THE INFLUENCE OF AGE ON THE INDUCIBILITY OF CYTOCHROME *P*-450 SPECIES IN RABBIT LIVER

Susceptibility of the induced species to an unusual crosslinking reaction

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1. Introduction

We are investigating the molecular organization of the liver microsomal monooxygenase system by using chemical reagents in vitro to crosslink neighbouring protein species. Studies carried out on intact microsomes using conventional crosslinking reagents such as the lysinereactive agent, dimethyl suberimidate, do not yield useful information because of the lack of specificity of such reagents. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of microsomal proteins after treatment of microsomes with dimethyl suberimidate results in wide, poorly-resolved protein bands with much protein material failing to enter the gel, presumably due to the formation of large aggregates (McIntosh and Freedman, unpublished observations). We have not obtained any evidence that small oligomers of cytochrome *P*-450 species or of other microsomal proteins are formed in response to treatment of microsomes with dimethyl suberimidate. However, we have found that treatment of microsomes with copper phenanthroline followed by analysis on standard reducing SDS–PAGE gels, leads to the formation of dimers of certain microsomal proteins in a very specific and reproducible manner [1]. This type of crosslinking brought about by copper phenanthroline, an oxidizing agent, and seen on reducing gels is unusual in that it cannot result from the oxidation of free thiol groups on the interacting protein species to form intermolecular disulphide bonds, which is the usual mechanism by which the reagent induces crosslinking. The crosslinking of microsomal proteins by such an oxidative mechanism is not demonstrated on the reducing gels used in the present study due to the inevitable reversal of the process, although evidence for oxidative crosslinking by copper phenanthroline has been obtained on non-reducing gels (McIntosh and Freedman, unpublished observations).

When liver microsomes from adult rabbits induced with the polycyclic aromatic hydrocarbon, β-naphthoflavone (BNF), are treated with copper phenanthroline and analysed on reducing SDS–PAGE gels, material is lost from two BNF-induced bands of est. mol. wt 57 000 (band D) and 53 000 (band E). Three new bands appear at est. mol. wt 107 000 (band A), 103 000 (band B) and 100 000 (band C). We have suggested that the 3 new bands are crosslinked dimers of the two BNF-induced proteins; i.e., A = D·D, B = D·E, C = E·E. The electrophoretic mobilities of other microsomal proteins in reducing SDS gels are not influenced by copper phenanthroline-treatment, and SDS–PAGE gels of treated material are well-resolved.

In order to obtain more information regarding the identity of the proteins represented by bands D and E, we have investigated the relative induction of these bands in rabbits of different age groups. Other authors have demonstrated that the principal species induced by BNF and certain other polycyclic aromatic hydrocarbons in the livers of adult rabbits is a particular cytochrome *P*-450 species (form 4), whereas the principal species induced in neonates is another form of cytochrome *P*-450 (form 6) [2–4]. We infer from our present findings that band D represents the cyto-

chrome *P*-450 species, form 6, and that band E represents the cytochrome *P*-450 species, form 4. We find that band D is exclusively, (or predominantly) induced by BNF in neonatal rabbits, and that only band A is formed in vitro after treatment of microsomes from neonates with copper phenanthroline. This is evidence that band A represents the homodimer of species D, i.e., form 6 cytochrome *P*-450. The mechanism of these unconventional crosslinking effects of copper phenanthroline is not yet established. Their significance, however, is considerable in that they are extremely reproducible and specific and therefore permit the investigation of interactions between specific proteins in intact microsomal membranes.

2. Experimental

2.1. *Animals*

New Zealand white rabbits obtained from Hop Rabbits Ltd, Chilham, Kent, were used. The animals were classified according to age as follows:

(i) neonates: age 8 days and weight 0.14–0.19 kg;
(ii) young: median age 45 days and weight 1.1–1.5 kg;
(iii) prime: median age 98 days and weight 2.8–3.6 kg;
(iv) old: median age 164 days and weight 3.7–4.2 kg.

The sex of the individual neonates was not determined; all the other animals were males.

