PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, 152, 568-572 (1976)

Impaired Hepatic Heme Synthesis in the Phenobarbital-Stimulated Selenium-Deficient Rat (39442)

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In 1957 Schwarz and Foltz (1) described an association between dietary selenium deficiency and hepatic necrosis. However, although the dietary requirement for selenium is established, its possible role in the maintenance of normal hepatic function remains speculative. Diplock *et al.* (2, 3) have recently suggested that selenium may be incorporated into a nonheme iron containing microsomal protein active in the NADPH-dependent electron transfer chain. It is conceivable, therefore, that the integrity of the hepatic microsomal mixed-function oxidase system may in part be dependent on an adequate dietary supply of selenium.

We have recently described an impaired inducibility by phenobarbital of cytochrome P_{450} and cytochrome b_5 in the selenium deficient rat (4). The normal inducibility of the flavoprotein component of the mixed function oxidase system, NADPH cytochrome *c* reductase, in these animals suggested that the impaired response in these selenium deficient rats may be limited to the heme-containing microsomal proteins. Preliminary observations (4) indicated that the apoprotein component of cytochrome P_{450} was induced by phenobarbital in the selenium deficient rats. The present study is a continuing investigation of the possible discoordinated synthesis of heme and apoprotein in the selenium deficient phenobarbital-stimulated rat.

Materials and methods. Male Holtzman rats weighing 60–90 g were placed on a selenium deficient torula yeast diet (5) and water *ad lib*. Two dietary groups were formed by the addition of supplemental selenium, 0.5 mg/kg as Na_2SeO_3, to the diet of one group of animals. Thus, a "selenium replete" and "selenium deficient" group was formed; the animals were maintained on this diet for 3 months, at which stage animals in both groups weighed 300–350 g.

Four animals from each group were then subdivided into two further groups. Within each dietary group two animals received a daily intraperitoneal injection of phenobarbital (80 mg/kg in 0.9 NaCl) for 5 days, the remaining two animals receiving only the vehicle. The rats were then fasted overnight and each animal was given 10 μCi of 5-δ-amino-[^{14}C]levulinic acid (Amersham Radiochemical Centre, 14.4 mCi/mmole) by intraperitoneal injection. The animals were killed by exsanguination 2 hr later. The livers were removed and homogenized in 4 vol of cold 0.25 *M* sucrose, 0.01 *M* Tris-HCl pH 7.4 in a Potter-Elvejham homogenizer with Teflon pestle at 600 rpm. Subcellular fractions were separated by differential centrifugation by the method of Novikoff and Heus (6), and the microsomal subfraction was resuspended to 25 ml in 0.05 *M* Tris-HCl pH 7.4.

Aliquots of homogenate and the microsomal subfractions were used for the extraction of and determination of heme. Lipids were first extracted as described by Druyan and Kelly (7). Hemes were then extracted twice from these samples with 20 ml of acidified acetone (2.5 ml of 31% HCl/100 ml acetone), the pooled extracts were flash evaporated and the hemes dissolved in 5 ml of alkaline pyridine (pyridine–1 *M* NaOH–water, 5:1:5 by volume, pH 13). An aliquot was taken for the determination of hemes as described by Greim *et al.* (8) and 100 μl was added to 10 ml of toluene-Omnifluor for determination of radioactivity in a Packard Tricarb Model 2425 scintillation spectrometer. Quench data were analyzed by a Control Data Corporation 6400 computer.

The turnover of cytochrome P_{450} apoprotein in selenium deficient animals was assessed in other animals from the two dietary groups. Microsomal fractions were prepared from phenobarbital-injected and saline-injected selenium deficient animals. The microsomal fractions were washed by the

method of Weihing *et al.* (9) to remove adsorbed and intracisternal proteins and equal amounts (50 μg) of sodium dodecyl sulfate (SDS) solubilized microsomal protein from these animals were applied to a 7.5% polyacrylamide-SDS-6 *M* urea slab gel prior to electrophoresis according to the method of Laemmli (10). The gel was fixed and stained in Coomassie blue 0.1% in 12.5% trichloroacetic acid, 50% methanol overnight and destained in 10% acetic acid. The tentative identification of the protein of mol wt 50,000–60,000 as cytochrome P_{450} apoprotein within this system has been described previously (11) and the problems associated with identification discussed (12).

