PHENOBARBITAL AND ITS SODIUM SALT

This substance was considered by previous working groups, in 1976 (IARC, 1977) and 1987 (IARC, 1987). Since that time, new data have become available, and these have been incorporated into the monograph and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

Phenobarbital

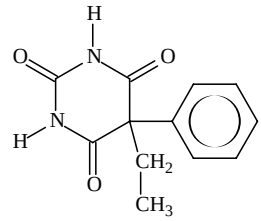
Chem. Abstr. Serv. Reg. No.: 50-06-6
Deleted CAS Reg. Nos: 11097-06-6; 46755-67-3
Chem. Abstr. Name: 5-Ethyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione
IUPAC Systematic Name: 5-Ethyl-5-phenylbarbituric acid
Synonyms: Phenobarbitone; phenobarbituric acid; phenylethylbarbituric acid; 5-phenyl-5-ethylbarbituric acid; phenylethylmalonylurea

Sodium phenobarbital

Chem. Abstr. Serv. Reg. No.: 57-30-7
Deleted CAS Reg. Nos: 125-36-0; 8050-96-2
Chem. Abstr. Name: 5-Ethyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione, monosodium salt
IUPAC Systematic Name: 5-Ethyl-5-phenylbarbituric acid, sodium salt
Synonyms: 5-Ethyl-5-phenylbarbituric acid sodium; phenobarbital sodium; phenobarbitone sodium; sodium ethylphenylbarbiturate; sodium 5-ethyl-5-phenylbarbiturate; sodium phenobarbitone; sodium phenylethylbarbiturate; sodium phenylethylmalonylurea; sol phenobarbital; sol phenobarbitone; soluble phenobarbital; soluble phenobarbitone

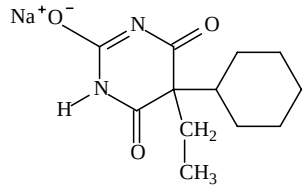
1.1.2 *Structural and molecular formulae and relative molecular masses*

Phenobarbital



$C_{12}H_{12}N_2O_3$ Relative molecular mass: 232.24

Sodium phenobarbital



$C_{12}H_{11}N_2NaO_3$ Relative molecular mass: 254.22

1.1.3 *Chemical and physical properties of the pure substances*

Phenobarbital

(*a*) *Description*: White, crystalline plates with three different phases (Gennaro, 1995; Lide & Milne, 1996; Budavari, 2000)
(*b*) *Melting-point*: 174 °C (Lide & Milne, 1996)
(*c*) *Spectroscopy data*: Infrared [prism (483), grating (21015)], ultraviolet (171), nuclear magnetic resonance [proton (6644), C-13 (4431)] and mass spectral data have been reported (Sadtler Research Laboratories, 1980; Lide & Milne, 1996).
(*d*) *Solubility*: Slightly soluble in water (1 g/L); insoluble in benzene; soluble in alkali hydroxides, carbonates, diethyl ether and ethanol (Gennaro, 1995; Lide & Milne, 1996; Budavari, 2000)
(*e*) *Dissociation constants*: pK_1, 7.3; pK_2, 11.8 (Chao *et al.*, 1978)

Sodium phenobarbital

(*a*) *Description*: Slightly hygroscopic crystals or white powder (Gennaro, 1995; Budavari, 2000)

(*b*) *Spectroscopy data*: Infrared [prism (8775), grating (28039), ultraviolet (19554) and nuclear magnetic resonance [proton 14710] and spectral data have been reported (Sadtler Research Laboratories, 1980).

(*c*) *Solubility*: Very soluble in water (1 kg/L) and ethanol; insoluble in chloroform and diethyl ether (Budavari, 2000)

1.1.4 *Technical products and impurities*

Phenobarbital is available as 8-, 16-, 32-, 65- and 100-mg tablets, as a 16-mg capsule and as a 15- or 20-mg/5 mL elixir. Sodium phenobarbital is available as 30-, 60-, 65- and 130-mg/mL injections and as a sterile powder in 120-mg ampules (Gennaro, 1995).

Trade names for phenobarbital include Adonal, Agrypnal, Amylofene, Barbenyl, Barbiphenyl, Barbipil, Barbita, Barbivis, Blu-phen, Cratecil, Dormiral, Doscalun, Duneryl, Eskabarb, Etilfen, Euneryl, Fenemal, Gardenal, Gardepanyl, Hysteps, Lepinal, Lepinaletten, Liquital, Lixophen, Lubergal, Lubrokal, Luminal, Neurobarb, Noptil, Nunol, Phenaemal, Phenemal, Phenobal, Phenoluric, Phenonyl, Phenyral, Phob, Sedonal, Sedophen, Sevenal, Somonal, Stental Extentabs, Teolaxin, Triphenatol and Versomnal. Trade names for sodium phenobarbital include Gardenal sodium, Linasen, Luminal sodium, PBS, Phenemalum, Phenobal sodium and Sodium luminal.

1.1.5 *Analysis*

Several international pharmacopoeias specify infrared absorption spectrophotometry with comparison to standards, thin-layer chromatography, high-performance liquid chromatography (HPLC) with ultraviolet detection and colorimetry as the methods for identifying phenobarbital; HPLC and titration with ethanolic potassium hydroxide are used to assay its purity. In pharmaceutical preparations, phenobarbital is identified by infrared absorption spectrophotometry, HPLC and colorimetry; HPLC and titration with ethanolic potassium hydroxide or silver nitrate are used to assay for phenobarbital content (British Pharmacopoeia Commission, 1993; Society of Japanese Pharmacopoeia, 1996; Council of Europe, 1997; US Pharmacopeial Convention, 1999).

Several international pharmacopoeias specify infrared absorption spectrophotometry with comparison to standards, thin-layer chromatography and HPLC as the methods for identifying sodium phenobarbital; HPLC with ultraviolet detection and potentiometric titration are used to assay its purity. In pharmaceutical preparations, sodium phenobarbital is identified by infrared absorption spectrophotometry and HPLC; HPLC with ultraviolet detection is used to assay for sodium phenobarbital content (British Pharmacopoeia Commission, 1993; Council of Europe, 1997; US Pharmacopeial Convention, 1999).

1.2 Production

The introduction of barbital in 1903 and phenobarbital in 1912 initiated the predominance of barbiturates, and for over half a century they reigned as the pre-eminent sedative-hypnotic agents. Although several so-called 'non-barbiturates' were introduced to displace barbiturates from time to time, it was not until chlordiazepoxide was marketed in 1961 that their position was challenged seriously. During the ensuing quarter of a century, the benzodiazepines displaced the barbiturates as the sedative-hypnotics of choice (Hardman *et al.*, 1996).

Two general methods of synthesis have been used for phenobarbital. The first is based on condensation of α-ethylbenzenepropanedioic acid ester (methyl or ethyl ester) with urea in the presence of sodium ethoxide. The second comprises condensation of benzeneacetonitrile with diethyl carbonate in ether solution to form α-cyanobenzene-acetic ester, followed by ethylation of this ester to α-cyano-α-ethylbenzeneacetic acid ester, which is further condensed with urea to yield iminobarbituric acid, and hydrolysis of iminobarbituric acid to phenobarbital (Chao *et al.*, 1978).

Sodium phenobarbital is obtained by dissolving phenobarbital in an ethanolic solution of an equivalent quantity of sodium hydroxide and evaporating at low temperature (Gennaro, 1995).

Information available in 2000 indicated that phenobarbital was manufactured by three companies each in China and India, by two companies in the Russian Federation and by one company each in Armenia, Germany, Hungary, Latvia, Switzerland and the USA (CIS Information Services, 2000a).

Information available in 2000 indicated that phenobarbital and its salts (unspecified) were formulated as a pharmaceutical by 31 companies in India, 18 companies in France, 12 companies in the USA, 11 companies each in Chile and South Africa, 10 companies each in Taiwan, Turkey and the United Kingdom, nine companies in Argentina, eight companies each in Brazil, Italy and Spain, six companies each in China and Poland, five companies each in Hungary and Japan, four companies each in Canada, Germany, the Islamic Republic of Iran, Portugal, the Republic of Korea and Switzerland, three companies each in Australia, Belgium, Bulgaria, Ecuador, Greece, Peru and Venezuela, two companies each in the Czech Republic, Egypt, Lithuania, Mexico, Romania, the Russian Federation, Thailand and Viet Nam and one company each in Hong Kong, Indonesia, Ireland, Malta, Norway, the Philippines, Singapore, the Slovak Republic, Surinam, Sweden, the Ukraine and Yugoslavia (CIS Information Services, 2000b).

Information available in 2000 indicated that sodium phenobarbital was manufactured by one company each in China, Germany, Hungary, Latvia and the United Kingdom (CIS Information Services, 2000a) and that it was formulated as a pharmaceutical by seven companies in India, four companies in Italy, two companies each in Argentina, Australia, Chile, Ecuador, France, the United Kingdom and the USA and one company each in Belgium, Bulgaria, Germany, Hungary, Indonesia, the Islamic

Republic of Iran, Japan, the Philippines, Poland, South Africa, Taiwan, Thailand and Venezuela (CIS Information Services, 2000b).

1.3 Use

Phenobarbital is a sedative, hypnotic and anti-epileptic drug. In appropriate doses, it is used in the treatment of neuroses and related tension states when mild, prolonged sedation is indicated, as in hypertension, coronary artery disease, functional gastrointestinal disorders and pre-operative apprehension. In addition, it has specific use in the symptomatic therapy of epilepsy, particularly for patients with generalized tonic–clonic seizures (grand mal) and complex partial (psychomotor) seizures. Phenobarbital is also included in the treatment and prevention of hyperbilirubinaemia in neonates. Because of its slow onset of action, phenobarbital is not generally used orally to treat insomnia but is used to help withdraw people who are physically dependent on other central nervous system depressants. With the exception of metharbital and mephobarbital, it is the only barbiturate effective in epilepsy (Gennaro, 1995).

Sodium phenobarbital, because it is soluble in water, may be administered parenterally. It is given by slow intravenous injection for control of acute convulsive syndromes (Gennaro, 1995).

The usual adult oral dose of phenobarbital as a sedative is 30–120 mg in two to three divided doses, that as a hypnotic is 100–320 mg and that as an anticonvulsant is 50–100 mg two or three times a day. The usual dose is 30–600 mg/day. The usual paediatric oral dose of phenobarbital as a sedative is 2 mg/kg bw or 60 mg/m^2 three times a day, that as a hypnotic is individualized by the physician and that as an anticonvulsant or antidyskinetic is 3–5 mg/kg bw or 125 mg/m^2 a day until a blood concentration of 10–15 μg/mL is attained (Gennaro, 1995).

The usual adult oral dose of sodium phenobarbital as a sedative is the same as that for phenobarbital, that as an intramuscular or intravenous sedative is 100–130 mg, that as an anticonvulsant is 200–300 mg repeated within 6 h if necessary, that as a pre-operative medication is 130–200 mg every 6 h and that as a post-operative sedative is 32–100 mg. The usual paediatric dose of sodium phenobarbital as an intramuscular sedative is 60 mg/m^2 three times a day, that as an anticonvulsant is 125 mg/m^2 per dose, that as a pre-operative medication is 16–100 mg and that as a post-operative sedative is 8–30 mg (Gennaro, 1995).

Phenobarbital ranked 63rd among the 200 generic drugs most frequently sold by prescription in the USA in 1999 (Anon., 2000).

1.4 Occurrence

1.4.1 *Occupational exposure*

According to the 1981–83 National Occupational Exposure Survey (National Institute for Occupational Safety and Health, 2000), about 23 000 workers, including 20 000 nurses, nursing aides, health aides, pharmacists and laboratory workers in health services and 2900 chemical industry workers, were potentially exposed occupationally to phenobarbital in the USA.

1.4.2 *Environmental occurrence*

No data were available to the Working Group.

1.5 Regulations and guidelines

Phenobarbital is listed in the pharmacopoeias of Austria, China, the Czech Republic, France, Germany, Italy, Japan, Poland, the United Kingdom and the USA, and in the European and International pharmacopoeias (US Pharmacopeial Convention, 1999; Royal Pharmaceutical Society of Great Britain, 2000; Swiss Pharmaceutical Society, 2000; Vidal, 2000). It is registered for human use in Ireland, the Netherlands, Norway, Spain and Sweden (Irish Medicines Board, 2000; Medical Products Agency, 2000; Medicines Evaluation Board Agency, 2000; Norwegian Medicinal Depot, 2000; Spanish Medicines Agency, 2000).

Sodium phenobarbital is listed in the pharmacopoeias of Austria, China, the Czech Republic, Germany, Italy, Japan, Poland, the United Kingdom and the USA, and in the European and International pharmacopoeias (Royal Pharmaceutical Society of Great Britain, 2000; Swiss Pharmaceutical Society, 2000).

2. Studies of Cancer in Humans

2.1 Cohort studies

2.1.1 *Studies of patients with seizures* (see Table 1)

A cohort of (initially) 9136 patients hospitalized for epilepsy in the Filadelfia treatment community in Denmark between 1933 and 1962 was followed for cancer incidence from 1943 to 1967 (Clemmesen *et al*., 1974). Throughout the study period, phenobarbital was the basic therapeutic drug, given in daily doses of 100–300 mg. In the 1940s, phenytoin was introduced at the treatment centre, either alone or in combination with phenobarbital, and in the mid-1950s primidone was also used. Primidone is partly metabolized to phenobarbital. The files of the Danish Cancer Registry, established in

Table 1. Cohort studies of cancer incidence or mortality in epilepsy patients, generally treated with phenobarbital

Country (reference)	Population; outcome measure (size); recruitment period/ follow-up period	Treatment modality	Cancer site	Relative risk (95% CI)		Comments
Studies of patients with seizures						
Denmark (Clemmesen & Hjalgrim-Jensen, 1981) (Update of Clemmesen *et al*., 1974)	Epilepsy patients; SIR (8077); 1933–62/1943–76	Anticonvulsants including phenobarbital	All cancers	[1.1]	[1.0–1.2]	
			Brain	[5.3]	[4.1–6.6]	Benign brain tumours included; initial 4 weeks of follow-up excluded
			Latency period (years):			
			0–9	[12]	[8.5–16]	
			10–14	[5.4]	[2.9–9.2]	
			15–19	[1.8]	[0.5–4.5]	
			20–24	[2.7]	[0.9–6.2]	
			25–29	[1.4]	[0.2–5.1]	
			30–34	[2.1]	[0.2–7.6]	
			≥ 35	[0.0]	[0.0–5.8]	
			Liver	[3.8]	[2.0–6.5]	Thorotrast exposure in 10/13 liver cancer cases
			Lung	[1.3]	[1.0–1.7]	
Denmark (Olsen *et al*., 1989) (update of Clemmesen *et al*., 1974)	Epilepsy patients; SIR (7864); 1933–62/1943–84	Anticonvulsants including phenobarbital	All cancers	1.2	(1.1–1.3)	Followed-up from date of first hospitalization for epilepsy; 140 patients known to have received Thorotrast were excluded.
			Brain	5.7	(4.7–6.8)	
			Latency period (years):			
			0–9	20	(16–26)	
			10–19	4.1	(2.5–6.2)	
			20–29	1.5	(0.7–2.8)	
			≥ 30	1.3	(0.5–2.8)	
			All except brain	1.0	(0.97–1.1)	
			Lung	1.4	(1.2–1.7)	
			Liver	1.9	(0.9–3.6)	
			Thyroid	1.2	(0.3–3.2)	
			Non-Hodgkin lymphoma	1.4	(0.8–2.3)	
			Leukaemia	0.8	(0.4–1.4)	
			Bladder	0.6	(0.3–0.9)	
			Melanoma	0.5	(0.2–1.0)	

Table 1 (contd)

Country (reference)	Population; outcome measure (size); recruitment period/ follow-up period	Treatment modality	Cancer site	Relative risk (95% CI)	Comments
England (White *et al*., 1979)	Epilepsy patients; SMR (1980); 1931–71/1951–77	Anticonvulsants (long term) including phenobarbital	All cancers	1.5 (1.2–1.9)	
			Brain	4.1 (1.5–8.9)	
			All except brain	1.4 (1.1–1.8)	
			Lung	1.4 (0.9–2.1)	
			Liver	0.0 (0.0–12)	~ 0.3 cases expected
			Lymphoma and leukaemia	1.3 (0.5–2.1)	
USA, Minnesota (Shirts *et al*., 1986)	Patients with seizure disorders; SIR (959); 1935–79/1935–82	Not specified	All cancers	1.4 (1.1–1.8)	
			Brain	24 (14–39)	
			Length of follow-up (years)		
			0–4	47 (26–82)	
			5–9	12 (1.4–43)	
			≥ 10	5.9 (0.7–21)	
			All except brain	1.1 (0.8–1.4)	
			Lung	2.7 (1.2–5.2)	Seven of nine lung cancers diagnosed during first 5 years of follow-up
			Liver	No cases observed	
			Lymphoma and leukaemia	2.9 (1.0–5.0)	
		Medication:	All except brain		Primarily phenobarbital and phenytoin
		Yes		0.9 (0.6–1.4)	
		No		1.3 (0.8–2.0)	

Table 1 (contd)

Country (reference)	Population; outcome measure (size); recruitment period/ follow-up period	Treatment modality	Cancer site	Relative risk (95% CI)		Comments
Transplacental exposure						
Denmark (Olsen *et al*., 1990)	Offspring of epilepsy patients; SIR (3727) 1933–62/1943–86	Born after mother's first admission for epilepsy (2579)	All cancers	1.0	(0.6–1.7)	
			Brain	1.4	(0.3–4.0)	
		Born before mother's first admission (1148)	All cancers	0.9	(0.6–1.2)	

1942, were used to identify incident cases of cancer, including benign brain tumours and bladder papillomas (Olsen *et al.*, 1989), in the patients. The expected numbers of cases were calculated from national cancer incidence rates, similarly based on the files of the Cancer Registry. In two updated reports with extended follow-up (Clemmesen & Hjalgrim-Jensen, 1977, 1981), the original roster was revised to include 8078 and 8077 subjects, respectively. The reduction of the cohort by 12% was due to exclusion of non-Danish citizens, patients who died before 1943 when the follow-up began, duplicate registrations and persons whose diagnosis of epilepsy was not sustained. In the most recent update (Clemmesen & Hjalgrim-Jensen, 1981), the period of follow-up was from 4 weeks after first admission to the treatment community or 1 January 1943, whichever came last, to the day of death or the end of 1976, whichever came first. A total of 467 cases of cancer were observed when 419.5 were expected [yielding an overall standardized incidence ratio (SIR) of 1.1; 95% confidence interval (CI), 1.0–1.2; or 1.25 (95% CI, 1.1–1.4) among men and 0.99 (95% CI, 0.87–1.13) among women]. The overall increase was due mainly to the observation of 71 tumours of the brain and nervous system when 13.4 were expected [yielding a significantly increased SIR of 5.3; 5.8 among men and 4.8 among women]. Excess rates of brain tumour were seen in particular during the first 15 years of follow-up [with SIRs of 12, 5.4, 1.8, 2.7, 1.4, 2.1 and 0.0 for latency periods of 0–9, 10–14, 15–19, 20–24, 25–29, 30–34 and ≥ 35 years, respectively]. The authors suggested that the seizures of some patients were early symptoms of their brain tumours. Thirteen cases of primary liver cancer were observed, when 3.44 cases were expected [SIR, 3.8; 95% CI, 2.0–6.5]. This excess of liver cancer, which was particularly evident in patients followed for 15 years or more, was ascribed to use of radioactive thorium dioxide (Thorotrast) for cerebral angiography in a subgroup of patients during diagnostic work-up. Only three of the 13 patients with primary liver cancer had had no documented exposure to Thorotrast, which was in agreement with the 3.4 cases expected among all cohort members. Finally, the increased SIR for lung cancer among male patients [SIR, 1.3; 95% CI, 0.94–1.7] was considered most likely to be due to a higher prevalence of smokers.