2.2. *Treatment with β-naphthoflavone*

Each experimental age group consisting of 4–6 rabbits was divided into two subgroups and treated as follows:

(i) induced animals: injected i.p. with a suspension of BNF (Aldrich) in Mazola corn oil to a final dosage of 60 mg/kg body wt, 24 h before death.
(ii) control animals: injected similarly with an equivalent volume of Mazola alone.

2.3. *Preparation of liver microsomal fractions*

The animals were killed by cervical dislocation. Microsomal fractions from livers pooled from each subgroup were prepared by the method in [5] with omission of butylated hydroxytoluene from buffers. Final microsomal pellets were resuspended in a small volume of buffer A (0.01 M Tris (pH 7.4); 0.1 M KCl; 1 mM EDTA; 20% (v/v) glycerol) to a final conc. 15–25 mg protein/ml, then used immediately or frozen at −20°C. Freezing and storage for up to several months did not adversely affect the subsequent electrophoretic separation of the microsomal proteins or the crosslinking reaction, except in the case of material from neonates which was found to be sensitive to storage at −20°C for more than a few weeks.

2.4. *Electrophoretic separation of microsomal proteins*

Separation was by SDS–PAGE essentially according to [6] except for the omission of a sample gel and modification of the composition of the sample buffer.

Sample solutions were prepared as follows: 35 μl microsomal fraction + 75 μl water + 200 μl sample buffer (0.25 mM Tris (pH 6.8); 8% (w/v) SDS) + 30 μl 2-mercaptoethanol + 10 μl bromophenol blue (0.1% solution) + 100 μl glycerol. The final concentrations of the principal constituents were: ~1.5 mg/ml protein; 3.5% (w/v) SDS; 6.7% (v/v) 2-mercaptoethanol; 0.11 M Tris.

Samples (20 μl) were electrophoresed on 7.5% acrylamide slab gels 1.5 mm thick. Staining was with Coomassie brilliant blue R (Gurr, stain 009120). Gels were scanned spectrophotometrically at 570 nm with a Gilford 240 spectrophotometer.

2.5. *Treatment with copper phenanthroline*

Incubation mixtures were made up as follows: 35 μl microsomal fraction + 40 μl water + 25 μl $CuCl_2$ (1 mM) + 10 μl 1:10-phenanthroline (2.5 mM) (BDH Chemicals Ltd). The concentration of the active species during incubation was: ~6 mg protein/ml; 0.23 mM Cu^{2+}; 0.23 mM 1:10-phenanthroline. Reactions were carried out for 15 min on ice and stopped by the addition of 200 μl sample buffer. For electrophoresis, 2-mercaptoethanol, bromophenol blue and glycerol were added in the same volumes as above.

2.6. *Molecular weight estimation*

Molecular weight standards used for calibration of the SDS–PAGE gels were: aldolase (Boehringer), 40 000; beef liver glutamate dehydrogenase (Boehringer), 53 000; bovine serum albumin (BDH Chemicals Ltd), 68 000; phosphorylase-*a* (Sigma), 94 000; β-galactosidase (Sigma), 130 000.

3. Results and discussion

3.1. *Induction of microsomal proteins by BNF in rabbits of different ages*

Examination of SDS–PAGE gels of microsomal material from control rabbits and rabbits induced with BNF very clearly reveals that band D is the major induced band in the neonatal animals (fig.1a, track 3), whereas band E is the major induced band in all the older animals (fig.1b–d, track 3). Band D remains inducible as a minor induced species throughout life. Band E is only inducible to a very limited extent in the neonatal group shown in fig.1a; other comparable batches of neonates have shown that band E may not be induced at all (fig.2, track 2). Presumably, the inducibility of band E is first permitted in the immediate post-natal period, and it becomes established as the major induced species in all older animals.

Scanning spectrophotometry of the gels yields absorbance profiles from which the effects of induction can be determined by subtraction of the appropriate gel scans (See fig.3a–d). Such procedure substantiates the finding that band D is the predominant induced species in neonates whereas band E is the major induced species in older animals (fig.4a–d). Molecular weight calibration with standards on the SDS–PAGE gels provided the following estimates: band D, 57 000; band E, 53 000.