The double isotope-polyacrylamide gel electrophoresis technique (13) was utilized as previously described (11) to examine the effect of phenobarbital administration on the synthesis of cytochrome P_{450} apoprotein in selenium deficient rats. For 5 days prior to sacrifice, one selenium deficient rat was given phenobarbital (80 mg/kg/day) while another received 0.9% NaCl only. On the fifth day of treatment, and after a 12-hr fast, the phenobarbital treated animal received 200 μCi of [4,5-^{3}H]leucine (Amersham Radiochemical Centre, 1 Ci/mmol) by intraperitoneal injection while the other saline-treated animal simultaneously received 50 μCi of [^{14}C]leucine (Amersham Radiochemical Centre, 348 mCi/mmole). Six hours later both animals were killed by exsanguination and a microsomal fraction obtained by differential centrifugation of the combined liver homogenate. The microsomes were washed (9), suspended in 1% SDS and 7.5% preparative polyacrylamide gel electrophoresis performed as above. After overnight freezing the gel was sliced transversely into 1.5-mm consecutive slices and the ^{3}H and ^{14}C radioactivity in each slice and its relationship to Coomassie blue stained bands was determined as previously described (11).

Data were analyzed where appropriate by two way analysis of variance with replication (14).

Results. Basal hepatic heme content and synthesis in the selenium deficient rats did not appreciably differ from the levels in selenium replete animals. However, in the selenium deficient animals hepatic heme synthesis did not increase with phenobarbital treatment to the extent documented in selenium replete rats. Total hepatic heme content (determined as mn*M*/total liver homogenate) increased to 168% of the control value in selenium replete phenobarbital treated rats, while a 10% increase only was observed in the selenium deficient group (Fig. 1). Changes in δ-amino-[^{14}C]levulinic acid incorporation paralleled those of the hepatic heme content. Thus, δ-amino-[^{14}C]levulinic acid incorporation into heme in the selenium replete phenobarbital animals was 158% of control, but no increase was demonstrable in the selenium deficient rats.

Figure 2 illustrates the microsomal heme content and δ-amino-[^{14}C]levulinic acid incorporation following phenobarbital treatment. After correction for inefficiency or incompleteness of microsomal recovery, determined by glucose-6-phosphatase recovery (13), microsomal heme accounted for 60–70% of total hepatic heme. Total microsomal heme content and radioactive incorporation were increased by phenobarbital administration in the selenium replete animals (respectively, 148 and 145% of control, Fig. 2). In contrast, this increase was appreciably less apparent in the selenium deficient animals treated with phenobarbital (values, respectively, 112 and 114% of con-

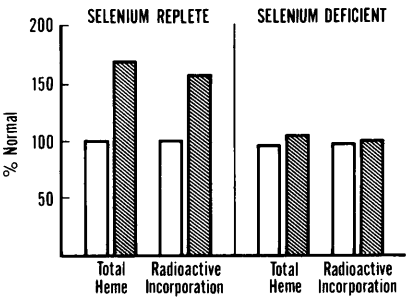

FIG. 1. Total hepatic heme and δ-amino-[^{14}C]levulinic acid incorporation in selenium replete and deficient rats. Heme was extracted and measured as described under methods. Each value, expressed as a percentage of selenium replete control, represents the mean of two animals. Clear bars, saline treated; hatched bars, 5 day phenobarbital treated (80 mg/kg/day).

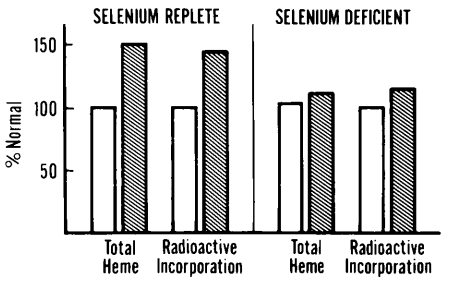

FIG. 2. Microsomal heme content and Δ-amino-[^{14}C]levulinic acid incorporation in selenium replete and deficient rats. Heme was extracted and measured as described under methods. Each value, the mean from two animals, is expressed as a percentage of selenium replete control (960 nm *M* heme/liver; 0.7 × 10^6 dpm/liver). Clear bars, saline treated; hatched bars, phenobarbital treated.

