasing duration of nutritional deficiency. Fasting animals also exhibited an initial elevation of heme at 24 hours followed by the steady decline. By 72 hours of nutritional deficiency, heme levels in the isocaloric protein deficient group were significantly lower than heme

levels in the fasting group. Amino acid administration after 72 hours of either nutritional regimen produced no effect, and at this time point (i.e., 96 hours), fasting and isocaloric protein deficient groups exhibited no difference in their depressant effect on microsomal heme levels.

3. Microsomal and Cytosolic Protein Levels

The effect of nutritional regimen on microsomal protein is shown in Figure 11. A significant decrease in microsomal protein levels was seen after 72 hours of fasting, with a significant increase occurring following the administration of a protein meal by gavage (96 hour time point). In the isocaloric protein deficient group, microsomal protein concentration was reduced by 35% by 72 hours of isocaloric protein deficiency, and returned to control values following the protein meal.

As shown in Figure 12, fasting resulted in a significant increase in cytosolic protein concentration after 24 and 48 hours. By 72 hours of fasting, however, cytosolic protein had fallen to control values. There was no rebound in cytosolic protein following the administration of a protein meal by gavage. There was no significant effect of isocaloric protein deficiency on cytosolic protein levels.

4. P450 Levels in Various Organs

The effect of isocaloric protein deficiency on microsomal cytochrome P450 in hepatic and extrahepatic sites was examined in a separate experiment as noted in section D.1. Animals were maintained for 60 hours on an isocaloric protein deficient

diet; a fasting "control" group was not included in this experiment as it had been determined in several experiments by this time that rats in the isocaloric protein deficient group ingest adequate quantities of the 20% glucose diet to safely consider them as "isocaloric protein deficient" rather than fasting. Figure 13 illustrates the effect of 60 hours of isocaloric protein deficiency on the liver as well as on 4 extrahepatic sites of P450 expression. Isocaloric protein deficient animals exhibited a significant loss of both hepatic and renal total microsomal P450, while total P450 in the lungs of isocaloric protein deficient rats was significantly elevated when compared with controls. The adrenal glands and spleens from the animals comprising the control-fed and isocaloric protein deficient groups, respectively, were pooled in order to provide an adequate amount of tissue for determination of total microsomal P450. While this data is not appropriate for statistical analysis, it appears that total P450 in the adrenals of isocaloric protein deficient rats is higher than in the control-fed group. The spleens pooled from the isocaloric protein deficient group appear to have a slightly lower level of total P450 than those pooled from the control-fed animals.

5. Cytochrome P450

Total hepatic cytochrome P450 levels following dietary deficiency are illustrated in Figure 14. Microsomal P450 was significantly decreased after 24 hours of isocaloric protein deficiency. There was no significant difference between control-fed and isocaloric protein deficient rats at time points following 24 hours. isocaloric

FIGURE 11.

Effect of isocaloric protein deficiency on microsomal protein concentration. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. The mean control value at 24 hours was 6.26±0.5 mg/ml, at 48 hours was 6.95±1.0 mg/ml, at 72 hours was 10.7±1.0 mg/ml, and at 96 hours was 6.08±0.8 mg/ml. Two-way analysis of variance determined significance for nutrition ($p<.05$) but not for time, with interaction between the variables ($p<.05$). One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal protein concentration expressed as percent control.

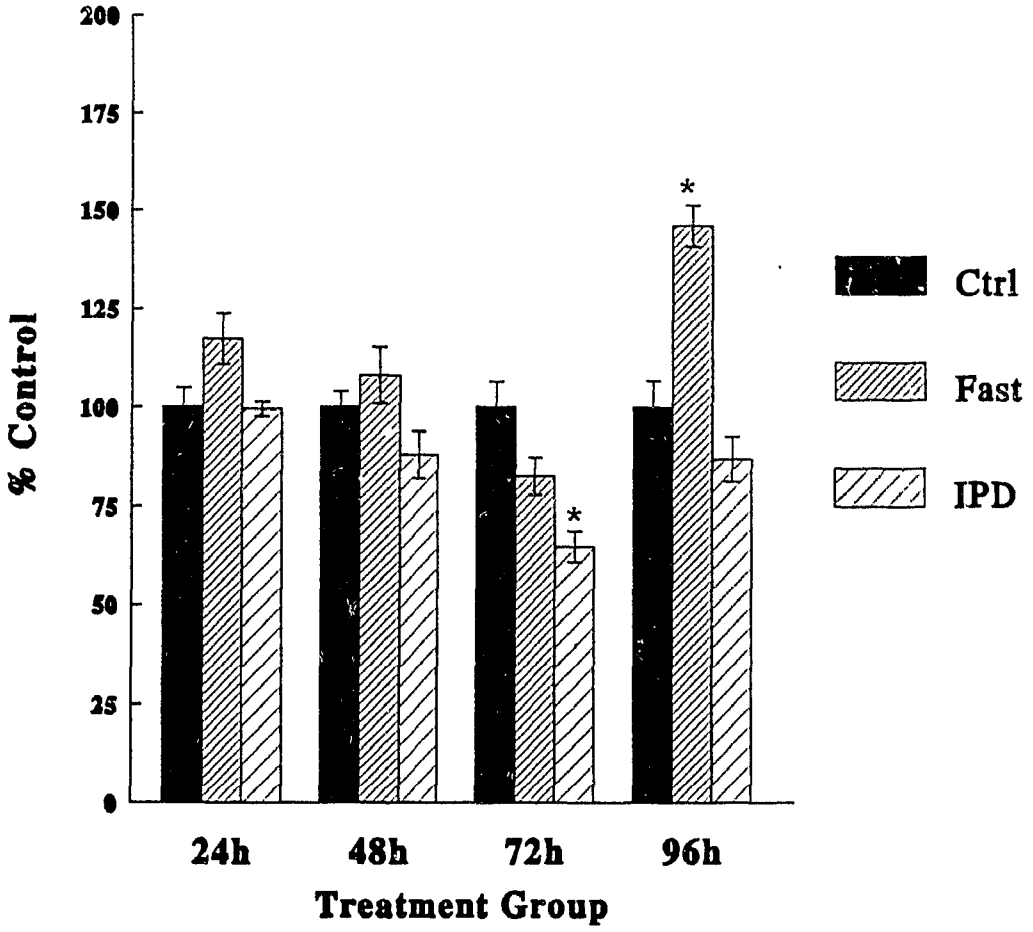

200
175
150
125
100
75
50
25
0
% Control
*
*
Ctrl
Fast
IPD
24h
48h
72h
96h
Treatment Group

FIGURE 11.

FIGURE 12.

Effect of isocaloric protein deficiency on cytosolic protein concentration. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. The mean control value at 24 hours was 22.6±2.1 mg/ml, at 48 hours was 27.0±1.1 mg/ml, at 72 hours was 27.9±.75 mg/ml, and at 96 hours was 30.7±6.0 mg/ml. Two-way analysis of variance determined significance for nutrition ($p<.0050$) and for time ($p<.0025$), with interaction between the variables ($p<.0005$). One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with cytosolic protein concentration expressed as percent control.

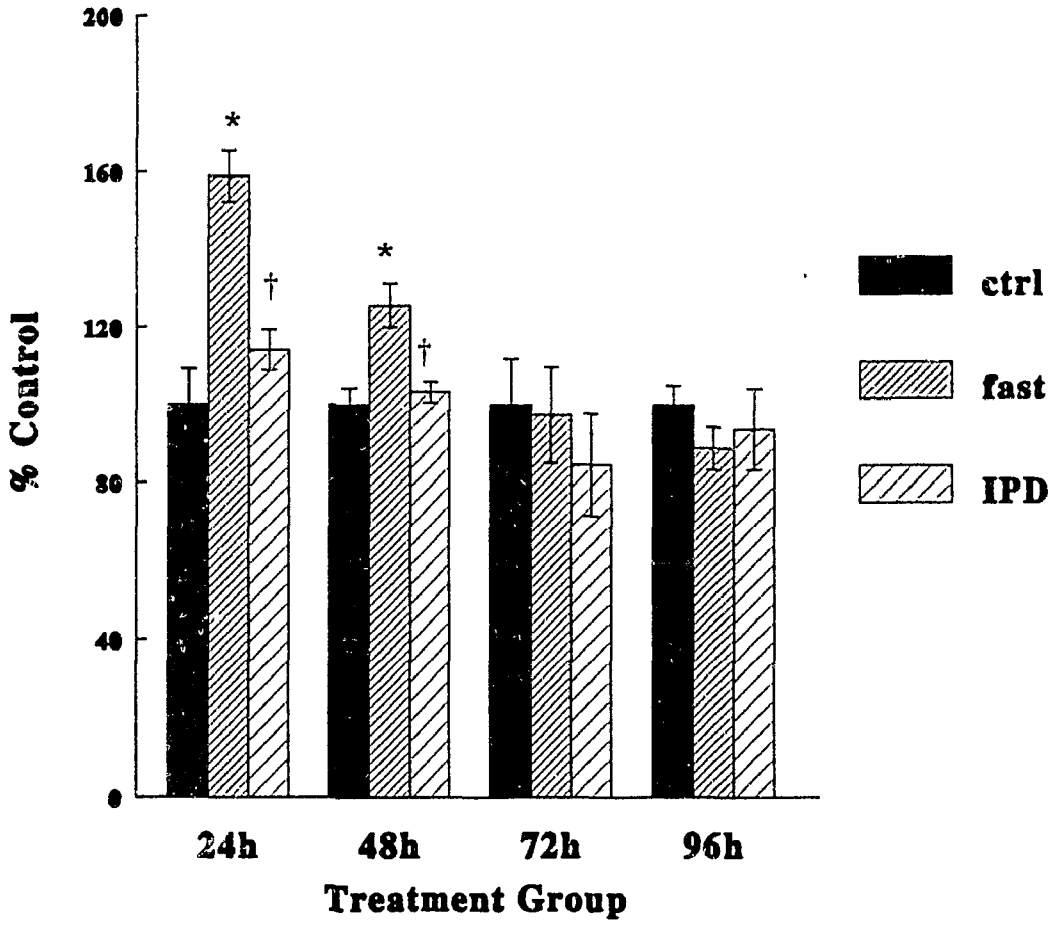

200
160
120
80
40
0
% Control
*
*
†
†
24h
48h
72h
96h
Treatment Group
ctrl
fast
IPD

FIGURE 12.

FIGURE 13.

Effect of isocaloric protein deficiency on the level of microsomal P450 in hepatic and extra-hepatic sites. Rats were control-fed or isocaloric protein deficient for 60 hours prior to sacrifice. Microsomal P450 was determined for liver, kidney, lung, spleen and adrenal glands, and is expressed as a percent of control values (n=5). The mean control value for hepatic P450 was .86±0.1, for renal P450 was .10±.01, and for pulmonary P450 was .08±.001. The pooled control value for adrenal P450 was 0.32, and for splenic P450 was 0.11. Both hepatic (p=.0020) and renal P450 (p=.0060) were significantly depressed in the isocaloric protein deficient group, while pulmonary P450 was increased in isocaloric protein deficient animals (p=.0140). Statistical comparisons were not carried out for spleen or adrenal glands as these samples were pooled within each nutritional group.