The Danish cohort was further evaluated by Olsen *et al*. (1989), who extended follow-up for cancer incidence from 1976 through 1984. Exclusion of additional duplicate admissions, non-Danish citizens and patients for whom the data were incomplete and the inclusion of patients hospitalized for fewer than 4 weeks reduced the cohort to 8004 patients. Of these, 140 had had documented exposure to Thorotrast. Linkage of the records of the remaining 7864 patients not known to have received this contrast medium with the files of the national Cancer Registry resulted in identification of 789 cancers, with 663.7 expected (SIR, 1.2; 95% CI, 1.1–1.3). A sixfold increased risk was seen for brain cancer on the basis of 118 observed cases; 43 were seen within 1 year of admission (SIR, 88). The risk for brain tumours in childhood was especially high. A significant excess also occurred for cancer of the lung, but with no clear trend over time. Non-significantly increased risks were seen for cancers of the liver (SIR, 1.9) and biliary tract (SIR, 1.7) on the basis of 9 and 11 observed cases, respectively. It was

suggested by the authors that these non-significant increases in risk among patients not known to have received Thorotrast might be related to undocumented exposure to this hepatocarcinogen. Four cases of thyroid cancer were observed, with 3.2 cases expected (SIR, 1.2; 95% CI, 0.3–3.2). Significant deficits of bladder cancer (SIR, 0.6; 18 cases) and melanoma (SIR, 0.5; 7 cases) were observed. In the subgroup of 140 patients with documented exposure to Thorotrast, 17 cases of liver cancer (SIR, 202) and three cases of biliary-tract cancer (SIR, 28) were reported.

White *et al*. (1979) evaluated mortality among patients admitted to one treatment centre for epilepsy in England between 1931 and 1971. Patients were included in the study if their notes stated a diagnosis of epilepsy not due to trauma or progressive disease and if they had been prescribed long-term anti-convulsant drug therapy. Most of the patients severely affected by epilepsy had been treated with phenobarbital and phenytoin. After exclusion of patients who were known to have discontinued anti-convulsant drug therapy within 6 months of starting, 2099 subjects were linked to the files of the National Health Service Central Register of the United Kingdom for information on vital status and migration; for those who had died, a copy of the death certificate was provided by the Office of Population Censuses and Surveys. Personal data on 38 subjects (2%) could not be verified, and 81 were known to have died before the period of follow-up (1951–77), leaving 1980 patients for the analysis of mortality. A total of 78 deaths from cancer was recorded when 51.5 were expected on the basis of the age-, sex- and calendar year-specific rates for England and Wales (standardized mortality ratio [SMR],1.5; 95% CI, 1.2–1.9). Neoplasms of the brain and central nervous system accounted for six of the deaths, with 1.5 expected (SMR, 4.1; 95% CI, 1.5–8.9). When these neoplasms were excluded as possibly being associated with epilepsy, the SMR was 1.4, which was still significantly high (95% CI, 1.1–1.8), but there was no trend over time and no significant excess of deaths was associated with cancer at a particular site. A non-significantly increased risk of 1.4 was seen for lung cancer.

Through a medical records system in Rochester, Minnesota (USA), providing access to all medical contacts of citizens of the area covered by the system, Shirts *et al*. (1986) identified 959 patients in whom an unprovoked seizure disorder had been diagnosed between 1935 and 1979. On the basis of the same records system, patients were followed for new diagnoses of cancer from the date of the initial diagnosis of seizure to death, last contact or the end of 1982: on average, 13 years. A total of 65 primary cancers were diagnosed when 45.9 were expected from the age- and sex-specific incidence rates of the background population, yielding an overall SIR of 1.4 (95% CI, 1.1–1.8). The increased risk was largely attributable to 17 primary brain cancers, for which the expected number was < 1; an SIR of 47 (13 cases) during the first 5 years of follow-up decreased gradually to 12 (two cases) and 5.9 (two cases) in the subsequent 5–9 years and 10 or more years of follow-up, respectively. The risk for lung cancer was also significantly elevated, with an SIR of 2.7 (95% CI, 1.2–5.2); however, seven of the nine cases were observed during the first 5 years of follow-up, limiting the excess to this early period. Marginally significant increased risks were

seen for breast cancer (SIR, 2.0; 95% CI, 0.98–3.8; 10 cases) and cancers of the lymphatic and haematopoietic system (SIR, 2.9; 1.0–5.0; seven cases), with no clear trends in risk over time. [The Working Group noted that no information was given on the completeness of follow-up.] The SIR for all cancers except primary brain cancers was 0.9 (95% CI, 0.6–1.4) for users of anticonvulsants and 1.3 (0.8–2.0) for non-users.

2.1.2 *Studies of transplacental exposure*

Annegers *et al*. (1979) reported on the occurrence of brain tumours in 177 individuals born in Rochester, Minnesota (USA), between 1939 and 1976 who had been exposed *in utero* to anti-convulsants during the first trimester of gestation. Barbiturates had been prescribed for the mothers of 135 of these patients. No case of brain tumour was observed during follow-up. [The Working Group noted that the expected number of brain tumours was not estimated, and that the number of person–years of follow-up was not provided]

Olsen *et al*. (1990) conducted a separate study of the incidence of cancer among 3727 offspring of 3758 women admitted for epilepsy to the Filadelfia treatment community in Denmark between 1933 and 1962. A survey of drug use by 130 of the patients indicated that 76% had been treated with phenobarbital and 30% with primidone. The records of the offspring, who were identified from hospital charts, population listings and parish registers, were linked with the files of the Danish Cancer Registry in order to follow-up for cancer through 1986. The expected age-, sex- and calendar time-specific cancer incidence rates for the general population were also derived from the Registry. Overall, 49 cancers were identified, with 53.8 expected, yielding an SIR of 0.9 (95% CI, 0.7–1.2). Among the 2579 children born after their mother's first admission for epilepsy, and thus presumably exposed *in utero* to anti-convulsant drugs, 14 cases of cancer were identified (average follow-up period, 22.4 years; maximum, 50 years), with 13.8 expected (SIR, 1.0; 95% CI, 0.6–1.7). This subcohort of offspring showed no excess risk for any specific tumour type, including brain tumours (3 observed cases, 2.2 expected). Among the group of 1148 offspring born before the first admission of the mother to the treatment community, 35 developed cancer (average follow-up period, 37.5 years; maximum, 65 years), with 40.0 expected (SIR, 0.9; 95% CI, 0.6–1.2). No significant increase in the risk for any cancer was found.

2.1.3 *Studies in the general population*

Phenobarbital was included in a hypothesis-generating cohort study designed to screen a large number (215) of drugs for possible carcinogenicity, which covered more than 140 000 subscribers enrolled between July 1969 and August 1973 in a prepaid medical care programme in northern California (USA). Computer records of persons to whom at least one drug prescription has been dispensed were linked to the cancer

records of hospitals covered by the medical care programme and the regional cancer registry. The observed numbers of cancers were compared with those expected, standardized for age and sex, for the entire cohort. Three publications summarized the findings for follow-up periods of up to 7 years (Friedman & Ury, 1980), up to 9 years (Friedman & Ury, 1983) and up to 15 years (Selby *et al*., 1989). Among 5834 persons who received phenobarbital, mostly as a sedative, associations were noted in the 7-year report for cancers of the lung (44 cases observed, 28.9 expected; $p < 0.05$), ovary (seven cases observed, 2.7 expected; $p < 0.05$) and gall-bladder (four cases observed, 1.0 expected; $p < 0.05$) and in the 9-year report for cancer of the lung [figures not given] and gall-bladder and biliary tract (six cases observed, 1.8 expected; $p < 0.05$). In the 15-year report, associations were noted with cancers of the gall-bladder (eight cases observed, 3.2 expected; $p < 0.05$) and bone (three cases observed, 0.6 observed; $p < 0.05$), but not for cancer of the lung. [The Working Group noted, as did the authors, that, since some 12 000 comparisons were made in this hypothesis-generating study, the associations should be verified independently. Data on duration of use were generally not provided.]

In a post-hoc evaluation of the finding of lung cancer in the 7-year follow-up, Friedman (1981) merged this group with 2156 users of pentobarbital and 2884 users of secobarbital, two other commonly used barbiturates. In the combined group of 9816 users of one of these three barbiturates, the author observed 87 cases of lung cancer when 50.2 were expected (SIR, 1.7; $p < 0.002$). Data on smoking habits, collected at regular health check-ups, were available for 49% of the members of the combined cohort, and information on histological subtype of lung cancer was obtained from the medical charts of cancer patients. The resulting SIRs for lung cancer among barbiturate users were 1.5 [95% confidence interval (CI), 0.4–3.8] for non-smokers, 1.4 [0.5–3.0] for ex-smokers and 1.6 [1.1–2.3] for smokers. There was no change in the risk for lung cancer after the incorporation of a lag time of 1 or 2 years in the analysis. There was no particular association with any of the major sub-types of lung cancer.

The risk pattern for lung cancer was evaluated by Friedman and Habel (1999), who extended follow-up through to 1992 and further added users of mixtures of barbiturates, resulting in a group of 10 213 exposed individuals. An initially elevated SIR of 1.6 (95% CI, 1.3–1.9) for lung cancer for the combined group of barbiturate users with 3–7 years of follow-up, unadjusted for smoking habits, gradually decreased and stabilized at about 1.3 [1.2–1.4] after 11–15 years of follow-up. An initial, non-significant increase in the risk for lung cancer of 80% among people who had never smoked decreased to near unity in later periods of follow-up. A dose–response trend was observed, on the basis of the number of prescriptions dispensed, with an SIR of 3.4 (95% CI, 2.0–5.4) for individuals receiving the highest dose (20 or more prescriptions of barbiturates). Adjustment for smoking habits in a Cox model in the subgroup for which this information was available reduced, but did not eliminate, the dose–response trend. [The Working Group noted that the last two analytical studies were post-hoc evaluations of a finding of lung cancer in a large surveillance study with multiple

testing. These studies did not include the results for phenobarbital users specifically but rather for the combined group of barbiturate users.]

In a companion study, based on the 10 368 users of barbiturates, Habel *et al*. (1998) observed 34 cases of bladder cancer in the 1992 follow-up, yielding an overall SIR of 0.71 (95% CI, 0.51–1.0). The SIRs for current smokers and former smokers were 0.56 (0.23–1.2) and 0.68 (0.27–1.4), respectively, whereas the SIR for people who had never smoked was 1.04 (0.48–2.0), indicating an inverse association between barbiturate treatment and bladder cancer risk only among current and former smokers.

2.2 Case–control studies (see Table 2)

In order to evaluate the relationships between cancer in children and drugs given to their mothers during pregnancy, Sanders and Draper (1979) studied 11 169 matched case–control pairs of children aged up to 15 years included in the Oxford Survey of Childhood Cancers. A history of epilepsy was reported by 39 case mothers [0.35%] and 22 control mothers [0.20%]; a review of available medical records (for 30 case mothers and 18 control mothers) showed no difference in the proportions of mothers in the two groups who had received phenobarbital (67% and 67%). Six of the 39 tumours in children of mothers with epilepsy were lymphomas, when four cases would have been expected on the basis of the proportion of lymphomas among childhood cancers in the population. [The Working Group noted that the number of children with brain tumours of the 39 mothers with epilepsy was not given.]

In a case–control study by Gold *et al*. (1978), all children under 20 years of age in whom brain tumours had been diagnosed in the Baltimore area, Maryland (USA), between 1965 and 1975 were ascertained from multiple data sources, including hospital tumour registries and death certificates. Of a total of 127 children who were eligible for the study, 84 were included (response rate, 66%) after completion of an interview with the parents. The parents of 76 population controls [response rate not provided] selected from birth certificates and matched to case children by race, sex and date of birth and 112 cancer controls [response rate not provided] selected from the same data sources as the cases and matched by race, sex and date and age at diagnosis were also interviewed [the items included in the interview were not fully characterized]. These subjects formed 73 matched pairs of brain tumour patients and population controls and 78 matched pairs of brain tumour patients and cancer controls, which were analysed separately. In the substudy in which population controls were used, maternal intake of barbiturates during the index pregnancy was associated with an odds ratio of 2.0 (95% CI, 0.3–22). Use of barbiturates by the children themselves was associated with an odds ratio of 2.5 (0.4–26). In the sub-study of matched pairs with cancer controls, the association with prenatal exposure became significant (lower 95% confidence bound, 1.5). Any use of barbiturates pre- or postnatally was significantly associated with brain tumours in the analysis with cancer controls (odds ratio, 5.5; 95% CI, 1.2–51), but not in that with population controls (3.0; 0.8–17). [The Working Group noted that neither

Table 2. Case–control studies of barbiturates, primarily phenobarbital, by cancer site

Country (reference)	Subjects	Exposure estimates	Odds ratio (95% CI)		Comments
Childhood cancers					
England (Sanders & Draper, 1979)	11 169 patients 11 169 cancer controls	Epilepsy in mother	[0.35%] [0.20%]		Formal risk estimates were not provided. Similar proportions of case and control mothers with epilepsy took phenobarbital.
Childhood brain tumours					
USA, Maryland (Gold *et al*., 1978)	73 patients	Prenatal (maternal) intake of barbiturates	2.0	(0.3–22)	No information available on specific use of phenobarbital
	73 population controls	Postnatal	2.5	(0.4–26)	
		Both	3.0	(0.8–17)	
	78 patients	Prenatal	∞	(1.5–∞)	
	78 cancer controls	Postnatal	2.5	(0.4–26)	
		Both	5.5	(1.2–51)	
USA, California (Goldhaber *et al*., 1990)	237 patients 474 controls	Prenatal	1.0	(0.5–1.9)	Based on records for 86 mothers; phenobarbital was the predominant barbiturate used.
		Postnatal			
		Unadjusted	1.8	(1.2–2.7)	
		Adjusted	1.4	(0.9–2.2)	Epilepsy of the child included in the adjusted estimate

Table 2 (contd)

Country (reference)	Subjects	Exposure estimates	Odds ratio (95% CI)		Comments
Childhood neuroblastoma					
USA (Kramer *et al*., 1987)	104 patients 104 controls	Mothers' phenobarbital use Neurally active drugs	[2.9%] [0.0%] 2.8	(1.3–6.0)	Formal risk estimates were not provided. 90% CI
Lung cancer					
Denmark (Olsen *et al*., 1993)	104 patients 200 controls	Phenobarbital treatment Ever versus never 1–749 g ≥ 750 g	 1.2 1.6 1.0	 (0.7–2.2) (0.8–3.0) (0.5–1.8)	
Bladder cancer					
Denmark (Olsen *et al*., 1993)	18 patients 33 controls	Phenobarbital treatment Ever versus never 1–749 g ≥ 750 g	 0.3 0.6 0.2	 (0.1–0.9) (0.1–2.7) (0.0–0.9)	
Primary liver cancer					
Denmark (Olsen *et al*., 1995)	26 patients 49 controls	Phenobarbital treatment Ever versus never 5–749 g ≥ 750 g	 2.0 0.4 3.2	 (0.5–7.2) (0.1–3.4) (0.7–14)	

Table 2 (contd)

Country (reference)	Subjects	Exposure estimates	Odds ratio (95% CI)		Comments
Biliary-tract cancer					
Denmark	13 patients	Phenobarbital treatment			
(Olsen *et al*., 1995)	24 controls	Ever versus never	1.5	(0.4-6.7)	
		5–749 g	1.3	(0.3-7.0)	
		≥ 750 g	1.6	(0.3-8.9)	
Malignant lymphoma					
Denmark	21 patients	Phenobarbital treatment			
(Olsen *et al*., 1995)	98 controls	Ever versus never	1.5	(0.5–5.0)	

the type nor duration of the exposure of mothers or children to barbiturates was described.]

In a population-based study in the USA (Kramer *et al*., 1987), 181 children with newly diagnosed, histologically confirmed neuroblastomas were identified from either the files of the Greater Delaware Valley Pediatric Tumour Registry or the Children's Hospital of Philadelphia for the period 1970–79. Of the 139 children eligible for study, 18 could not be traced and 17 refused, leaving 104 for inclusion (response rate, 75%). One population control per case was selected by random-digit dialling and matched to the case by area of residence, race and date of birth (plus or minus 3 years). The response rate of those eligible and invited was 57%. Interviews, conducted over the telephone with mothers of study subjects, included questions on health history and exposure to alcohol, drugs and other treatments. Three case mothers and no control mothers reported use of phenobarbital at some time during pregnancy. When use of phenobarbital was combined with use during pregnancy of other 'neurally active drugs', defined by the authors to include other barbiturates, amphetamines, narcotics, tranquillizers, diet pills and muscle relaxants, there was a statistically significant, positive association with neuroblastoma in the children, with an odds ratio for the matched pairs of 2.8 (90% CI, 1.3–6.0).

Goldhaber *et al*. (1990) identified 304 children aged 0–19 years, notified with malignant intracranial or spinal cord tumours between 1960 and 1983, from a computerized information system on patients discharged from hospitals run by a prepaid medical care programme in northern California (USA) and the files of the Cancer Registry of the San Francisco Bay Area. The 237 that were included were those for which the diagnosis had been confirmed in a medical record review and whose family had belonged to the programme for at least 6 months. For each study child, two control children were selected from the membership list and matched to the case on year of birth, sex and initial date of membership of the health care programme. The medical charts of the mothers, from the respective birth departments (inside or outside the medical care programme), were reviewed for information on barbiturate use during pregnancy, and the available medical charts on the children after birth were reviewed. Fifty-five cases (23%) and 72 (15%) controls had a history of childhood exposure to barbiturates, yielding an odds ratio of 1.8 (1.2–2.7). In a subgroup of 86 women for whom prenatal records were available, there was no difference between cases and controls with regard to exposure to barbiturates, 19 (22%) case mothers and 39 (23%) control mothers having taken barbiturates during pregnancy, yielding a matched-pair odds ratio of 1.0 (95% CI, 0.5–1.9). Phenobarbital, alone or in combination, was the predominant barbiturate used. Gastrointestinal disorder was the most common indication for barbiturate use for the mothers of both cases (38%) and controls (29%). Epilepsy in the child was associated with an odds ratio for brain cancer of 5.1 (1.8–14). Adjustment for epilepsy in a conditional logistic regression model reduced the odds ratio for brain cancer associated with barbiturate use from 1.8 to 1.4 (0.9–2.2).

Olsen *et al*. (1993) conducted a case–control study of lung and bladder cancer nested in the Danish cohort of epileptic patients described above (Olsen *et al*., 1989), in order to address the effects of phenobarbital specifically. A total of 111 cases of lung cancer (SIR, 1.5; 95% CI, 1.2–1.8) and 19 cases of bladder cancer (0.6; 0.4–0.9) observed during follow-up of cohort members through 1984 were each matched to two cancer-free cohort members on the basis of sex, year of birth and time from year of first admittance to the treatment centre for epilepsy. Eight cases (6.2%) and 13 controls (5.0%) were excluded because medical records could not be obtained. An additional 14 controls for which the case had been excluded were also dropped, leaving 104 lung cancer cases with 200 lung cancer controls and 18 bladder cancer cases with 33 bladder cancer controls for study. Information on use of phenobarbital, primidone and other anti-convulsants was abstracted from the medical records at the epilepsy centre, and indications of exposure to Thorotrast were obtained from the files of the Danish Thorotrast study. In a conditional logistic regression analysis for matched sets, with adjustment for concurrent use of other anti-convulsants, any use of phenobarbital was associated with odds ratios of 1.2 (95% CI, 0.7–2.2) for lung cancer and 0.3 (0.1–0.9) for bladder cancer. Dose–response analyses revealed no consistent relationship between lung cancer and cumulative exposure to phenobarbital. The risk for bladder cancer declined significantly with increasing cumulative exposure to phenobarbital. Exclusion from the analysis of five cases of lung cancer and two controls for cases of bladder cancer with exposure to Thorotrast did not change the results appreciably.

