Defective utilization of haem in selenium-deficient rat liver

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We have previously suggested that an inherent defect in hepatic haem utilization was responsible for the rapid stimulation of hepatic microsomal haem oxygenase activity observed in selenium-deficient rats given phenobarbital, a well known inducer of haem formation. To test this hypothesis, hepatic haem content was deliberately raised in selenium-deficient rats by administration of either tryptophan or allylisopropyl-acetamide, or by injecting haem itself. We now report that selenium-deficient rats are apparently relatively less efficient in utilizing hepatic haem than normal controls. The findings detailed in the present paper thus indicate that stimulation of hepatic microsomal haem oxygenase activity is indeed a manifestation of abnormal haem utilization in selenium deficiency. This suggests a novel role for selenium in hepatic haem metabolism.

Phenobarbital administration to selenium-deficient rats rapidly and markedly stimulates the activity of hepatic microsomal haem oxygenase, the rate-limiting and haem-inducible enzyme in the oxidative degradation of haem (Correia & Burk, 1978). On investigating the cause for this stimulation, we have found that it was not associated with accelerated turnover of hepatic cytochrome *P*-450, as might have been expected (Bissell & Hammaker, 1976*a,b*), but apparently with a relative excess of haem which results from a rapid increase in hepatic haem formation coupled with a lag and/or a defect in its utilization in the assembly of hepatic haemoproteins (Correia & Burk, 1978).

Quantitatively, cytochrome *P*-450 exerts the greatest demand for haem synthesized in the liver by virtue of its relatively high hepatic content and its rapid turnover rate (Meyer & Schmid, 1978). Although much less abundant than cytochrome *P*-450, the cytosolic haemoprotein tryptophan pyrrolase, because of its relatively short half-life, apparently also utilizes a significant portion of hepatic haem (Feigelson & Greengard, 1961; Schimke *et al.*, 1965; Meyer & Schmid, 1978). Thus, in selenium-deficient rats given phenobarbital, defec-

tive utilization of haem by apo-(tryptophan pyrrolase) coupled with delayed induction of apo-(cytochrome *P*-450) apparently resulted in a substantial excess of 'free' haem, and in stimulation of hepatic haem oxygenase activity (Correia & Burk, 1978). If utilization of hepatic haem is indeed impaired in selenium-deficiency, then compounds that produce a net increase in hepatic haem content should mimic phenobarbital and, as a result, stimulate hepatic microsomal haem oxygenase activity. This hypothesis was tested and the findings are described in the present paper.

Materials and methods

Materials

ALA, tryptophan, ATP, NADPH, glutathione, pyridoxal 5'-phosphate, coenzyme A and haemin hydrochloride were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). AIA was a gift from the Hoffman-La Roche Company (Nutley, NJ, U.S.A.). [1,4-¹⁴C]Succinic acid was obtained from New England Nuclear Company. Succinyl thio kinase was obtained from Porphyrin Products (Logan, UT, U.S.A.).

Methods

Animals. From the time of weaning, male Holtzman rats were fed *ad libitum* a semi-synthetic selenium-deficient diet containing torula yeast and corn oil as protein and fat components respectively,

Abbreviations used: ALA, δ -aminolaevulinic acid; AIA, allylisopropylacetamide.

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and a supplement of 100 i.u. of vitamin E per kg of diet. Control rats were fed the same diet supplemented with 0.5 mg of selenium as Na_2SeO_3 per kg of diet. All experiments were carried out with rats (250–300 g) that had been fed the respective diets for at least 8 weeks. The activity of the selenoenzyme glutathione peroxidase in liver cytosol of rats fed this selenium-deficient diet for 6 weeks is about 1% of that of controls, indicating that the rats used in these experiments were severely selenium-deficient (Lawrence & Burk, 1976). Selenium-deficient animals and controls (selenium-adequate) were selected from the same litter and thus were age-matched in each experiment.

In all experiments, rats were starved overnight (18 h) before administration of test compounds. After the rats were killed, livers were perfused *in situ* with ice-cold 1.15% KCl and then excised and weighed, except where indicated otherwise.

Treatment. Animals were injected intraperitoneally with tryptophan (1.5 g/kg body wt.), subcutaneously with AIA (400 mg/kg body wt.) or intravenously with haem (2.5 $\mu\text{mol/kg}$ body wt.) as methaemalbumin (haem/albumin molar ratio, 33:1) at 0 h and killed at the times indicated.