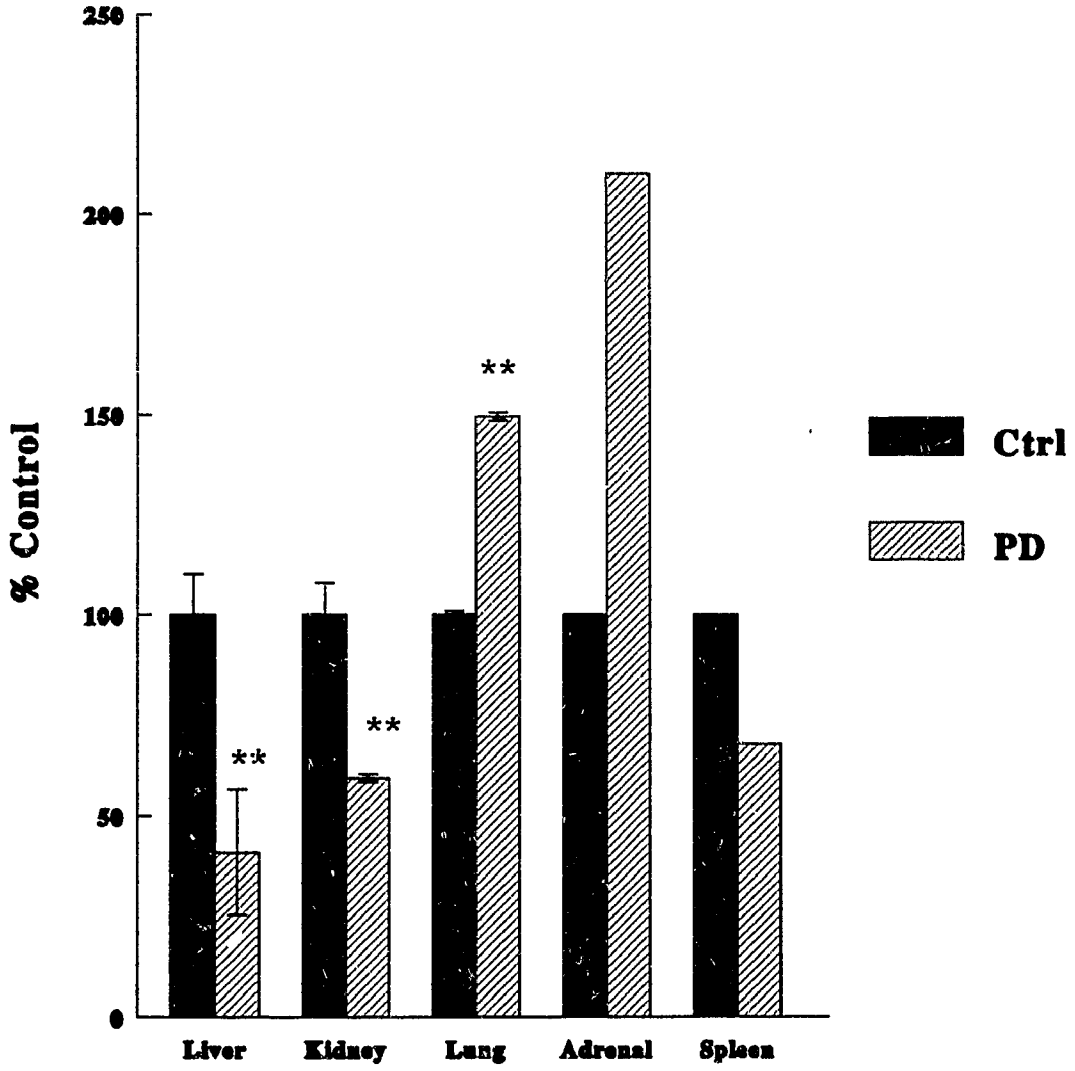

250
200
150
100
50
0
% Control
**
**
**
Liver
Kidney
Lung
Adrenal
Spleen
Ctrl
PD

FIGURE 13.

FIGURE 14.

Effect of isocaloric protein deficiency (IPD) on total microsomal P450. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. Two-way analysis of variance determined significance for nutrition (p=.00006) and for time (p=.0279), with no interaction between the variables. One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for p< .05, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for p< .05, .01, or .001, respectively. Data are presented as means ± standard error, with total microsomal P450 expressed as nmol/mg microsomal protein.

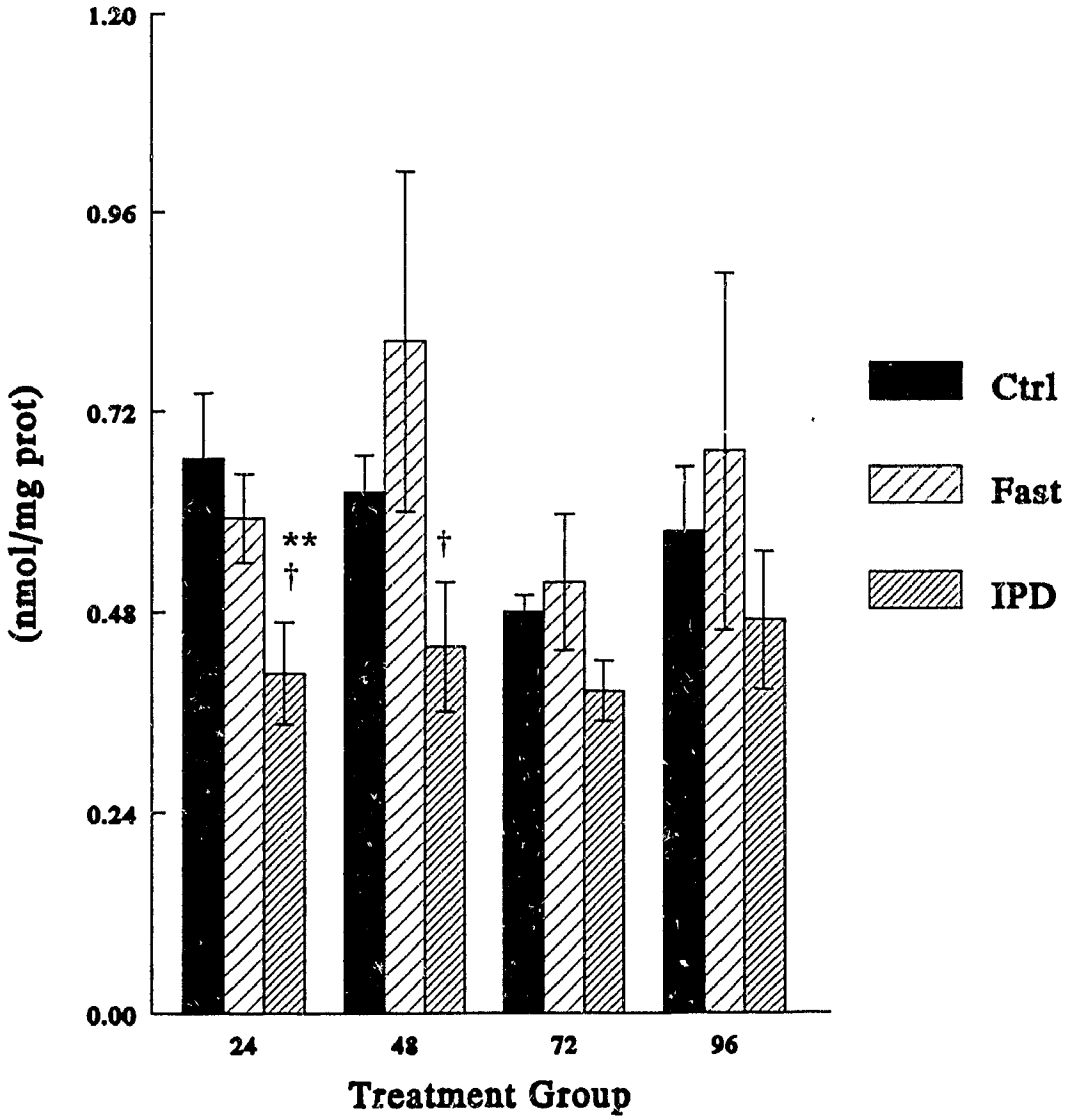

1.20
0.96
0.72
0.48
0.24
0.00
(nmol/mg prot)
**
†
†
24
48
72
96
Treatment Group
Ctrl
Fast
IPD

FIGURE 14.

FIGURE 15.

Effect of isocaloric protein deficiency (IPD) on microsomal EROD activity. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. Two-way analysis of variance determined significance for nutrition ($p<.00001$) but not for time ($p=.0852$), with no interaction between the variables. One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal EROD activity expressed as nmol product/mg microsomal protein/minute.

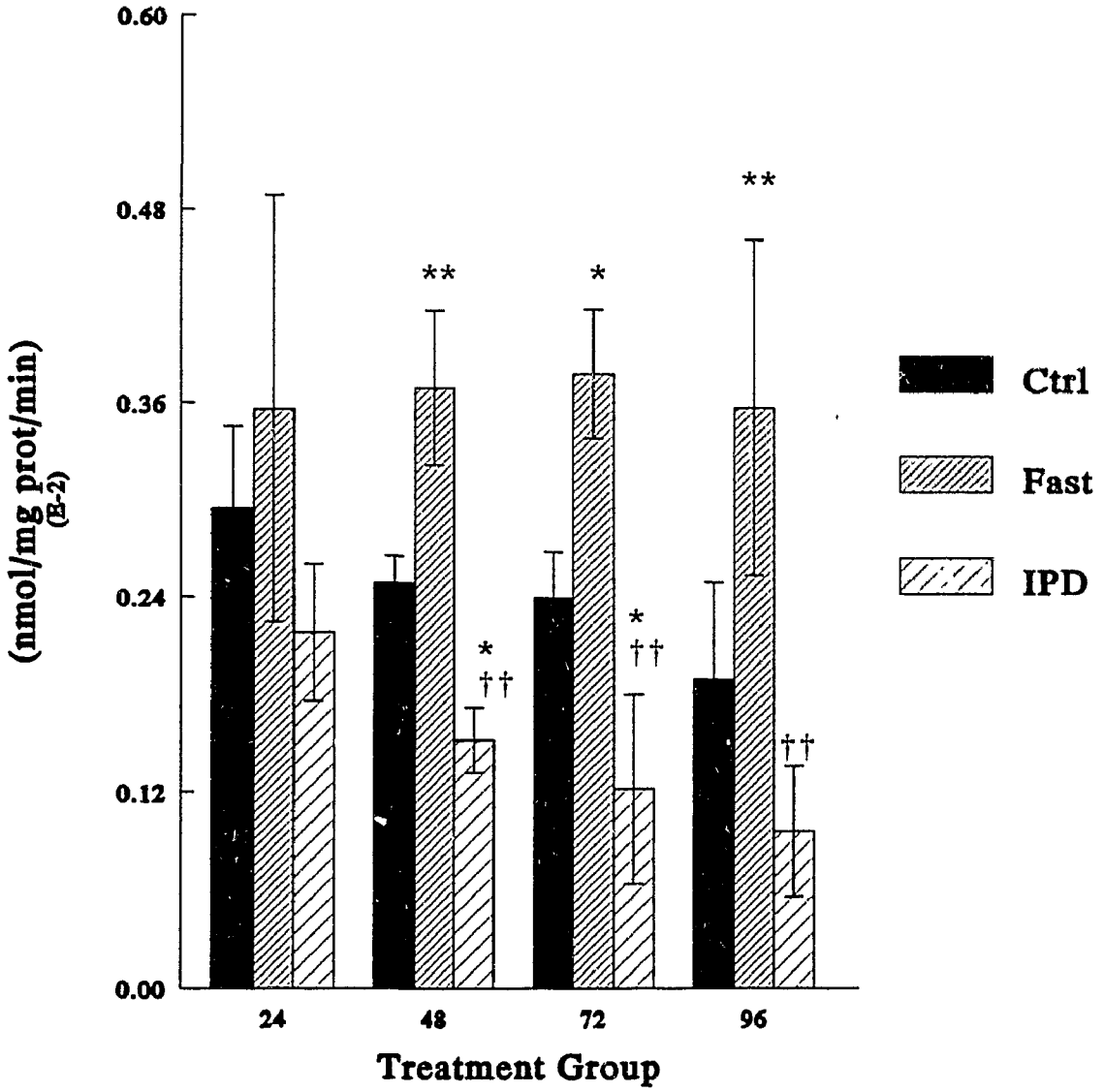

0.60
0.48
0.36
0.24
0.12
0.00
(nmol/mg prot/min)
(E-2)
**
*
**
*
††
*
††
††
24
48
72
96
Treatment Group
Ctrl
Fast
IPD

FIGURE 15.

protein deficient rats demonstrated significantly lower levels of P450 than fasting rats at 24, 48, and 72 hours, suggesting that the "isocaloric protein deficient" group consumed sufficient calories from 20% glucose to produce an isocaloric protein deficient state rather than a hypocaloric, or fasting, protein deficiency condition. The administration of amino acids to control–fed, fasting or isocaloric protein deficient animals (*i.e.*, the "96 hour" groups) produced no further changes in hepatic cytochrome P450 levels in either fasting or isocaloric protein deficient animals.

6. Microsomal EROD Activity

Microsomal ethoxyresorufin–O–deethylase activity was determined in microsomes from control, fasting or isocaloric protein deficient animals as illustrated in Figure 15. Significantly lower levels of EROD activity were observed after 48 and 72 hours of isocaloric protein deficiency. Levels of EROD activity were significantly different in isocaloric protein deficient and fasting rats at 48, 72, and 96 hours, suggesting that the isocaloric protein deficient group is indeed in an "isocaloric" and not "hypocaloric" protein deficient state. The fasting condition resulted in significantly elevated levels of EROD activity after 48, 72, and 96 hours of nutritional deficiency. The administration of amino acids to control–fed, fasting or isocaloric protein deficient animals produced no further changes in microsomal EROD activity.

FIGURE 16.

Effect of isocaloric protein deficiency (IPD) on microsomal N-demethylase activity. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. Two-way analysis of variance determined significance for nutrition ($p=.00001$) and for time ($p<.00001$), with no interaction between the variables. One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal N-demethylase activity expressed as nmol product/mg microsomal protein/minute.