Other authors have observed that a cytochrome *P*-450 species of similar molecular weight to band E is the major microsomal protein in adult animals induced by BNF and the polycyclic aromatic hydrocarbons, 3-methylcholanthrene and 2,3,7,8-tetra-

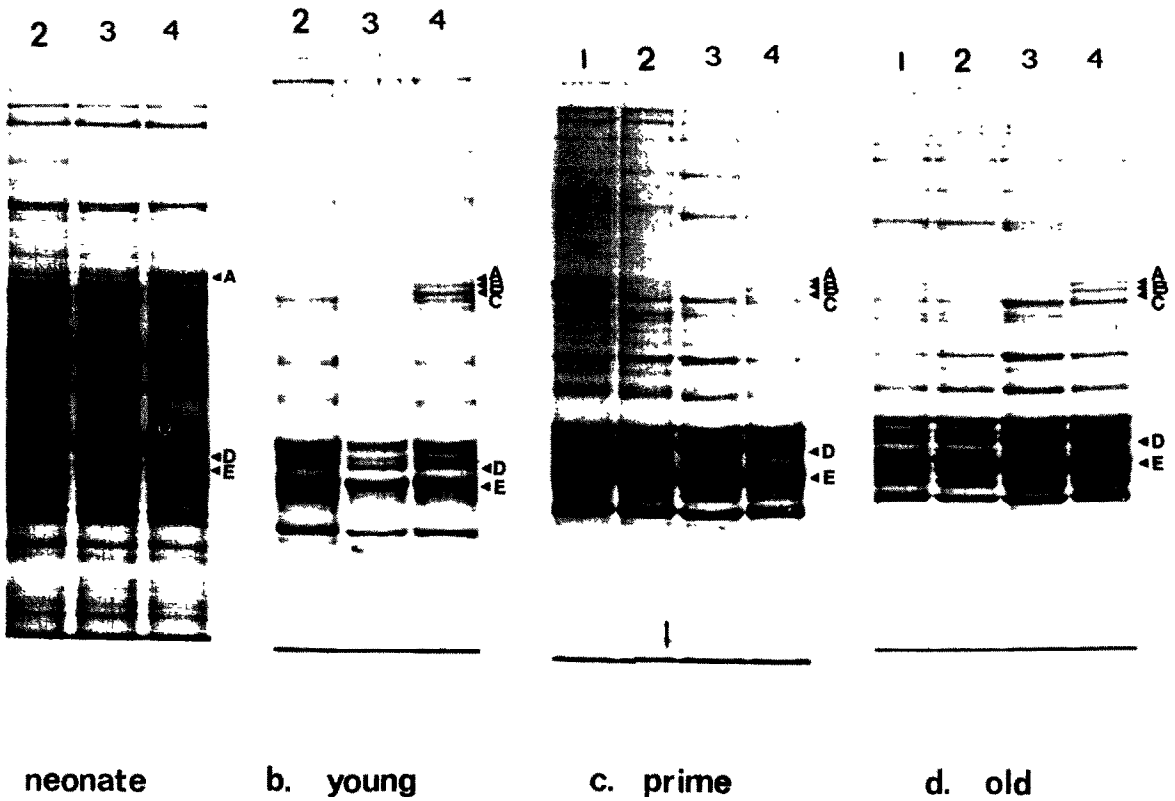


Fig.1. SDS–PAGE gels of rabbit liver microsomal proteins. Tracks (1): copper phenanthroline-treated microsomes from control rabbits. Tracks (2): untreated microsomes from control rabbits. Tracks (3): untreated microsomes from BNF-induced rabbits. Tracks (4): copper phenanthroline-treated microsomes from BNF-induced rabbits. The direction of electrophoresis is downwards.