Analytical procedures. ALA synthase was assayed by the method of Strand *et al.* (1972), except that liver homogenates in 0.1 M-pyridoxal phosphate/0.9% NaCl/0.01 M-Tris buffer, pH 7.4, were used as recommended by Yoda *et al.* (1974). Tryptophan pyrrolase activity was determined by the method of Feigelson & Greengard (1961) as described by Badawy & Evans (1975), except that livers were excised and weighed before perfusion *ex situ* (Correia & Burk, 1978). Microsomal haem oxygenase activity was measured in 18000 g liver supernatant as described previously (Correia & Schmid, 1975). Protein was measured by the method of Lowry *et al.* (1951). Cytochrome P-450 content was determined by the method of Estabrook *et al.* (1972).

Results

Hepatic haem content was deliberately raised by treatment of animals with: (i) tryptophan, which has been shown to increase synthesis of haem (Greengard & Feigelson, 1961; Marver *et al.*, 1966; Badawy, 1977) without affecting cytochrome P-450 content (M. A. Correia & R. F. Burk, unpublished work); (ii) AIA, which increases haem synthesis, apparently in response to its selective destruction of cytochrome P-450 haem (Marver *et al.*, 1966; Marver, 1969; Satyanarayana Rao & Padmanaban, 1973; Badawy, 1977); or (iii) haem itself as methaemalbumin, in a dose insufficient to induce hepatic microsomal haem oxygenase in control rat liver. The effects of these experimental manipu-

Fig. 1. Effects of tryptophan and AIA on hepatic ALA synthase activity in selenium deficiency

Selenium-deficient (continuous lines) and control (broken lines) rats were treated with either tryptophan (●) or AIA (■) and killed at the times indicated by the points. Values represent the means $\pm$ s.d. (indicated by the bars) of three individual experiments. Basal (zero-time) activities were 226.7 ± 19.3 and 195.4 ± 60.5 (pmol of ALA formed/mg of protein per 30 min) for the selenium-deficient and control animals respectively.

lations on formation, utilization and catabolism of hepatic haem were determined in selenium-deficient rats as described below.

Effects of tryptophan and AIA on haem synthesis in the liver

Tryptophan progressively increased hepatic ALA synthase activity, the rate-limiting enzyme in the haem synthetic pathway, both in selenium-deficient and control rats (Fig. 1). The magnitude of this increase was consistently greater in controls than in selenium-deficient rats, although this difference between the two groups failed to reach statistical significance (Fig. 1). On the other hand, after AIA administration, hepatic ALA synthase activity first exhibited a transient increase in both groups of rats. The values at 2 and 4 h of treatment

Fig. 2. Effects of tryptophan on rat liver tryptophan pyrrolase activity in selenium deficiency

Animals were treated with tryptophan and killed at the times indicated by the points. Liver homogenates were prepared and assayed for tryptophan pyrrolase activity. Filled and stippled columns indicate tryptophan pyrrolase (holoenzyme) activity in selenium-deficient and control rats respectively. Open columns represent apo-(tryptophan pyrrolase) activity determined after addition of exogenous haem *in vitro*. Values represent means of three individual experiments. Basal (zero-time, mean $\pm$ S.D.) values for holoenzyme activity were 1.74 ± 0.69 and 1.41 ± 0.37 μ mol of kynurenine formed/g of liver per h, in selenium-deficient and control animals respectively. Significant differences are indicated by * ($P < 0.05$) and ** ($P < 0.02$) between corresponding values in the two groups. The bars represent $\pm$ S.D.

were consistently and significantly higher in controls than in selenium-deficient rats (Fig. 1). Thus these findings indicate that both tryptophan and AIA augment hepatic haem formation in controls to a similar or at times greater extent than in selenium-deficient rats.

Effects of tryptophan and AIA on hepatic tryptophan pyrrolase activity

In rat liver cytosol, tryptophan pyrrolase normally appears partly as haem-occupied holoenzyme and partly as haem-free apoenzyme. The hepatic apoenzyme can be functionally reconstituted to its full enzymic potential by administration of haem *in vivo*, or by addition of haem to liver homogenate or cytosol *in vitro* (Feigelson & Greengard, 1961; Schimke *et al.*, 1965). Furthermore, its substrate, tryptophan, is found to activate and stabilize the

Fig. 3. Effects of AIA on hepatic tryptophan pyrrolase activity in selenium-deficiency

Selenium-deficient and control animals were treated with AIA and killed after the time intervals indicated. Liver homogenates were prepared and assayed as in Fig. 2. Values are means of three individual experiments. Basal (zero-time, mean $\pm$ S.D.) values for holoenzyme activity were 1.57 ± 0.56 and 0.65 ± 0.32 μ mol of kynurenine formed/g of liver per h in selenium-deficient and control animals respectively. The key to the columns is given in the legend to Fig. 2.