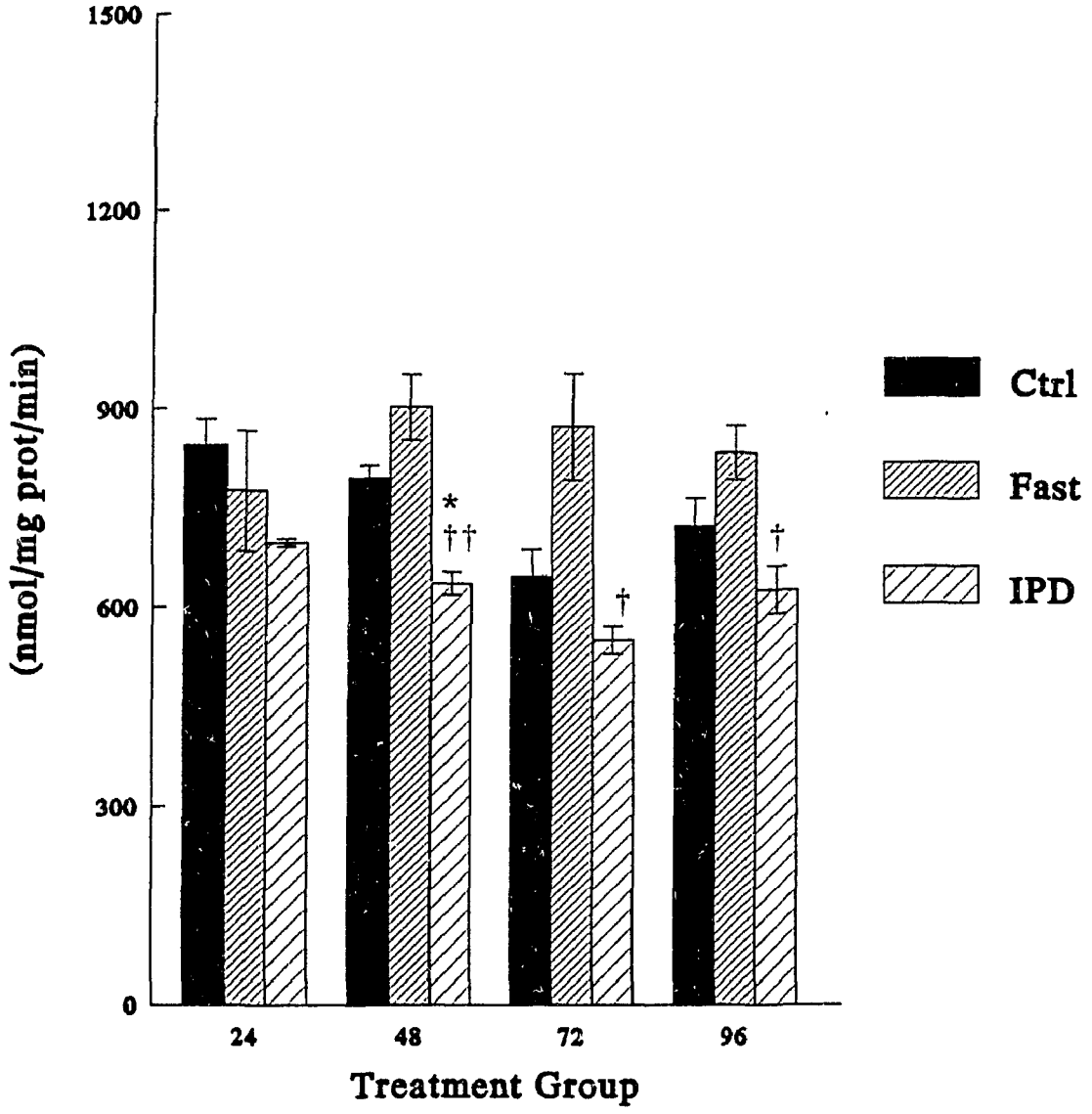

1500
1200
900
600
300
0
(nmol/mg prot/min)
*
††
†
†
24
48
72
96
Treatment Group
Ctrl
Fast
IPD

FIGURE 16.

7. Microsomal N-Demethylase Activity

Microsomal N-demethylase activity was determined in microsomes from control, fasting or isocaloric protein deficient animals as shown in Figure 16. Relative to controls, N-demethylase activity was significantly depressed after 48 hours of isocaloric protein deficient. After 48, 72 and 96 hours of nutritional deficiency, isocaloric protein deficient rats exhibited significantly different N-demethylase levels from those observed in fasting animals. Fasting resulted in no significant difference when individual time points were compared via one-way ANOVA, however, when the data were pooled in two-way ANOVA, the apparent modest elevations of N-demethylase activity in the fasting group were significantly different from the control animals ($p<.01$). Amino acid administration produced no further effect on N-demethylase levels.

8. Microsomal Para-Nitrophenol Hydroxylase (PNPH) Activity

The effect of nutritional regimen on microsomal p-nitrophenol hydroxylation is shown in Figure 17. After 48 hours of isocaloric protein deficiency, PNPH activity exhibited a small significant decrease relative to control-fed rats. Fasting rats exhibited significantly elevated PNPH activity at this time point. At 24 and 72 hours of treatment, there was no difference in PNPH activity for the three nutritional regimens. Administration of amino acids produced no significant effect on PNPH activity for either nutritional regimen.

FIGURE 17.

Effect of isocaloric protein deficiency (IPD) on microsomal PNPH activity. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. Two-way analysis of variance determined significance for nutrition (p=.0142) and for time (p=.0079), with interaction between the variables (p=.0058). One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for p< .05, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for p< .05, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal PNPH activity expressed as nmol product/mg microsomal protein/minute.

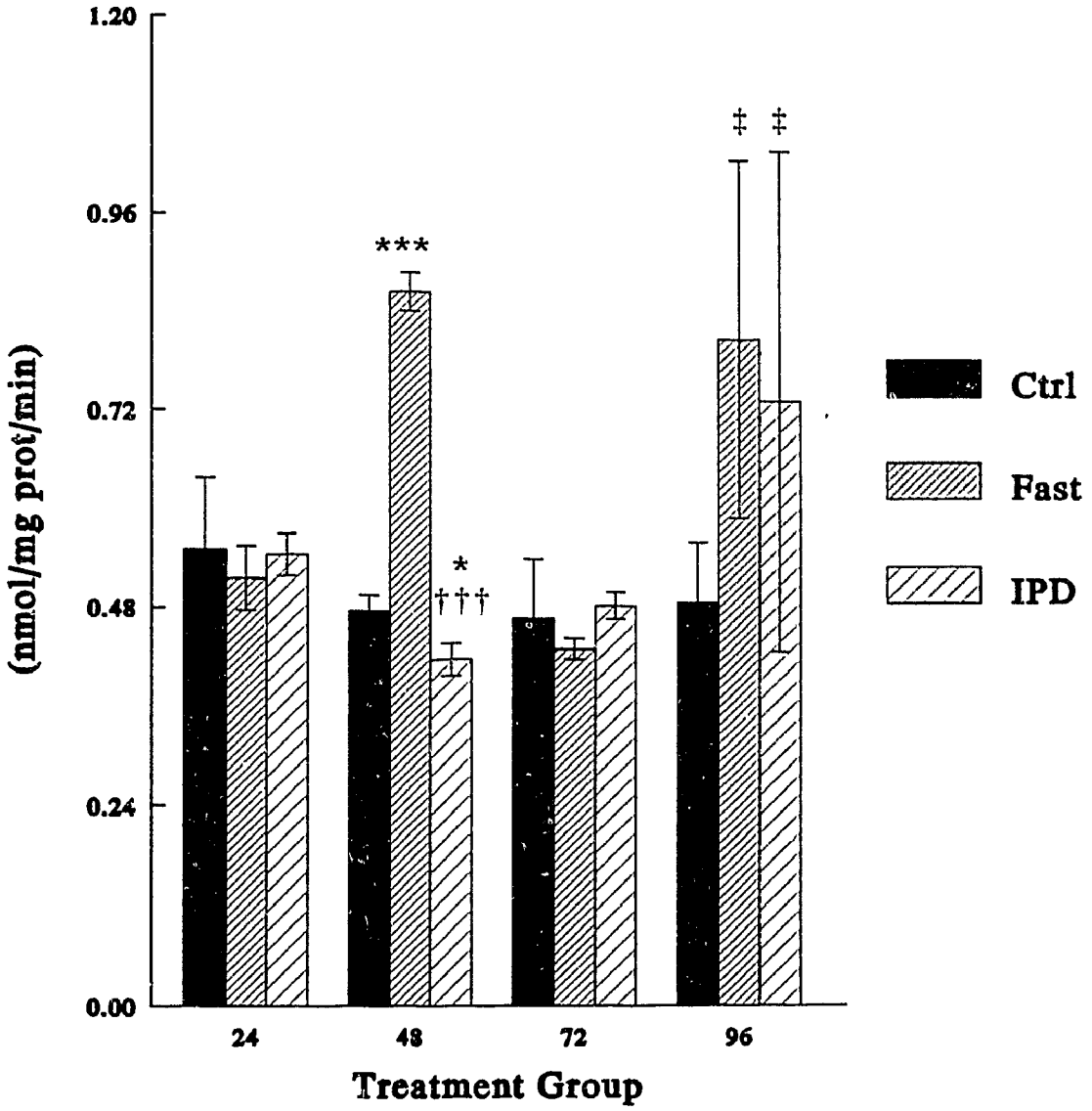

1.20
0.96
0.72
0.48
0.24
0.00
(nmol/mg prot/min)
***
*
†††
‡ ‡
24
48
72
96
Treatment Group
Ctrl
Fast
IPD

FIGURE 17.

FIGURE 18.

P4502C11 expression during isocaloric protein deficiency and following Poly IC administration. Microsomes from control–fed male rats (lanes 1 and 2), from control–fed female rats (lanes 3 and 4), from isocaloric protein deficient male rats (lanes 5 and 6), and from Poly IC–treated (20 mg/kg) male rats (lanes 7 and 8) were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. P4502C11 was detected with the use of a specific, monoclonal antibody and HRP–linked secondary antibody.

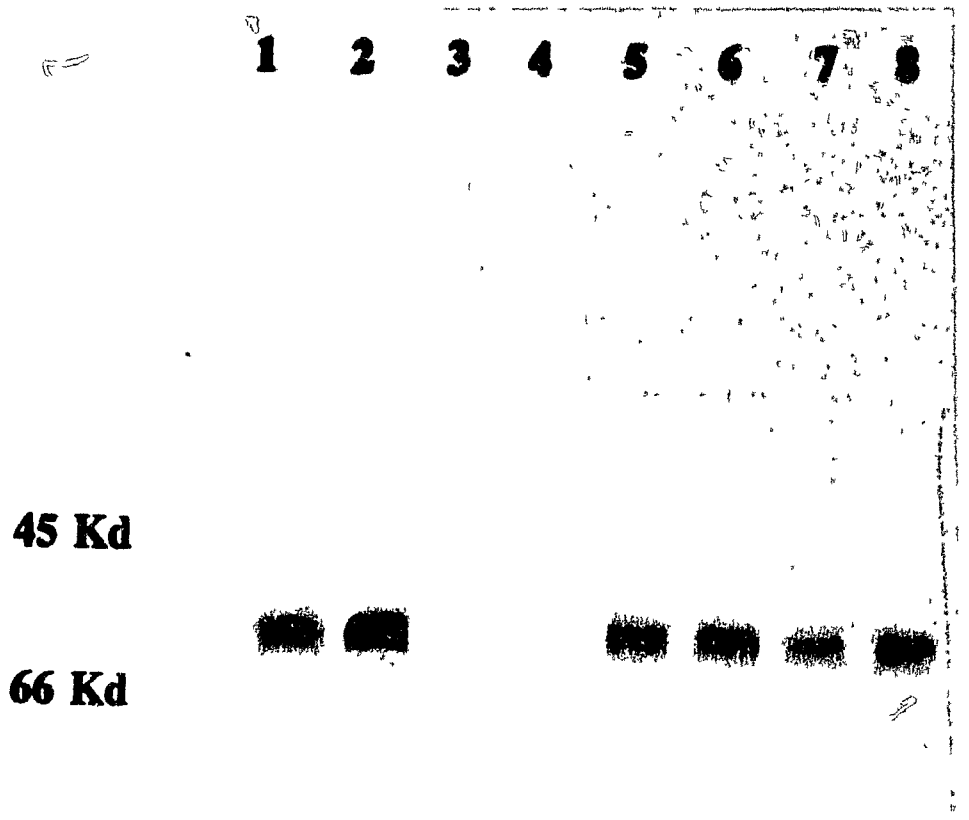


FIGURE 18.

