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STRUCTURAL AND FUNCTIONAL ASSEMBLY OF RAT INTESTINAL CYTOCHROME P-450 ISOZYMES

EFFECTS OF DIETARY IRON AND SELENIUM

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Abstract—We have reported previously that both dietary iron and selenium regulate intestinal cytochrome P-450 content by modulating the synthesis of its prosthetic heme moiety. Whether these elements are required for synthesis and/or viability of its apocytochrome moiety is unknown. We have examined the effects of intraluminal deprivation of these elements on the apocytochrome moieties of the constitutive (P-450) and the β-naphthoflavone inducible (P-448) intestinal isozymes. The relative content of intestinal apocytochrome P-450 moieties generated by dietary deprivation of iron and/or selenium was assessed indirectly by complexing with exogenous heme *in vitro*, to reassemble the holocytochromes which could be monitored spectrally and catalytically. We now report that, whereas both intraluminal iron and selenium are required for maintenance of the prosthetic apocytochrome moiety of the constitutive intestinal isozyme, only intraluminal selenium is required for the viability of apocytochrome P-448. The latter apparently survives in the absence of intraluminal iron and can be assembled to the holocytochrome, with exogenously added heme. The mechanistic basis of the critical requirement of intestinal apocytochromes for intraluminal selenium is unclear. It is intriguing, however, that the deleterious effects of selenium deprivation are principally exerted in cell systems actively synthesizing protein and inexorably dependent on their extracellular milieu for their nutriment.

The small intestinal mucosa serves as the primary portal of entry for orally ingested nutrients and adventitious material, and it is hardly surprising that it has consequently developed the potential to biotransform such absorbed compounds to more water soluble, often less toxic, and ultimately excretable products. Such biotransformations are catalyzed in the intestinal mucosa largely by a microsomal mixed-function oxidase (MFO†) system, of which cytochrome P-450 and NADPH-cytochrome P-450 reductase are key components [1–9]. The biotransformation potential of the intestinal mucosa appears to closely parallel its absorptive capacity as indicated by the descending gradient in mucosal MFO activity from the duodenum to the colon [1, 9, 10]. Similarly, a progressive enrichment in cytochrome P-450 content and MFO activity occurs as the mucosal crypt cells mature during their migration to the villous tip, where they are believed to function primarily as absorptive units [9, 11–13].

Intestinal cytochrome P-450, in common with its hepatic isozymes, is induced by a wide variety of xenobiotics. However, in the intestinal mucosa, the polycyclic aromatic hydrocarbons are more potent inducers of cytochrome P-450 (P-448) than the phenobarbital type of inducers [1–5, 7, 14–19]. Intestinal cytochrome P-450 is also regulated by dietary nutrients. For instance, not only intraluminal iron but, as recently shown by us, selenium is also critically required for basal maintenance of intestinal cytochrome P-450 content and its dependent MFO activity [9, 20]. Accordingly, acute deprivation of either element for as short a period as a single day reduces basal intestinal cytochrome P-450 content by limiting the formation and/or availability of its prosthetic heme [20]. Whether intraluminal iron and/or selenium also regulate cytochrome P-450 by modulating the formation and/or availability of the apocytochrome moiety is unknown. Normally, short-term deprivation of either iron or selenium has no appreciable effect on hepatic cytochrome P-450 in intact rats [21, 22]. However, selenium has been shown recently to be required for induction of apocytochrome P-450 by phenobarbital in hepatocytes in monolayer culture [23]. Since in its extensive dependency on the intraluminal environment, the mature enterocyte at the villous tip behaves like a hepatocyte in culture, we investigated whether acute dietary deprivation of iron and/or selenium, which limits the availability of cytochrome P-450 heme, has any concurrent effect on intestinal apocytochrome P-450. In the liver, apocytochrome P-450 formation can be dissociated from heme synthesis. When endogenous heme is limiting, the hepatic apocytochrome can combine with exogenous heme to form the functional holocytochrome [24]. It is unknown whether, in the intestinal mucosa, the syntheses of the two cytochrome P-450 components are coordinated, or whether formation of apocytochrome P-450 can be similarly dissociated from that of heme. We therefore investigated whether, under conditions of limited intestinal heme availability

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† Abbreviations: MFO, mixed-function oxidase; β-NF, β-naphthoflavone; GSH, glutathione; DTE, dithioerythritol; BaP, benzo[*a*]pyrene; AHH, aryl hydrocarbon hydroxylase; EROD, 7-ethoxyresorufin *O*-deethylase; ECOD, ethoxycoumarin *O*-deethylase; BSA, bovine serum albumin; and HSA, human serum albumin.

elicited by dietary iron deprivation, the intestinal apoprotein would exist independently of heme. We have also examined whether its occurrence in the "uncommitted" or "heme-free" form could be monitored following its structural and functional assembly with exogenous heme *in vitro* to the holocytochrome. In addition, employing this approach we have explored whether, in such iron-deprived rats, β-naphthoflavone, an inducer of cytochrome P-448, would increase the relative fraction of "heme-free" and, therefore, constitutable apocytochrome P-448. Such an increase in apocytochrome P-488 would permit us to examine in detail the extent of the heme-dependent structural and functional assembly of the holocytochrome P-448 *in vitro*.

EXPERIMENTAL PROCEDURE

Animals. All experiments were performed with adult male Sprague–Dawley rats (200–240 g) from Simonsen Laboratories (Gilroy, CA). The animals were housed in hanging stainless steel cages in rooms maintained at 25° with controlled 12 hr diurnal lighting, and were protected from contact with pesticides and cigarette smoke, which are known to influence cytochrome P-450-dependent MFO activity [2, 25].

Diets. Animals were routinely weaned from the standard laboratory chow diet onto a specific dietary regimen by feeding the semi-synthetic casein-based diet deficient in the experimental variable for 2 days. This regimen was instituted to permit sufficient time for a complete crypt to villous tip cell transformation, sloughing of tip cells from the mucosa, and consequent expurgation of natural cytochrome P-450 inducers normally present in the laboratory chow [1, 5, 15]. The animals were then pair-fed the specific purified diets, deficient in or supplemented with iron, selenium, and/or the cytochrome P-448 inducer β-naphthoflavone (β-NF).