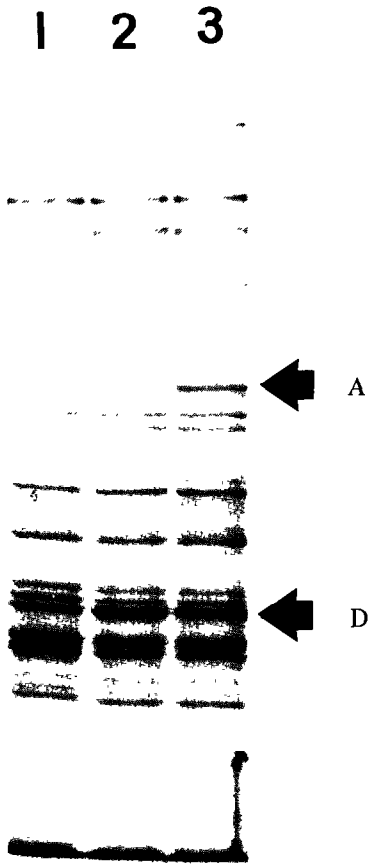


Fig.2. SDS–PAGE gel of liver microsomal proteins from a second batch of neonatal rabbits. Track (1): untreated microsomes from control animals. Track (2): untreated microsomes from BNF-induced animals. Track (3): copper phenanthroline-treated microsomes from BNF-induced animals.

chlorodibenzo-*p*-dioxin (TCDD) [2–4]. Johnson and Muller-Eberhard [7] report findings from their own laboratory and from that of Coon, that this induced cytochrome *P*-450 species, usually referred to as form 4 or LM_4, is immunologically indistinguishable regardless of whether it has been induced by BNF, TCDD or 3-methylcholanthrene. This species is quite distinct from the predominant induced species in neonates, which has been referred to as form 6 cytochrome *P*-450 (table 1). It is clear that the similarities in inducibility with respect to age and in estimated molecular weights make it probable that bands D and E are both cytochrome *P*-450 species, being forms 6 and 4, respectively, in the nomenclature of [2].

3.2. *The effect of copper phenanthroline on microsomes in vitro*

It can be seen that treatment with copper phenanthroline of microsomes from neonatal rabbits induced with BNF results in a single new band (band A) and loss of material predominantly from band D (fig.1a, track 4; fig.2, track 3; fig.3a–d; fig.4e). In all other groups, similar treatment of microsomes from BNF-induced rabbits results in 3 new bands (bands A–C) and substantial loss in material from both band D and E (fig.1b–d; fig.4f–h). The estimated molecular weights of the protein species represented by bands A, B and C are 107 000, 103 000 and 100 000, respectively. Taking into account some imprecision in molecular weight calibration on SDS–PAGE gels, these values of molecular weight strongly imply that bands A, B and C are dimers derived from the cytochrome *P*-450 species represented by bands D (57 000 daltons) and E (53 000 daltons). Since A can be derived exclusively from D in the neonate (fig.2), we conclude that D is a homodimer of A, in confirmation of our suggestion [1]. We also infer from these findings that C is likely to be a homodimer of E and B is a heterodimer of both D and E. Thus, A = D.D ≡ form 6·form 6; B = D.E ≡ form 4·form 6; C = E.E ≡ form 4·form 4.

The treatment of microsomes from control animals with copper phenanthroline did not result in the appearance of dimers in the neonatal, young (data not shown) or prime (fig.1c, track 1) age groups. This implies that form 4 and form 6 cytochromes *P*-450 are not constitutive in these animals. However, a limited appearance of dimers was seen when microsomes from control rabbits in the old group were treated (fig.1d, track 1). This implies that these forms of cytochrome *P*-450 become minor constitutive species in advanced age.

We do not understand the mechanism by which copper phenanthroline causes the form 4 and form 6 cytochromes *P*-450 to form dimers. The specificity of the reaction, which only takes place with these 2 protein species, suggests that they may share some common molecular feature, although they have been shown to be quite distinct by peptide fingerprinting [10] and immunological criteria [11]. One feature the 2 forms do share is the susceptibility of their catalytic activity to inhibition by α-naphthoflavone [2].