9. Cytochrome P4502C11 Western Analysis and ELISA

Cytochrome P4502C11 is a constitutively expressed, male-specific P450 isoform. In order to confirm antibody specificity, microsomes from male control and female control rats as well as from male isocaloric protein deficient (60 hours) rats or Poly IC- (an interferon inducer) treated male rats were assessed by Western analysis. Figure 18 illustrates the result of probing the membrane with a polyclonal antibody directed against rat P4502C11. A single band corresponding to a molecular weight of approximately 50 kDa appeared for microsomes from male but not female rats. When densitometry was performed it was found that the average density of the two bands from the isocaloric protein deficient rats amounted to 79% of the control male rats while the Poly IC treated rats exhibited a density of 64% of the control value. Microsomes from female rats produced a density equal to 2% of control values.

An enzyme linked immunosorbent assay was established for the quantitation of P4502C11 levels in fasting, isocaloric protein deficient and control fed animals. As shown in Figure 19, it was found that for both fasting and isocaloric protein deficient feeding regimens, P4502C11 levels were decreased relative to controls. This down-regulation was manifest by 24 hours, but was neither time-dependent nor influenced by the administration of a protein meal by gavage (96 hour time point).

FIGURE 19.

Effect of isocaloric protein deficiency on the level of microsomal P4502C11. Rats were maintained on control, fasting or isocaloric protein deficient feeding regimens for 24, 48 or 72 hours prior to sacrifice, or maintained for 72 hours before given an amino acid "meal" by gavage followed by sacrifice at the 96 hour time point; n=4 for each treatment group at each time point. Microsomal P4502C11 levels were quantitated by ELISA. Two-way analysis of variance determined significance for nutrition (p=.0010) but not for time (p=.0630), with no interaction between the variables. One-way analysis of variance determined differences between nutritional regimens within each time point grouping. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for p< .05, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for p< .05, .01, or .001, respectively. Data are presented as mean absorbance ± standard error.

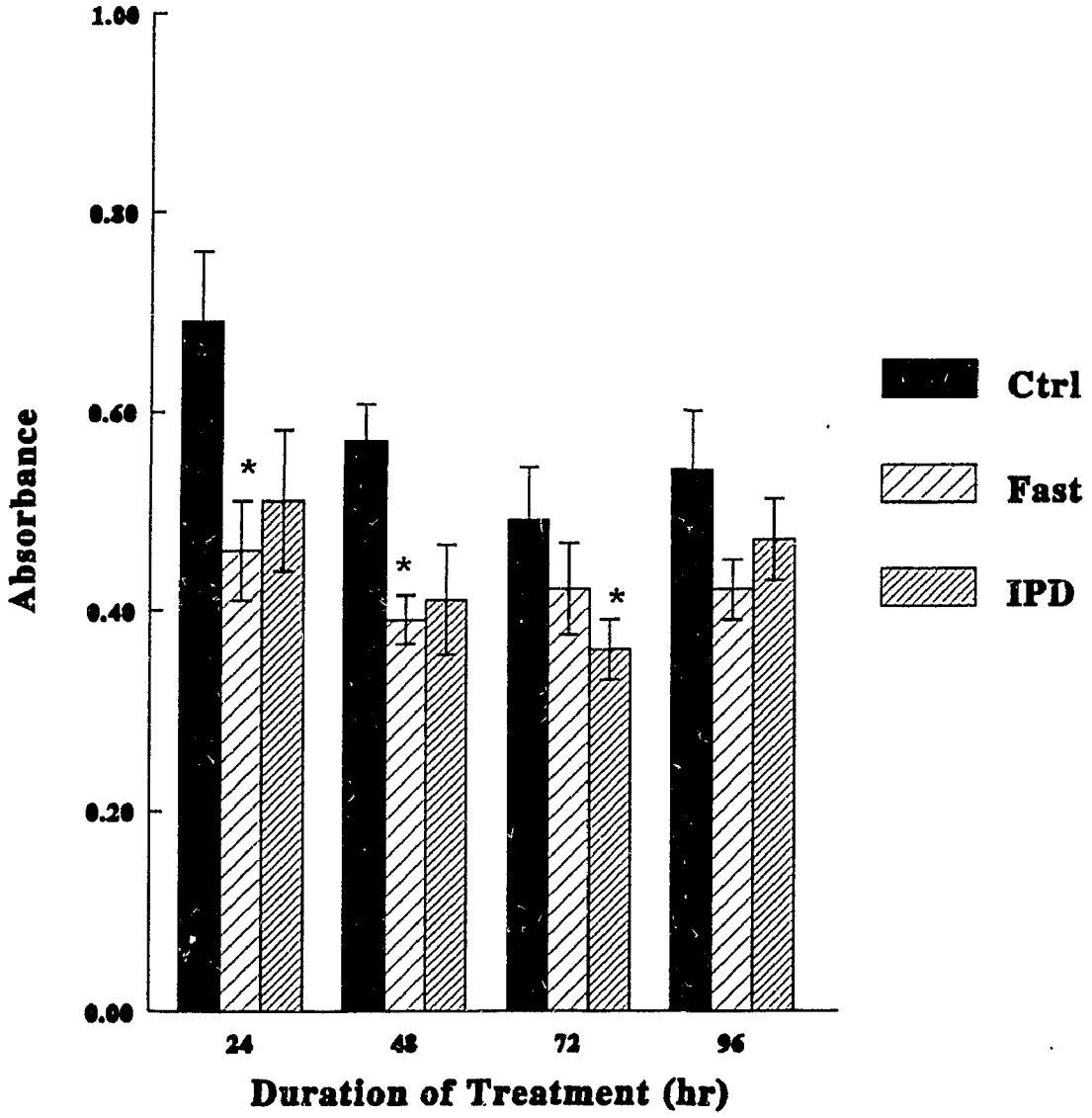

1.00
0.80
0.60
0.40
0.20
0.00
Absorbance
*
*
*
24
48
72
96
Duration of Treatment (hr)
Ctrl
Fast
IPD

FIGURE 19.

FIGURE 20.

Effect of isocaloric protein deficiency on the induction of total microsomal cytochrome P450 by clofibrate. Rats were placed on control–fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours at which time they were treated with olive oil or 500 mg/kg clofibrate (i.p.) and sacrificed 24 hours later (giving a total of 60 hours of fasting or isocaloric protein deficiency); n=4 for each of 6 treatment groups. Two–way analysis of variance determined significance for nutrition (p=.0016) but not clofibrate induction (p=.0627), with significant interaction between the variables (p=.0201). One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for p< .05, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for p< .05, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal cytochrome P450 content expressed as nmol/mg microsomal protein.

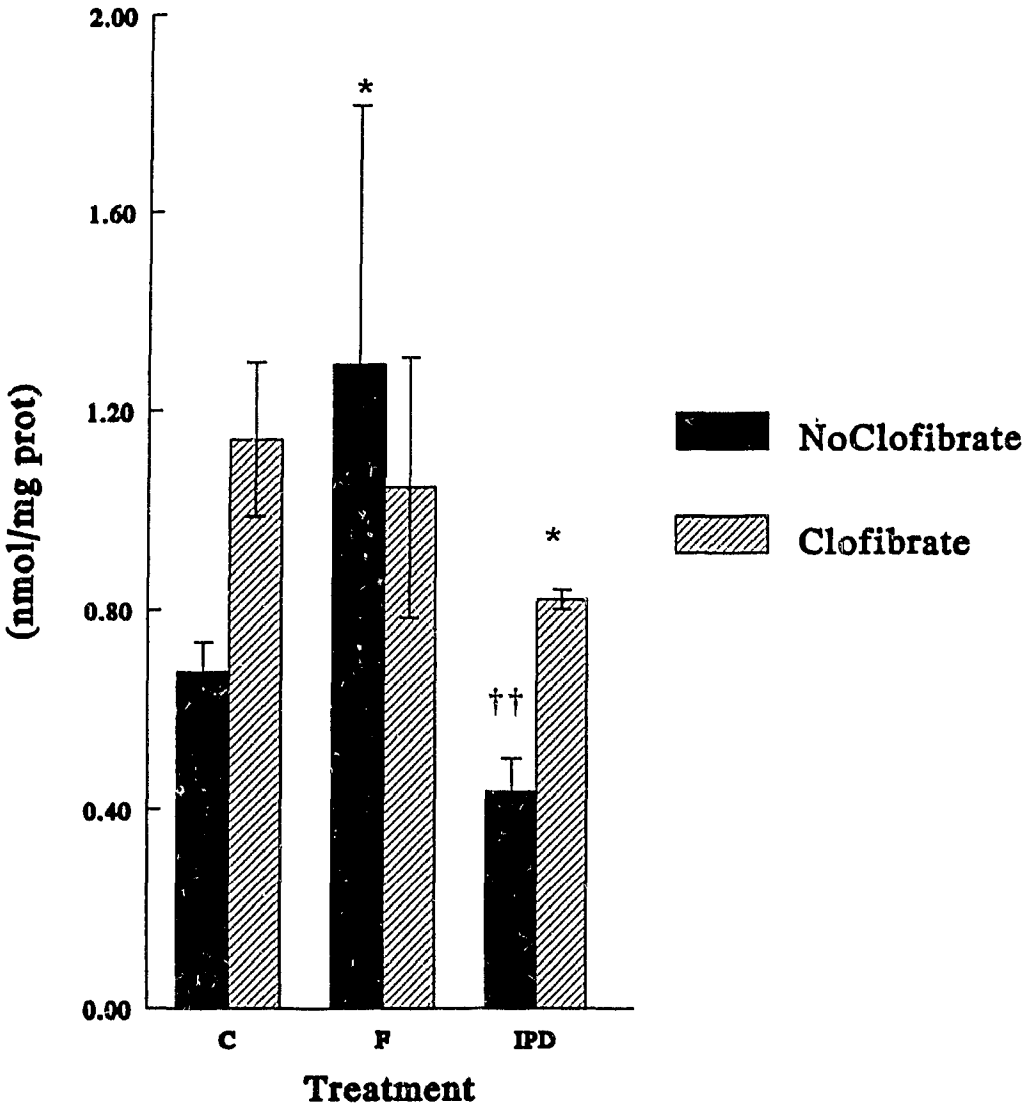

2.00
1.60
1.20
0.80
0.40
0.00
(nmol/mg prot)
*
*
††
C
F
IPD
Treatment
NoClofibrate
Clofibrate

FIGURE 20.

E. Effect of isocaloric protein deficiency (IPD) on the clofibrate-mediated induction of hepatic cytochrome P450 content and activities.

Experiments designed to examine the effect of isocaloric protein deficiency on the transcriptional induction of cytochrome P4504A1 employed the peroxisome proliferating agent, clofibrate. The induction protocol utilised is one characterised by Hardwick, *et al.* (1987) where clofibrate, at a dose of 500 mg/kg, is given 24 hours prior to sacrifice. In the experiments to be described, rats were maintained on control-fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours before clofibrate administration, and sacrificed 24 hours later (giving a total of 60 hours nutritional deficiency).

1. Cytochrome P450

The effect of clofibrate induction and nutritional regimen on microsomal cytochrome P450 content is shown in Figure 20. In uninduced animals, isocaloric protein deficiency produced no effect relative to control-fed rats, however, isocaloric protein deficient rats exhibited significantly lower total P450 levels than fasting rats, indicating that this group was in a state of isocaloric, rather than hypocaloric protein deficiency. Clofibrate administration produced a significant induction of total P450 in control-fed and isocaloric protein deficient rats, although the levels in the isocaloric protein deficient group were significantly lower than levels in control-fed animals. Clofibrate treatment did not increase P450 levels in fasting animals above the elevated levels seen in uninduced fasting rats.

FIGURE 21.

Effect of isocaloric protein deficiency on the induction of microsomal EROD activity by clofibrate. Rats were placed on control–fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours at which time they were treated with olive oil or 500 mg/kg clofibrate (i.p.) and sacrificed 24 hours later (giving a total of 60 hours of fasting or isocaloric protein deficiency); n=4 for each of 6 treatment groups. Two–way analysis of variance determined significance for nutrition ($p=.00006$) and for clofibrate induction ($p=.0434$), with no significant interaction between the variables. One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal EROD activity expressed as nmol product/mg microsomal protein/minute.