The following diets were used: (i) *semi-synthetic iron and selenium deficient diet* (*−Fe −Se*), consisting of vitamin-free casein, 27%; corn starch, 56%; hydrogenated vegetable oil, 14%; salt mixture without added ferrous sulfate or sodium selenite, 3%; and ICN vitamin diet fortification mixture, 1% (ICN Nutritional Biochemicals, Cleveland, OH). The iron and selenium contents of this diet were determined to be below 0.1 mg and 0.005 mg per 100 g diet respectively; (ii) *semi-synthetic iron supplemented, selenium deficient diet* (*+Fe −Se*), consisting of the above diet with 10 mg iron as ferrous sulfate added per 100 g diet; (iii) *semi-synthetic iron deficient, selenium supplemented diet* (*−Fe +Se*), consisting of −Fe −Se diet supplemented with 0.05 mg selenium as sodium selenite per 100 g diet; (iv) *semi-synthetic iron and selenium supplemented diet* (*+Fe +Se*), consisting of −Fe −Se diet with iron and selenium supplements as in diets (ii) and (iii); (v) *semi-synthetic iron deficient, selenium supplemented, β-NF-supplemented diet* (*−Fe +β-NF*), consisting of −Fe +Se diet (ii) supplemented with β-NF, 0.1 g per 100 g diet; (vi) *semi-synthetic iron and selenium supplemented, β-NF supplemented diet* (*+Fe +β-NF*), consisting of the +Fe +Se diet (iv) supplemented with β-NF, 0.1 g per 100 g diet. Following weaning, animals were divided into experimental groups, each fed *ad lib*. one of the above diets and given free access to distilled water for 3–5 days. Regardless of the dietary regimen, the animals were found to gain weight uniformly during this period.

The intracellular selenium status of the intestinal mucosa of rats fed the above diets was assessed by determining the activity of the cytosolic selenium-dependent glutathione peroxidase [26]. Unless otherwise specified (diets i and ii), all diets contained adequate selenium.

Intestinal cell preparations. Intestinal segments and villous cell populations were prepared as previously described [20, 27] except that the small intestine was sectioned 12 or 20 cm distal to the pylorus, for preparation of everted intestinal sacs or for subcellular fractionation respectively.

Preparation of cell fractions. Whole mucosa or villous cell fractions from rats fed the semi-synthetic diet were pooled from two animals in order to obtain sufficient enzyme containing material, while mucosa or villous cells from rats fed the β-NF-supplemented diets were used without pooling. Tissues were homogenized in a glass–Teflon Potter–Elvehjem homogenizer with 20 strokes at 200 rpm, in 0.05 M Tris/20% glycerol (v/v)/1.15% KCl (w/v) buffer (pH 7.8), supplemented with trypsin inhibitor (5 mg/g wet weight of small intestine) and heparin (3 units/ml) to decrease degradation and agglutination of the microsomal MFO proteins [8]. For *in vitro* examination of cytochrome P-450 assembly in subcellular fractions, histidine HCl, (0.1 M final concentration, pH 7.8 [28]) was added to the homogenate. In such studies, whole homogenate or its 9000 *g* supernatant fraction, obtained after a 20-min centrifugation at 4°, was incubated with hemin, after which 9000 *g* supernatant fractions were prepared and/or centrifuged at 105,000 *g* for 60 min at 4°. The microsomal pellet was collected and finely dispersed in 0.05 M Tris/20% glycerol/1.15% KCl buffer (pH 7.8) by ten passes through a 16 gauge needle.

In an attempt to remove non-specifically bound heme from microsomes, the microsomal suspension was incubated at 37° for 4 min following addition of human serum albumin (HSA, 60 μM), with or without dithioerythritol (DTE, 1 mM) in 0.05 M Tris buffer (pH 7.8) [29]. However, this procedure not only removed adventitious heme, but prevented the increase in both intestinal microsomal cytochrome P-450 content and EROD activity observed when hemin was incubated with 9000 *g* supernatant fractions from rats fed the iron-deficient diet supplemented with β-NF (data not shown). Similarly, incubation of 9000 *g* supernatant fraction from rats fed the +Fe +β-NF diet with HSA also reduced intestinal microsomal cytochrome P-450 content and EROD activity, to levels seen in intestinal microsomes from iron-deprived rats. These findings indicate that, in addition to eliminating adventitious heme, HSA also removed prosthetic heme from intestinal cytochrome P-450, thereby underscoring the instability of intestinal cytochrome P-450 [8] as compared to the much more stable hepatic isozyme [29].

In all studies to be reported, microsomes were "washed" by resuspension and recentrifugation at 105,000 *g* for 60 min at 4°. The final microsomal

pellet was resuspended and homogenized in the 0.05 M Tris/20% glycerol/1.15% KCl buffer with ten strokes by hand in a glass–Teflon homogenizer, to yield approximately 2 mg protein/ml buffer for uninduced microsomes, and 1 mg/ml for β-NF-induced microsomes.

Protocol for hemin-dependent cytochrome P-450 assembly. Assembly of intestinal cytochrome P-450 with hemin was examined in two separate *in vitro* systems: subcellular fractions of intestinal mucosal cells and everted intestinal sacs.

Incubation mixtures contained whole homogenate or 9000 *g* supernatant fractions of villous cells (10 ml, final volume) or microsomes (2 ml, final volume) at 4.5 to 5.0 mg protein/ml. Hemin (ferriprotoporphyrin IX hydrochloride) was dissolved in 0.1 N NaOH and buffered with 0.05 M Tris, pH 7.8. Aliquots of the hemin solution were added to the cellular fractions and incubated at 37° at various concentrations and for different time periods (Results). At the end of the incubation, the homogenate or 9000 *g* supernatant fractions were either mixed with equal volumes of ice-cold homogenizing buffer and centrifuged to obtain microsomes, or were directly assayed for MFO activity as soon as possible (Results). On the other hand, following incubation with hemin, microsomes were immediately assayed for MFO activity.