The copper phenanthroline crosslinking reaction is currently under investigation in this laboratory. We

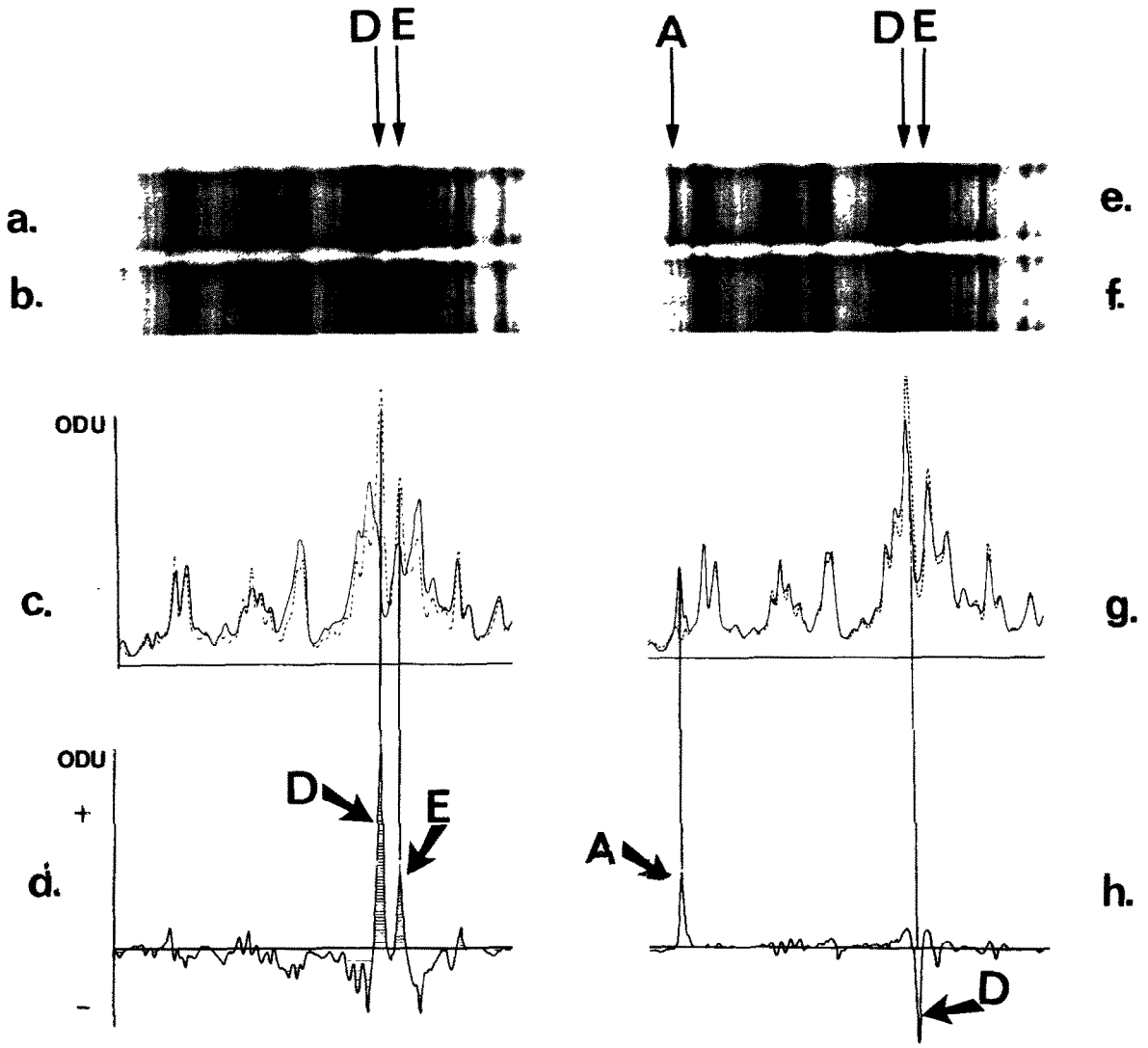
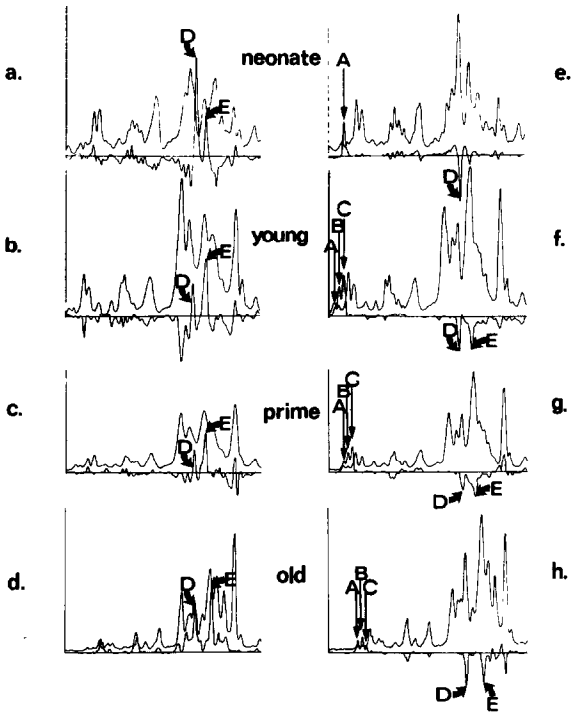