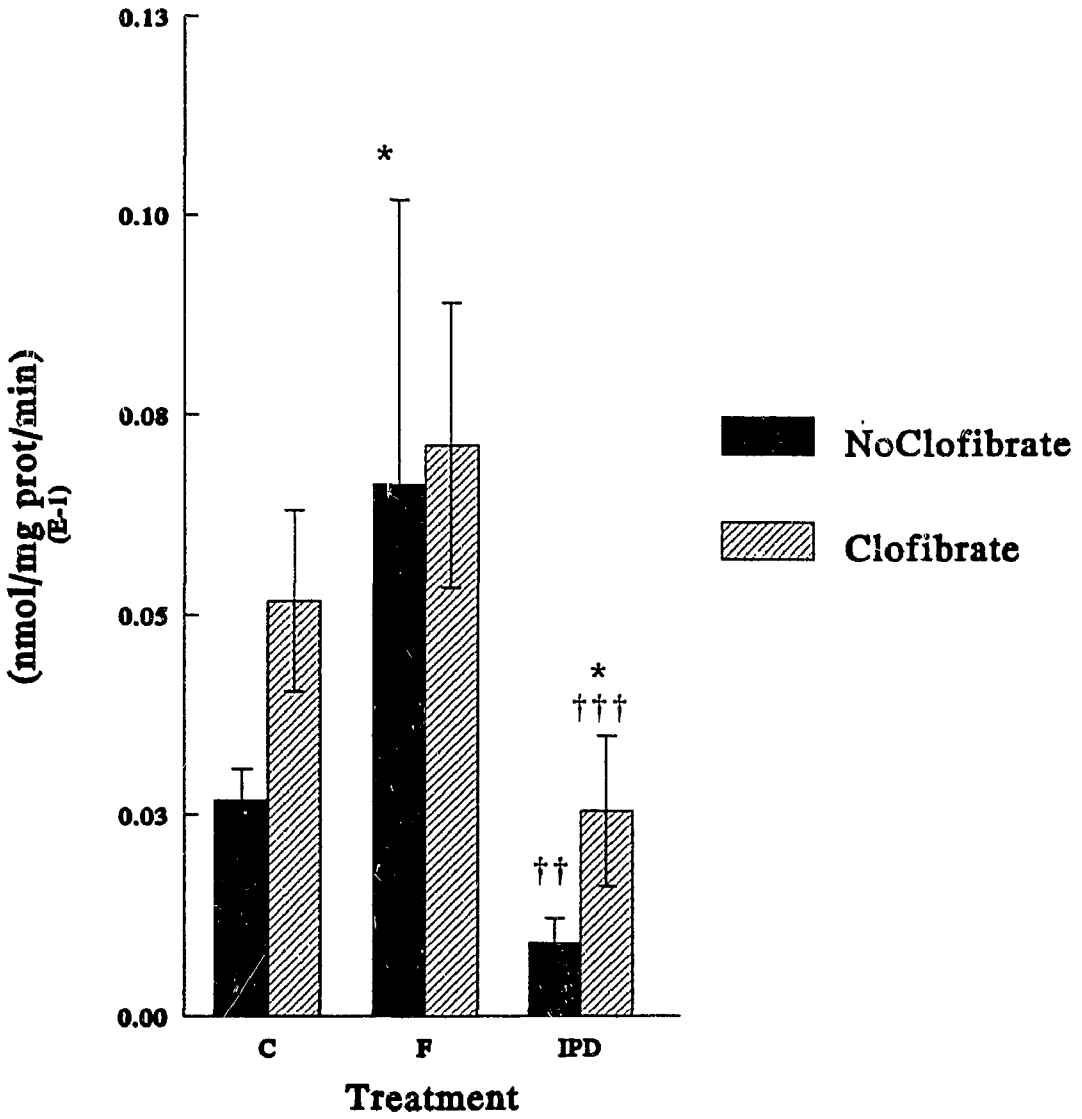

0.13
0.10
0.08
0.05
0.03
0.00
(nmol/mg prot/min)
(E-1)
*
*
†††
††
C
F
IPD
Treatment
NoClofibrate
Clofibrate

FIGURE 21.

2. Microsomal EROD Activity

The effect of nutritional deficiency on the induction of EROD activity by clofibrate is illustrated in Figure 21. In uninduced animals, isocaloric protein deficiency produced no effect relative to control–fed rats, however, uninduced isocaloric protein deficient rats exhibited significantly lower levels of EROD activity than uninduced fasting rats. Clofibrate significantly induced EROD activity in both control–fed and isocaloric protein deficient rats, however, induced levels of EROD activity were lower in isocaloric protein deficient rats than in control–fed rats. Clofibrate treatment did not increase EROD activity in fasting rats above the increase seen in uninduced fasting animals.

3. Cytochrome P4501A1 Western Analysis

Microsomes from one representative animal from each experimental group were separated by gel electrophoresis, and transferred to nitrocellulose membrane. The choice of representative animals was based on EROD activities so as to best represent the pattern of results depicted in Figure 21. The result of probing with a monoclonal antibody specific for P4501A1 is shown in Figure 22. Lanes 1 to 3 contain microsomes from uninduced rats from control–fed, fasted and isocaloric protein deficient rats, respectively, and show no binding of the anti–1A1 antibody. Lanes 4 through 6 contain microsomes from corresponding induced nutritional groups. No 1A1 protein was detected in clofibrate–treated rats. Lane 7 contains a standard

FIGURE 22.

P4501A1 expression following clofibrate administration. Microsomes from a representative rat from each of the uninduced or induced nutritional groups were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. P4501A1 was detected with the use of a specific, monoclonal antibody and HRP–linked secondary antibody. Lanes 1 through 6 contain microsomes from each of the experimental groups, and lane 7 contains microsomes from a β–naphthoflavone–induced control rat.

1 2 3 4 5 6 7

45 Kd

66 Kd

FIGURE 22.

FIGURE 23.

Effect of isocaloric protein deficiency on the induction of microsomal LAH activity by clofibrate. Rats were placed on control–fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours at which time they were treated with olive oil or 500 mg/kg clofibrate (i.p.) and sacrificed 24 hours later (giving a total of 60 hours of fasting or isocaloric protein deficiency); n=4 for each of 6 treatment groups. Two–way analysis of variance determined no significance for nutrition, but a significant effect of clofibrate induction ($p<.00001$), with significant interaction between the variables ($p=.0170$). One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal LAH activity expressed as nmol product/mg microsomal protein/minute.

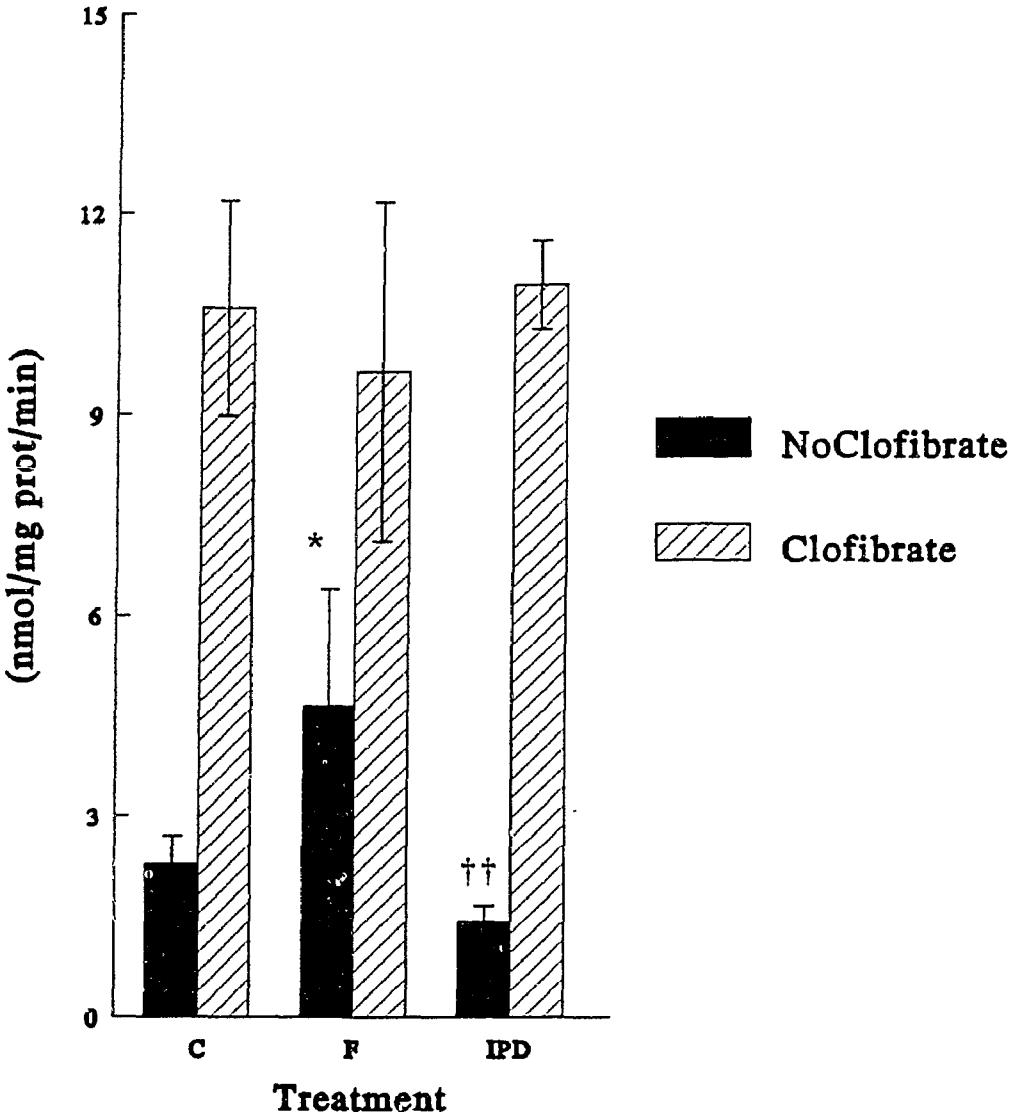

15
12
9
6
3
0
(nmol/mg prot/min)
*
††
C
F
IPD
Treatment
NoClofibrate
Clofibrate

FIGURE 23.

consisting of microsomes from a ß–naphthoflavone–treated male rat, revealing the expected strong induction of P4501A1 protein.

4. Microsomal Lauric Acid Hydroxylase (LAH) Activity

The effect of clofibrate induction and isocaloric protein deficiency on LAH activity is shown in Figure 23. In uninduced animals, isocaloric protein deficiency produced no effect relative to control–fed rats, however, isocaloric protein deficient rats exhibited significantly lower LAH activity than fasting rats. Fasting produced a significant induction of LAH activity without clofibrate treatment. Clofibrate produced a significant induction of LAH activity in al 3 nutritional groups, with no differences between nutritional groups for the absolute levels of induced LAH activity.

F. Effect of isocaloric protein deficiency (IPD) on the induction of hepatic cytochrome P450 content and activities by pyridine.

Experiments designed to examine the effect of isocaloric protein deficiency on the translational induction of cytochrome P4502E1 were conducted using pyridine as the inducing agent. The induction protocol utilised is one characterised by Kim and Novak (1990) where pyridine, at a dose of 200 mg/kg, is given 12 hours prior to sacrifice. In the experiments to be described, rats were maintained on control–fed, fasting or isocaloric isocaloric protein deficient feeding regimens for approximately 36 hours before pyridine administration, and sacrificed 12 hours following treatment with the inducer (giving a total of 48 hours of nutritional deficiency).

FIGURE 24.

Effect of isocaloric protein deficiency on the induction of total microsomal cytochrome P450 by pyridine. Rats were placed on control–fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours at which time they were treated with saline or 200 mg/kg pyridine (i.p.) and sacrificed 12 hours later (giving a total of 48 hours of fasting or isocaloric protein deficiency); n=4 for each of 6 treatment groups. Two–way analysis of variance determined significance for nutrition ($p<.00001$) and for pyridine induction ($p<.00001$), with significant interaction between the variables ($p=.0004$). One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal cytochrome P450 content expressed as nmol/mg microsomal protein.