In preliminary experiments, in an attempt to maximize hemin-mediated assembly of cytochrome P-450 in intestinal 9000 *g* supernatant fractions from β-NF-treated rats, hemin incubation was performed in the presence of various sulfhydryl compounds. This procedure has been found to maximize the reconstitution of bacterial cytochrome $P\text{-}450_{cam}$ and its reactivation from cytochrome $P\text{-}420_{cam}$ [30–32]. Sulfhydryl agents also have been shown to enhance the reconstitution of hepatic cytochrome P-450 [24]. In contrast to findings of studies with bacterial and hepatic cytochrome P-450, supplementation of incubations of intestinal 9000 *g* supernatant fractions from − Fe+β-NF fed rats with the sulfhydryl compounds cysteine, GSH, or DTE not only inhibited the hemin-mediated assembly of the intestinal holocytochrome, but in fact substantially reduced the basal cytochrome content and its functional activity (data not shown). Incubation of intestinal microsomes from the +Fe + β-NF fed rats with cysteine also decreased cytochrome P-448 content by 27%. Because of these findings, sulfhydryl agents were omitted from all incubations of 9000 *g* supernatant fractions with hemin.

Everted intestinal sacs were prepared by modification of the methods of Wilson and Wiseman [33] and Chowhan and Amaro [34]. Intestinal segments (12 cm) were excised, placed in carbogen (95% O_2, 5% CO_2)-gassed ice-cold incubation buffer, and everted over a glass rod at 4°. The intestinal segments were attached at the pyloric end to a flared glass tube passed through a rubber stopper. They were tied off at the distal end, placed in ice-cold carbogen-gassed incubation media in 50 ml tubes, and distally anchored to the bottom of the tube. Carbogen inlet and gas outlet consisted of 18 gauge needles passed through the rubber stopper, with PE 190 tubing sleeved over the inlet.

Everted sacs were incubated at 37° by gentle shaking in a water bath with constant gentle bubbling of carbogen in one of the following media: (i) Tyrode's buffer, pH 7.8 [35]; (ii) modified Krebs–Henseleit (K–H) bicarbonate buffer, pH 7.8 [36], consisting of Na^+ (0.143 M), K^+ (5.9 mM), Cl^- (0.128 M), Mg^{2+} (1.2 mM), Ca^{2+} (2.6 mM), $H_2PO_4^-$ (2.2 mM), HCO_3^- (24.9 mM), and SO_4^{2-} (1.2 mM), supplemented with 0.2% glucose; (iii) the above K–H bicarbonate buffer supplemented with 1.2% NaCl (205 mM); and (iv) the above K–H bicarbonate buffer supplemented with 1.2% NaCl and 6.5% glucose. The addition of 1.2% NaCl and 6.5% glucose to the modified K–H incubation media was intended to sufficiently change the osmolality of the media in order to prevent net fluid movement across the mucosa [37]. In turn, this was expected to overcome the loss of mucosal cellular membrane integrity [3] and possibly that of the membrane-bound cytochrome P-450 during everted intestinal sac incubations (Results).

During hemin incubations, the everted sac medium was supplemented with glutamine (15 mM), the major mucosal respiratory "fuel" [39], and cysteine (6 mM), for protection of protein sulfhydryl groups [31, 32]. Hemin was dissolved in 0.1 N NaOH, adjusted to pH 7.8 with 0.1 N HCl, buffered in 0.1 M phosphate buffer (sodium and potassium salts), and added to 50 ml of incubation media after a 5-min preincubation at 37°. Incubations were carried out for 20 min with or without added hemin (15 μM), at the end of which HSA (0.1 mM) and DTE (1.0 mM) were added to the incubation medium and further incubated for 4 min to remove excess heme present in the medium and/or non-specifically bound to the mucosal cells [29].

At the end of all incubations, the sacs were removed from the apparatus and rinsed twice in 50 ml of ice-cold K–H buffer (pH 7.8), whole mucosa were separated from the submucosa, and homogenates were prepared as described under "Intestinal cell preparation". Individual cell populations could not be used because an increased tendency for cell sloughing occurred post-incubation of the everted sacs, which made intestinal cell separation difficult by the scraping technique.

Assays: Intestinal cytochrome P-450. Microsomal cytochrome P-450 content was determined by the method of Omura and Sato [40] modified as previously described [20]. The method of Johannesen and DePierre [41] was used with β-NF-induced microsomes, which employs the reduced difference spectrum of CO-bubbled microsomes (extinction coefficient, 105 $mM^{-1}\,cm^{-1}$). This was not employed in studies with non-induced microsomes due to marked interference at 452 nm from the disproportionately greater absorption of reduced cytochrome b_5 at 426 nm. The amount of sodium dithionite required for reducing the pigment was carefully controlled since excess dithionite appears to cause microsomal enzyme inactivation with consequent loss of spectrally detectable cytochrome P-450.

MFO activity. In preliminary experiments, incubation of heme with intestinal preparations resulted in a proportionately greater contamination of intestinal microsomes with non-prosthetically bound heme than that observed after corresponding hemin

incubation of liver preparations. To avoid possible interference by non-prosthetically bound heme in substrate oxidation or during subsequent product analysis from such hemin-constituted intestinal preparations, MFO assays had to be pre-screened for the effect of hemin. Assays of MFO activity in the 9000 *g* supernatant fraction were conducted with a radiometric aryl hydrocarbon hydroxylase (AHH) assay using [^{3}H]benzo[a]pyrene (BaP) as substrate [42]. The radiometric AHH assay was originally designed to avoid heme-mediated quenching of 3-OH BaP fluorescence [43]. EROD activity was chosen for monitoring MFO activity in the microsomal fraction because its basal values in the intestinal mucosa are sufficiently high for easy detection and it was most markedly stimulated following induction by dietary inducers (Table 1). In preliminary studies, heme was found to quench the fluorescence of resorufin (0.4% decrease in fluorescence per nmole hemin per ml); however, in our EROD assay system, the supernatant obtained following protein precipitation of incubations of 9000 *g* supernatant fraction or microsomes with hemin did not contain sufficient heme to produce such quenching.