On the basis of the same cohort of 8004 epileptic patients, Olsen *et al*. (1995) conducted a nested case–control study of hepatobiliary cancer and malignant lymphoma. A total of 26 cases of primary liver cancer (SIR, 4.7; 95% CI, 3.2–6.8), 14 of biliary-tract cancer (2.2; 1.2–3.5), 17 of non-Hodgkin lymphoma (1.5; 0.9–2.3) and six of Hodgkin disease (0.9; 0.4–2.0) observed during follow-up of cohort members through 1984 were each matched to two (hepatobiliary cancers) or five (lymphomas) cancer-free controls on the basis of sex, year of birth and time from year of first admittance to the treatment centre for epilepsy. Three cases (4.8%) and 12 controls (6.2%) were excluded because medical records were missing; an additional 12 controls that were no longer matched to a case were excluded, leaving 60 cases and 171 controls for study. Overall, administration of phenobarbital, adjusted for the effect of other anti-convulsant therapy, was associated with non-significantly increased rates for cancers of the liver (odds ratio, 2.0) and biliary tract (odds ratio, 1.5). A separate, but unadjusted, matched analysis after exclusion of individuals exposed to Thorotrast revealed no increase in risk for liver cancer (odds ratio, 1.0) or for biliary-tract cancers (odds ratio, 0.8) in association with exposure to phenobarbital. The relative risk for malignant lymphomas was 1.5 (95% CI, 0.5–5.0).

3. Studies on Cancer in Experimental Animals

Phenobarbital has been evaluated previously (IARC, 1977). The Working Group was aware of numerous studies involving long-term oral administration of phenobarbital to mice and chose a number of well-conducted studies of carcinogenicity in various strains, in which adequate numbers of animals, several doses and an adequate duration were used.

3.1 Oral administration

Mouse: Groups of 17–37 male and 16–39 female C3Hf/Anl (C3H) mice, 1–3 months of age, were fed a control diet or a diet containing 0.05% phenobarbital [purity not specified] for 12 months. Male mice on the control diet had a higher incidence of hepatic tumours (neoplastic hepatic nodules) than females, and an increased tumour incidence in animals of each sex was found when the number of mice per cage was decreased from five to one (control males: for five mice/cage, 7/17 (41%); and for one mouse/cage, 25/37 (68%); control females: for five mice/cage, 1/16 (6%); and for one mouse/cage, 5/39 (13%)). Dietary administration of phenobarbital increased the incidence of hepatic tumours in animals of each sex (males: 35/36 (97%) for one mouse/cage and 16/17 (94%) for five mice/cage; females: 29/29 (100%) for one mouse/cage and 10/16 (63%) for five mice/cage). An increase in the multiplicity of tumours was also observed in phenobarbital-treated mice of each sex (males: 6.33 versus 1.62 for one mouse/cage and 4.47 versus 0.41 for five mice/cage; female: 6.66 versus 0.13 for one mouse/cage and 1.44 versus 0.06 for five mice/cage). Treatment with phenobarbital did not affect the histological characteristics or degree of differentiation of hepatic tumours (Peraino *et al*., 1973a). [The Working Group noted that no statistical methods were used to compare the tumour incidence or multiplicity in treated and untreated groups.]

Groups of 30 male and 30 female CF-1 mice, 4 weeks of age, were fed a diet containing sodium phenobarbital (purity > 97%) at 500 mg/kg for up to 109 weeks. The control groups comprised 45 animals of each sex. Liver tumours were found in 11/45 male and 10/44 female controls and in 24/30 males and 21/28 females treated with phenobarbital. Histologically, the tumours were classified as type A (tumours in which the parenchymal structure was basically retained) and type B (tumours in which the parenchymal structure was distorted). In the treated group, 16 type A tumours and eight type B tumours were found in males and 12 type A and nine type B tumours in females, whereas only two type B tumours were found in control males, and all the other tumours in the control group were type A (Thorpe & Walker, 1973). [The Working Group interpreted type A tumours as adenomas and type B tumours as carcinomas.]

Groups of male and female CF-1 mice were given drinking-water containing 0.05% sodium phenobarbital [purity not specified] (112 males, 74 females) or normal water (49 males and 47 females) from the time of weaning until 120 weeks of age. The

average age at death of mice with hepatomas was much lower in treated males (84.9 weeks) than in the controls (106 weeks). The incidences of hepatomas in treated mice were much higher than those in the control groups (treated males, 77/98; treated females, 45/73; control males, 12/44; control females, 0/47). The first hepatoma was found in a treated male at 48 weeks of age, and the first hepatoma was seen in a control male at 79 weeks (Ponomarkov *et al.*, 1976). [The Working Group noted that no statistical methods were used to compare the results in treated and control groups.]

Groups of 30 male and 30 female BALB/c mice, 8 weeks of age, were given drinking-water containing 0.05% sodium phenobarbital (purity, 99%) for life. Fifty male and 50 female mice were used as untreated controls. No liver tumours were observed in either treated or control mice during their lifetime (110–120 weeks). The incidence of lung tumours in treated males (8/30) and females (6/30) was not statistically different from that of control males (19/50) and females (7/50) (Cavaliere *et al.*, 1986).

Groups of male A^{vy}/A (yellow) and agouti *A/a* (C3H/HeN-MTV/Nctr × VY/WffC3Hf/Nctr-A^{vy})F_1 mice, 7–8 weeks of age, were fed a diet containing sodium phenobarbital [purity not specified] at a concentration of 500 mg/kg for 510–593 days. Index groups of 12 treated yellow mice were killed after 12, 15 and 18 months of treatment. No significant difference was seen in the incidence of hepatocellular adenomas between untreated yellow and agouti males at terminal sacrifice. Sodium phenobarbital increased the incidence of hepatocellular adenomas in yellow male mice from 23/193 (12%) in controls to 105/192 (55%) in treated animals. The incidence of hepatocellular adenomas in treated yellow males was significantly greater than that in treated agouti males (105/192 versus 46/192) [*p* value not given in table or text]. Treatment with sodium phenobarbital decreased the incidence of carcinoma significantly ($p = 0.0001$) from that observed in the untreated groups of both yellow (6/192 treated versus 26/193 untreated; $p = 0.0002$) and agouti (15/192 treated versus 28/189 untreated; $p = 0.03$) mice (Wolff *et al.*, 1986).

Groups [initial numbers not specified] of male germ-free (Gf) and conventional (Cv) C3H/He mice, 6 weeks of age, were given an irradiated basal diet containing phenobarbital [purity not specified] at 200 mg/kg until 12 months of age. The incidence and number of liver nodules per mouse in treated Gf mice was significantly higher than that in untreated Gf animals (67% (14/21) versus 30% (42/139); $p < 0.01$; tumour nodules/mouse, 2.0 versus 0.4; $p < 0.001$). The incidence of liver tumour nodules and their average number in phenobarbital-treated Cv mice were also significantly higher than those in untreated mice (100% (31/31) versus 75% (42/56); $p < 0.01$; average number, 4.5 versus 1.3; $p < 0.001$) (Mizutani & Mitsuoka, 1988).

Groups of male C3H/He and C57BL/6 mice, 8 weeks old, were given diets containing sodium phenobarbital [purity not specified] to provide a daily intake of 85 mg/kg bw. Groups of five control and five treated mice of each strain were killed at 5, 30, 40 and 80 weeks, and two additional groups of 20 control and 20 treated animals of each strain were killed at 60 weeks. Further groups of 190 C3H/He and C57BL/6 control and 90 C3H/He and 125 C57BL/6 treated mice were killed

in extremis or at the end of the respective experiments, i.e. at 91 weeks for C3H/He and 100 weeks for the C57BL/6 mice. In addition, 25 animals of each strain were treated for 60 weeks and then returned to the control diet, and the survivors were killed at the end of the respective experiments. Nodules were seen in both treated and control C3H/He mice as early as 30 weeks, and were numerous in both these groups at the final kill at 91 weeks. In control animals, all the nodules were of the basophilic type, while in the treated group both basophilic and eosinophilic nodules were found. The majority of treated animals bore eosinophilic nodules. By 91 weeks, 80% (16/20) of the control animals and 40% (8/20) of the treated animals bore basophilic nodules, while all the treated animals and none of the controls (0/20) also developed multiple eosinophilic nodules (20/20). C3H/He mice given sodium phenobarbital for 60 weeks and then returned to the control diet bore fewer nodules at 91 weeks than treated mice killed at 60 or 91 weeks. Nodules did not develop in C57BL/6 mice until week 60 in the treated group and not until week 100 among control animals. In C57BL/6 mice treated with sodium phenobarbital, 2/20 mice developed eosinophilic nodules by week 60, and 50% of them bore this type of nodule at 100 weeks; however, fewer eosinophilic nodules were found than in C3H/He mice at 91 weeks (26 in 10 C57 BL/6 mice versus 68 in 20 C3H/He mice). The cumulative incidence of carcinomas in control C3H/He and C57BL/6 mice was 28 and 4%, respectively. The incidence of carcinomas was not increased by treatment with sodium phenobarbital in either strain (30% in C3H/He and 5% in C57BL/6 treated mice). The authors concluded that the two strains of mouse reacted in a qualitatively similar manner to administration of sodium phenobarbital, although they showed considerable quantitative differences in terms of the time and number of nodules (Evans *et al*., 1992).

Genetically modified mouse: Groups of single transgenic c-*myc* mice (overexpressing the c-*myc* oncogene), double transgenic c-*myc*/HGF mice (overexpressing c-*myc* and co-expressing the hepatocyte growth factor) and wild-type mice [initial numbers and sex not specified] were given a diet containing 0.05% phenobarbital from 3 weeks to 10 months of age. At 6 months, the incidence of hepatocellular adenomas was 5/10 in c-*myc* mice fed phenobarbital, 0/5 in wild-type mice and 0/10 in c-*myc* mice on basal diet. At 8 months, the incidence of hepatocellular adenomas was 8/10 in c-*myc* mice fed phenobarbital, 0/5 in wild-type mice and 2/10 in c-*myc* mice on basal diet. At 10 months, the incidence of hepatocellular adenomas was 10/10 in c-*myc* mice fed phenobarbital, 0/5 in wild-type mice and 4/12 in *c-myc* mice on basal diet. At 8 months, hepatocellular carcinoma occurred only in c-*myc* mice fed phenobarbital (2/10). At 10 months, the incidence of hepatocellular carcinomas was 4/10 in c-*myc* mice fed phenobarbital, 0/5 in wild-type mice and 1/12 in c-*myc* mice fed basal diet. In contrast to the single transgenic c-*myc* mice, no liver tumours were found in 10 c-*myc*/HGF mice killed at 6, 8 or 10 months after treatment with phenobarbital, nor were there any liver tumours in the control c-*myc*/HGF animals (10 mice killed at 6 and 8 months and 12 mice at 10 months) (Thorgeirsson & Santoni-Rugiu, 1996; Thorgeirsson *et al*., 1997).

Groups of 15 male and 15 female *p*53 heterozygous (C57BL/6TacfBR-[KO]*p*53$^{+/-}$) and wild-type (C57BL/6TacfBR) mice [age unspecified] were fed diets containing phenobarbital at 0, 500 or 1000 mg/kg for 26 weeks. All groups, including those fed normal diet, had a 97% survival rate. All mice fed phenobarbital showed a significant increase in absolute and relative liver weights; no difference in the liver weights was seen between heterozygous and wild type mice. There were no tumours in mice of either sex or genotype treated with phenobarbital, although the livers of all animals showed moderate to marked centrilobular hepatocellular hypertrophy (Sagartz *et al*., 1998).

Groups of 15 transgenic male MT42 mice harbouring a mouse metallothionein promoter and a human transforming growth factor α (TGFα) cDNA transgene, and 15 non-transgenic male CD-1 mice were given drinking-water containing 0.05% (w/v) sodium phenobarbital from 6 weeks of age for 26 weeks. Five mice from each group were killed at 12, 24 and 32 weeks. The TGFα-transgenic MT42 mice had no tumours at 12 weeks, and the incidence of hepatocellular tumours (adenomas plus carcinomas) after 24 weeks was 4/5 (2/5 mice with carcinomas) and that after 32 weeks was 3/4 (all with carcinomas). No liver tumours appeared in the non-transgenic CD-1 mice at any time during the experiment (Takagi *et al.*, 1993).

Rat: Groups of 34–36 male and female Wistar rats, 7 weeks of age, were given drinking-water containing 0 (control) or 500 mg/L sodium phenobarbital up to 152 weeks of age, when the survivors were killed. No significant differences were found in body-weight gain or survival between groups. Sodium phenobarbital induced hepatocellular adenomas late in life, the first tumour being diagnosed at 77 weeks. The average age at death of rats with liver tumours was 132 weeks for males and 125 weeks for females. In males and females, respectively, the liver tumour incidences were 1/22 and 2/28 before 99 weeks of age, 5/18 and 2/19 between 100 and 129 weeks and 7/8 and 5/12 from 130 weeks. The cumulative incidences of liver adenomas throughout the study were 0/35 for control and 13/36 for treated males and 0/32 for control and 9/29 for treated females. Among older rats, the numbers of nodules per rat in the treated groups were 11.0 ± 5.5 for males and 14.2 ± 6.0 for females. The hepatocellular nodules were larger in treated females than in males (10.1 ± 3.1 mm versus 5.3 ± 1.1 mm) [no statistical analysis provided] (Rossi *et al*., 1977).

Fifty male Fischer 344 rats [age unspecified] were placed on a diet containing sodium phenobarbital at 500 mg/kg for 1 week, after which the concentration was increased to 1000 mg/kg of diet and was maintained at this level for 103 weeks. Twenty-five male rats maintained on a normal diet for 2 years served as controls. Of the 33 treated rats that lived 80 weeks or more, 11 (33%) developed small foci of nodular hyperplasia; none developed in the controls. Only one treated animal killed at 102 weeks had a lesion, which compressed the surrounding liver without local invasion or metastasis [an adenoma by recent criteria] (Butler, 1978).

Two groups of 30 male Fischer 344/NCr rats (874 days of age on average) were given drinking-water containing 500 mg/L sodium phenobarbital for up to 233 days. Although there was no significant difference in the number of rats with hepatocellular

adenomas between the control (10/30) and the treated group (14/30), the total numbers of hepatocellular adenomas were greater in the treated than the control group (sodium phenobarbital: five basophilic, 64 eosinophilic; control: 14 basophilic, two eosinophilic). Of the 47 hepatocellular adenomas in treated rats that were examined, 36 were positive for γ-glutamyl transpeptidase (γ-GT), while none of the 11 adenomas in control rats were positive for this enzyme. The hepatocellular carcinomas in sodium phenobarbital-treated (2/30) and control rats (2/30) were all negative for the enzyme (Ward, 1983).

Groups of 20 male Fischer 344/DuCrj rats [age unspecified] were fed diets containing sodium phenobarbital at a concentration of 0 (control), 8, 30, 125 or 500 mg/kg for 104 weeks. No treatment-related changes in clinical signs, survival rates, body weight, food consumption or haematological or blood biochemical end-points were observed at any concentration; however, significantly elevated liver weights (relative to body weight) were noted in groups fed 125 and 500 mg/kg of diet ($2.50\% \pm 1.24$ and $3.03\% \pm 0.19$ versus $2.30\% \pm 0.30$, $p < 0.05$ and 0.01, respectively). Hypertrophy of hepatocytes was also seen at these concentrations. Regenerative hyperplasia was observed in 11/20 rats at the highest concentration. Although foci positive for glutathione *S*-transferase (placental form) were found in all groups at termination, the numbers per cm^2 and areas (mm^2/cm^2) in rats fed the two higher concentrations of sodium phenobarbital were significantly higher than control values (average number, > 25 and > 30 versus > 11, $p < 0.01$ for both groups; average area, > 4 and > 8 versus > 1, $p < 0.01$ for both groups). No hepatocellular adenomas were found, and hepatocellular carcinomas occurred in only one rat each at 8 and 125 mg/kg of diet. At the concentrations given, no changes were observed in any other organ (including thyroid) (Hagiwara *et al*., 1999).

3.2 Exposure *in utero*

Mouse: In a study to determine the possible carcinogenic effects of sodium phenobarbital on the offspring of mice exposed both before and during gestation, 12 BALB/c/Cb/Se mice, 10 weeks of age, were given 1 mg of sodium phenobarbital per day by stomach tube for 10 days before and throughout gestation. Twelve mice of the same sex and age given water alone under identical conditions served as controls. During the observation period of 80 weeks, no increase in the incidence of tumours of the liver (control, 0/56; sodium phenobarbital, 0/60 [males and females combined]), lung (control, 13/56; sodium phenobarbital, 18/60) or any other organ was found in exposed offspring compared with those of controls (Cavaliere *et al.*, 1985). [The Working Group noted the small numbers of animals.]

3.3 Administration with known carcinogens and modifying agents

Numerous studies have shown the tumour promoting activity of phenobarbital in mouse and rat liver and rat thyroid, although there is less evidence of such activity in rat lung (Pollard & Luckert, 1997), bladder (Wang *et al*., 1983; Imaida & Wang, 1986; Diwan *et al*., 1989a) and male accessory glands (Pollard *et al*., 1995). The results of the numerous studies of initiation–promotion show that the primary promoting effect of phenobarbital in mice and rats is on the liver and thyroid.

In mice, inhibition or enhancement of hepatocarcinogenesis by phenobarbital depends on the strain, sex, age at the start of exposure and type of initiator used (Uchida & Hirono, 1979; Diwan *et al*., 1984; Pereira *et al*., 1985, 1986; Klaunig *et al*., 1988a,b; Weghorst *et al*., 1989, 1994).

Selected studies of liver and thyroid tumour promotion are summarized below, while studies of initiation–promotion by phenobarbital in the liver of various species are summarized in Table 3. Table 4 lists similar studies on the thyroid, and Table 5 shows those on other organs.

3.3.1 *Promotion in mouse liver*

(*a*) *Studies in adult mice*

Groups of 10–12 male mice of strains C57 BL/6NCr (C57BL/6), C3H/HeNCr^{MTV-} (C3H), and DBA/2NCr (DBA/2), 8 weeks of age, were given a single intraperitoneal injection of 90 mg/kg bw *N*-nitrosodiethylamine (NDEA). Beginning 2 weeks later, groups of mice were given drinking-water containing 0.05% phenobarbital, and 10 mice per group were killed at 12, 24, 36 and 52 weeks of age. Phenobarbital significantly increased the incidence of hepatocellular tumours after 24 weeks of treatment in NDEA-initiated C3H mice (from 20% to 70%) and DBA/2 mice (from 0% to 90%). When the mice were 36 weeks of age, the incidence of liver tumours in mice given NDEA alone was 10% for DBA/2, 10% for C57 BL/6 and 50% for C3H mice and those in mice given NDEA plus phenobarbital was 90% in DBA/2 and 100% in C3H mice, but no such increase was observed in C57BL/6 mice. At 52 weeks, the low incidence of hepatocellular tumours in C57BL/6 mice given NDEA was not significantly increased by subsequent exposure to phenobarbital (from 20% to 30%), but DBA/2 mice were especially susceptible (incidence increased from 40% to 100%) (Diwan *et al*., 1986a).