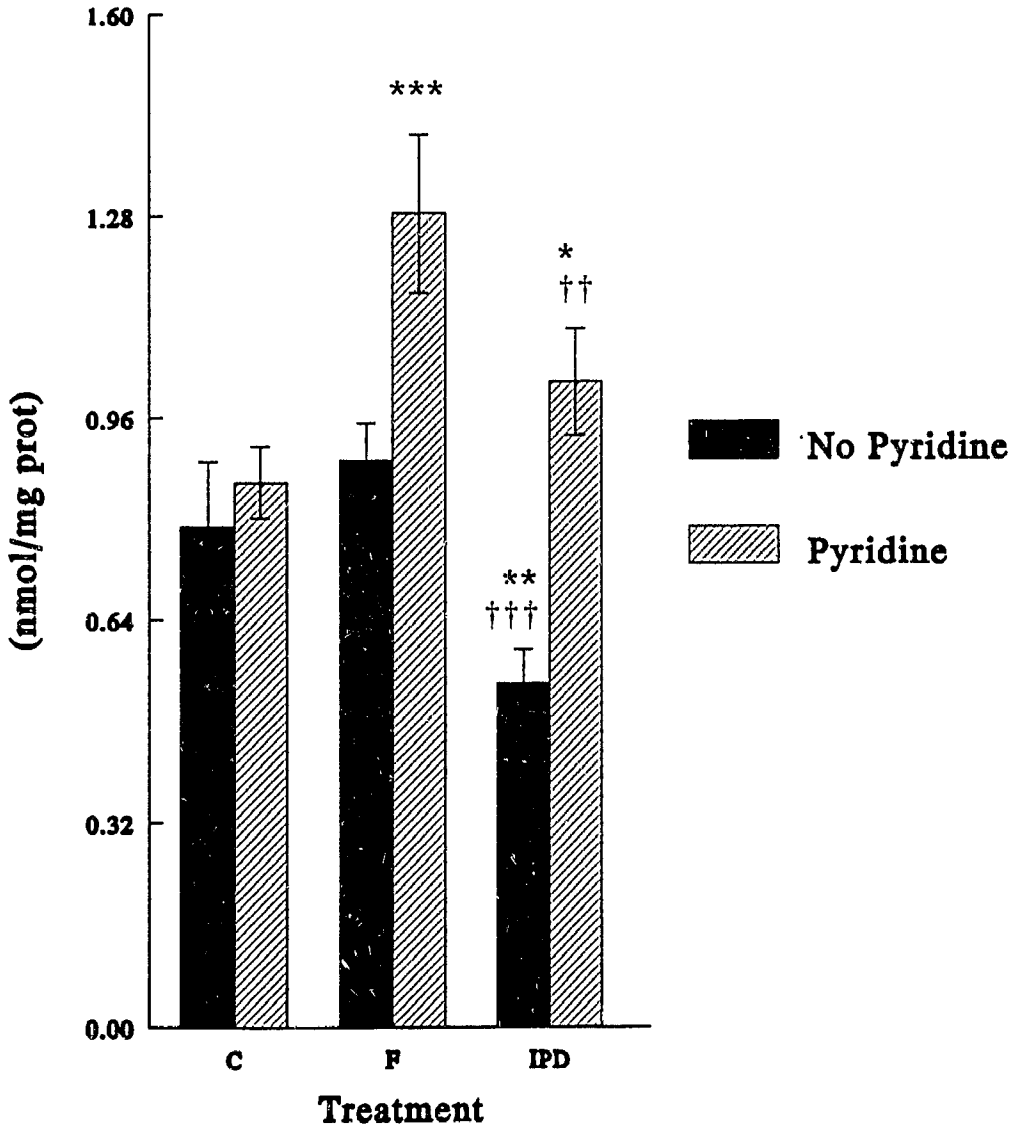

1.60
1.28
0.96
0.64
0.32
0.00
(nmol/mg prot)
***
*
††
**
†††
C
F
IPD
Treatment
No Pyridine
Pyridine

FIGURE 24.

1. Cytochrome P450

The effect of pyridine treatment and nutritional regimen on total P450 content is illustrated in Figure 24. In uninduced rats, isocaloric protein deficiency significantly depressed total P450 levels, while fasting had no significant effect. Fasting and isocaloric protein deficient rats exhibited significantly different effects on total P450 levels in uninduced animals. Pyridine treatment failed to induce total P450 levels in control--fed rats. Total P450 levels were significantly increased in both fasting and isocaloric protein deficient rats following pyridine treatment, however, isocaloric protein deficient P450 levels were significantly lower than in fasting animals.

2. Microsomal EROD Activity

The effect of pyridine treatment and nutritional regimen on microsomal EROD activity is presented in Figure 25. There was no significant effect of fasting ($p=.06$) or of isocaloric protein deficiency ($p=.06$) in uninduced rats. Pyridine induced all 3 nutritional groups, however, isocaloric protein deficiency produced lower levels of EROD activity than those seen in the fasting group.

3. Cytochrome P4501A1 Western Analysis

Microsomes from one representative animal from each experimental group were separated by gel electrophoresis, and transferred to nitrocellulose membrane. The

FIGURE 25.

Effect of isocaloric protein deficiency on the induction of microsomal EROD activity by pyridine. Rats were placed on control–fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours at which time they were treated with saline or 200 mg/kg pyridine (i.p.) and sacrificed 12 hours later (giving a total of 48 hours of fasting or isocaloric protein deficiency); n=4 for each of 6 treatment groups. Two–way analysis of variance determined significance for nutrition ($p=.0167$) and for pyridine induction ($p<.00001$), with no significant interaction between the variables ($p=.1272$). One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal EROD activity expressed as nmol product/mg microsomal protein/minute.

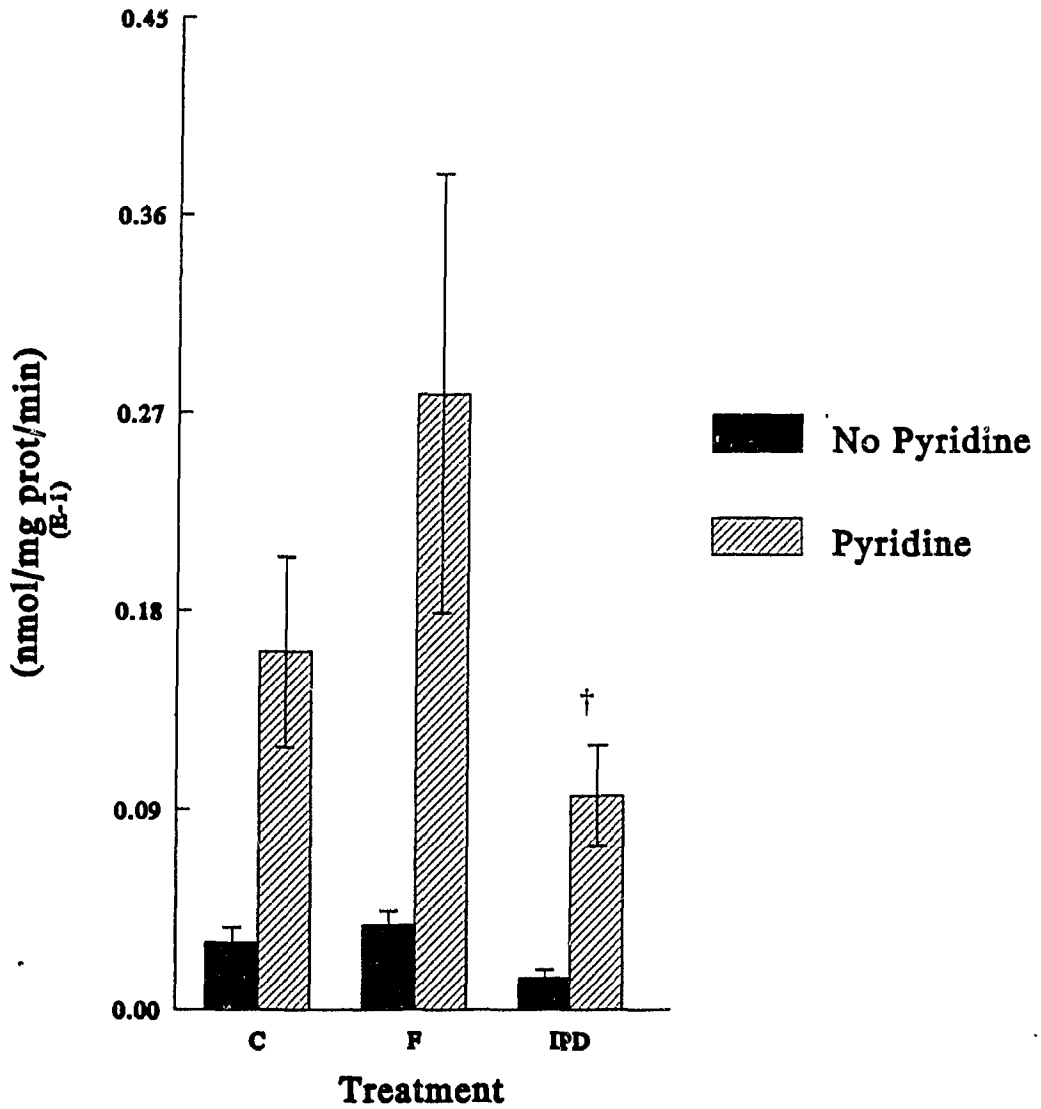

0.45
0.36
0.27
0.18
0.09
0.00
(nmol/mg prot/min)
(E-1)
†
C
F
IPD
Treatment
No Pyridine
Pyridine

FIGURE 25.

FIGURE 26.

P4501A1 expression following pyridine administration. Microsomes from a representative rat from each of the uninduced or induced nutritional groups were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. P4501A1 was detected with the use of a specific, monoclonal antibody and HRP–linked secondary antibody. Lanes 1 through 3 contain microsomes from uninduced control, fasted and isocaloric protein deficient rats, respectively, while lanes 4 through 6 contain microsomes from induced control, fasted and isocaloric protein deficient rats, respectively. Lane 7 contains microsomes from a β–naphthoflavone–induced control rat.

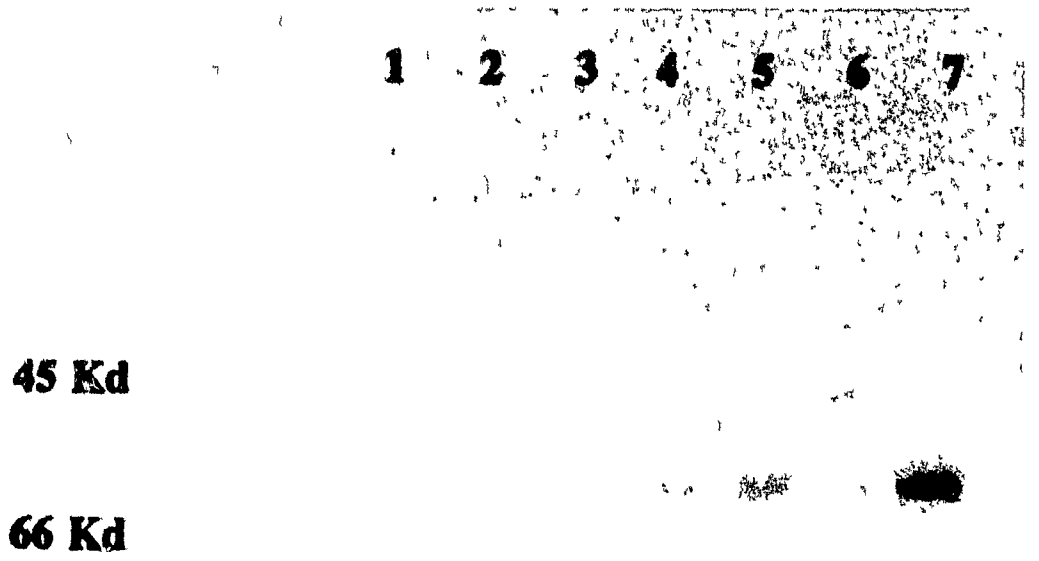


FIGURE 26.

TABLE 11. P4501A1 Western Analysis in Pyridine–induced and –uninduced Isocaloric protein deficient, Fasted and Control–fed Rats. (Corresponds to Figure 26).

Treatment Group	Densitometer Units
Control–fed, no Pyridine	undetectable
Fasted, no Pyridine	undetectable
Protein Deficient, no Pyridine	undetectable
Control–fed, Pyridine	300
Fasted, Pyridine	3500
Protein Deficient, pyridine	50
Standard	22000

choice of representative animals was based on EROD activities so as to best represent the pattern of results depicted in Figure 25. The result of probing with a monoclonal antibody specific for P4501A1 is shown in Figure 26. Lanes 1 to 3 contain microsomes from uninduced rats from each of the three nutritional groups, and show no binding of the anti–1A1 antibody. Lane 4 contains microsomes from an induced rat in the control–fed group, lane 5, a rat from the induced fasting group, and lane 6 from the induced isocaloric protein deficient group. Lane 7 contains a standard consisting of microsomes from a ß–naphthoflavone– treated male rat. The Western blot revealed that antibody binding in the induced fasting animal (Lane 5) was detectably greater than for the induced control (Lane 4), while that of the induced isocaloric protein deficient rat (Lane 6) was lower than for the control. This is supported by densitometry (Table 11).

4. Microsomal PNPH Activity

The effect of pyridine treatment and nutritional status on microsomal PNPH activity is shown in Figure 27. In uninduced rats, PNPH activity was not significantly different from controls in the isocaloric protein deficient group, while uninduced fasting rats exhibited increased levels of PNPH activity relative to both control–fed and isocaloric protein deficient rats. Pyridine treatment significantly induced PNPH activity in all three nutritional groups, however, the absolute levels of PNPH activity were higher in fasting rats relative to both isocaloric protein deficient and control rats.

FIGURE 27.

Effect of isocaloric protein deficiency on the induction of microsomal PNPH activity by pyridine. Rats were placed on control-fed, fasting or isocaloric protein deficient feeding regimens for approximately 36 hours at which time they were treated with saline or 200 mg/kg pyridine (i.p.) and sacrificed 12 hours later (giving a total of 48 hours of fasting or isocaloric protein deficiency); n=4 for each of 6 treatment groups. Two-way analysis of variance determined significance for nutrition ($p=.00002$) and for pyridine induction ($p<.00001$), with significant interaction between the variables ($p=.0029$). One-way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal PNPH activity expressed as nmol product/mg microsomal protein/minute.