Aryl hydrocarbon hydroxylase (AHH). Activity was determined by a modification of the radiometric assay of Van Cantfort *et al.* [42]. Assays were carried out at 37° in scintillation vials with 0.2 to 0.5 mg of 9000 *g* supernatant protein in a final volume of 0.5 ml of 0.05 M Tris buffer (pH 7.4) containing NADPH (0.5 mM), NADH (0.5 mM), and $MgCl_2$ (5 mM). Substrate concentrations in assays of both induced and non-induced enzyme were routinely 80 μM, which were found to be saturating for hepatic cytochrome P-450 [44]. After a 3-min preincubation at 37°, reactions were started with 20 μl substrate, spiked with 150,000–200,000 cpm [^{3}H]BaP, and terminated after 15 min with 1 ml of 0.15 M KOH in 85% dimethylsulfoxide (DMSO). Assay blanks consisted of the same incubation mixture, to which the KOH/DMSO mixture was added at "0" time, i.e. before incubation. Products were extracted twice into hexane (5 ml) by vortexing for 1 min and centrifuging for 2 min at 2000 rpm. Aliquots (0.5 ml) of the final aqueous phase were radioquantitated in 15 ml of Dimilume. Activity was expressed as pmoles BaP hydroxylated per mg protein per min.

7-Ethoxyresorufin-O-deethylase (EROD). Activity was determined by a modification of the fluorimetric assays described [45, 46] as previously described [20]. Reactions with uninduced microsomes contained 0.5 to 1.0 mg of protein, 1 nmole of 7-ethoxyresorufin (0.33 mM in DMSO), and were carried out at 37° for 10 min; reactions with β-NF-induced microsomes contained 0.1 to 0.2 mg of protein, 7-ethoxyresorufin (5 nmoles), and were carried out at 37° for 2 min. Because we suspected that the resorufin commercially supplied was impure, it was further purified and the standard stock solution quantitated spectrophotometrically using an extinction coefficient of $4.00 \times 10^4\ M^{-1}\ cm^{-1}$ at 572 nm [45]. Spectral quantitation of the standard stock solution we believe is an important precaution to be taken in this assay and accounts for the discrepancy in the magnitude of the values presented in this study and those previously reported [20], wherein resorufin concentrations

Table 1. Effects of β-naphthoflavone (β-NF) on intestinal microsomal cytochrome P-450 and related MFO activity*

β-NF†	Cytochrome P-450 (pmoles/mg protein)	Ethoxyresorufin *O*-deethylase (EROD) (pmoles/mg protein/min)	Ethoxyresorufin *O*-deethylase (EROD) (pmoles/pmole P-450/min)	Ethoxycoumarin *O*-deethylase (ECOD) (pmoles/mg protein/min)	Ethoxycoumarin *O*-deethylase (ECOD) (pmoles/pmole P-450/min)
−	25.0 ± 5.0	2.31 ± 0.48	0.09 ± 0.02	8.5 ± 1.2	0.34 ± 0.1
+	150.0 ± 25.0	2540 ± 210	16.9 ± 1.38	2450 ± 250	16.3 ± 1.6

* Values are mean ± S.E.M., N = 4; each N represents a pool of tissue from two non-induced animals, or individual tissue samples from β-NF-treated animals.

† β-NF (0.1%) supplemented in the semi-synthetic diet, replete in iron and selenium.

were quantitated on the basis of purified sample weight.

7-Ethoxycoumarin-O-deethylase (ECOD). Activity was determined by the method of Greenlee and Poland [47] with the modifications of Guengerich [48]. The assay was performed in scintillation vials, in a volume of 1 ml containing NADPH (0.5 mM), NADH (0.5 mM), $MgCl_2$ (5 mM), and BSA (1 mg/ml) in 0.05 M Tris buffer (pH 7.4). Reactions contained 0.1 ml of 9000 *g* supernatant fraction and were started with 7-ethoxycoumarin (0.5 nmole in 0.025 ml of 50% aqueous methanol). After a 3-min preincubation, reactions were carried out at 37° for 10 min with vigorous shaking. Reactions were stopped with 0.1 ml of 2 N HCl and 3 ml of chloroform, transferred to 12 ml centrifuge tubes, vortexed, and centrifuged for 1 min at 2000 rpm to separate the layers. A 1.5-ml aliquot of the organic phase was extracted with 2.5 ml sodium borate (30 mM, pH 9.2). Fluorescence of the aqueous phase was monitored in an Aminco–Bowman spectrofluorimeter (excitation at 368 nm and emission at 456 nm) and compared with a standard curve obtained with 10 pmoles to 5 nmoles 7-OH coumarin. Assay blanks consisted of the complete reaction mixture to which substrate was added after addition of HCl and chloroform. Product recovery ranged from 80 to 95%.

Other assays. Heme content was monitored by the reduced minus oxidized difference (557–575 nm) spectra by the pyridine–hemochromogen method, employing an extinction coefficient of 34.7 mM^{-1} cm^{-1} [49]. Cytochrome b_5 concentration was determined spectrally from the NADH-reduced minus oxidized difference between 424 and 409 nm using an extinction coefficient of 185 mM^{-1} cm^{-1} [50]. Glutathione peroxidase activity was monitored in the cytosolic fraction as described by Lawrence and Burk [26]. Protein was assayed by the method of Lowry *et al.* [51].

Statistical analysis. All experiments utilized animals *pair-fed* the specific diets, deficient in or supplemented with iron, selenium, and/or β-NF; each group of animals fed the experimental diets were further subdivided into two groups, one for *in vitro* hemin incubation studies and one to serve as the corresponding non-hemin-treated control. Statistical analysis employed Student's paired *t*-test for differences between the paired observations. Student's unpaired *t*-test was used to analyze differences between means of samples with an unequal number of observations, as indicated in the tables.