enzyme, thereby enhancing its activity (Greengard & Feigelson, 1961; Schimke *et al.*, 1965). Consistent with this, tryptophan indeed increased both the holoenzyme-dependent activity and apoenzyme-dependent activity (determined on addition of haem to liver homogenate *in vitro*) in control and selenium-deficient rats. However, tryptophan-mediated increases in both these parameters were significantly ($P < 0.05$) less in selenium-deficient than in control rats during the first 4 h of administration (Fig. 2). At 6 h of tryptophan administration, however, apotryptophan pyrrolase activity declined in control rats after reaching a maximum at 4 h, whereas at 6 h it was still increasing in selenium-deficient rats, suggesting a lag in its induction. Similarly, after AIA administration the AIA-induced enhancement of the holoenzyme- and apoenzyme-dependent activities were consistently ($P < 0.05$) lower in the selenium-deficient rats than in corresponding controls (Fig. 3). Collectively, these findings suggest impaired stimulation of tryptophan pyrrolase activity and/or defective utilization of haem by apotryptophan pyrrolase in selenium-deficient rat liver, at least during the initial 4 h period

Table 1. *Effects of tryptophan and AIA on hepatic microsomal haem oxygenase activity in selenium-deficient rats*

Selenium-deficient or control rats were treated with either tryptophan (1.5 g/kg body wt.; intraperitoneal) or AIA (400 mg/kg body wt.; subcutaneous) and killed at designated times. Values are means $\pm$ s.d. of at least three individual experiments. N.S., not statistically significant compared with corresponding animals.

Time after treatment (h)	Microsomal haem oxygenase activity (nmol of bilirubin formed/mg of protein per 10 min)	
	Control	Selenium-deficient
Tryptophan		
0	0.12 $\pm$ 0.01	0.15 $\pm$ 0.01 (N.S.)
2	0.18 $\pm$ 0.06	0.19 $\pm$ 0.04 (N.S.)
4	0.19 $\pm$ 0.07	0.46 $\pm$ 0.00 ($P < 0.01$)
6	0.28 $\pm$ 0.01	1.01 $\pm$ 0.26 ($P < 0.001$)
AIA		
0	0.14 $\pm$ 0.07	0.16 $\pm$ 0.07 (N.S.)
1	0.14 $\pm$ 0.09	0.27 $\pm$ 0.15 (N.S.)
2	0.11 $\pm$ 0.06	0.37 $\pm$ 0.09 ($P < 0.05$)
4	0.09 $\pm$ 0.03	0.35 $\pm$ 0.14 ($P < 0.05$)
6	0.07 $\pm$ 0.03	0.18 $\pm$ 0.13 (N.S.)

of tryptophan or AIA administration. It must be noted that, whereas these findings in control animals are in agreement with those of Marver *et al.* (1966), they only partially agree with those of Badawy & Morgan (1980), who, unlike Marver *et al.* (1966) and us, observed a decrease in holoenzyme activity without any increase in total tryptophan pyrrolase activity after similar treatment of rats with AIA.

Effects of tryptophan and AIA on hepatic haem degradation

The activity of hepatic microsomal haem oxygenase, the rate-limiting enzyme in oxidative haem catabolism, was employed as a determinant of hepatic haem degradation. At 4 h after tryptophan administration, haem oxygenase activity was significantly increased in selenium-deficient rat liver, but not in controls (Table 1). In selenium-deficient rats, this enzyme activity showed a further increase at 6 h after administration of the test compound, whereas it was only slightly stimulated in control animals (Table 1).

In contrast with this protracted effect of tryptophan, AIA markedly, albeit transiently, increased hepatic microsomal haem oxygenase activity in selenium-deficient rats, whereas it caused a time-dependent decrease in this activity in controls (Table 1). This decrease is consistent with the well known effect of the drug in selectively destroying the haem of cytochrome *P*-450 by pathways that circumvent

Fig. 4. *Effects of haem (methaemalbumin) on hepatic microsomal haem oxygenase activity in selenium-deficiency*

Selenium-deficient (●) and control (○) animals were injected with haem as methaemalbumin (2.6 μ mol of haem/kg body wt.; intravenous), and animals were killed after the time intervals indicated. Values represent means $\pm$ s.d. of at least three individual experiments.

bilirubin formation (Unseld & De Matteis, 1976; Ortiz de Montellano *et al.*, 1978).