In order to analyse the genetics of susceptibility to promotion of hepatocarcinogenesis in DBA/2NCr (susceptible) and C57BL/6NCr (resistant) mice by phenobarbital, groups of 40 reciprocal F_1 hybrid male B6D2F_1 and D2B6F_1, 5 weeks of age, were given an intraperitoneal injection of 90 mg/kg bw NDEA or an equal volume of tricaprylin. Two weeks later, the groups received 0.05% phenobarbital continuously in the drinking-water or drinking-water alone. Ten mice from each group were killed at 33 weeks of age, and the remaining mice were killed when found moribund or at

Table 3. Promotion of preneoplastic and neoplastic liver lesions by phenobarbital (PB) or sodium phenobarbital (NaPB) in mice, rats, hamsters and monkeys initiated by various carcinogens

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Mouse					
C57BL/6, C3H and DBA2 (M)	NDEA, 90 mg/kg bw, ip × 1	2 weeks	PB, 0.05% in water, 52 weeks	Percentage of mice with tumours C57BL/6: NDEA, 20%; NDEA + PB, 30% C3H: NDEA, 90%; NDEA + PB, 100% DBA/2: NDEA, 40%; NDEA + PB, 100%	Diwan *et al*. (1986a)
D2B6F$_1$ (M)	NDEA, 90 mg/kg bw, ip × 1	2 weeks	PB, 0.05% diet up to 53 weeks (NDEA alone, 110 weeks)	Percentage of mice with tumours NDEA: adenomas, 97%; carcinomas, 40%; hepatoblastomas, 10% NDEA + PB: adenomas, 96%; carcinomas, 90%; hepatoblastomas, 77%	Diwan *et al*. (1995)
C3H, C57BL/6 and BALB/c (M)	NDEA 20 h after PH, 20 mg/kg bw, ip × 1	6 h	PB, 0.05% diet, 20 weeks	Total volume of EAIs: C3H: NDEA, 710/cm^3; NDEA + PB, 83824/cm^3 C57BL/6: NDEA, 83/cm^3; NDEA + PB, 131/cm^3 BALB/c: NDEA, 140/cm^3; NDEA + PB, 5452/cm^3	Lee *et al*. (1989)
B6C3F$_1$ (M)	NDEA, 35 mg/kg bw, ip × 2/week/8 weeks	12 weeks	PB, 0.05% diet, 60 days	Altered foci per liver (NDEA, 407; NDEA + PB, 696); PB removed for 30 days (NDEA + PB, 95)	Kolaja *et al.* (1996a)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Transgenic mice					
TGF-α transgenic (MT42) and non-transgenic CD1 (M)	NDEA, 5 mg/kg bw, ip × 1 at 15 days of age	2 weeks	PB, 0.05% diet, 35 weeks	MT42: NDEA or NDEA + PB, 80–100% carcinomas; PB alone, 33%; CD1: NDEA + PB, 40% carcinomas; NDEA or PB alone, no tumours	Tamano *et al.* (1994)
$Cx32^{y/+}$, $Cx32^{y/-}$	NDEA, 90 mg/kg, ip × 1	NR	PB, 0.05% diet, 39 weeks	Promotion of liver tumours in $Cx32^{y/+}$ mice but not in $Cx32^{y/-}$ mice	Moennikes *et al.* (2000)
Rat					
SD/Anl [Anl66] (NR)	AAF, 0.02% diet, 11, 16, 21 or 26 days	None	PB, 0.05% diet, 260 days	Highest incidence of hepatomas in group initiated for 26 days (AAF, 27/103; AAF + PB, 86/108)	Peraino *et al.* (1971)
Sprague-Dawley (M)	AAF, 0.02% diet, 18 days	Various intervals (up to 120 days)	PB, 0.05% diet, up to 407 days	Final tumour incidences influenced by duration of post-AAF treatment and not length of treatment-free intervals	Peraino *et al.* (1977)
Fischer 344 (NR)	AAF, 0.02% for 13 weeks + iron-loading diet	0 days	PB, 0.05% diet, 24 weeks	Iron accumulation-resistant foci; when AAF discontinued, foci disappear; AAF followed by PB, foci reappear	Watanabe & Williams (1978)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Sprague-Dawley (M)	B[*a*]P, 4 mg/rat ig × 6 (24 h after PH)	2 weeks	PB, 0.05% diet, 50 weeks	B[*a*]P given ig followed by PB produced tumours in 6/15 rats; PB alone, 0/10	Kitagawa *et al.* (1980)
Donryu (M)	3′-Me-DAB, 0.06% diet, 3 weeks	2 weeks	PB, 0.05% diet with initiator and/or given as a promoter for 35 weeks	Inhibition of EAIs when given with initiator; promotion when given after initiation	Narita *et al.* (1980)
CD1 (M)	AAF, 0.02% diet, 2 weeks	1 week	PB, 0.002–0.25% diet (various concentrations), 78–84 weeks	Dose-dependent tumour-promoting effects	Peraino *et al.* (1980)
ACI (M and F)	Cycasin, 100 mg/kg bw, ig × 1	1 week	PB, 0.05% diet, up to 480 days	γ-GT-positive foci; incidence significantly greater in females than in males	Uchida & Hirono (1981)
CD (F)	*N*-OH-AABP or *N*-OH-FABP, 0.4 mmol/kg bw, ip × 1 (24 h after PH)	2 days	PB, 0.05% diet, 64 weeks	γ-GT-positive foci; no growth with PB alone; growth initiated by PH + PB	Shirai *et al.* (1981)
Sprague-Dawley (M)	NDEA, 40 or 50 mg/kg bw, ip (18 h after PH)	1 week	PB 0.06% with choline-sufficient diet for 6 or 7 weeks or choline-deficient diet for 8 weeks	γ-GT-positive foci; choline-sufficient + PB, increased; choline-deficient + PB, synergistic promoting action	Shinozuka *et al.* (1982)
Wistar (M)	NDEA, ~ 10 mg/kg bw in water, 2, 4 or 6 weeks	1 week	PB, ~ 15 mg/kg bw per day in water, 4, 6 or 8 weeks	PB treatment reduced latency for development of neoplastic nodules by 3–6 months	Barbason *et al.* (1983)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Sprague-Dawley (M)	NMOR, 120 mg/L, in water, 7 weeks	0 days	PB, 0.75, 0.075 or 0.0075 g/L, drinking-water, 16 weeks	γ-GT, G6PDH, ATPase-deficient foci; dose-dependent promotion by PB	Moore *et al*. (1983)
Fischer 344 (M)	FANFT, 0.2% diet, 6 weeks	1 week	PB, 0.05% diet, 86 weeks	No phenotype given; promotion by PB	Wang *et al*. (1983)
Sprague-Dawley (M)	NMOR, 80 mg/L, in water, 7 weeks	5 weeks	PB, 0.05% diet for 10 weeks, before or after NMOR	γ-GT-positive foci; slight inhibition before NMOR; promotion after NMOR	Schwarz *et al*. (1983)
Sprague-Dawley (M)	NMOR, 200 mg/L, in water, 3 weeks	1 week	PB, 0.05% diet, 12, 24, 36 or 48 weeks	γ-GT-, G6PDH-, ATPase-positive foci; time-dependent in number and size of foci; PB increased homogeneity and number of foci; increased G6PDH activity	Ito *et al*. (1984)
Donryu (M)	3′-Me-DAB, diet, 3 weeks	None	PB, 5–500 mg/kg of diet, 21 weeks	Dose-dependent increase in number and size of EAIs	Kitagawa *et al*. (1984)
Fischer 344 (M)	AAF, 0.02% diet, 8 weeks	4 weeks	PB, 0.05% diet, for 24 weeks, after or before AAF (4-week interval between PB and AAF)	PB had promoting but not syncarcinogenic effect.	Williams & Furuya (1984)
Fischer 344 (M and F)	MNU, 0.05 mmol/kg bw, iv × 1/week, 4 weeks	2 weeks	PB, 0.05% in water, 71 weeks	MNU alone, no liver tumours; MNU + PB, 50% males and 40% females developed liver tumours	Diwan *et al*. (1985)
Sprague-Dawley (F)	NDEA, 10 mg/kg bw, ig × 1, 24 h after PH	2–6 weeks	PB, 0.00005–0.2% diet, 7–8 months	Dose-dependent increase in number and size of foci; NOAEL at 0.001%	Goldsworthy *et al*. (1984)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Fischer 344 (M)	NDEA, 200 mg/kg bw or *N*-OH-AAF, 30 mg/kg bw or AFB_1, 0.5 or 1.0 mg/kg bw, ip	2 weeks	PB, 0.05% diet, 6 weeks; PH end of third week of the experiment	γ-GT-positive foci; growth only in NDEA-initiated rats	Shirai *et al*. (1985)
Wistar (M)	NDEA 10 mg/kg bw per day + PB 15 mg/rat, for 2, 4 or 6 weeks	None (simultaneous administration)	PB, 15 mg/rat, for 2, 4 or 6 weeks	PAS-positive; at 6 weeks significant decrease in number of foci with NDEA + PB	Barbason *et al*. (1986)
Wistar (M)	NDEA, 1.1, 3.3, 10 or 30 mg/kg bw, ip × 1	1 week	NaPB, 40, 100 or 1000 µg/mL in water, 12–18 months	Liver carcinomas (38%) seen only after 1000 µg/mL PB promotion and 30 mg/kg bw NDEA	Driver & McLean (1986a)
CD (F)	NDEA, 10 mg/kg bw, ig, 20 h after PH	1 week	PB, 0.05% diet, 6 months; PB withdrawn for 10 days	γ-GT-positive foci; growth with PB; decrease in number and size of foci after withdrawal of PB	Glauert *et al*. (1986)
	NDEA, 10 mg/kg bw, ig, 20 h after PH	1 week	PB, 0.05% diet, 3 months, then high- or low-fat diet for 8 months	At 3 months, promotion of mostly γ-GT-positive foci; high- or low-fat diet decreased number and size of foci	
Fischer 344 (F)	NDEA, 10 mg/kg bw ig × 1, 24 h after PH	1 week	PB, 0.05% diet, 4 months; groups of rats withdrawn from PB for 5–180 days; re-administration of PB for 10–90 days	Altered hepatocellular foci; withdrawal of PB decreased total number; re-administration of PB increased number	Hendrich *et al*. (1986)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Fischer 344 (M)	NBHPA, 1000 mg/kg bw ip × 1 + (250 mg/kg bw ip × 3, fortnightly, 3 weeks later)	1 week	PB, 0.05% in water, 22 weeks	γ-GT-positive foci; promotion by PB	Moore *et al.* (1986)
Sprague-Dawley (M/F)	NDEA, 4, 8 or 16 mg/kg bw, ip at day 1	20 days	PB, 0.05% diet, 8 weeks	γ-GT-positive foci, increase in number and size of foci (similar in both sexes); γ-GT-positive/Fe-resistant foci, greater in F	Peraino *et al.* (1987)
Fischer 344 (F)	NDEA, 10 mg/kg bw, ig × 1, 24 h after PH	2 weeks	PB, 0.001–0.5% diet (various concentrations), 6 months	Dose-dependent increase in number and volume of foci; threshold at 0.005%	Pitot *et al.* (1987)
Fischer 344 (M)	NBHPA, 0.2% in water, 1 week	1 week	PB, 0.05% diet, 50 weeks	No phenotype given; promotion by PB	Shirai *et al.* (1988)
Fischer 344 (M)	IQ, 0.025, 0.05 or 0.1% diet, 2 weeks (PH at end of first week)	1 week	PB, 0.05% diet, 83 weeks	IQ, no carcinomas; IQ + PB, 35–50% carcinomas; increase in γ-GT-positive foci	Tsuda *et al.* (1988)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Fischer 344 (M)	NDEA, 1 × 100 mg/kg bw (day 1) + MNU, 4 × 20 mg/kg bw (days 2, 5, 8, 11), ip + NBHPA, 0.1% in water, 2 weeks (weeks 3–4)	0 day	NaPB, 0.05% diet, 14 or 20 weeks	GST-P-positive foci; increase in number and size of foci	Shibata *et al.* (1990)
Fischer 344 (M and F)	NDEA, 10 mg/kg bw × 1 (24 h after PH) at 4 weeks, 6 or 12 months of age, ig	2 weeks	PB, 0.05%, 6 months	γ-GT-positive foci; growth more effective at 6 months than when initiated at 4 weeks or 12 months; males more susceptible	Xu *et al.* (1990)
Wistar (F)	NDEA, 10 mg/kg bw, ig	10 days	PB, 20, 50, 100, 200 and 200 mg/L in water for 70, 23, 11, 9, 5 weeks, respectively	ATPase-deficient foci; not increased at 20 mg/L; no effect at 50, 100 mg/L; inhibitory effect at 200 mg/L	Appel *et al.* (1991)
Fischer 344 (M)	NBHPA, 1000 mg/kg bw × 2 (week 1), ip; NEHEA, 1500 mg/kg bw × 2 (week 2), ip; DMAB, 75 mg/kg bw (week 3), sc × 2	1 week	PB, 0.05% diet, 12 weeks	GST-P-positive foci; promotion by PB	Uwagawa *et al.* (1992)
Fischer 344/DuCrj (M)	NDEA, 100 mg/kg bw, ip	1 week	PB, 1, 4, 16, 75, 300 or 1200 mg/L in water, 39 weeks	γ-GT-positive, GST-P-positive foci; dose-dependent promotion by PB from 75 to 1200 mg/L	Maekawa *et al.* (1992)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
SPF Wistar (M/F)	AFB_1, 2 mg/kg bw (M), 5 mg/kg bw (F), ig	3 weeks	PB, 50 mg/kg diet for 70 weeks (F) and 55–59 weeks (M)	GST subunits; mostly eosinophilic clear-cell foci with elevated levels of mu (Yb1, Yb2) and pi (Yp) family subunits	Grasl-Kraupp *et al.* (1993)
Fischer 344 (M)	PB, 0.1% in water, AFB_1, 1 mg/kg bw, ip	–	PB, 1 week before AFB_1	GST-P-positive foci; inhibition of AFB_1-induced foci by PB	Gopalan *et al.* (1993)
Fischer 344 (M)	NBHPA, 2000 mg/kg bw, sc	1 week	PB, 1000 mg/kg bw or (PB, 500 mg/kg bw diet + thiourea, 0.05% in water), 19 weeks	Eosinophilic, basophilic, clear-cell foci; increase with PB; synergistic with thiourea	Shimo *et al.* (1994)
Sprague-Dawley (F)	NDEA, 10 mg/kg bw	1 week	PB, 500 mg/kg diet for 170 or 240 days; + TCDD (150 µg/kg diet) from 170 to 240 days or 240 to 450 days	Eosinophilic foci; PB alone, no effect; PB + TCDD, increase in number and size	Sills *et al.* (1994)
Wistar (M)	Tamoxifen, 420 mg/kg diet, 3 months	0 days	PB, 0.1% in water for 3, 6, 9, 12 months or lifetime	GST-P-positive foci; increased at 6 and 9 months	Carthew *et al.* (1995)
Fischer 344 (M)	^{192}Ir seeds	None	PB, 0.05% diet, 20, 40 or 60 weeks	PB promoted development of EAIs initiated by ^{192}Ir	Ida *et al.* (1995)
Sprague-Dawley (F)	NDEA, 10 mg/kg bw, ip at 5 days of age	16 days	PB, 10, 100 or 500 mg/kg diet and/or mestranol, 0.02 or 0.2 mg/kg diet, 8 months	GST-P-positive foci; growth at low mestranol + 10 or 100 mg/kg diet PB; no growth at low mestranol + 500 mg/kg diet PB; high mestranol effective only with 100 mg/kg diet PB	Dragan *et al.* (1996)

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Fischer 344 (M)	NDEA, 150 mg/kg bw, ip × 2	4 months	PB, 10, 100 or 500 mg/kg diet for 7, 30 or 60 days	Eosinophilic foci; growth at 100 (7 and 60 days), 500 (30 and 60 days) mg/kg diet; at 10 mg/kg diet, PB was ineffective	Kolaja *et al.* (1996b)
Fischer 344 (M)	NDEA, 100 mg/kg bw, ip × 1/week, 3 weeks	1 week	PB, 500 mg/kg diet, 20 weeks or PB + 100 mg/kg diet MMTS, 20 weeks	GST-P-positive foci; promotion with PB; inhibition with PB + MMTS	Sugie *et al.* (1997)
Fischer 344 (M)	NDEA, 200 mg/kg bw, ip; 2 weeks later, D-galactosamine, 300 mg/kg bw, ip at the end of weeks 2 and 5	2 weeks	PB, 500 mg/kg diet, weeks 3–8	GST-P-positive foci; promotion by PB; multiple injection of D-galactosamine as effective as PH	Kim *et al.* (1997)
Fischer 344 (M)	NDEA, 200 mg/kg ip, PH at week 3	2 weeks	PB, 1, 2, 4, 7.5, 15 or 500 mg/kg diet, 6 weeks	GST-P- and TGF-α-positive foci; promotion at 500 mg/kg diet; inhibition at 1, 2, 4, 7.5 mg/kg diet	Kitano *et al.* (1998)
			PB, 0.01, 0.1 or 0.5 mg/kg diet, 6 weeks	No promotion	
			PB, 1, 2, 4, 7.5, 15, 30, 60, 125, 250 or 500 mg/kg diet, 6 weeks	GST-positive foci; promotion at doses higher than 15 mg/kg diet; inhibition at 2 and 4 mg/kg diet	

Table 3 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose and duration of PB or NaPB and route	Promoting or anti-promoting effects on preneoplastic or neoplastic hepatocellular lesions	Reference
Hamster					
Syrian golden (M)	NDEA, 100 mg/kg bw, ip × 1 or MAMA, 20 mg/kg bw × 1	2 weeks	PB, 0.05% in water, up to 62 weeks	Percentage of mice with tumours: NDEA alone, 37% adenomas; MAMA alone, 63% adenomas, 11% carcinomas; NDEA + PB, 27% adenomas; MAMA + PB, 67% adenomas, 11% carcinomas	Diwan *et al*. (1986b)
Syrian golden (NR)	NDMA, 6 mg/kg bw, ip × 1	1 week	PB, 0.05% diet, 31 weeks	NDMA alone, 1/15 adenomas; NDMA + PB, 3/15 adenomas	Tanaka *et al*. (1987)
Monkey					
Erythrocebus patas (M and F)	NDEA (to pregnant monkeys), 0.6–3.2 mmol/kg bw (cumulative), iv	4 years	PB (to mother and offspring), 15 mg/kg bw per day in water, up to 43 months	NDEA alone, no tumours; NDEA + PB, 11.6 adenomas per mother, 5.6 adenomas per offspring	Rice *et al*. (1989)
	NDEA, 0.1–0.4 mmol/kg bw, ip (at 14-day intervals) × 20	2 weeks	PB, 15 mg/kg bw in water, 9 months	NDEA alone, 1.6 adenomas per monkey and 0.3 carcinomas per monkey; NDEA + PB, 17.25 adenomas per monkey and 2.0 carcinomas per monkey	

Table 3 (contd)

AAF, 2-acetylaminofluorene
AFB_1, aflatoxin B_1
B[*a*]P, benzo[*a*]pyrene
DMAB, 3,3′-dimethyl-4-aminobiphenyl
EAI, enzyme-altered island
F, female
FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide
G6PDH, glucose 6-phosphate dehydrogenase
γ-GT, γ-glutamyl transferase
GST, glutathione *S*-transferase
GST-P, glutathione *S*-transferase placental form
ig, intragastrically
ip, intraperitoneally
IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline
iv, intravenously
M, male
MAMA, methylazoxymethanol acetate
3′-Me-DAB, 3′-methyl-4-(dimethylamino)azobenzene
MMTS, *S*-methyl methanethiosulfonate
MNU, *N*-methyl-*N*-nitrosourea
NBHPA, *N*-nitrosobis(2-hydroxypropyl)amine
NDEA, *N*-nitrosodiethylamine
NEHEA, *N*-nitrosoethyl-*N*-hydroxyethylamine
NMOR, *N*-nitrosomorpholine
NOAEL, no-observed-adverse-effect level
N-OH-AABP, *N*-hydroxy-4-acylaminobiphenyl
N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene
N-OH-FABP, *N*-hydroxy-4-formylaminobiphenyl
NR, not reported
PAS, periodic acid-Schiff
PH, partial hepatectomy
sc, subcutaneously
SPF, specific pathogen-free
TCDD, 2,3,7,8-tetrachloro-*para*-dibenzodioxin
TGF-α, transforming growth factor α