RESULTS

Characteristics of intestinal cytochrome P-450 and mixed-function oxidases. In preliminary studies, basal "constitutive" cytochrome P-450 content and MFO activity of intestinal microsomes from rats fed a purified semi-synthetic diet, devoid of the natural inducers commonly found in standard rat chow [1, 9], were examined (Table 1). When these rats were fed a semi-synthetic diet supplemented with β-NF for 4 days, a 6-fold increase in cytochrome P-450 content was observed. In parallel, both EROD and ECOD activities increased markedly, but the rise in EROD activity was strikingly higher than that in ECOD activity regardless of whether activity was expressed on the basis of protein or cytochrome P-450 content.

In rats fed the inducer-free diet, the basal "constitutive" form of intestinal cytochrome P-450 exhibited an absorption maximum at 452.5 nm. β-NF supplementation of that diet resulted in a 6-fold increase in intestinal cytochrome P-450 content, with a concomitant blue shift of the absorption maximum of the chromophore to 448.5–449 nm. Further indication that the β-NF-inducible cytochrome is inherently different from the constitutive form is provided by the markedly different K_m values obtained for intestinal AHH activity: 2.4×10^{-5} M and 1.1×10^{-3} M for β-NF-treated and control rats respectively.

Assembly of cytochrome P-450 with hemin in cellular fractions. The occurrence of "heme-free" apocytochrome P-450 in the intestinal mucosa was verified by monitoring its structural assembly to the catalytically active holocytochrome after *in vitro* incubation of various subcellular fractions of the tissue with exogenous heme. We have found a similar methodological approach to be successful in monitoring the structural assembly of hepatic holocytochrome P-450 *in vitro* from its constitutive moieties [24, 52]. Initial attempts to assemble intestinal cytochrome P-450 from rats fed the selenium-supplemented diets with or without Fe (diets iv or iii) by incubating mucosal *homogenates* (20%, w/v) with hemin resulted in complete inactivation of the enzyme. This occurred in spite of the fact that the homogenizing buffer contained sufficient glycerol and trypsin inhibitor to stabilize intestinal cytochrome P-450 and protect it from tryptic degradation [8]. Thus, the intestinal enzyme appeared to be inherently unstable under incubation conditions found suitable for successful cytochrome P-450 reconstitution in liver homogenates *in vitro* [24, 52].

Cytochrome P-450 assembly with heme in incubations of mucosal *microsomes* from rats fed diets iii or iv yielded erratic results. For example, EROD activity was decreased slightly in hemin-incubated microsomes from both −Fe and +Fe fed rats, while substantial increases were observed in AHH activity in the same preparations (data not shown). Moreover, various hemin concentrations (1–15 μM) and/or incubation times (5–20 min) in systems containing intestinal homogenates or microsomes similarly gave inconsistent results. Since in the EROD assay no heme was detected in the supernatant fraction after microsomal protein precipitation, fluorescence quenching by hemin was excluded as the explanation for the apparent discrepancies between the observed results of each of the two assays. Moreover, product inhibition of EROD activity [53] was also excluded as an explanation, since it was not confirmed even after inclusion of resorufin concentrations 20-fold higher than those reported to be inhibitory. It is possible, however, that the EROD-specific cytochrome P-450 species is selectively inactivated during the hemin preincubation, thereby accounting for such a discrepancy.

In contrast to the variable values of MFO activity encountered when hemin was incubated with intestinal *microsomes*, incubation of the *9000 g super-*

natant fraction for 20 min with hemin, in which AHH activity was assayed during the final 15 min, yielded reproducible results of hemin-stimulated MFO activity (data not shown). As with incubations of homogenates though, prolonged incubation of this subcellular fraction resulted in significant inactivation of the enzyme. Microsomes prepared after incubation of 9000 *g* supernatant fractions contained only 20% of MFO activity present in microsomes from corresponding non-incubated controls. Such inactivation was also reflected by the abolition of differences in AHH activity between rats fed −Fe and +Fe diets.

Assembly of cytochrome P-450 with hemin in everted intestinal sacs. Since *in vitro* incubation of homogenates or subcellular fractions with hemin did not permit detection of significant assembly of cytochrome P-450, the everted intestinal sac was employed as a model for the intact organ. The stability of cytochrome P-450 and MFO activity was examined following 1 hr of incubation of everted intestinal sacs at 37° in Tyrode's buffer, Krebs–Henseleit (K–H) bicarbonate buffer containing 0.2% glucose, or K–H bicarbonate buffer supplemented with 5% glucose and 1.2% NaCl. K–H bicarbonate buffer supplemented with NaCl and glucose was the only medium capable of maintaining a fully functional MFO enzyme system (Fig. 1), presumably by protecting intracellular membranes from hypertonic insult [37]. Incubation of everted intestinal sacs from rats fed either −Fe or +Fe diet with hemin (15 μM) for 20 min in NaCl and glucose-supplemented K–H bicarbonate buffer (pH 7.4) resulted in significant increases in microsomal cytochrome P-450 content and EROD activity (Table 2). The intestinal cytochrome content in iron-deprived (−Fe) rats was in fact raised to levels found in rats fed the +Fe diet. In parallel, EROD activity was also increased significantly although the magnitude of the increase was not as great as that of cytochrome P-450 (Table 2).

These findings indicate that, regardless of the dietary iron status of the rats, some "heme-free" apocytochrome P-450 apparently exists in the intestinal mucosa and can be constituted with exogenous heme. Furthermore, iron deficiency apparently produces no additional "free" apocytochrome that can be constituted by this procedure. Thus, the significantly

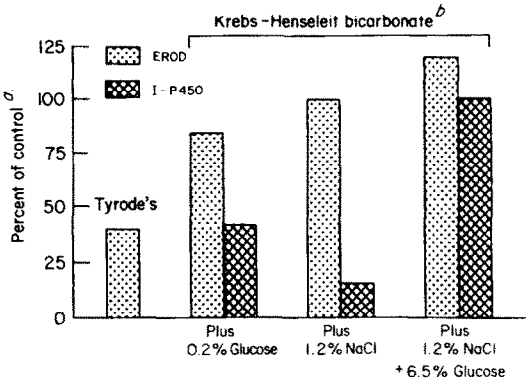

Fig. 1. Effects of various incubation media on intestinal cytochrome P-450 (I-P450) and ethoxyresorufin *O*-deethylase (EROD) activity in everted intestinal sac preparations. Everted intestinal sacs were prepared from rats fed +Fe +Se diet for 5 days, and incubated for 1 hr in various media. Controls consisted of intestinal segments (Experimental Procedure) immediately used for mucosal cell separation without incubation. (a) Control values for I-P-450: 23.9 ± 2.8 pmoles/mg microsomal protein; EROD: 2.67 ± 0.40 pmoles/mg microsomal protein/min. Values are mean ± S.D., N = 3; each N represents tissue pooled from two animals. (b) Krebs–Henseleit bicarbonate buffer supplemented with glucose and/or NaCl (Experimental Procedure).