Fig.3. Derivation of difference scans showing effects of BNF-induction and copper phenanthroline-treatment. (a) Portion of track loaded with untreated microsomes from BNF-induced neonates. (b) Portion of track loaded with untreated microsomes from control neonates. (c) Superimposed spectrophotometric scans of these tracks: control sample, solid line; BNF-induced sample, dashed line. (d) Difference between the above scans, showing material induced by BNF above abscissa. (e) Portion of track loaded with copper phenanthroline-treated microsomes from BNF-induced neonates. (f) Portion of track loaded with untreated microsomes from BNF-induced neonates (= (a)). (g) Superimposed spectrophotometric scans of the above tracks: untreated BNF-induced samples, dashed line; copper phenanthroline-treated BNF-induced sample, solid line. (h) Difference between the above scans, showing material lost following copper phenanthroline treatment below the abscissa, and material gained above the abscissa. Regarding superimposition of scans (e.g., steps (c) and (g) above), correction can be made for vagaries in amounts of material loaded on the tracks by manipulation of recorder range to normalize the size of chosen marker peaks.

Table 1
Comparison of properties of microsomal species induced in rabbits by BNF, 3-methylcholanthrene (MCA) and (TCDD)

Principal induced species in:	Molecular weight as estimated electrophoretically:	Inducing agent:	Species referred to as:	Available in isolated form?	Particularly active in catalysing:	Ref.
Adult	54 000	MCA	RbLvMc *P*-450$_{54}$	No	2-acetylaminofluorene hydroxylation	[3]
Adult	54 000 (55 300 by amino acid analysis)	BNF	Cytochrome *P*-450, LM$_4$	Yes	Acetanilide hydroxylation	[4,8,9]
Adult	54 500	TCDD	Cytochrome *P*-450, Form 4	Yes	Acetanilide hydroxylation	[2]
Adult	53 000	BNF	Band E	No	–	This study
Neonate	57 000	MCA	RbLvMc *P*-450$_{57}$	No	Benzo[a]pyrene hydroxylation, biphenyl-4-hydroxylation	[3]
Neonate	57 000	TCDD (trans-placentally)	Cytochrome *P*-450, Form 6	Yes	Benzo[a]pyrene hydroxylation	[2]
Neonate	57 000	BNF	Band D	No	–	This study



have so far established that free thiol groups are not involved in the crosslinking reaction and that the dimers are stable to reducing conditions. The reaction is completely inhibited by the presence of 1% SDS. Furthermore, a significant proportion of the interacting species can rapidly form dimers at temperatures as low as −10°C, at which it is to be expected that the bulk lipid phase of the membrane is essentially immobile [12]. The characteristics and specificity of the reaction make it appear likely that it will be of unique value in the analysis of the molecular organization of microsomal monooxygenases in situ.

Fig.4. Difference scans showing the effects of induction (a–d) and copper phenanthroline-treatment (e–h) for each age group. The difference scans are marked with arrows indicating the positions of bands A–E in each case. Unmarked curves are reference scans. These are: scans of untreated microsomes from control animals (a–d); scans of untreated microsomes from BNF-induced animals (e–h). The difference scans were derived as discussed in fig.3.

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