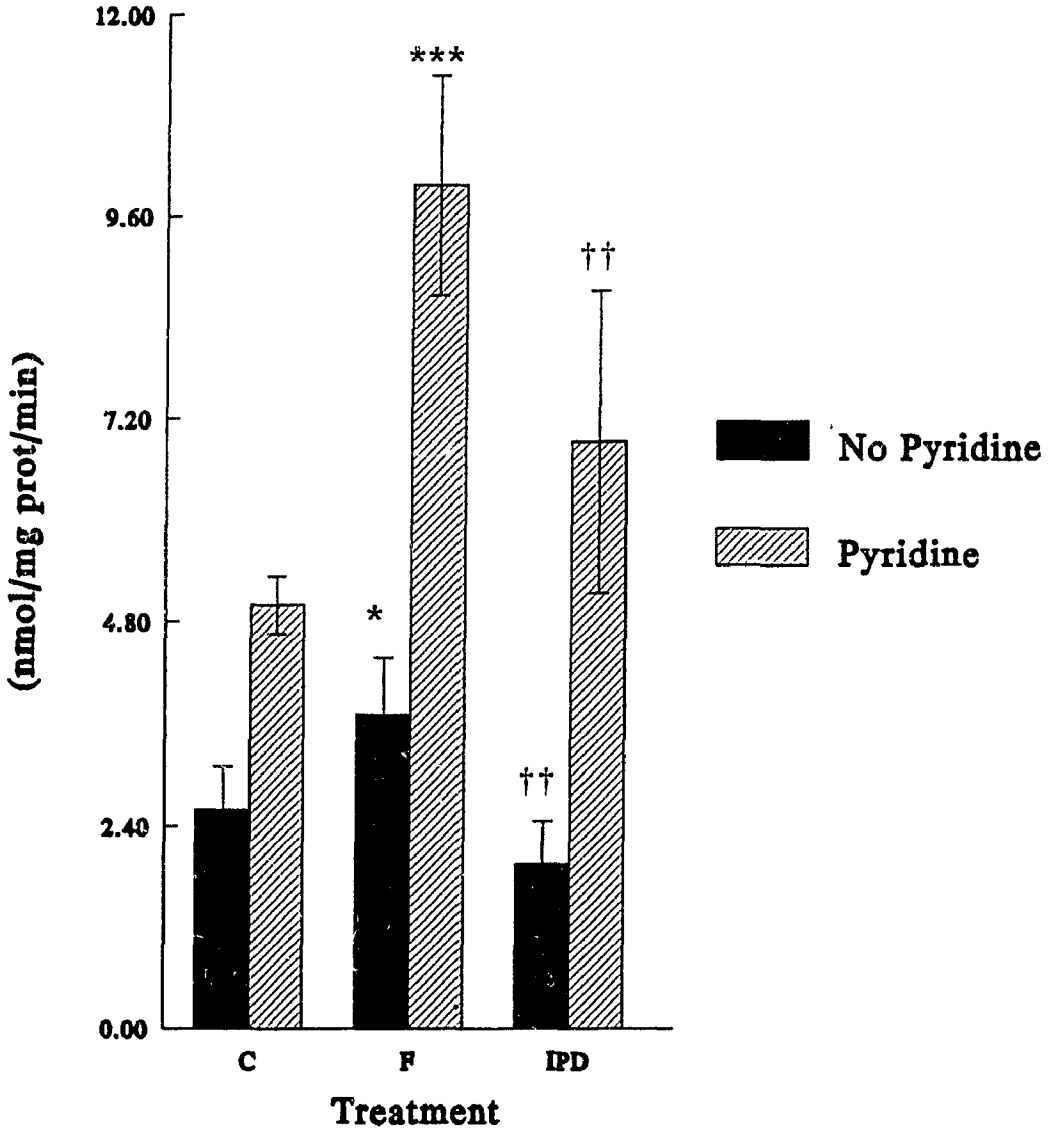

12.00
9.60
7.20
4.80
2.40
0.00
(nmol/mg prot/min)
***
*
††
††
C
F
IPD
Treatment
No Pyridine
Pyridine

FIGURE 27.

Control–fed and isocaloric protein deficient animals exhibited no differences in induced levels of PNPH catalysis.

5. Cytochrome P4502E1 Western Analysis

Microsomes from each uninduced and induced control–fed, fasted and isocaloric protein deficient rat were assessed for the level of P4502E1 protein by Western analysis. Microsomes from uninduced animals exhibited no binding. As shown in Figure 28, microsomes from induced rats exhibited differential binding of the 2E1 antibody according to nutritional group. Lanes 1 to 3 contain microsomes from induced control–fed animals, lanes 4 to 6 from induced fasting animals, and lanes 7 to 9 from induced isocaloric protein deficient animals. Antibody binding was greater in fasting rats relative to controls and roughly equal to controls in isocaloric protein deficient rats. This observation is supported by densitometry (Table 12).

G. Effect of isocaloric protein deficiency (IPD) on the induction of hepatic cytochrome P450 content and activities by acetone.

Experiments designed to examine the effect of isocaloric protein deficiency on the induction of cytochrome P4502E1 by acetone were carried out using the protocol of Song, *et al.* (1989), in which rats were induced by the administration of 5% acetone (v/v) in drinking water for a period of 10 days. In experiments designed to test the inductive effect of acetone in the isocaloric protein deficient state, animals were

FIGURE 28.

Effect of isocaloric protein deficiency on the level of microsomal P4502E1 in pyridine-induced rats. Microsomes from each induced and uninduced control-fed, fasted and isocaloric protein deficient rat were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with a monoclonal antibody specific for P4502E1. Upper lanes 1 through 9 contain microsomes from uninduced control-fed, fasted and isocaloric protein deficient rats. Lower lanes 1, 2, and 3 contain microsomes from isocaloric protein deficient pyridine-induced rats; lower lanes 4, 5, and 6 from fasted pyridine-induced rats, and lower lanes 7, 8, and 9 contain microsomes from control-fed pyridine-induced rats. Antibody binding produced a single band at a molecular weight of approximately 50 kilodaltons.

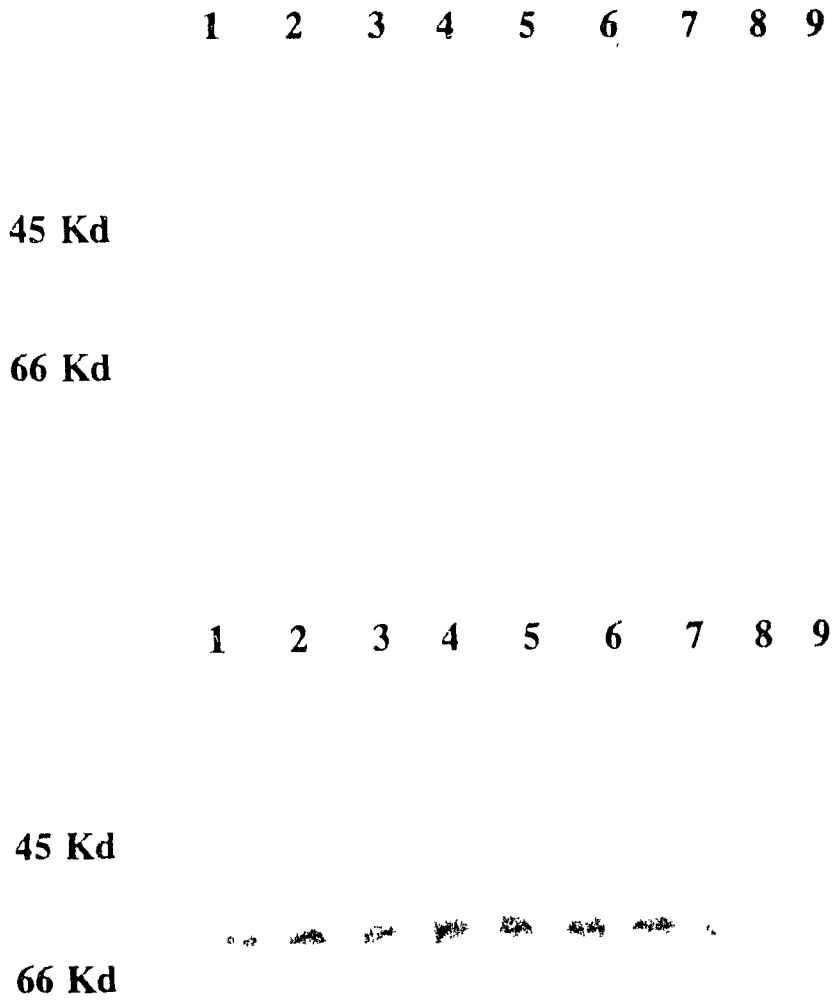


FIGURE 28.

TABLE 12. P4502E1 Western Analysis in Pyridine-induced and -uninduced Isocaloric protein deficient, Fasted and Control-fed Rats.
(Corresponds to Figure 28).

Treatment Group	Densitometer Units
Control-fed, no Pyridine	undetectable
Fasted, no Pyridine	undetectable
Protein Deficient, no Pyridine	undetectable
Control-fed, Pyridine	4600
Fasted, Pyridine	7000
Protein Deficient, Pyridine	5200

induced with acetone for 10 days as per the protocol, after which they were started on control, fasting, or isocaloric protein deficient feeding regimens, and left for a period of 36 hours before sacrifice.

1. Cytochrome P450

The effect of nutritional regimen and acetone administration on total hepatic P450 is shown in Figure 29. In uninduced rats, isocaloric protein deficiency resulted in a significant depression of total P450 relative to control–fed rats. Fasting resulted in no difference from controls in uninduced rats, however, the fasting and isocaloric protein deficient groups were significantly different. Acetone administration produced significant induction of total P450 levels in all three groups, however, fasting animals exhibited significantly higher absolute levels of total P450 than either isocaloric protein deficient or control--fed rats.

2. Microsomal EROD Activity

The effect of feeding regimen and acetone induction on microsomal EROD activity is shown in Figure 30. In uninduced animals, fasting and isocaloric protein deficient were not significantly different from control–fed rats, however, EROD levels in uninduced fasting and isocaloric protein deficient groups were significantly different from each other. Acetone treatment resulted in a small significant induction of EROD activity in control–fed rats, and no induction in the isocaloric protein deficient group.

FIGURE 29.

Effect of isocaloric protein deficiency on the induction of total microsomal cytochrome P450 by acetone. Rats were administered plain water or water containing 5% acetone (v/v) for 10 days before being placed on control–fed, fasting or isocaloric protein deficient feeding regimens for 36 hours prior to sacrifice; n=4 for each of 6 treatment groups. Two–way analysis of variance determined significance for nutrition ($p=.00002$) and for acetone induction ($p<.00001$), with no interaction between the variables ($p=.1360$). One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal cytochrome P450 content expressed as nmol/mg microsomal protein.