lower heme-constituted cytochrome P-450 values in rats deprived of dietary iron versus those in rats fed iron, suggest that iron deprivation, and consequently impaired heme formation, may have also limited the availability of intestinal apocytochrome P-450 for assembly to the holocytochrome. Alternatively, "free" apocytochrome deprived of intracellular heme might be unstable and undergo accelerated degradation and/or inactivation, thereby accounting for the lower heme constitution of intestinal cytochrome P-450 in rats deprived of dietary iron.

Requirement for dietary selenium in hemin-mediated cytochrome P-450 assembly. Deprivation of dietary selenium in rats results in decreased intestinal microsomal cytochrome P-450 and heme content, and EROD activity [20]. Everted intestinal sacs from

Table 2. Effect of exogenous hemin on microsomal cytochrome P-450 and MFO activity in everted intestinal sacs*

Diet	Cytochrome P-450 (pmoles/mg protein)		EROD (pmoles/mg protein/min)	
	−Hemin	+Hemin	−Hemin	+Hemin
−Fe +Se	15.7 ± 4.7	25.1 ± 5.0†	0.68 ± 0.22	1.11 ± 0.32‡
+Fe +Se	20.9 ± 2.7§	33.2 ± 10.3‡‖	2.40 ± 0.36§	2.81 ± 1.0¶

* Everted intestinal sacs from rats fed either −Fe or +Fe, Se-supplemented diet (diet iii or iv), were incubated with hemin (15 μM) at 37° for 20 min, as described (Experimental Procedure). Values are mean ± S.E.M., N = 4; each N represents a pool of tissue from two animals.

† $P < 0.1$ vs −hemin value.
‡ $P < 0.05$ vs −hemin value.
§ $P < 0.02$ vs −Fe −hemin value.
‖ $P < 0.05$ vs −Fe +hemin value.
¶ $P < 0.1$ vs −Fe +hemin value.

Table 3. Effect of exogenous hemin on microsomal cytochrome P-450 and MFO activity in everted sacs* from rats deprived of dietary selenium*

Diet	N	Cytochrome P-450 (pmoles/mg protein) −Hemin	Cytochrome P-450 (pmoles/mg protein) +Hemin	EROD (pmoles/mg protein/min) −Hemin	EROD (pmoles/mg protein/min) +Hemin
−Fe −Se	7	9.53 ± 1.6	13.45 ± 2.1†	0.17 ± 0.02	0.29 ± 0.03‡
+Fe −Se	5	22.58 ± 6.2§	23.86 ± 9.1‖	0.22 ± 0.02¶	0.33 ± 0.06‖

* Incubations with hemin (15 μM) were carried out as described (Experimental Procedure). Rats were pair fed either −Fe or +Fe, Se-deficient diet (diet i or ii). Values are mean ± S.E.M.; each N represents a pool of tissue from two animals.

† $P < 0.05$ vs −hemin value; not statistically different (NS) from corresponding +Fe value.

‡ $P < 0.001$ vs −hemin value.

§ $P < 0.02$ vs −Fe −hemin value.

¶ $P < 0.1$ vs −Fe −hemin value.

‖ NS vs −hemin value.

rats deprived of both dietary iron and selenium exhibited lower cytochrome P-450 content and EROD activity than those from rats deprived of only selenium (Table 3). However, when the basal values of the two variables in +Fe −Se fed rats (Table 3) are compared with the corresponding values in +Fe +Se fed rats (Table 2), the variability in cytochrome P-450 content is too great to permit noticeable differences of selenium deprivation between the two groups. This lack of significant difference is in contrast to our previous findings and probably arises from the simple fact that the animals in Table 2 were not pair fed with those in Table 3. Even so, the values for EROD activity, the more sensitive variable, exhibited marked differences between +Fe +Se and +Fe −Se fed rats in these groups (Tables 2 and 3). Moreover, intestinal EROD activity appears to be exquisitely sensitive to selenium deprivation. That is, the specific EROD activity (pmoles resorufin formed/pmoles cytochrome P-450/min) fell precipitously from 0.09–0.11 in +Fe +Se fed rats (Tables 1 and 2) to 0.01 in rats deprived solely of dietary selenium (Table 3).

Since intestinal cytochrome P-450 content and EROD activity decrease in rats deprived of dietary selenium [20], and since synthesis of the apocytochrome moiety of the rapidly turning over phenobarbital-inducible isozyme of cultured hepatocytes was reported to require selenium [23], we investigated whether dietary selenium was required for intestinal apocytochrome P-450 formation and/or viability.

Incubation of everted intestinal sacs from −Fe −Se fed rats with hemin resulted in slightly increased cytochrome P-450 content and EROD activity (Table 3). However, in +Fe −Se fed rats no such increases were apparent, indicating the absence of constitutable "free" apocytochrome in mucosa of these rats. This is in sharp contrast to the observed hemin-mediated increases in these variables in +Fe +Se fed rats (Table 2). Thus, selenium deprivation appears to reduce the extent of constitutable microsomal "heme-free" apocytochrome, which normally occurs in rats fed an iron-supplemented diet adequate in selenium (Table 2). In addition, although partial structural and functional assembly of microsomal cytochrome P-450 with exogenous hemin was observed in intestinal sacs from rats deprived of both iron and selenium (Table 3), it occurred to a much lesser extent than that seen in intestinal sacs from rats deprived of iron but supplemented with selenium (Table 2). This partial assembly may reflect reduced availability of constitutable apocytochrome P-450 which may result either from its impaired formation or lack of viability due to selenium deprivation. Thus, in addition to regulating *heme* synthesis, intraluminal iron and selenium may modulate intestinal *apocytochrome* P-450 availability. It is unclear whether such modulation is exerted by direct effects of these nutritional elements on apoprotein synthesis, or indirectly, via impairment of heme synthesis. In the second event, it could be inferred that formation of the "constitutive" form of intestinal cytochrome P-450 requires coordinated, interdependent synthesis of its two moieties.