Table 4. Promotion of thyroid tumours in rats initiated by various carcinogens and promoted by phenobarbital (PB) or sodium phenobarbital (NaPB)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose, route and duration of PB or NaPB	Promoting effects on preneoplastic and/or neoplastic thyroid lesions	Reference
Wistar (M)	NBHPA, 70 mg/kg bw per week, sc × 1 per week, 6 weeks	1 week	PB, 500 mg/kg diet, 12 weeks	Follicular-cell neoplasms; NBHPA, 23%; NBHPA + PB, 100%	Hiasa *et al*. (1982)
Wistar (M)	NBHPA, 2800 mg/kg bw, sc	1 week	PB, 500 mg/kg diet, 6, 12 or 19 weeks	Follicular-cell adenomas at 19 weeks: NBHPA, 37%; NBHPA + PB, 87%	Hiasa *et al*. (1983)
Wistar (M and F)	NBHPA, 2100 mg/kg bw (M), 4200 mg/kg bw (F), ip	1 week	PB, 20, 100, 500 or 2500 mg/kg diet, 19 weeks	Thyroid tumours; 500 mg/kg diet, 3-fold yield in M only; 2500 mg/kg diet, 8-fold in M and 3-fold in F	Hiasa *et al*. (1985)
Wistar (M), castrated at week 2 or at beginning	NBHPA, 2100 mg/kg bw, ip, week 1	1 week	PB, 500 mg/kg diet, 38 weeks	Follicular-cell adenomas; NBHPA, 20%; NBHPA + PB, 75%; castrated at week 2, 30%; castrated at beginning, 20%	Hiasa *et al*. (1987)
Fischer 344 (M)	NDEA, 75 mg/kg bw × 1, ip	2 weeks	PB, 500 mg/kg diet, 45–71 weeks	Follicular-cell tumours at 71 weeks NDEA, 0/15; NDEA + PB, 5/15	Diwan *et al*. (1988)
Fischer 344 (M and F)	MNU, 0.05 mmol/kg bw, iv × 1 per week, 4 weeks, or 0.2 mmol/kg bw, iv × 1	None, 2 or 5 weeks	PB, 0.05% in water with or following MNU	Follicular-cell tumours; PB promotion by both simultaneous and subsequent administration	Diwan *et al*. (1985)
Fischer 344 (M)	NBHPA, 1000 mg/kg bw, ip × 1 + (250 mg/kg bw, ip × 3, fortnightly, 3 weeks later)	1 week	PB, 0.05% in water, 22 weeks	Follicular-cell adenomas; NBHPA, 3/15; NBHPA + PB, 9/15	Moore *et al*. (1986)

Table 4 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose, route and duration of PB or NaPB	Promoting effects on preneoplastic and/or neoplastic thyroid lesions	Reference
CD (SD) BR (M and F)	NBHPA, 700 mg/kg bw × 1 per week, sc, 5 weeks	2 weeks	PB, 500 mg/kg diet and/or thyroxine, 15 weeks	Follicular-cell adenomas; M: NBHPA, 6/16; NBHPA + PB, 15/18; NBHPA + PB + thyroxine, 5/20; F: NBHPA + PB, 1/20	McClain *et al.* (1988)
Fischer 344 (M)	NBHPA, 0.2% in water	1 week	PB, 500 mg/kg diet, 50 weeks	Follicular-cell adenomas	Shirai *et al.* (1988)
Fischer 344 (M)	IQ, 0.025, 0.05 or 0.1% diet, 2 weeks	1 week	PB, 0.05% diet	Papillar adenomas and carcinomas; IQ (0.1%), 5.3%; IQ + PB, 44–64%	Tsuda *et al.* (1988)
Fischer 344 (M)	NDMA.OAc, 0.05 nmol/kg bw, ip	2 weeks	PB, 500 mg/L in drinking-water, 50 or 78 weeks	Follicular-cell tumours at 78 weeks; NDMA.OAc, 1/15; NDMA.OAc + PB, 7/15	Diwan *et al.* (1989c)
Fischer 344/DuCrj (M)	NBHPA, 2800 mg/kg bw, sc	1 week	PB, 0.05% in water, 25 weeks	Multiple thyroid neoplasms; NBHPA, 3/30; NBHPA + PB, 14/30	Kanno *et al.* (1990)
Fischer 344 (M)	NDEA, 100 mg/kg bw, ip × 1 (day 1) + MNU, 20 mg/kg bw, ip × 4 (day 2, 5, 8 and 11) + NBHPA, 0.1% in water, 2 weeks (weeks 3–4)	0 day	NaPB, 500 mg/kg diet, 20 weeks	Follicular-cell hyperplasia and adenoma: initiated rats, 5/15 and 4/15; initiated rats given NaPB, 14/15 and 12/15	Shibata *et al.* (1990)

Table 4 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose, route and duration of PB or NaPB	Promoting effects on preneoplastic and/or neoplastic thyroid lesions	Reference
Fischer 344 (M)	NBHPA, 1000 mg/kg bw, ip × 2 (week 1) + NEHEA, 1500 mg/kg bw, ig × 2 (week 2) + NEHEA, 75 mg/kg bw, sc × 2 (week 3)	1 week	PB, 0.05% in diet, 12 weeks	Follicular-cell hyperplasia (no adenoma): NBHPA + NEHEA + DMAB, 0/15; NBHPA + NEHEA + DMAB + PB, 5/15	Uwagawa *et al*. (1992)

DMAB, 3,3′-dimethyl-4-aminobiphenyl
F, female
ip, intraperitoneally
IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline
iv, intravenously
M, male
MNU, *N*-methyl-*N*-nitrosourea
NBHPA, *N*-nitrosobis(2-hydroxypropyl)amine
NDEA, *N*-nitrosodiethylamine
NDMA.OAc, *N*-nitrosomethyl(acetoxymethyl)amine
NEHEA, *N*-nitrosoethyl-*N*-hydroxyethylamine
sc, subcutaneously

Table 5. Tumours in other organs initiated by various carcinogens and promoted by phenobarbital (PB) or sodium phenobarbital (NaPB)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose, route and duration of PB or NaPB	Promoting effects on pre-neoplastic and/or neoplastic lesions	Reference
Lung					
Mouse					
Swiss (M and F)	Urethane, 4%, 1 mL, sc × 1	0 day	PB, 2%, 0.1 mL, sc × 1/day, 6 days, before urethane; PB, 2%, 0.1 mL, sc × 1/day, 8 days	PB before urethane: inhibition of tumour formation; PB after urethane: no effect	Adenis *et al*. (1970)
ddy (pregnant)	ENU, 50 mg/kg bw, ip × 1, on gestation day 16	4 weeks	PB, 0.05% diet to offspring, 4 weeks to 6 months of age	No promotion by PB	Tsuchiya *et al*. (1984)
Rat					
Wistar (NR)	MNU, 30 mg/kg bw, iv × 1	7 months	PB, 0.5% diet or NaPB, 0.05% in water, 15–16 months	Promotion by PB and NaPB	Pollard & Luckert (1997)
Bladder					
Mouse					
C57BL/6 (M/F)	NBHPA, 0.022% or NDBA, 0.024% in water for life	None	NaPB, 1 mg/mL in water, starting 1 week before initiation and continued concurrently	No effect on NBHPA-induced tumours; prolonged tumour induction by NDBA	Bertram & Craig (1972)
Rat					
Fischer 344 (M)	FANFT, 0.2% diet, 6 weeks	1 week	PB, 0.05% diet, 86 weeks	PB promoted bladder carcinogenesis	Wang *et al*. (1983)

Table 5 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose, route and duration of PB or NaPB	Promoting effects on pre-neoplastic and/or neoplastic lesions	Reference
Fischer 344 (M)	FANFT, 0.2% diet or NDBA, 0.005% in water, 4 weeks	1 week	NaPB, 0.05% or 0.15% diet, 95 weeks	NaPB promoted bladder carcinogenesis initiated with FANFT but not with NDBA	Imaida & Wang (1986)
Fischer 344 (M)	FANFT, 0.2% diet, 2 or 6 weeks	2 weeks	NaPB, 0.1% diet, 60 weeks	NaPB promoted preneoplastic bladder lesions in rats fed FANFT for 6 weeks	Diwan *et al*. (1989a)
Skin					
Mouse					
HRS/J/Anl (F)	DMBA, 250 µg, topical × 1/week, 6 weeks	1 week	PB, 0.05% diet, 49 weeks	PB had no effect on skin tumour development	Grube *et al*. (1975)
	DMBA, 250 µg, topical × 1/week, 12 weeks	None	PB, 0.05% diet, 49 weeks (starting week 7)	PB decreased skin tumour development after 10 weeks of DMBA	
Brain					
Rat					
Fischer 344 (pregnant)	ENU, 3.5 mg/kg bw, iv × 1, day 20 of gestation	4 weeks	PB, 0.05% in water, 74 weeks	PB had no effect on ENU-induced neurogenic tumours	Walker & Swenberg (1989)

Table 5 (contd)

Strain (sex)	Initiator, dose, route and duration	Interval between initiator and promoter	Dose, route and duration of PB or NaPB	Promoting effects on pre-neoplastic and/or neoplastic lesions	Reference
Male accessory glands					
Rat					
Wistar (M)	MNU, 30 mg/kg bw, iv × 1	7 months	PB, 500 mg/kg diet, 7 months	PB promoted MNU-induced male accessory gland carcinogenesis (prostate and seminal vesicle)	Pollard *et al.* (1995)
Gastrointestinal tract					
Rat					
Wistar (M)	MNNG, 100 mg/L in water, 8 weeks	0 day	PB, 0.05% diet, 32 weeks	PB had not effect on MNNG-induced gastro-duodenal tumours	Takahashi *et al.* (1984)
Fischer 344 (M)	NDMA.OAc, 0.05 nmol/kg bw, ip × 1	2 weeks	PB, 0.05% in water, 78 weeks	PB had no effect on NDMA.OAc-induced intestinal tumours	Diwan *et al.* (1989c)

Table 5 (contd)

DMBA, 7,12-dimethylbenz[*a*]anthracene
ENU, *N*-ethyl-*N*-nitrosourea
F, female
FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide
ip, intraperitoneally
iv, intravenous;y
M, male
MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
MNU, *N*-methyl-*N*-nitrosourea
NBHPA, *N*-nitrosobis(2-hydroxypropyl)amine
NDBA, *N*-nitrosodibutylamine
NDMA.OAc, *N*-nitrosodimethyl(acetoxymethyl)amine
NR, not reported
sc, subcutaneously

47 weeks of age. At 33 weeks, 9/10 D2B6F_1 mice given NDEA followed by phenobarbital had hepatocellular adenomas (3.8 ± 1.0/mouse) versus 2/10 (1.0 ± 0) with NDEA alone. Administration of phenobarbital significantly increased the incidence (from 30% to 100%, $p < 0.05$) and the number of tumours (average number, 5.2 versus 2.0/mouse, $p < 0.05$) initiated by NDEA in the reciprocal F_1 cross B6D2F_1 mice. By 47 weeks, all mice of reciprocal F_1 hybrids D2B6F_1 and B6D2F_1 that had received phenobarbital after administration of NDEA had multiple hepatocellular tumours, including both adenomas (12.5 and 15/mouse, respectively) and carcinomas (2.6 and 2.7/mouse, respectively). Thus, the susceptibility to promotion of hepatocarcinogenesis by phenobarbital was a dominant trait in crosses between DBA/2 and C57BL/6, and the two reciprocal F_1 hybrids responded similarly to promotion by phenobarbital. Interestingly, however, 8/10 D2B6F_1 mice but only 1/10 B6D2F_1 mice given phenobarbital after NDEA developed single or multiple (1.75 ± 0.4) hepatoblastomas between 33 and 47 weeks. No hepatoblastomas were found in mice given only NDEA or phenobarbital (Diwan *et al*., 1989b).

Eight groups of 30 male weanling C3H/HeN mice were given either a normal diet or a diet containing 1.0% choline chloride, 1.5% DL-methionine or both DL-methionine and choline chloride with or without 0.05% phenobarbital for 52 weeks. A further eight groups of 30 mice each were given a single intraperitoneal injection of 150 mg/kg bw NDEA and received the same dietary supplements with or without 0.05% phenobarbital. Treatment with NDEA resulted in a 63% suppression in the body weight gained at 15 weeks (maximum growth period) when all groups of mice receiving NDEA were combined and compared with all groups not receiving NDEA (6.0 versus 16.2 g weight gain, respectively [no *p* value given]). NDEA decreased the survival time of mice in all treated groups ($p < 0.005$ compared with untreated controls) except for one NDEA-treated group without phenobarbital and supplemented with methionine only. The first death from liver cancer occurred at 20 weeks after initiation for the group given NDEA plus phenobarbital, at 25 weeks for the group given NDEA only, at 42 weeks for that given phenobarbital only and at 49 weeks for the untreated control group. Animals given phenobarbital only with both methionine and choline had longer survival than mice receiving no supplementation when analysed on the basis of deaths with tumours ($p < 0.0005$). Groups receiving the initiating dose of NDEA and no phenobarbital showed similar trends. Combined treatment with methionine lowered the relative liver weights of the mice given NDEA plus phenobarbital from 19.5% ± 1.6 to 13.8% ± 1.5 of body weight ($p < 0.05$). Treatment with phenobarbital only resulted in incidences of hepatocellular carcinoma of 79% in animals on the normal diet, 74% in those on choline-supplemented diet, 60% with methionine supplementation and 31% with methionine plus choline supplementation. In mice initiated with NDEA and promoted with phenobarbital, dietary supplementation with methionine and choline also protected against the formation of liver carcinomas; however, the total incidence of liver tumours (adenomas and carcinomas) was not altered. Metastases of hepatocellular carcinomas to the lungs were

found only in mice receiving NDEA plus phenobarbital; the incidence was reduced from 16% in the group receiving no supplement to 6% in the group receiving choline supplementation and to 0% in groups of mice receiving diets supplemented with methionine alone or with choline and methionine (Fullerton *et al*., 1990).

To confirm the promoter-dependent development of hepatoblastomas in mice, groups of 30 male D2B6F$_1$ mice, 5 weeks of age, were given a single intraperitoneal injection of 90 mg/kg bw NDEA and then 2 weeks later were given either a normal diet or a diet containing 500 mg/kg phenobarbital for 53 weeks. Mice exposed to NDEA alone and to phenobarbital alone were maintained for 110 weeks. Hepatocellular tumours (adenomas and carcinomas) occurred in 97% of D2B6F$_1$ mice given NDEA alone. The incidence of hepatocellular carcinomas in NDEA-treated mice (37%) was significantly enhanced by subsequent administration of phenobarbital (96%). Multiple hepatocellular adenomas and carcinomas developed in 77% of mice exposed to phenobarbital alone. Only 10% of the mice treated with NDEA alone developed hepatoblastomas, while subsequent administration of phenobarbital resulted in an increased incidence (77%) and multiplicity (2.8 ± 1.5) of such tumours. Multiple hepatoblastomas also occurred in 11/30 (37%) mice that received phenobarbital only. Thus, in D2B6F$_1$ mice, the development of hepatoblastoma from its precursor cells (adenoma and carcinoma cells) is strongly increased in the presence of a promoting agent (Diwan *et al*., 1995).

(*b*) *Studies in juvenile mice*

Groups of male and female pups of DDD strain mice [total initial number not given] were given either an intraperitoneal injection of 0.025 mL of 0.12% *N*-nitrosodimethylamine (NDMA) 24 h after birth followed by 0.05% phenobarbital in the drinking-water from 4 weeks of age (36 newborn mice), NDMA alone (24 newborn mice), a single intraperitoneal injection of saline (0.9% NaCl) followed by phenobarbital (38 newborn mice) or a single intraperitoneal injection of saline alone (24 newborn mice). The experiment was terminated 16 weeks after birth. Survival was not affected in any group. Liver tumours occurred in 27/35 (77%) mice exposed to NDMA plus phenobarbital, with a multiplicity of 4.3 per mouse. Twenty-four had type A tumours (simple nodular growth of liver parenchymal cells) and three male mice had type B tumours (areas of papilliform or adenoid growth of tumour cells with a distorted parenchymal structure). None of the tumours metastasized. In mice that received NDMA alone, liver tumours (all type A) occurred in 8/24 (33%) mice, with an average number of 0.4 tumours per mouse. No sex difference was found in the incidence, type or multiplicity of tumours in mice given NDMA alone or NDMA plus phenobarbital. None of the mice exposed to saline and phenobarbital or saline alone developed tumours (Uchida & Hirono, 1979). [The Working Group noted the short duration of exposure.]

Groups of 40 male B6C3F$_1$ mice, 15 days of age, were given a single intraperitoneal dose of 5 mg/kg bw NDEA. Starting 2 weeks later, groups of mice received 500 mg/L

phenobarbital continuously in the drinking-water until 36 weeks of age. Three animals from each group were killed at 4, 20 and 28 weeks, and six animals from each group were killed at 12, 36 and 44 weeks of age. Half of the remaining animals were killed at 52 weeks and the remainder at 60 weeks of age. NDEA alone induced multiple focal hepatic lesions, including hepatocellular foci, adenomas (average, 34/mouse at 44 weeks, 16/mouse at 52 weeks and 13/mouse at 60 weeks) and carcinomas (3/mouse at 44 weeks, 8/mouse at 52 weeks and 12/mouse at 60 weeks). Subsequent exposure to phenobarbital suppressed the development of focal hepatic lesions, decreased the number of adenomas (5/mouse at 44 weeks, 6/mouse at 52 weeks and 8/mouse at 60 weeks) and carcinomas (0 at 44 weeks, 0 at 52 weeks and 1/mouse at 60 weeks) and prolonged the latency or significantly slowed the rate at which hepatocellular tumours developed in these mice (Diwan *et al*., 1984).

Groups of CD-1 mice [initial numbers unspecified; sex ratio presumably equal], 15 days of age, received an intraperitoneal injection of 0, 5 or 20 mg/kg bw *N*-ethyl-*N*-nitrosourea (ENU). At 5 weeks of age, they received 500 mg/L sodium phenobarbital in the drinking-water until 51 weeks of age, and the experiment was terminated 1 week later. ENU induced lung and liver tumours in a dose-dependent fashion. Sodium phenobarbital promoted the hepatocarcinogenesis initiated by ENU in females (at the high dose of ENU: 6/33 adenomas ($p \leq 0.01$), 7/33 carcinomas ($p \leq 0.01$; adenomas plus carcinomas); at the low dose of ENU: 4/32 adenomas ($p \leq 0.05$), 2/32 carcinomas; ($p \leq 0.05$; adenomas plus carcinomas); ENU alone: no liver tumours). Males were more susceptible than females to the carcinogenicity of ENU, and subsequent treatment with sodium phenobarbital increased the hepatocellular carcinoma incidence (high dose of ENU: 22/30 adenomas and 10/30 carcinomas; low dose of ENU: 8/39 adenomas and 2/39 carcinomas; high dose of ENU plus sodium phenobarbital: 22/25 adenomas, 17/25 carcinomas ($p \leq 0.05$); low dose of ENU plus sodium phenobarbital, 14/36 adenomas, 10/36 carcinomas; $p \leq 0.01$). Subsequent treatment with sodium phenobarbital also promoted the development of spontaneous liver tumours. Sodium phenobarbital treatment did not, however, alter the incidence of lung tumours induced by ENU (Pereira *et al*., 1985).