Stimulation of hepatic haem oxygenase activity in selenium-deficiency by a 'substimulatory' dose of haem

To confirm that the haem-binding capacity of the selenium-deficient liver is limiting, haem itself was

administered as methaemalbumin, at one-tenth the dose ordinarily required to induce haem oxygenase activity in control rats. This dose of haem had no effect on hepatic haem oxygenase activity in controls. In marked contrast, however, methaemalbumin induced hepatic haem oxygenase rapidly within 1 h of its administration in selenium-deficient rats (Fig. 4).

Discussion

The availability of utilizable haem in the so-called free haem pool in the liver is determined by finely attuned control of its synthesis and degradation (Tschudy & Bonkowsky, 1972; Schmid, 1973; Bissell & Hammaker, 1976*a,b*, 1977). Utilization of haem from this pool, however, is critically dependent on availability of either apo-haemoproteins capable of incorporating haem prosthetically or proteins such as albumin, haemopexin and ligandin, capable of haem binding. Defective coupling of haem with available apo-haemoproteins in the liver, and/or reduced availability of haem-binding proteins, would ostensibly limit the hepatic haem-binding capacity. This inevitably would result in underutilization of hepatic haem and in a consequent excess of 'free' or unutilizable haem. Under these circumstances, disposal of excessive haem is readily accomplished by stimulation of hepatic haem oxygenase activity. Accordingly, we suggested that such impaired utilization of hepatic haem in selenium-deficient rats given phenobarbital was causally responsible for the rapid stimulation of hepatic haem oxygenase observed in these animals (Correia & Burk, 1978).

Experimental evidence reported in the present paper provides further support for this hypothesis. Tryptophan, an essential amino acid that increases hepatic haem formation without affecting hepatic cytochrome *P*-450 content (Marver *et al.*, 1966), and AIA, which is an agent known to stimulate haem synthesis presumably in response to its selective degradation of cytochrome *P*-450 haem to covalent AIA-porphyrin adducts (Marver, 1969; Satyanarayana Rao & Padmanaban, 1973; Unseld & De Matteis, 1976; Ortiz de Montellano *et al.*, 1978), were both found to stimulate hepatic haem oxygenase activity only in selenium-deficient rats. Concurrently, hepatic apo-(tryptophan pyrrolase) activity was significantly reduced, indicating defective haem-utilization in these animals. Thus, although less haem is apparently formed in selenium-deficient rats relative to that in controls after tryptophan or AIA treatment, its underutilization still results in an excess of haem, which is rapidly disposed of by induction of microsomal haem oxygenase. In confirmation of this theory, haem, in doses normally insufficient to induce hepatic haem oxygenase in control rats, rapidly and significantly

induced hepatic haem oxygenase in selenium-deficient animals (Fig. 4). These findings indicate that the selenium-deficient rat liver is incapable of utilizing even small amounts of haem, presumably because of a highly compromised haem-binding potential.

It is unclear whether this haem-binding potential of the selenium-deficient rat liver is impaired because of reduced availability of haem-binding proteins and/or defective coupling of haem by the available apo-haemoproteins. Although reduced availability of haem-binding proteins would account for the immediacy with which induction of microsomal haem oxygenase occurs after administration of phenobarbital (Correia & Burk, 1978) or the other test compounds examined, this induction persists beyond apparent correction and/or restoration of hepatic apo-haemoproteins in selenium-deficient rats to levels comparable with those in control animals (Burk & Correia, 1977). As such, with repeated phenobarbital administration, despite comparably increased rates of apo-(cytochrome *P*-450) synthesis and proportionately induced levels of apocytocrome in selenium-deficient and control rats (MacKinnon & Simon, 1976; Burk & Correia, 1977), induction of cytochrome *P*-450 is greatly impaired in the selenium-deficient rat liver (Burk and Masters, 1975; MacKinnon and Simon, 1976; Burk and Correia, 1977). Furthermore, this impairment is not corrected when haem is incubated *in vitro* with liver homogenates or mitochondrion-rough-endoplasmic-reticulum complexes from phenobarbital-treated selenium-deficient rats, which should have been predictably enriched in apo-(cytochrome *P*-450) (Correia & Burk, 1980). These findings suggest that impaired induction of cytochrome *P*-450 in selenium deficiency is not associated with limited availability of haem, caused by its enhanced degradation, or of apo-(cytochrome *P*-450). They are, however, consistent with the concept that impaired induction results from an inherent defect in the haem-apo-(cytochrome *P*-450) assembly, from generation of a faulty, highly unstable cytochrome species, or from a critical structural alteration in the key apo-cytochrome species. The latter possibility is particularly attractive given the recent finding that selenium is required for the formation of phenobarbital-inducible apo-(cytochrome *P*-450) species in rat hepatocytes in monolayer cultures (Newman & Guzelian, 1982). Defective coupling or impaired apo-cytochrome formation would result not only in reduced levels of inducible cytochrome *P*-450, but also in a relative excess of haem in the 'free' haem pool and consequently in the rapid and persistent stimulation of microsomal haem oxygenase activity observed in the selenium-deficient rat liver (Burk & Correia, 1977; Correia & Burk, 1978).