Effects of dietary iron and selenium deprivation on apocytochrome P-448 formation. To test whether intestinal apocytochrome P-448 formation is similarly affected in the absence of heme, a relative excess of "free" apocytochrome was deliberately generated as follows. Rats were deprived of dietary iron in order to restrict intracellular heme formation, while they were concomitantly fed β-NF, a cytochrome P-448 inducer, which was expected to increase apocytochrome synthesis. Under such dual dietary regimen, any "heme-free" apocytochrome P-448 generated in excess would be expected to be spectrally quantifiable after complexation with exogenous hemin.

Iron deprivation reduced intestinal microsomal cytochrome P-448 content and its dependent MFO activity in β-NF-fed rats (Table 4). Incubation of 9000 *g* supernatant fractions of intestinal mucosa of these rats with hemin reversed the reduction of cytochrome P-448 content nearly completely. In parallel, EROD and AHH activities were restored to almost basal (+Fe +β-NF) levels (Table 4).

Essentially similar findings were obtained after hemin incubation of everted intestinal sacs from rats fed a β-NF containing diet, supplemented with or without iron (Table 5). However, the relative magnitude of the hemin-mediated increases of cytochrome P-448 content and EROD activity was lower in this system than in that containing intestinal 9000 *g* supernatant fraction. This discrepancy is largely due to the fact that values for cytochrome P-450 content

Table 4. Effect of incubation of 9000 *g* supernatant fraction with exogenous hemin on microsomal cytochrome P-450 and MFO activity in β-NF-fed rats*

Diet	Cytochrome P-450† (pmoles/mg protein) −Hemin	Cytochrome P-450† (pmoles/mg protein) +Hemin	EROD† (nmoles/mg protein/min) −Hemin	EROD† (nmoles/mg protein/min) +Hemin	AHH‡ (pmoles/mg protein/min) −Hemin	AHH‡ (pmoles/mg protein/min) +Hemin
−Fe +β-NF	76.3 ± 18.0	132.6 ± 19.2§	0.91 ± 0.21	1.24 ± 0.13‖	117.9 ± 19.3	191.6 ± 34.4¶
+Fe +β-NF	155.8 ± 20.2**	147.3 ± 24.1	1.55 ± 0.21**	1.71 ± 0.21	210.1 ± 28.5**	254.9 ± 16.7

* Values are mean ± S.E.M. Statistical differences were determined with Student's paired *t*-test unless stated otherwise.

† Reaction mixtures contained 9000 *g* supernatant fraction (2 mg protein/ml) in a final volume of 10 ml and were incubated with or without hemin (15 μM) at 37° for 20 min. Cytochrome P-450 content and activity were determined in microsomal fractions. N = at least five individual animals.

‡ The 9000 *g* supernatant fraction (10 mg protein/ml) was preincubated with hemin (15 μM) for 5 min before assay. Activity was determined in the 9000 *g* supernatant fraction. N = three individual animals.

§ $P < 0.001$ vs −hemin value. Not significantly different (NS) vs +Fe values.

‖ $P < 0.02$ vs −hemin value.
$P < 0.05$ vs +Fe values, Student's *t*-test.

¶ $P < 0.05$ vs −hemin value.
NS vs +Fe +hemin value.

** $P < 0.05$ vs −Fe −hemin value.

and MFO activity of intestinal sacs reflect a mixed pool of microsomes from villous tip and crypt cells, whereas the 9000 *g* supernatant fractions were obtained from villous tip cells, which are relatively enriched in cytochrome P-450 and MFO activity. To a lesser extent, such a discrepancy may result from the inability of the enterocyte, during the short incubation period, to take up hemin in sufficient quantities to meet the increased demand for intracellular heme following induction of the apocytochrome by β-NF.

To determine whether dietary selenium deprivation also affects intestinal apocytochrome P-448 formation, rats were fed for 5 days with iron-supplemented diets to which β-NF was added as in diet vi, but which either lacked selenium (Se < 0.002 ppm) or contained selenium (0.5 ppm). Microsomal EROD activity was determined following incubation of 9000 *g* supernatant fractions of intestinal mucosa pooled from two rats, with or without hemin (15 μM). In two separate determinations, intestinal EROD activity in selenium-deprived rats averaged 1.18 nmoles resorufin formed/mg protein/min when incubated in the absence of hemin and 1.33 nmoles resorufin formed/mg protein/min in its presence, as compared with corresponding values of 1.94 and 2.05 nmoles resorufin formed/mg protein/min in selenium-supplemented β-NF-fed rats. These results indicate that selenium deprivation also reduces availability of intestinal apocytochrome P-448.

DISCUSSION

We have reported previously that intestinal cytochrome P-450 content and function are acutely reduced within a single day of dietary selenium deprivation, and that such reduction was independent of intestinal seleno-dependent GSH-peroxidase activity [20]. In part, such reduction was due to diminished intestinal heme availability, resulting from impaired activity of ferrochelatase, the terminal heme synthetic enzyme [20]. We now report that intracellular selenium also appears to determine the availability of the apoprotein moieties of both constitutive cytochrome P-450 and cytochrome P-448 in the intestinal mucosa. Thus, dietary selenium appears to regulate basal intestinal cytochrome P-450 content and function by modulating the syntheses of both the heme and apocytochrome moieties of these intestinal isozymes. It is possible, however, that intracellular selenium is critically required for synthesis of all rapid turnover proteins. Accordingly, reduced formation of both heme and apocytochrome might merely reflect impairment of protein synthesis by selenium deprivation. This possibility is consistent with the

Table 5. Effect of exogenous hemin on microsomal cytochrome P-450 and MFO activity in everted intestinal sacs from β-NF-fed rats*

Diet	N	Cytochrome P-450 (pmoles/mg protein) −Hemin	Cytochrome P-450 (pmoles/mg protein) +Hemin	EROD (nmoles/mg protein/min) −Hemin	EROD (nmoles/mg protein/min) +Hemin
−Fe +β-NF	5	66.9 ± 7.1	82.2 ± 19.2†‡	0.41 ± 0.09	0.71 ± 0.19†§
+Fe +β-NF	7	115.1 ± 9.1‖	102.8 ± 16.0	2.05 ± 0.15‖	1.40 ± 0.12†

* Everted sacs were incubated with hemin (15 μM) at 37° for 20 min, as described (Experimental Procedure). Values are mean ± S.E.M., obtained from individual animals.