In a study to compare the effect of sodium phenobarbital on the development of liver tumours in juvenile and adult mice, 6-week-old male B6C3F_1 mice (20–24 per group) received 15 or 45 mg/L NDEA in the drinking-water for 4 weeks. One week later, they were given 500 mg/L sodium phenobarbital in the drinking-water until termination of the study at 50 weeks of age. In a second experiment, 15-day-old male BALB/c and B6C3F_1 mice received a single intraperitoneal injection of 25 mg/kg bw NDEA or the vehicle alone. At 4 weeks of age, they were given 500 mg/L sodium phenobarbital in the drinking-water until 20 or 28 weeks of age. In the first experiment, both concentrations of NDEA induced hepatocellular adenomas (25 and 65%, respectively) and carcinomas (13 and 30%, respectively). Subsequent treatment with sodium phenobarbital increased the incidence of both hepatocellular adenomas (100% with both initiating concentrations of NDEA; $p \leq 0.01$ at 15 mg/L) and carcinomas (81 and 70% at 45 and 15 mg/L of

NDEA, respectively; $p \leq 0.01$). In the second experiment, BALB/c mice given NDEA alone had a high incidence (66%) of hepatocellular adenomas (2.4 ± 0.72 adenomas/mouse) at 28 weeks. Subsequent administration of sodium phenobarbital increased both the incidence (88% at 20 weeks, $p \leq 0.01$, and 100% at 28 weeks) and the number of adenomas per mouse (1.70 ± 0.82 at 20 weeks, $p \leq 0.05$, and 18.9 ± 1.23 at 28 weeks, $p \leq 0.01$). Sodium phenobarbital alone did not produce any liver tumours. In $B6C3F_1$ mice, NDEA alone induced both hepatocellular adenomas (100%) and carcinomas (30%) by 28 weeks. Subsequent administration of sodium phenobarbital decreased the incidence of hepatocellular carcinomas (0%) and the number of adenomas per mouse (51.8 ± 3.0 versus 7.0 ± 0.56, $p \leq 0.01$). The authors concluded that inhibition or enhancement of hepatocarcinogenesis by phenobarbital is dependent on both the mouse strain and the age at the start of exposure (Pereira *et al*., 1986).

Groups of infant male BALB/c mice, 15 days of age, received a single intraperitoneal injection of 2.5, 10, 25 or 50 mg/kg bw NDEA, and at weaning (28 days) were given either tap-water or water containing 500 mg/L sodium phenobarbital for 40 weeks. Ten mice per group were killed at 12 weeks, 15 at 24 weeks and 20 at 40 weeks after weaning. No significant differences were seen in the body weights of the groups. Both NDEA and sodium phenobarbital alone increased the liver: body weight ratios at all times examined. At 12 weeks, hepatic adenomas were seen only with the highest dose of NDEA alone, but when NDEA treatment was followed by sodium phenobarbital, mice in all treated groups developed hepatocellular adenomas (4/10 at 2.5 mg/kg bw and 80–100% at higher doses). Sodium phenobarbital thus decreased the latency to hepatic adenoma formation in NDEA-initiated mice. Hepatocellular trabecular carcinomas occurred at 40 weeks in 1/20 (5%) and 2/20 (10%) mice exposed to 25 and 50 mg/kg bw of NDEA, respectively; phenobarbital treatment decreased the time to appearance of carcinomas and increased the incidence (20 and 30%, respectively) of such lesions over that in mice exposed to NDEA alone, but this effect was not significant. Subsequent administration of phenobarbital did not alter the incidence or multiplicity of lung adenomas induced by NDEA (Klaunig *et al*., 1988a).

Groups of 10 male $B6C3F_1$ mice, 15 days of age, were given a single intraperitoneal injection of either NDEA or NDMA (5 mg/kg bw) and, after weaning at 4 weeks of age, were exposed to 500 mg/L phenobarbital in the drinking-water or given tap-water for 24 weeks. Control groups received a single intraperitoneal injection of saline at 15 days of age and at weaning were exposed to either tap-water or 500 mg/L phenobarbital. No significant difference in body weights was seen between different groups. Exposure to NDEA only induced a 100% incidence of hepatocellular adenomas, with a mean of 14.8 adenomas/mouse; subsequent administration of phenobarbital significantly decreased this number to 6.4/mouse ($p < 0.05$). In contrast, phenobarbital treatment after exposure to NDMA significantly increased the incidence (from 60% to 100%; $p < 0.05$) and number of adenomas per liver (0.80 to 5.70; $p < 0.05$). Phenobarbital treatment increased the percentage of eosinophilic adenomas in both NDEA- (from 8% to 20%) and NDMA- (from 0% to 72%) treated

mice. No hepatocellular foci or adenomas were seen in groups given phenobarbital only or no treatment. The type of initiator therefore appears to be important in determining whether 15-day-old initiated male $B6C3F_1$ mice respond to the promoting effects of phenobarbital (Klaunig *et al*., 1988b). [The Working Group noted the short duration of exposure and the small number of animals per group.]

Groups of male C57BL, C3H and $B6C3F_1$ mice [total initial number not given], 15 days of age, were given either a single intraperitoneal injection of 5 mg/kg bw NDEA or an equal volume of saline. At 28 days of age (at weaning), they received either normal drinking-water (controls) or drinking-water containing 500 mg/L phenobarbital for 28 weeks. In a second study, NDMA was used as the initiator instead of NDEA under identical experimental conditions. All three strains of mice exposed to phenobarbital after NDEA developed hepatocellular foci, but their incidence, number and size did not differ from those in mice given NDEA only. All C3H mice exposed to NDEA only or NDEA plus phenobarbital developed hepatocellular adenomas, but the number of adenomas in the latter group (52.5 ± 18.2) was significantly higher ($p < 0.05$) than that in mice given NDEA only (29.8 ± 13.6). $B6C3F_1$ mice exposed to NDEA plus phenobarbital, however, showed a significant decrease ($p < 0.05$) in the number of hepatic adenomas (6.4 ± 4.1) as compared with the group given NDEA only (15.0 ± 5.4), although no difference was found in the incidence or size of the tumours. In C57BL/6 mice, phenobarbital treatment decreased the incidence of adenomas in those given NDEA from 90% to 50% ($p < 0.05$). The number (18.5 ± 5.4) and size of the adenomas (20.8 ± 6.5 mm) in C3H mice given NDMA plus phenobarbital were significantly greater than in the NDMA-treated group (number, 1.7 ± 1.0, $p < 0.05$; size, 12.3 ± 3.4 mm, $p < 0.05$). In $B6C3F_1$ mice, the number but not the size of adenomas in animals given NDMA plus phenobarbital was significantly greater than in mice given NDMA only (6.2 ± 4.3 versus 0.8 ± 0.8; $p < 0.05$). In C57/BL mice treated with NDMA plus phenobarbital, the size of the adenomas was significantly decreased as compared with the group given NDMA only (6.5 ± 1.1 versus 11.0 ± 2.8 mm; $p < 0.05$). Thus, the strain of the mouse and the initiating carcinogen determine the ability of phenobarbital to either inhibit or promote hepatocellular carcinogenesis in 15-day-old mice (Weghorst *et al*., 1989). [The Working Group noted the small number of mice per group.]

Groups of male and female $B6C3F_1$ mice, 15 days of age, were given either a single intraperitoneal injection of 5 mg/kg bw NDEA or an equal volume of saline. At weaning (28 days of age), some groups received drinking-water containing 500 mg/L phenobarbital, while others received deionized water, for 24 weeks. All mice were killed at 28 weeks of age. Hepatocellular foci and adenomas were found only in groups that received NDEA or NDEA plus phenobarbital. In males, NDEA plus phenobarbital caused a significant decrease ($p < 0.05$) in the total number and size of hepatocellular adenomas when compared with the group given NDEA only (number, 15.5 ± 4.8 with NDEA only, 6.4 ± 4.1 with NDEA plus phenobarbital; size, 13.9 ± 1.7 with NDEA only and 10.7 ± 3.0 with NDEA plus phenobarbital). None of the female mice exposed to NDEA only developed adenomas, but 100% of those exposed to NDEA plus phenobarbital had

multiple hepatocellular adenomas which were exclusively eosinophilic. In male mice exposed to NDEA plus phenobarbital, the percentage of basophilic adenomas was smaller than in mice given NDEA only (65% versus 97% of all adenomas). The authors concluded that the sex of mice was important in determining their susceptibility to promotion by phenobarbital (Weghorst & Klaunig, 1989). [The Working Group noted the small number of mice per group.]

Groups of male and female C3H/HeNCr mice [total initial number not given], 15 days of age, were given either a single intraperitoneal injection of 5 mg/kg bw NDEA or saline, and at 4 weeks of age, received either 500 mg/L phenobarbital in the drinking-water or normal drinking-water for 24 weeks. At 28 weeks of age, 8/10 male and 0/10 female mice exposed to NDEA only developed adenomas. The adenomas in phenobarbital-promoted male (10/10) and female mice (7/10) were predominantly eosinophilic, while these lesions in mice given NDEA only were typically basophilic. Treatment with phenobarbital significantly increased the number and size of hepatocellular adenomas in NDEA-initiated male mice, the number of adenomas with NDEA only being 7.1 ± 5.7 and that with NDEA plus phenobarbital being 19.8 ± 14.8 ($p < 0.05$); the volume (mm^3) of adenomas with NDEA only was 1.33 ± 0.47 and that with NDEA plus phenobarbital was 1.83 ± 0.18 ($p < 0.05$). Thus, both male and female young C3H/HeNCr mice are susceptible to the promoting effect of phenobarbital (Weghorst *et al*., 1994).

(*c*) *Studies in transgenic mice*

Groups of 110 TGF-α transgenic MT42 mice and 112 CD-1 mice [sex unspecified], 15 days of age, were given a single intraperitoneal injection of 5 mg/kg bw NDEA and either a normal diet or, 2 weeks later, a diet containing 0.05% phenobarbital, from 4 weeks of age for 35 weeks. Control groups received a single intraperitoneal injection of saline alone and normal diet or the diet containing phenobarbital. At 10, 23 and 37 weeks after the start of the experiment, 5–10 mice from each group were killed. The mean body weights of MT42 mice given NDEA plus phenobarbital decreased from week 16, while that of mice given NDEA only decreased from 26 weeks. No remarkable changes in body weight were seen in CD-1 mice. The first death from a liver tumour was that of an MT42 mouse given NDEA plus phenobarbital at week 21, and the effective numbers of animals were determined on the basis of those alive after that time. Almost all (9/10) MT42 mice given NDEA plus phenobarbital died of liver tumours in weeks 21–34, and 8/8 given NDEA only died of liver tumours in weeks 29–34. All CD-1 mice and all control MT42 mice lived to 37 weeks. A time-related increase in the liver weights of MT42 mice given NDEA plus phenobarbital or NDEA only was observed which was due mainly to a large number of coalescing liver tumours. The livers of MT42 control mice were also heavier than those of control CD-1 mice. Hepatocellular carcinomas developed in 10/10 MT42 mice given NDEA plus phenobarbital by week 23, and in only 2/5 CD-1 mice by week 37. Hepatocellular carcinomas were also observed at week 23 in 8/10 MT42 mice given NDEA only, but the number per liver and the volume per cent

of hepatocellular carcinomas were significantly greater in those receiving NDEA plus phenobarbital (number, 11.7 ± 1.0 versus 4.0 ± 1.1, $p < 0.001$; volume %, 54.2 ± 3.6 versus 20.8 ± 5.5, $p < 0.001$). Adenomas and preneoplastic foci were induced by phenobarbital alone at week 23, while only one control MT42 mouse developed foci at that time. By week 37, 5/5 MT42 mice given NDEA only and 2/6 given phenobarbital only had developed hepatocellular carcinomas and adenomas. In CD-1 mice, NDEA initiation and phenobarbital promotion significantly increased the numbers per liver and volume per cent of adenomas and foci at 23 weeks when compared with those given NDEA alone (number of foci, 30.7 ± 9.8 versus 5.4 ± 3.8, $p < 0.05$; foci volume, 0.16% ± 0.06 versus 0.03% ± 0.02, $p < 0.05$; number of adenomas, 4.1 ± 1.4 versus 0.6 ± 0.3, $p < 0.05$). No tumours or adenomas were found in CD-1 mice given NDEA alone or phenobarbital alone. The number per liver and volume per cent of hepatocellular carcinomas in MT42 mice given NDEA only or NDEA plus phenobarbital were significantly higher than those in CD-1 mice at weeks 23 and 37. The proliferating cell nuclear antigen-labelling indices of the foci and adenomas in MT42 mice given NDEA alone or NDEA plus phenobarbital were significantly higher than those in CD-1 mice (Tamano *et al*., 1994). [The Working Group noted the inadequate number of animals per group.]

3.3.2 *Promotion in rat liver*

(*a*) *Effects of subsequent administration of phenobarbital*

Groups of (SD/Anl[Anl 66]) rats [initial number, sex and age not specified] were given diets containing 0.02% 2-acetylaminofluorene (AAF) for 11, 16, 21 or 26 days. At each of these intervals, 36 rats were transferred to the control diet and another 36 were transferred to a diet containing 0.05% phenobarbital. Four rats from each group were killed at 21-day intervals starting 91 days after the beginning of the experiment. The subsequent treatment with phenobarbital for up to 260 days increased the incidence of hepatomas at each of the four periods of AAF treatment (AAF 11 days, 2/105; AAF 11 days followed by phenobarbital, 17/106; AAF 16 days, 7/101; AAF 16 days followed by phenobarbital, 42/104; AAF 21 days, 18/103; AAF 21 days followed by phenobarbital, 64/102; AAF 26 days, 27/103; AAF 26 days followed by phenobarbital, 86/108; two-way analysis of variance for duration of AAF feeding, $p < 0.05$ and for phenobarbital treatment, $p < 0.01$). At each sacrifice interval, more rats given both AAF and phenobarbital had tumours. The hepatomas found at the early sacrifice intervals were seen only in the groups that had been fed AAF for the two longer periods (Peraino *et al*., 1971).

To investigate the effects of varying the time of exposure to phenobarbital on enhancement of hepatocarcinogenesis, groups of 106–109 male Sprague-Dawley rats, 22 days of age, were given diets containing 0.02% AAF for 3 weeks and were then fed a diet containing 0.05% phenobarbital for various times: AAF diet for 18 days then phenobarbital diet; AAF diet for 18 days then phenobarbital diet for 5 days then control

diet; AAF diet for 18 days then phenobarbital diet for 20 days then control diet; AAF diet for 18 days then control diet for 10 days then phenobarbital diet; AAF diet for 18 days then control diet for 30 days then phenobarbital diet. Beginning 101 days after the cessation of AAF feeding, 12 rats from each experimental group were killed at 3-week intervals and examined for tumours. Continuous treatment with phenobarbital, beginning immediately after 18 days of AAF feeding caused a threefold increase (73/109 versus 22/106) in the incidence of tumours of all sizes and an eightfold increase in that of larger (≥ 10 mm) tumours (46/109 versus 5/106). Treatment with phenobarbital for only 5 days had no effect on the incidence of tumours but produced a 60% increase in the number of animals with larger tumours (8/106 versus 5/106). When administration of phenobarbital was increased to 20 days, it had a slightly greater effect (35/108 versus 22/106). In rats that received normal diet for 10 days and then phenobarbital, the effect on tumour incidence was similar to that in animals that received phenobarbital in the diet immediately after 18 days of AAF feeding (78/108 versus 73/109). When the treatment-free interval was increased to 30 days, a slight reduction was seen in the enhancing effect of phenobarbital (68/106 versus 73/109). When tumours of all sizes were taken into account, the rates of increase in the percentage of rats with tumours were parallel in the groups given AAF and AAF plus phenobarbital after 120 days, the tumour incidence in the latter group being threefold greater than that in the AAF group. The percentage of rats with larger tumours, however, increased at a higher rate in the group given AAF plus phenobarbital than in that given AAF. An increased rate of appearance of new tumour foci was also seen in rats given AAF plus phenobarbital, the largest increase occurring for tumours ≥ 10 mm (Peraino *et al*., 1973b). [The Working Group noted that no statistical analysis was provided.]

Groups of male Donryu rats [initial numbers not specified], 21 days old, were fed a basal diet containing 600 mg/kg 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) for the first 3 weeks and then a diet containing 5–500 mg/kg phenobarbital. Groups of 5–10 animals were killed at 12 and 24 weeks of age. A dose-dependent effect of phenobarbital was clearly seen on both the number and size of enzyme-altered islands at concentrations > 10 mg/kg of diet. The increase in the total number of islands in these groups was significant ($p < 0.05$ or 0.01). The numbers of enzyme-altered islands in the largest size class (about 1000 μm) were significantly increased at 100 and 500 mg/kg of diet ($p < 0.05$ and < 0.01, respectively), while those in the next two lower size classes (500–999 and 250–499 μm) were significantly increased at the highest dietary concentration ($p < 0.01$) (Kitagawa *et al*., 1984). [The Working Group noted the small number of animals per group and the short duration of exposure.]

(*b*) *Effects of simultaneous administration of phenobarbital*

Six groups of 50 (SD/AnI[AnI66]) rats received either normal diet or a diet containing 0.05% phenobarbital, 0.01% AAF, 0.05% phenobarbital plus 0.01% AAF,

0.02% AAF or 0.05% phenobarbital plus 0.02% AAF. Three rats per group were killed at regular intervals over a 6-month period. Simultaneous feeding of AAF and phenobarbital reduced the incidence of hepatic tumours when compared with that in rats given AAF only [incidences not given]. Highly differentiated hepatomas began to appear by day 72 in rats exposed to 0.02% AAF, and all rats in this group had multiple hepatomas after 120 days. Rats receiving both phenobarbital and 0.02% AAF did not develop hepatomas until 60 days after tumours appeared in rats receiving AAF alone. Furthermore, the livers of rats receiving phenobarbital and AAF were less cirrhotic than those receiving AAF alone [data for 0.01% AAF and phenobarbital not shown] (Peraino *et al.*, 1971).

Groups of 5–10 male Donryu rats, 21 days of age, were given a diet containing 5–500 mg/kg phenobarbital simultaneously with 100 mg/kg 3′-Me-DAB. The animals were killed at 12 or 24 weeks of age. A concentration-dependent increase in the number of enzyme-altered islands was seen at 12 and 24 weeks of age (at 12 weeks, 20–500 mg/kg phenobarbital, 0.11 ± 0.09 to 0.32 ± 0.10 islands/cm^2; at 24 weeks, 20–500 mg/kg phenobarbital, 0.63 ± 0.12 to 0.97 ± 0.79 islands/cm^2; and 3′-Me-DAB alone, 0.04 ± 0.04 and 0.17 ± 0.13 islands/cm^2 at 12 and 24 weeks; $p < 0.01$ and < 0.05, respectively). Thus, phenobarbital given simultaneously with a low concentration of initiating carcinogen enhanced carcinogenesis at all the concentrations tested (Kitagawa *et al.*, 1984). [The Working Group noted the small number of animals per group and the short duration of exposure.]

(*c*) *Time- and dose-related responses in phenobarbital promotion*

Groups of male Sprague-Dawley rats [numbers and age not specified] received drinking-water containing 200 mg/L *N*-nitrosomorpholine (NMOR) or normal drinking-water for 3 weeks and 1 week later were placed on either basal diet or a diet containing 0.05% phenobarbital up to week 52. Some rats [number not stated] in both experimental and control groups were killed at week 4, and 7–13 animals per group were killed at weeks 16, 28, 40 and 52. The incidence of hepatocellular carcinomas in animals exposed to NMOR alone was 9.5% (2/21; data at 40 and 52 weeks combined), and subsequent administration of phenobarbital increased this incidence to 28.6% (6/21) [no p value given]. No hepatocellular carcinomas were found in rats exposed to phenobarbital alone or in those that were untreated. A time-related increase in the number and average size of altered hepatocellular foci was seen in groups receiving NMOR alone or NMOR plus phenobarbital. Subsequent administration of phenobarbital did not markedly increase the number of foci over that in the group treated with NMOR alone; however, phenobarbital increased the homogeneity of the histochemical reaction and increased the activity of glucose-6-phosphate dehydrogenase. This was associated with an increase in the acidophilic and mixed-cell character of the lesions (Ito *et al.*, 1984).