trol, Fig. 2). The value of the interaction term for microsomal heme and radioisotope incorporation between selenium deficient and replete animals treated with phenobarbital was significant ($P < 0.01$) in each case. While hepatic incorporation of δ-amino-[^{14}C]levulinic acid in the selenium deficient rats was not augmented by phenobarbital pretreatment, the specific activity of hepatic heme did not differ between the selenium replete and deficient groups (250–290 dpm/nmole of heme). In all animals the specific activity of heme from microsomal fractions was greater than that of the total hepatic homogenate (800 dpm/mmole of heme).

Figure 3 demonstrates that even in the selenium deficient rat there is an appreciable increase in the density of staining of protein migrating in the mol wt 50,000–60,000 region of a polyacrylamide gel after phenobarbital treatment. Cytochrome P_{450} apoprotein represents a substantial proportion (estimated as 50%, (13)) of polypeptides in this region and an identical increase in staining density after phenobarbital treatment in normal rats suggests that selenium deficiency may not impair the inductive response of cytochrome P_{450} apoprotein.

This impaired response in heme synthesis together with a normal pattern of increase in gel protein staining indicated that discoordinated synthesis of apoprotein and heme was occurring in the phenobarbital treated selenium deficient rats. We therefore investigated the effect of phenobarbital on synthe-

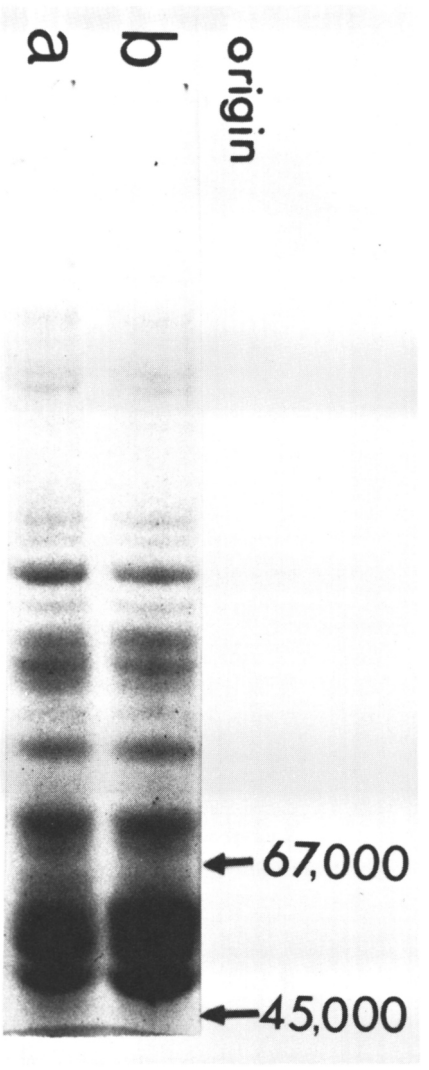

FIG. 3. Polyacrylamide slab gel electrophoresis. Microsomal protein fractions from selenium deficient rats were solubilized in 1% sodium dodecyl sulfate (SDS) and electrophoresed on 7.5% polyacrylamide slab gel as described in the text. Protein bands were stained with Coomassie-blue. Equal amounts (50 μg) of microsomal protein from individual samples were applied: (a) Microsomes from selenium deficient, saline treated animal, (b) Microsomes from selenium deficient animal treated for 5 days with phenobarbital 80 mg mg/kg/day. Molecular weight markers 67,000 (albumin), 45,000 (ovalbumin).

sis rate of apoprotein cytochrome P_{450} in selenium deficiency by a double isotope method. Figure 4 demonstrates a marked increase in ^{3}H/^{14}C ratios in the mol wt