Collectively, our studies on hepatic haem and

haemoprotein metabolism in selenium-deficiency have revealed a novel and critical role for selenium in hepatic haem homeostasis. Since the only other known biochemical role of the element is its prosthetic function in glutathione peroxidase (Oh *et al.*, 1974), our findings are of considerable biological significance.

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References

- Badawy, A. A.-B. (1977) *Life Sci.* **21**, 755–767
- Badawy, A. A.-B. & Evans, M. (1975) *Biochem. J.* **150**, 511–520
- Badawy, A. A.-B. & Morgan, C. J. (1980) *Biochem. J.* **186**, 763–772
- Bissell, D. M. & Hammaker, L. E. (1976a) *Arch. Biochem. Biophys.* **176**, 91–92
- Bissell, D. M. & Hammaker, L. E. (1976b) *Arch. Biochem. Biophys.* **176**, 103–112
- Bissell, D. M. & Hammaker, L. E. (1977) *Biochem. J.* **166**, 301–304
- Burk, R. F. & Correia, M. A. (1977) *Biochem. J.* **168**, 105–111
- Burk, R. F. & Masters, B. S. S. (1975) *Arch. Biochem. Biophys.* **170**, 124–131
- Correia, M. A. & Burk, R. F. (1978) *J. Biol. Chem.* **253**, 6203–6210
- Correia, M. A. & Burk, R. F. (1980) in *Microsomes, Drug Oxidations and Chemical Carcinogenesis* (Coon, M. J. *et al.*, eds.), pp. 583–586, Academic Press, New York
- Correia, M. A. & Schmid, R. (1975) *Biochem. Biophys. Res. Commun.* **65**, 1378–1384
- De Matteis, F. (1973) *Drug Metab. Dispos.* **1**, 267–272
- Estabrook, R. W., Peterson, J., Baron, J. & Hildebrandt, A. (1972) *Methods Pharmacol.* **2**, 303–350
- Feigelson, P. & Greengard, O. (1961) *J. Biol. Chem.* **236**, 153–157
- Granick, S., Sinclair, P., Sassa, S. & Grieninger, G. (1975) *J. Biol. Chem.* **250**, 9215–9225
- Greengard, O. & Feigelson, P. (1961) *J. Biol. Chem.* **236**, 158–161
- Lawrence, R. A. & Burk, R. F. (1976) *Biochem. Biophys. Res. Commun.* **71**, 952–958
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacKinnon, A. M. & Simon, F. R. (1976) *Proc. Soc. Exp. Biol. Med.* **152**, 568–572
- Marver, H. S. (1969) in *Microsomes and Drug Oxidations* (Gillette, J. R., Conney, A. H., Cosmides, G. F., Estabrook, R. W., Fouts, J. R. & Mannering, G. J., eds.), pp. 495–511, Academic Press, New York
- Marver, H. S., Tschudy, D. P., Perlroth, M. G. & Collins, A. (1966) *Science* **154**, 501–502
- Meyer, U. A. & Schmid, R. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.), 4th edn., pp. 1166–1220, McGraw-Hill Book Co., Inc., New York
- Newman, S. & Guzelian, P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2922–2926
- Oh, S. H., Ganther, H. E. & Hoekstra, W. G. (1974) *Biochemistry* **13**, 1825–1829
- Ortiz de Montellano, P. R., Mico, B. A. & Yost, G. S. (1978) *Biochem. Biophys. Res. Commun.* **83**, 132–137
- Satyanarayana Rao, M. R. & Padmanaban, G. (1973) *Biochem. J.* **134**, 859–868
- Schimke, R. T., Sweeney, F. W. & Berlin, C. M. (1965) *J. Biol. Chem.* **240**, 322–331
- Schmid, R. (1973) *Drug Metab. Dispos.* **1**, 256–258
- Strand, L. J., Swanson, A. L., Manning, J., Branch, S. & Marver, H. S. (1972) *Anal. Biochem.* **47**, 457–470
- Tschudy, D. P. & Bonkowsky, H. L. (1972) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **31**, 147–159
- Unsold, A. & De Matteis, F. (1976) in *Porphyrins in Human Disease* (Doss, M., ed.), pp. 71–75, S. Karger, Basel
- Yoda, B. Schacter, B. A. & Israels, L. G. (1974) *Biochim. Biophys. Acta* **372**, 478–481