Groups of male Wistar-derived rats [initial number not specified], weighing 80–120 g, were given a single intraperitoneal injection of 0, 1.1, 3.3, 10 or 30 mg/kg bw NDEA. One week later, some of these groups received sodium phenobarbital in

the drinking-water at 0, 40, 100 or 1000 μg/mL for 12–18 months. Sodium phenobarbital alone given for 12 months produced a few clear-cell foci, but NDEA at all doses caused both clear-cell foci and hyperplastic nodules. The number of lesions per animal showed a dose-related trend, those given 1.1 or 3.3 mg/kg bw NDEA having only one or two nodules per liver, while those given 30 mg/kg bw had > 20 nodules per liver. The incidence of basophilic foci showed a dose–response relationship: 0% at 1.1 mg/kg bw NDEA, 14% at 3.3 mg/kg bw, 50% at 10 mg/kg bw and 54% at 30 mg/kg bw. Among animals exposed to 30 mg/kg NDEA and no phenobarbital, 7/18 (36%) had hyperplastic nodules; the incidence of nodules increased to 22/28 (80%) with 1000 μg/mL phenobarbital. Since the dose of carcinogen used (30 mg/kg bw) induces clear-cell foci, basophilic foci and hyperplastic nodules in the absence of any promotion, no clear dose–response relationship for sodium phenobarbital promotion was discernible; however, the highest dose of sodium phenobarbital was essential for carcinoma development. Animals given sodium phenobarbital developed only clear-cell foci. Administration of the highest dose of sodium phenobarbital 43 weeks after the single dose of NDEA also resulted in carcinoma development in 38% of the animals, confirming the persistence of initiated cells. The authors concluded that basophilic foci may be more important than hyperplastic nodules in carcinoma formation (Driver & McLean, 1986a).

Groups of male Wistar-derived rats [numbers not specified], weighing 60–100 g, were given protein-free diets for 3 days, then placed on a high-protein diet (50% casein) for 3 days and then returned to 41B stock pellet diet; 18 h after commencing the high-protein diet, groups of rats were given a single intraperitoneal injection of 15 mg/kg bw NDMA in saline. The total length of the experiment was 20 months. Two groups were fed 41B diet throughout and given NDMA. Beginning on day 10, some groups of animals received phenobarbital in the drinking-water at 40, 100 or 1000 μg/mL for the remainder of the 20 month-experiment. Rats given the protein-free or high-protein diet plus NDMA, followed by 40 or 100 μg/mL phenobarbital, showed no increase in the incidence of any lesions over that in the group given the diets and NDMA alone; however, when the phenobarbital concentration was increased to 1000 μg/mL, the numbers of animals with nodules (5/8) and of nodules per animal increased greatly, and half of the animals (4/8) developed hepatocellular carcinomas. None of eight animals given 41B diet, NDMA and the highest dose of phenobarbital developed hyperplastic nodules, but the incidence of carcinomas was similar (3/8). The effect of phenobarbital does not appear to be related to its ability to induce enzyme activity in the liver, as lower doses (40 or 100 μg/mL) given for 2 months did not promote liver tumour development in spite of being adequate for the induction of cytochrome P450 (CYP) enzymes and ethoxyresorufin deethylase (Driver & McLean, 1986b). [The Working Group noted the small number of animals per group and that ethoxyresorufin deethylase does not specifically represent the activity of phenobarbital-specific CYPs.]

(*d*) *Effect of age and sex on promotion by phenobarbital*

Groups of weanling, 6- and 12-month-old male and female Fischer 344 rats [initial number not specified] were subjected to a 70% partial hepatectomy and 24 h later were given NDEA by intubation in a single dose of 10 mg/kg bw. Two weeks later, the animals received 0.05% phenobarbital in the diet for 6 months. Although altered hepatic foci and neoplastic nodules were present in the livers of almost all animals that were initiated with NDEA and promoted with phenobarbital, the incidence of neoplastic nodules was much lower in rats initiated with NDEA at weaning (males, 4/17; females, 3/10) or at 6 months of age (males, 2/7; females, 3/6) than in those initiated at 12 months (males, 6/8; females, 8/8). The incidence of hepatocellular carcinomas was much lower in rats initiated with NDEA at 6 months (males, 2/9; females, 1/9) or 12 months (males, 0/8; females, 1/5) followed by phenobarbital promotion than in those initiated at weaning (males, 10/11; females, 4/10). The authors concluded that the stage of initiation and promotion at which phenobarbital acts in hepatocarcinogenesis in rats is altered by both the age and sex of the animal (Xu *et al*., 1990). [The Working Group noted the small number of animals per group.]

(*e*) *Promotion by phenobarbital after multi-organ (broad-spectrum) initiation*

Groups of 30 male Fischer 344 rats, 6 weeks of age, were given drinking-water containing 0.01% *N*-nitrosobutyl-*N*-(4-hydroxybutyl)amine (NBHBA) for 4 weeks and were then fed either a basal diet or a diet containing 0.05% phenobarbital for 32 weeks. Control groups of 30 rats each received either phenobarbital alone or remained untreated. Hyperplastic nodules developed in 8/30 rats that received the nitrosamine plus phenobarbital ($p < 0.01$ compared with the group given NBHBA) but in none of the other groups. Phenobarbital did not promote bladder tumours induced or initiated by NBHBA (Ito *et al*., 1980). [The Working Group noted that size of the nodules and the numbers per rat were not given.]

Groups of 20–21 male Fischer 344 rats, 7 weeks of age, were given drinking-water containing 0.1% *N*-nitrosoethyl-*N*-hydroxyethylamine (NEHEA) for 2 weeks and were then placed either on a basal diet or a diet containing 0.05% phenobarbital for 32 weeks. A control group of 20 animals was given the diet containing phenobarbital. At the end of week 3, all rats were subjected to unilateral nephrectomy. The final weights of the rats given NEHEA plus phenobarbital or NEHEA alone were essentially similar to those of the controls [actual body weights not given]. All rats exposed to the nitrosamine with or without phenobarbital developed multiple hyperplastic hepatocellular nodules, although the incidence of hepatocellular carcinomas was significantly higher in the group given NEHEA plus phenobarbital than in the group given NEHEA only (14/17 versus 7/21, $p < 0.01$). No hepatocellular tumours were found in the group given phenobarbital only. Subsequent administration of phenobarbital had no significant effect on the incidence of

kidney neoplastic nodules or renal-cell tumours induced by NEHEA (Hirose *et al*., 1981).

Groups of male Fischer 344 rats [initial number not specified], weighing 100–120 g, were fed either a diet containing 0.02% AAF or given drinking-water containing 0.01% NBHBA for 4 weeks. They were then fed either basal diet or a diet containing 0.05% phenobarbital for 32 weeks. Subsequent administration of phenobarbital significantly increased the average number of hyperplastic nodules (5.5 versus 3.6/cm^2, $p < 0.05$) and their area (3.5 mm^2 versus 1.9 mm^2/cm^2 of liver, $p < 0.05$) in AAF-treated rats. No hepatocellular carcinomas occurred in rats given AAF only but were found in 5/24 (21%) rats exposed to AAF followed by phenobarbital. Although NBHBA alone or phenobarbital alone induced no liver tumours, 8/30 (27%) rats exposed to NBHBA plus phenobarbital had hyperplastic nodules. Phenobarbital had no effect on bladder carcinogenesis initiated by AAF or NBHBA (Nakanishi *et al*., 1982).

Groups of male and female Fischer 344 rats [initial numbers not specified], 4 weeks of age, received intravenous injections of *N*-methyl-*N*-nitrosourea (MNU) either as 0.05 mmol/kg bw once a week for 4 weeks (total dose, 0.2 mmol/kg bw) or as a single injection of 0.2 mmol/kg bw. Two weeks after the last injection, some groups of rats received drinking-water containing 0.05% phenobarbital until 52 or 80 weeks of age. Rats in one group received similar treatment with phenobarbital from the day of the first MNU injection. Between 53 and 80 weeks of age, subsequent exposure to phenobarbital promoted hepatocarcinogenesis, resulting in the development of hepatocellular adenomas and carcinomas in 50% of males and 40% of females exposed to multiple doses of MNU. Concurrent administration of phenobarbital with divided doses of MNU significantly enhanced the yield of hepatocellular foci/cm^2 but did not affect hepatic tumour development (MNU plus phenobarbital: 2/10 males and 1/10 females; MNU only: 0/10 males and females). Phenobarbital also promoted thyroid tumours initiated by MNU, but no other tumours initiated or induced by this nitrosourea were affected by phenobarbital (Diwan *et al*., 1985).

Groups of male Fischer 344 rats [initial number not specified], 4 weeks of age, were given a single intraperitoneal injection of 0.05 nmol/kg bw *N*-nitrosomethyl(acetoxymethyl)amine. Two weeks later, the rats were given either tap-water or water containing 500 mg/L phenobarbital for 78 weeks. None of the rats exposed to the nitrosamine alone developed liver tumours; however, subsequent phenobarbital treatment resulted in a significant increase in the incidence (5/15, $p < 0.05$) of hepatocellular tumours (three adenomas and two carcinomas). Phenobarbital promoted the development of thyroid tumours but not of any other tumours initiated by *N*-nitrosomethyl(acetoxymethyl)-amine (Diwan *et al*., 1989c).

(*f*) *Effects of phenobarbital on promotion and progression stages of hepatocarcinogenesis*

In a three-stage initiation–promotion–progression model, groups of 7–12 female Sprague-Dawley rats, 5 days of age, were initiated with a single intraperitoneal injection

of 10 mg/kg bw NDEA, fed a diet containing phenobarbital at 0.05% at weaning for 6 months, subjected to partial hepatectomy at 6 months and given a putative progressor agent (100 mg/kg bw ENU 24 h later or 3 × 150 mg/kg bw hydroxyurea 20, 30 and 40 h later) intraperitoneally. Phenobarbital was discontinued after the progressor agent was given, and animals were killed 6 months after administration of the progressor. The number of promoter-independent altered hepatic foci in the group given NDEA plus phenobarbital (4900 ± 250 per liver) was increased to 18 500 ± 1500 per liver by the addition of ENU and 6600 ± 700 per liver by the addition of hydroxyurea. Hepatocytes isolated from animals exposed to ENU in this protocol showed a greater degree of chromosomal damage and aneuploidy than those from animals not given a second initiator. In a variation of the model in which the promoting agent was maintained after administration of the progressor agent, the number of heterogeneous altered hepatic foci (foci-in-foci) increased significantly after administration of either ENU or hydroxyurea. The incidence of hepatocellular carcinoma was 3/14 in rats given NDEA plus phenobarbital, 1/12 in those given NDEA plus phenobarbital plus hydroxyurea and 2/17 in those given NDEA plus phenobarbital plus ENU. The incidence was increased when promotion was maintained until sacrifice, carcinomas being seen in 1/8 animals receiving NDEA plus phenobarbital, 6/9 given NDEA plus phenobarbital plus hydroxyurea and then phenobarbital and 8/9 in those given NDEA plus phenobarbital plus ENU and then phenobarbital (Dragan *et al*., 1993). [The Working Group noted the small number of animals per group.]

3.3.3 *Promotion in hamster liver*

Groups of male Syrian golden hamsters [initial numbers not specified], 5 weeks of age, received a single intraperitoneal injection of either 100 mg/kg bw NDEA or 20 mg/kg bw methylazoxymethanol acetate (MAMA) and 2 weeks later were given either tap-water or drinking-water containing 500 mg/L phenobarbital for 18, 45 or 62 weeks, and killed. Between 18 and 62 weeks, hepatocellular adenomas were induced by both NDEA (11/30, 37%) and MAMA (19/30, 63%), but carcinomas were seen only in MAMA-treated hamsters (2/18, 11%). Subsequent administration of phenobarbital for up to 45–62 weeks had no significant effect either on the incidence (adenoma: NDEA plus phenobarbital, 27%; MAMA plus phenobarbital, 67%; carcinoma: MAMA plus phenobarbital, 11%) or on the histological appearance of preneoplastic or neoplastic hepatocellular lesions in either NDEA- or MAMA-initiated hamsters. Phenobarbital treatment alone did not induce hepatocellular lesions (Diwan *et al*., 1986b).

Groups of male Syrian golden hamsters, 5–6 weeks of age (initial number, 163), were given subcutaneous injections of 500 mg/kg bw *N*-nitrosobis(2-hydroxypropyl)-amine (NBHPA) once a week for 5 weeks, after which they were fed a basal diet alone or a diet containing 0.05% phenobarbital for 30 weeks. Control groups were given subcutaneous injections of saline once a week for 5 weeks followed by either a basal

diet or diet containing 0.05% phenobarbital for 30 weeks. Phenobarbital treatment significantly decreased the final body weight of hamsters initiated with NBHPA (average, 135 g versus 145 g; $p < 0.05$). No significant effect was seen on the liver weights of animals exposed to phenobarbital or NBHPA plus phenobarbital. Hepatic hyperplastic nodules were found in 100% of animals exposed to the nitrosamine with or without phenobarbital. Hepatocellular carcinomas occurred in 13/24 (54%) hamsters given NBHPA plus phenobarbital but in only 2/15 (13%) given the nitrosamine alone ($p < 0.02$). Treatment with phenobarbital did not affect the development of bile-duct, gall-bladder or pancreatic lesions induced or initiated by NBHBA (Makino *et al*., 1986).

Groups of male Syrian golden hamsters, 6 weeks of age, received an intraperitoneal injection of 6 mg/kg bw NDMA and were then fed a basal diet (15 animals), the same dose of NDMA and 1 week later a diet containing 500 mg/kg diet phenobarbital for 30 weeks (11 animals), 1 week of basal diet and then the diet containing phenobarbital for 30 weeks (14 animals; controls), phenobarbital or basal diet continuously for 31 weeks (13 animals; untreated controls). All groups developed altered hepatic foci. The number (foci/cm^2) and size (mm$^2 \times 10^{-2}$) of foci induced by NDMA did not significantly change after subsequent treatment with phenobarbital (NDMA: number, 6.3 ± 3.4; size, 10.8 ± 8.9; NDMA plus phenobarbital: number, 7.3 ± 3.4, size 11.5 ± 9.0). Animals treated with phenobarbital alone developed significantly more foci than the untreated group (1.8 ± 0.8 versus 0.2 ± 0.3 foci/cm^2, $p < 0.01$). One hamster exposed to NDMA alone developed three adenomas, while three given NDMA plus phenobarbital developed one adenoma each. Treatment with phenobarbital alone did not result in tumour formation, but enlarged hepatocytes with abundant cytoplasm were observed. Thus, phenobarbital failed to promote liver carcinogenesis initiated by NDMA in hamsters (Tanaka *et al*., 1987).

3.3.4 *Promotion in monkey liver*

In a first study, nine pregnant patas monkeys (*Erythrocebus patas*) were injected intravenously twice weekly with NDEA to provide a cumulative dose of 0.6–3.2 mmol/kg bw. None of the nine mothers or six offspring developed tumours during 4 years of subsequent observation. At that time, three mothers and three offspring were given drinking-water containing phenobarbital at a concentration providing a dose of 15 mg/kg bw per day for the remainder of their lives or up to 43 months. Within less than 2 years, multiple hepatocellular neoplasms had developed in both offspring (5.6 adenomas and 0.3 carcinomas per animal) and mothers (11.6 adenomas per animal) given NDEA followed by phenobarbital, but none were found in those given NDEA alone. A few hepatocellular foci (0.3 foci/cm^2) were detected in monkeys treated with NDEA only. The authors concluded that, in patas monkeys, hepatocellular carcinogenesis could be initiated by prenatal exposure to NDEA and that the initiated cells could remain dormant for years. In a second study,

young male and female patas monkeys [initial numbers not specified] received intraperitoneal injections of 0.1–0.4 mmol/kg bw NDEA in phosphate-buffered saline at 14-day intervals for a total of 20 injections. Fifteen days later, four of the seven survivors were given drinking-water containing phenobarbital at a concentration providing a dose of 15 mg/kg bw per day for 9 months. A significant increase in the multiplicity of hepatocellular neoplasms was found among animals given NDEA plus phenobarbital (adenomas, 17.25 per animal; carcinomas, 2.0 per animal) when compared with those given NDEA alone (adenomas, 1.6 per animal; carcinomas, 0.3 per animal), and the increase in the incidence of adenomas was statistically significantly greater than that with NDEA plus phenobarbital [*p* value not given]. Animals exposed to phenobarbital alone had no tumours or foci. Thus, phenobarbital is also an effective promoter of hepatocellular neoplasia in this non-rodent species (Rice *et al*., 1989).

3.3.5 *Tumour promotion in the thyroid*

Table 4 summarizes studies of the promotion of tumours in the thyroid with phenobarbital.

Mouse: Pregnant B10.A mice were given a single intravenous injection of 0.5 mmol/kg bw ENU in citrate buffer on day 18 of gestation and were allowed to deliver normally. After sexing at 3 weeks of age, equal numbers of pups from each litter of dams treated with ENU were given ENU alone or ENU concomitantly with drinking-water containing 0.05% phenobarbital from 4 to 80 weeks of age. Postnatal administration of phenobarbital more than tripled the incidence of thyroid follicular-cell adenomas in ENU-treated offspring (ENU: males, 1/14; females, 1/14; ENU plus phenobarbital: males, 5/14; females, 2/14; 7% versus 25% in males and females combined, $p < 0.05$). Postnatal administration of phenobarbital had no effect on the development of intestinal or renal tumours induced or initiated by ENU (Diwan *et al*., 1989d).

Rat: A total of 231 male Wistar rats, 6 weeks of age, were given a subcutaneous injection of 700 mg/kg bw NBHPA once a week for 4 or 6 weeks, and 1 week later were given either basal diet or a diet containing phenobarbital at 500 mg/kg during weeks 5–16 or weeks 7–18. Control groups received phenobarbital alone for similar durations. The experiment was terminated at 20 weeks. No significant effects were observed on the mean final body weights of rats given four injections of NBHPA, but those given six injections with or without phenobarbital weighed significantly less than the corresponding control groups ($p < 0.05$). The mean weights of the thyroid glands of rats exposed to phenobarbital alone or with NBHPA were higher than those of the corresponding control groups ($p < 0.05$). The incidence of thyroid tumours was 66% in the group exposed to phenobarbital after four injections of NBHPA, but no thyroid tumours were found in rats given four injections of NBHPA alone or phenobarbital alone. Thyroid tumours were found in 24% (5/21) of rats given six injections of

NBHPA and in 100% (21/21) given six injections of the nitrosamine followed by phenobarbital. Two rats given four doses of NBHPA followed by phenobarbital and 5/21 (24%) rats given six doses of NBHPA followed by phenobarbital developed malignant thyroid tumours. All rats given four or six doses of NBHPA followed by phenobarbital had multiple follicular adenomas (106 and 239, respectively) (Hiasa *et al*., 1982).