† $P < 0.05$ vs −hemin value, Student's paired *t*-test.

‡ $P \pm 0.05$ vs +Fe −hemin, not significant vs +Fe +hemin, Student's *t*-test.

§ $P < 0.01$ vs +Fe values, Student's *t*-test.

‖ $P < 0.005$ vs −Fe −hemin value, Student's *t*-test.

report that, in primary nonproliferating hepatocyte cultures, *de novo* synthesis of the rapid turnover phenobarbital-inducible cytochrome P-450 isozyme, but not that of the slow turning over constitutive isozyme, is dependent on selenium [23]. However, because of the dynamic cellular turnover of the intestinal mucosa, turnover of all intestinal cytochrome P-450 isozymes (including the constitutive one) is inherently rapid and therefore potentially dependent on intracellular selenium.

Additionally, findings detailed herein confirm previous reports from this laboratory [9, 20] that dietary iron deprivation of rats reduces their basal intestinal cytochrome P-450 content and its associated MFO activity. We now report that such a reduction can be largely reversed by incubation of everted intestinal sacs with exogenous heme *in vitro*. Thus, in the event of acute intracellular heme deficiency, ingestion of heme-containing compounds could similarly restore cytochrome P-450 content and function in the intestinal mucosa. However, the incompleteness of such restoration (Table 3) suggests that, in rats fed the inducer-free diet, dietary iron deprivation impairs the formation and/or viability of the constitutive intestinal apocytochrome P-450. If the syntheses of the two moieties were to be synchronously coordinated or interdependent, this impairment might possibly result from reduced heme availability. Alternatively, deprived of its heme, the constitutive apocytochrome might be relatively unstable and therefore nonviable. A third, albeit less likely, explanation for the observed relatively low extent of intestinal holocytochrome assembly in iron-deprived rats is that, after its *in vitro* assembly, subsequent methodological procedures may promote the dissociation of the newly constituted hemoprotein.

The ability of exogenous heme to structurally and functionally restore intestinal cytochrome P-450 following its uptake from the lumen raises the question of whether the enterocyte possesses a "free" heme pool analogous to that of the hepatocyte. In the liver, such a "free" heme pool not only supplies heme for hemoprotein formation but also regulates heme metabolism [54–57]. This is accomplished by a very finely attuned modulation of the key rate-limiting enzymes in heme synthesis and degradation, namely mitochondrial δ-aminolevulinic acid synthetase (ALAS) and microsomal heme oxygenase (MHO) respectively. Heme deprivation of the "free" heme pool derepresses and turns "on" heme synthesis, whereas heme surplus stimulates MHO and accelerates disposal of the excess heme. We have shown previously that intraperitoneal administration of exogenous heme to rats does indeed stimulate not only hepatic but intestinal MHO activity in rats [58]. Thus, exogenous heme does appear to gain access into a common pool in the enterocyte from where it can be utilized for intestinal hemoprotein formation and induction of intestinal MHO. The only apparent caveat to its existence is the fact that depletion of its heme following dietary iron deprivation is not associated with the expected derepression of intestinal ALAS. Apparently, in the enterocyte iron deprivation is found to decrease rather than increase ALAS activity [20]. Similar findings were also reported in the liver of rats fed low iron diet for 10 or 30 days [21, 59]. Our findings are in accordance with the conclusion of Muller-Eberhard and associates [21, 59] that iron itself plays a direct role in heme-mediated regulation of ALAS. The failure to observe derepression of intestinal ALAS following iron deprivation thus may reflect this particular feature, rather than the absence of a regulatory heme pool in the enterocyte.

The findings detailed also confirm previous reports that dietary β-NF induces cytochrome P-448, a characteristic species of intestinal cytochrome P-450 in rats [8]. This cytochrome P-450 species is spectrally and catalytically distinguishable from the constitutive isozyme. Moreover, the 1000-fold stimulation of EROD activity observed following β-NF administration (Table 1) suggests that the β-NF-inducible cytochrome is functionally much more active in ethoxyresorufin *O*-deethylation than its constitutive counterpart. In addition, the β-NF-inducible apocytochrome, generated in the absence of intestinal heme, is notable in that it can be assembled with exogenous heme to almost fully restore cytochrome P-448 content and activity in −Fe +β-NF-fed rats to values comparable to those in +Fe +β-NF-fed animals. This finding suggests that, unlike the constitutive apocytochrome P-450, apocytochrome P-448 is independent of the presence of heme for its formation and/or viability. Together, these findings suggest that formation of the constitutive intestinal cytochrome P-450 isozyme apparently involves coordinate or interdependent synthesis of its heme and apocytochrome moieties, whereas formation of the constituent moieties of β-NF-inducible cytochrome P-448 need not occur in a concerted fashion.

These findings are of note not only because of the rapidly expanding list of commonly prescribed drugs whose metabolism entails a significant "first pass" through the intestinal mucosa, but also because dietary iron deficiency is clinically common. In the latter event, the ability of the intestinal mucosa to metabolize drugs by a cytochrome P-450-dependent process may be jeopardized. If such metabolism of a xenobiotic predominantly occurs in the intestinal mucosa and incurs its pharmacological inactivation and/or detoxification, the individual may be at added pharmacological/toxicological risk during dietary iron deficiency. The findings presented herein, at least in theory, suggest an approach, i.e. intraluminal heme, to remedy such an impairment.

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