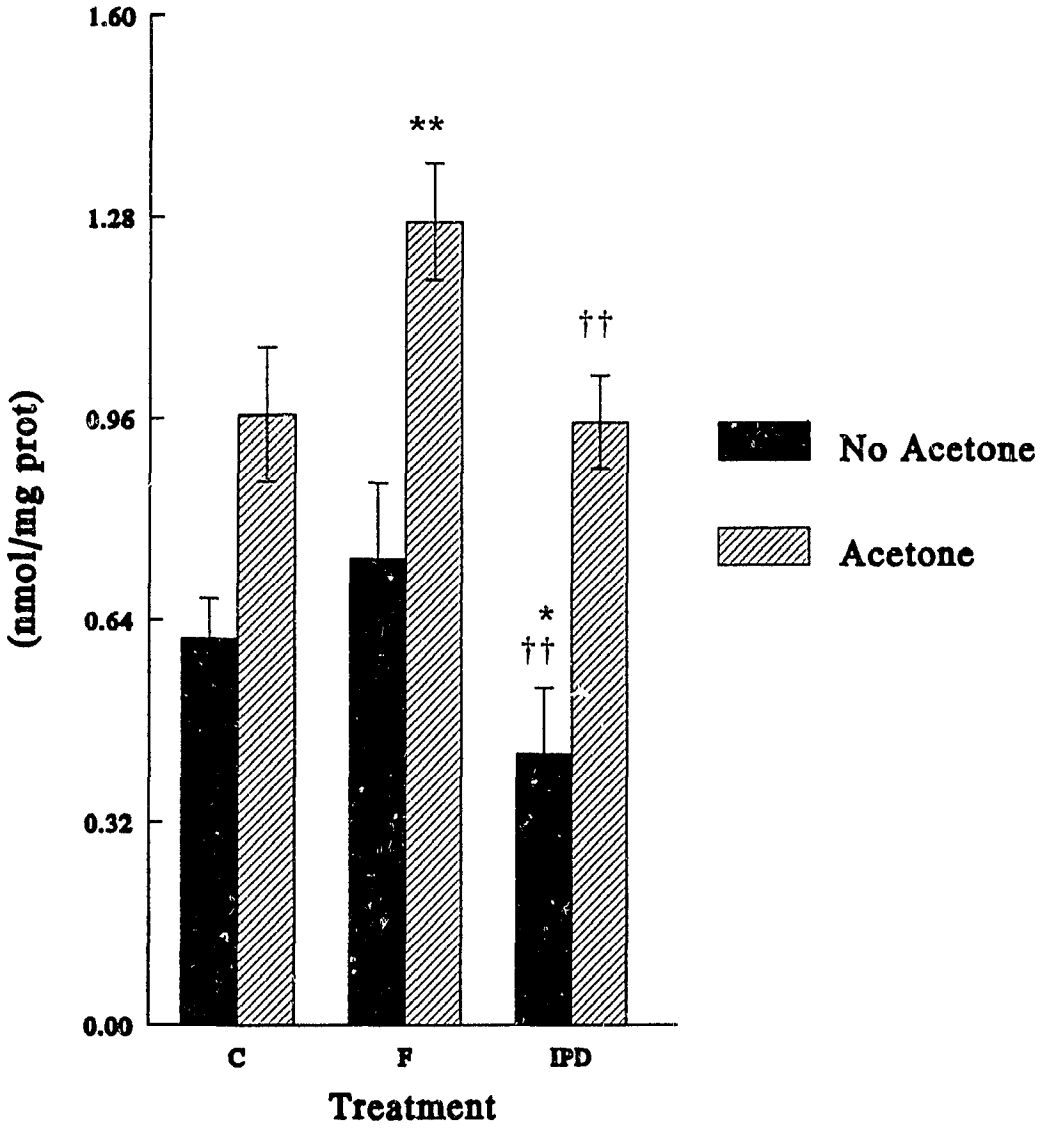

1.60
1.28
0.96
0.64
0.32
0.00
(nmol/mg prot)
**
††
*
††
C
F
IPD
Treatment
No Acetone
Acetone

FIGURE 29.

FIGURE 30.

Effect of isocaloric protein deficiency on the induction of microsomal EROD activity by acetone. Rats were administered plain water or water containing 5% acetone (v/v) for 10 days before being placed on control–fed, fasting or isocaloric protein deficient feeding regimens for 36 hours prior to sacrifice; n=4 for each of 6 treatment groups. Two–way analysis of variance determined significance for nutrition ($p<.00001$) and for acetone induction ($p<.00001$), with significant interaction between the variables ($p=.00002$). One–way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control–fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented as means ± standard error, with microsomal EROD activity expressed as nmol product/mg microsomal protein/minute.

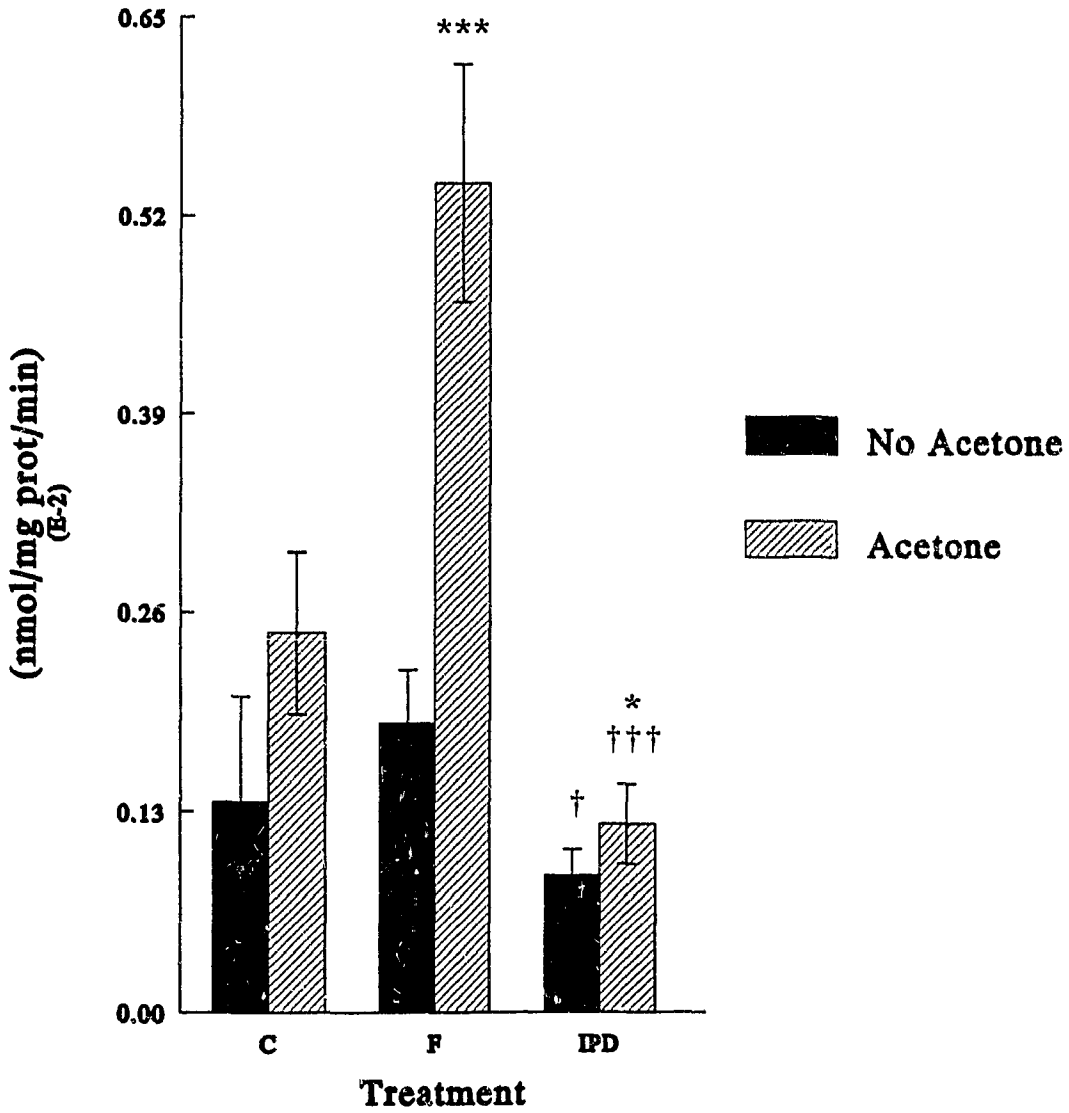

0.65
0.52
0.39
0.26
0.13
0.00
(nmol/mg prot/min)
(E-2)
***
*
†††
†
No Acetone
Acetone
C
F
IPD
Treatment

FIGURE 30.

FIGURE 31.

P4501A1 expression following acetone administration. Microsomes from a representative rat from each of the uninduced or induced nutritional groups were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. P4501A1 was detected with the use of a specific, monoclonal antibody and HRP–linked secondary antibody. Lanes 1 through 3 contain microsomes from uninduced control, fasted and isocaloric protein deficient rats, respectively, while lanes 4 through 6 contain microsomes from induced control, fasted and isocaloric protein deficient rats, respectively. Lane 7 contains microsomes from a β–naphthoflavone–induced control rat.

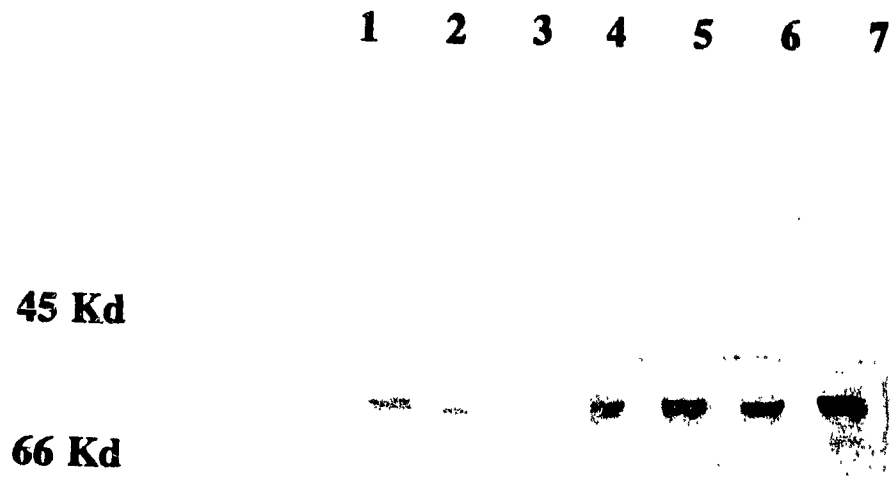


FIGURE 31.

TABLE 13. P4501A1 Western Analysis in Acetone-induced and -uninduced Isocaloric protein deficient, Fasted and Control-fed Rats.
(Corresponds to Figure 31).

Treatment Group	Densitometer Units
Control-fed, no Acetone	7000
Fasted, no Acetone	4700
Protein Deficient, no Acetone	2700
Control-fed, Acetone	10000
Fasted, Acetone	15000
Protein Deficient, Acetone	13000
Standard	20000

In the fasting group, acetone treatment resulted in substantially greater levels of EROD activity than those seen in either the isocaloric protein deficient or control-fed groups.

3. Cytochrome P4501A1 Western Analysis

Microsomes from one representative animal from each experimental group were separated by gel electrophoresis and transferred to nitrocellulose membrane. The result of probing with a monoclonal antibody specific for P4501A1 is shown in Figure 31. Lanes 1 to 3 contain microsomes from uninduced rats from each of the three nutritional groups, and show minimal binding of the anti-1A1 antibody. Lane 4 contains microsomes from an induced rat in the control-fed group, lane 5, a rat from the induced fasting group, and lane 6 from the induced isocaloric protein deficient group. Lane 7 contains a standard consisting of microsomes from a ß-naphthoflavone-treated male rat. The induction of cytochrome P4501A1 protein was not attenuated by fasting or isocaloric protein deficiency. In fact, it was found that acetone induction occurred to different extents according to nutritional regimen with control-fed rats exhibiting a 1.5 fold increase in P4501A1 protein, fasting rats a 3-fold induction of P4501A1, and isocaloric protein deficient animals a 4.5-fold induction.

4. Microsomal PNPH Activity

The effect of acetone treatment and feeding regimen on microsomal PNPH activity is shown in Figure 32. In uninduced rats, isocaloric protein deficiency resulted

in a significant increase in PNPH activity relative to both fasting and control–fed rats, while there was no difference between uninduced control–fed and fasting animals. This finding is not in keeping with the findings presented in Figures 17 or 27. As shown in Figure 17 it was found that isocaloric protein deficiency resulted in a small but significant depression of PNPH activity after 48 hours of nutritional deficiency, and furthermore, an elevation of PNPH in isocaloric protein deficient animals was not observed at any time point relative to either fasting or control–fed rats. Similarly, in Figure 27, uninduced, isocaloric protein deficient rats demonstrated significantly lower levels of PNPH activity than uninduced fasting rats. The discrepant result in isocaloric protein deficient rats is not due to insufficient intake of their 20% glucose feed as the level of PNPH activity in fasting and isocaloric protein deficient rats is significantly different. The elevated levels of PNPH activity in the isocaloric protein deficient group suggests that these animals were exposed to an inducing influence. One possibility is excessive exposure to acetone fumes in the room in which the animals were housed. It has been shown by others (Elovaara et al., 1991) as well as by the results in this thesis, that exposure to acetone fumes can produce significant P450 induction.

Acetone treatment resulted in significant induction of PNPH activity in all three nutritional groups, with no differences among them in the absolute levels of PNPH activity.

FIGURE 32.

Effect of isocaloric protein deficiency on the induction of microsomal PNPH activity by acetone. Rats were administered plain water or water containing 5% acetone (v/v) for 10 days before being placed on control-fed, fasting or isocaloric protein deficient feeding regimens for 36 hours prior to sacrifice; n=4 for each of 6 treatment groups. Two-way analysis of variance determined no effect of nutrition ($p=.1423$) and a significant effect for acetone induction ($p<.00001$), with significant interaction between the variables ($p=.0253$). One-way analysis of variance determined differences between nutritional regimens within the induced or uninduced groups, respectively. Significant differences between control-fed and fasting or isocaloric protein deficient rats are indicated with 1, 2 or 3 asterisks for $p< .05$, .01, or .001, respectively. Significant differences between fasting and isocaloric protein deficient rats are indicated with 1, 2 or 3 crosses for $p< .05$, .01, or .001, respectively. Data are presented means ± standard error, with microsomal PNPH activity expressed as nmol product/mg microsomal protein/ minute.