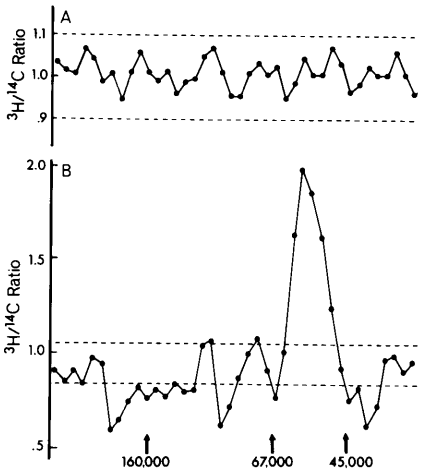

FIG. 4. Effect of phenobarbital on the relative rate of synthesis of apoprotein cytochrome P_{450} in selenium deficient rats. A, (upper panel) control experiment. Both ^{14}C- and ^3H-labeled leucine simultaneously delivered to untreated control rats. $^3H/^{14}C$ ratio for each gel slice was divided by homogenate $^3H/^{14}C$ ratio[4]. Dotted horizontal lines represent 95% confidence limits for variation from mean ratio. B, (lower panel) [^{14}C]leucine was administered to saline treated selenium deficient rat, [^{3}]leucine simultaneously administered to phenobarbital treated selenium deficient rat. Animals were killed 6 hr later and microsomes prepared from the combined liver homogenate were electrophoresed on 7.5% polyacrylamide (see text). Molecular weight markers 45,000 (ovalbumin), 67,000 (albumin), 160,000 (gammaglobulin). Dotted horizontal lines represent 95% confidence limits established in control experiment.

50,000–60,000 region of the gel similar to that previously demonstrated in normal phenobarbital treated rats (11).

Discussion. The determination of cytochrome P_{450} involves the spectrophotometric measurement of the heme moiety, and normally the apoprotein content is not determined. Siekevitz (15) recently presented evidence that in fetal and neonatal rats there is an apparent discoordination in the appearance of the heme and apoprotein moieties of cytochrome P_{450}. Thus, the previous demonstration (4) of impaired inducibility of cytochrome P_{450} by phenobarbital in selenium deficient rats, with, however, normal inducibility of the flavoprotein NADPH cytochrome c reductase, suggested that a comparable discoordination of synthesis of these two moieties may be present. An alternative explanation that heme transport to the microsomes rather than heme synthesis was impaired seemed unlikely in view of the observation of Murty *et al.* (16) that vitamin E deficiency impaired both bone marrow and hepatic heme synthesis.

The present study confirms the concept of discoordinated synthesis of heme and apoprotein in selenium deficient phenobarbital treated rats. Thus, the anticipated increase in δ-amino-[^{14}C]levulinic acid incorporation was not apparent in the selenium deficient rats, and the observation that total hepatic radioisotope incorporation as well as microsomal isotope incorporation were impaired indicated that the defect is one of impaired heme synthesis rather than a defect in intracellular transport of heme.

Two approaches were utilized to demonstrate that in the selenium deficient rats exposure to phenobarbital was associated with an increase in cytochrome P_{450} apoprotein. Thus, both polyacrylamide gel staining and radio-active leucine incorporation indicated that cytochrome P_{450} apoprotein induction was not impaired by selenium deficiency. In view of the present impossibility of the certain identification of cytochrome P_{450} apoprotein(s) this conclusion must be qualified. It may be stated, however, that the induction of proteins or polypeptides of mol wt 50,000–60,000 did not appear quantitatively different in selenium deficient rats and selenium replete controls, and since cytochrome P_{450} apoprotein represents the major protein in this molecular weight range in our polyacrylamide gel system (13) a workable extrapolation seems justifiable.

Thus, in the selenium deficient animal the synthesis of the heme and apoprotein moieties of cytochrome P_{450} do not respond identically to phenobarbital induction. The recent demonstration (15) of discoordinated synthesis of heme and apoprotein in the neonate argues against the proposal that induction of heme biosynthesis leads in turn to enhanced cytochrome P_{450} synthesis (17, 18), but rather favors a primary effect of phenobarbital in increasing P_{450} synthesis with limited availability of heme resulting in excess apocytochrome P_{450} (19, 20).

This investigation was supported in part by Grant AM 15851 from the United States Public Health Serv-

ice. We are most grateful for the excellent technical assistance provided by Mrs. Eileen Sutherland, and to R. F. Burk for providing selenium deficient animals.

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Received October 22, 1975. P.S.E.B.M. 1976, Vol. 152.