Groups of male Wistar rats (total, 200), 6 weeks of age, received a single subcutaneous injection of 2800 mg/kg bw NBHPA, and 2 weeks later were given either a basal diet or a diet containing phenobarbital at 500 mg/kg for 6, 12 or 19 weeks. The study was terminated at 20 weeks. Of the rats given NBHPA, 21/24 (87%) developed thyroid follicular-cell adenomas when phenobarbital was given for 19 weeks, 19/24 (79%) when phenobarbital was given for 12 weeks and 10/24 (42%) when phenobarbital was given for 6 weeks. The incidences of thyroid adenomas in NBHPA-initiated rats exposed to phenobarbital for 19 and 12 weeks were significantly higher ($p < 0.05$) than that in rats given the nitrosamine alone (9/24, 37%). Ten, 5.2 and 2.6 thyroid tumours per rat were found after exposure to phenobarbital for 19, 12 and 6 weeks, respectively, and the total numbers of follicular-cell adenomas were 237, 124 and 62 in NBHPA-treated rats exposed to phenobarbital for 19, 12 and 6 weeks, respectively. Papillary adenomas occurred in three rats exposed to phenobarbital for 6 weeks and one each in those exposed for 19 and 12 weeks after initiation with NBHPA. Nine (37%) rats exposed to NBHPA only developed tumours, consisting of 23 follicular-cell adenomas. None of the rats exposed to phenobarbital only developed any thyroid tumours (Hiasa *et al*., 1983).

Groups of 10 male and 10 female Fischer 344 rats, 4 weeks of age, received either a single intravenous injection of 0.2 mmol/kg bw MNU or four weekly injections of 0.05 mmol/kg bw MNU followed 2 weeks later by or concurrently with drinking-water containing 0.05% phenobarbital, which was continued until 52 or 80 weeks of age. At 52 weeks, phenobarbital given subsequent to MNU or concurrently with divided doses of MNU significantly enhanced the incidence of thyroid follicular-cell tumours only in males (MNU, 10–20%; MNU plus phenobarbital, 50%; MNU followed by phenobarbital, 60–70%). Between 53 and 80 weeks of age, the thyroid tumour incidence was 70% in male rats exposed to MNU plus phenobarbital, 80–100% in male rats that received MNU followed by phenobarbital and about 30–40% in groups that received MNU only. The incidence of thyroid tumours in females exposed to MNU with or before phenobarbital (60–70%) was not significantly different from that in males, but a higher multiplicity of these tumours in males (average, 3.5 versus 2.1 per tumour-bearing rat) persisted in the groups given phenobarbital. Several animals developed both follicular-cell adenomas and carcinomas after treatment with MNU and phenobarbital (Diwan *et al*., 1985). [The Working Group noted the small number of animals per group.]

To investigate the effects of dose and sex on the development of thyroid tumours, groups of 24 male and 24 female Wistar rats, 6 weeks of age, received a single

intraperitoneal injection of 2100 mg/kg bw (males) or 4200 mg/kg bw (females) NBHPA, and 1 week later were fed diets containing phenobarbital at a concentration of 20, 100, 500 or 2500 mg/kg for 19 weeks. Control groups received either NBHPA alone or various concentrations of phenobarbital in their diet. No significant differences were found in the body-weight gain of either male or female rats treated with NBHPA plus phenobarbital or phenobarbital alone, whereas rats exposed to NBHPA alone had lower body weights than controls. Thyroid follicular-cell tumours occurred only in groups that received NBHPA with or without phenobarbital. NBHPA treatment alone resulted in an 8% incidence of thyroid tumours in both male and female rats; with the addition of 500 mg/kg of diet phenobarbital, a threefold increase in tumour yield was found in male rats but no increase in females. At 2500 mg/kg of diet, a marked increase (about eightfold) in tumour yield was seen in male rats but a less than threefold increase in females. The incidences of follicular adenomas in rats given NBHPA followed by a concentration of 20, 100, 500 or 2500 mg/kg of diet phenobarbital were 8, 45, 70 and 66% for males and 12, 17, 50 and 58% for females, respectively. Papillary adenomas were seen in male rats only at 500 and 2500 mg/kg of diet phenobarbital (12% and 20%, respectively); only one female rat (4%) developed such a tumour when given 2500 mg/kg of diet. No thyroid tumours were found in control groups with or without phenobarbital treatment (Hiasa *et al*., 1985).

A total of 120 male Wistar rats, 6 weeks of age, were given a single intraperitoneal injection of 2100 mg/kg bw NBHPA at the end of the first experimental week. Groups of 20 of these rats were castrated either at the beginning of the experiment or at the end of the second week and received the basal diet containing phenobarbital at 500 mg/kg from week 3 to week 40 of the experiment. The other groups were given the basal diet without phenobarbital. Significant differences in mean body weight were found between groups that received NBHPA only or NBHPA plus phenobarbital and those of castrated rats. The mean weights of the thyroid of rats that received phenobarbital after NBHPA initiation with or without castration at the end of the second week were (not significantly) higher than that in the group exposed to NBHPA alone, while the thyroids of rats that were castrated at the beginning of the experiment weighed significantly less than those of rats that were similarly castrated and given phenobarbital ($p < 0.05$). The incidence of thyroid follicular-cell adenomas was 20% in animals that received NBHPA only, 75% in those that received the nitrosamine plus phenobarbital, 30% in those that were castrated at the end of second week and received NBHPA plus phenobarbital and 0% in those that were castrated at the end of second week and received NBHPA only. The incidences of these tumours were 20% and 0% in rats castrated at the beginning of the first week, treated with NBHPA and given phenobarbital or basal diet, respectively. The incidence of thyroid carcinomas was 10% with NBHPA only, 40% with NBHPA plus phenobarbital and 15% in animals that were castrated at the end of the second week and given phenobarbital. No thyroid carcinomas occurred in any other groups. The serum concentration of thyroid-stimulating hormone (TSH) in the group given NBHPA plus phenobarbital was significantly

higher than in groups that received NBHPA only or NBHPA plus phenobarbital with castration. Thus, castration inhibited the development of thyroid tumours in rats treated with NBHPA, and the inhibition was greater when the rats were castrated before receiving the nitrosamine (Hiasa *et al*., 1987).

Groups of 20 male and 20 female CD(SD)BR rats (weighing 204 g and 143 g, respectively) were injected subcutaneously with 700 mg/kg bw NBHPA or saline once a week for 5 weeks and were then fed a control diet or a diet containing 500 mg/kg phenobarbital and/or L-thyroxine (T4) at a dose of 50 μg/kg bw per day from week 6 through week 20, when all animals were killed. The dose of T4 was required to maintain the normal thyroid gland weight in phenobarbital-treated rats and induced a slight increase in the serum concentration of T4 and a slight decrease in that of triiodothyronine (T3). The food consumption in all groups was comparable, but slightly decreased body weight was observed in all groups that received NBHPA. All rats treated with phenobarbital had increased liver weights, and NBHPA and T4 did not affect this increase. In male rats, phenobarbital increased the weight of the thyroid gland over that of controls ($p \leq 0.05$). NBHPA alone did not alter thyroid weights (~ 30 mg), but subsequent administration of phenobarbital resulted in a marked increase in thyroid weight (~ 55 mg). T4 reduced this response in groups given phenobarbital with or without NBHPA (~ 30 mg). No significant differences were seen in the weights of the thyroid gland in female rats. Six of 16 male rats exposed to NBHPA alone developed thyroid follicular-cell adenomas (20 tumour foci), and this incidence was significantly increased by treatment with phenobarbital (15/18, $p \leq 0.05$) (107 tumour foci). T4 reduced the incidences of both adenomas (5/20) and tumour foci (11). Only one female rat given NBHPA plus phenobarbital developed an adenoma. None of the rats exposed to phenobarbital alone developed thyroid tumours (McClain *et al*., 1988). [The Working Group noted the short duration of exposure.]

Groups of 20 male Fischer 344 rats, 6 weeks of age, were given drinking-water containing 0.2% NBHPA for 1 week, and 1 week later were given either basal diet or a diet containing 0.05% phenobarbital, for 50 weeks. Controls received the diet containing 0.05% phenobarbital alone. The incidence of thyroid follicular-cell carcinomas in rats given NBHPA plus phenobarbital was significantly higher than that in rats given the nitrosamine alone (19/19 versus 14/20, $p < 0.05$). The mean number of tumours (adenomas and carcinomas) per rat given NBHPA plus phenobarbital was also significantly higher than in those given NBHPA only (2.63 ± 1.12 versus 1.56 ± 1.34, $p < 0.02$). One carcinoma in a rat given the combination metastasized to the lung. The difference in the incidence of thyroid adenomas in groups given NBHPA with and without phenobarbital (10/19 versus 7/20) was not statistically significant (Shirai *et al*., 1988).

Pregnant Fischer 344/NCr rats were given a single intravenous injection of 0.2 mmol/kg bw MNU in citrate buffer on day 18 of gestation. After sexing, equal numbers of pups from each litter of dams treated with MNU were given MNU alone with a normal diet and tap-water or with drinking-water containing 0.05% phenobarbital

from 4 to 80 weeks of age. Postnatal administration of phenobarbital had no effect on the incidence or latency of either neurogenic or renal tumours induced or initiated by MNU. However, phenobarbital given postnatally promoted the development of thyroid tumours of follicular cell origin (MNU: males, 1/19; females, 0/17; MNU plus phenobarbital: males, 13/17; females, 3/14), especially in male offspring exposed prenatally to MNU ($p < 0.01$). The multiplicity of thyroid tumours in the MNU plus phenobarbital group was 2.6 per rat in males and 1.3 per rat in females, while that with MNU only was 1 and 0 per rat, respectively. Thyroid follicular-cell carcinomas were found only in male rats exposed transplacentally to MNU and postnatally to phenobarbital (6/17) (Diwan *et al.*, 1989d).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

Phenobarbital is readily absorbed from the gastrointestinal tract in humans (Lous, 1954) and is eliminated by hepatic extraction and renal excretion. In the liver, phenobarbital is *para*-hydroxylated and subsequently conjugated (Butler, 1956). After oral administration of [^{14}C]phenobarbital (120 mg phenobarbital containing 31 μCi ^{14}C-label) to two healthy men, 78–87% of the dose was recovered in the urine over 16 days. Phenobarbital *N*-glucoside, *para*-hydroxyphenobarbital and unchanged phenobarbital accounted for approximately 27, 19 and 29% of the dose, respectively (average for the two subjects) (Tang *et al.*, 1979). Small amounts of the *O*-methylcatechol metabolite of phenobarbital were identified in the urine of a single individual given 300 mg orally (Treston *et al.*, 1987). In patients with epilepsy receiving long-term treatment, 57% of the daily dose was recovered in urine, 14% as the *N*-glucoside, 16% as *para*-hydroxyphenobarbital and 27% as unchanged phenobarbital (Bernus *et al.*, 1994).

4.1.2 *Experimental systems*

After an intravenous injection of 50 mg/kg bw [^{14}C]phenobarbital to rats, the total amount of radiolabel in the liver was higher than that in other organs. Urinary elimination of radiolabel associated with phenobarbital reached a peak after 6–8 h (Glasson *et al.*, 1959). The major pathways for biotransformation of phenobarbital in rodents appear to involve *para*-hydroxylation, with excretion in either the free form or as a glucuronide conjugate. The *N*-glucoside of phenobarbital was formed at < 1% and excreted in urine only in mice; it was not detected in the urine of rats, guinea-pigs, rabbits, cats, dogs, pigs or monkeys (Soine *et al.*, 1991). Less than 50% of a dose of

phenobarbital administered to rodents was excreted in urine (Maynert, 1965). In addition to urinary excretion, 18% of a dose of 75 mg/kg bw phenobarbital was excreted in the bile of Sprague-Dawley rats within 6 h, the majority as conjugated metabolites (Klaassen, 1971). Very small amounts of *meta*-hydroxyphenobarbital, a 3,4-dihydrodiol and a 3,4-catechol derivative were also detected in the urine of rats and guinea-pigs given 110 and 56 mg/kg bw, respectively, by intraperitoneal injection (Harvey *et al.*, 1972). When phenobarbital was administered at a dose of 15 mg/kg bw to beagle pups at 4, 10, 20, 40 and 60 days of age, no difference in the elimination half-time was observed with age, and there was no apparent formation of the *para*-hydroxylated metabolite (Ecobichon *et al*., 1988).

In male CFE rats and CF1 mice pretreated with phenobarbital, a total of 63% of phenobarbital and metabolites was excreted in 3 days in the urine and 30% with faeces in rats, and 72% and 11% in mice, respectively. After 2 weeks on a diet containing 0.1% (w/v) phenobarbital for rats and 0.05% (w/v) phenobarbital for mice, more phenobarbital and metabolites were excreted in the faeces of rats and less in those of mice than in animals that had not been pretreated. Most of the material excreted in urine was *para*-hydroxyphenobarbital and its conjugates, with lesser amounts of phenobarbital. The main effects of phenobarbital pretreatment on phenobarbital excretion in rats were increased urinary excretion of conjugated *para*-hydroxyphenobarbital, a similar decrease in the excretion of the free form of this metabolite and no change in total phenobarbital excretion. In mice, phenobarbital pretreatment caused a threefold increase in the urinary excretion of the free form of *para*-hydroxyphenobarbital, a small decrease in that of the conjugated form and about a twofold decrease in that of unchanged phenobarbital. The overall effect of phenobarbital pretreatment in mice appeared to be about a 50% increase in the amount of all forms of *para*-hydroxyphenobarbital, taking into account changes in urinary and faecal excretion. A minor urinary metabolite found in both species was not identified but was believed to be either the 3,4-dihydrodiol or the 3,4-catechol (Crayford & Hutson, 1980).

The half-time for clearance of phenobarbital was decreased to one-third by 70 weeks in C3H/He mice given phenobarbital at a dose of 85 mg/kg of diet for up to 90 weeks (Collins *et al.*, 1984).

4.1.3 *Comparison of animals and humans*

The *para*-hydroxylated metabolite of phenobarbital and its conjugates as well as unchanged phenobarbital have been identified in the urine of humans and rodents. The formation and urinary excretion of the *N*-glucoside of phenobarbital appears to occur selectively in humans, with minor amounts excreted in mice but not in other species examined.

4.2 Toxic effects

4.2.1 *Humans*

The most frequent side-effect of phenobarbital is sedation; however, tolerance develops after long-term treatment. Nystagmus and ataxia occur at excessive doses. Phenobarbital has produced irritability and hyperactivity in children and confusion in the elderly. Scarlatiniform or morbilliform rash, probably with other manifestations of drug allergy, occurred in 1–2% of patients (Hardman *et al.*, 1996).

Oppenheimer *et al*. (1968) showed increased T4 turnover in humans treated with phenobarbital. Ohnhaus and Studer (1983) determined that an induction sufficient to increase antipyrine clearance by at least 60% was required to change steady-state thyroid hormone levels in phenobarbital-treated patients. Induced patients had decreased concentrations of T4, but the concentrations of T3, TSH and the TSH response to thyrotropin-releasing hormone usually remained within normal limits. The concentrations of circulating free T3 and free T4 were reported to be reduced in children with epilepsy maintained on phenobarbital, although clinical hypothyroidism was not seen (Yüksel *et al*., 1993).

4.2.2 *Experimental systems*

The intraperitoneal LD_{50} of phenobarbital was reported to be 340 mg/kg bw in mice (Collins & Horlington, 1969), and the oral LD_{50} was 162 mg/kg bw in rats (Goldenthal, 1971). The oral LD_{50} in rats for sodium phenobarbital was 660 mg/kg bw (Stecher, 1968).

(*a*) *Cell proliferation and apoptosis*

Studies conducted during the 1960s suggested that phenobarbital causes liver enlargement by inducing both hyperplasia and hypertrophy of liver cells, mainly in the pericentral region of the liver (Ruttimann, 1972). Since that time, extensive studies have been conducted on the enhancement by phenobarbital of cell proliferation in the liver and in hepatocytes *in vitro*. The effects of the drug on cell proliferation after initiation by a carcinogen have also been investigated and the cell proliferation rates compared in hepatocytes in altered foci and in surrounding tissue.

(i) *Studies in the absence of carcinogen initiation*

Peraino *et al.* (1971) measured the labelling index, which is the percentage of cells with intense labelling due to incorporation of radioactive precursors in DNA during semiconservative replication (i.e. not unscheduled DNA synthesis), after administration to male Sprague-Dawley rats of a diet containing 0.05% (w/v) phenobarbital for up to 8 weeks. The labelling indexes for hepatocytes and littoral cells (the lining cells of the sinusoids) measured at 2, 4 and 8 weeks were not significantly different in the phenobarbital-fed rats and the controls; however, when the labelling index was

measured within about 3 days of phenobarbital administration, a marked increase was found in both hepatocytes and littoral cells, indicating that the stimulating effect of phenobarbital is relatively rapid and short-lived.

In another experiment, young male Sprague-Dawley rats were treated intraperitoneally with 83 mg/kg bw phenobarbital daily for up to 5 days, and the hepatocyte labelling index was measured 24 h after the last injection (Peraino *et al.*, 1975). The labelling index peaked at four times the control level in rats exposed to phenobarbital for 3 days and returned to control values by 5 days, despite continued phenobarbital administration. The liver weight increased continuously throughout the 5-day period.

Ward and Ohshima (1985) investigated the cell proliferation (measured by [^{3}H]thymidine incorporation) accompanying promotion by phenobarbital of 'naturally occurring' foci in aged Fischer 344 rats. Rats aged 26 months were given drinking-water containing phenobarbital at 0.05% (w/v) for up to 27 weeks. Phenobarbital increased the number and volume of eosinophilic and γ-glutamyl transpeptidase-positive foci, but not of basophilic foci. The labelling indexes were increased in both the eosinophilic and basophilic foci in comparison with normal hepatocytes but were higher in the eosinophilic foci than in the basophilic foci. The index was not significantly increased in phenobarbital-exposed normal hepatocytes over that in controls. Of the phenobarbital-exposed rats, 30% developed eosinophilic adenomas; the controls had none.

Büsser and Lutz (1987) gave male SIV-50 SD rats a dose of 2.3 or 23 mg/kg bw phenobarbital orally and measured the labelling index in the liver 24 h later. The higher dose increased the index 2.5-fold, but the lower dose had no appreciable effect. Smith *et al.* (1991) fed a diet containing 0.1% (w/w) phenobarbital to male and female Sprague-Dawley rats and CD-1 mice for 1 or 5 weeks and assessed cell proliferation from the incorporation into DNA of bromodeoxyuridine released from osmotic minipumps during the entire 1-week administration of phenobarbital or during the last week for the 5-week experiment. The labelling index was increased several-fold in the livers of rats and mice of each sex at 1 week, but there were no increases at 5 weeks. However, the liver weight was increased by phenobarbital treatment at 1 and 5 weeks: in rats, the increase was 20–40% above control values, and in mice the increase was 50–75%.

The effects of administration of large doses of phenobarbital on liver cell proliferation were compared in 6–8-week-old male and female Fischer 344 rats and B6C3F$_1$ mice (Klaunig *et al.*, 1991). The animals were treated with 500 mg/kg bw phenobarbital for 3, 7 and 14 days and evaluated for cell proliferation by measurement of radiolabelled thymidine. Phenobarbital enhanced cell proliferation in both species, with continued increases over the 14-day period. The effects were somewhat greater in males than in females.

Administration of 0.1% phenobarbital in the drinking-water of young male Fischer 344 rats increased the labelling index in hepatocytes from 0.1% to about 2.7% at 5 days, and the index remained elevated at 10 days. The plasma concentration of growth

factor had increased about threefold by day 3 and remained elevated up to day 7. The investigators proposed that the increase in hepatocyte growth factor mediated phenobarbital-enhanced cell proliferation (Lindroos *et al.*, 1992).

The effect of phenobarbital on DNA synthesis was also examined in 8-week-old B6C3F_1 mice and Fischer 344 rats given a diet containing phenobarbital at 10, 50, 100 or 500 mg/kg for 7, 14, 21, 28 and 90 days. An increase in hepatic DNA synthesis — measured by [^{3}H]thymidine incorporation — was seen in both rat and mouse liver after 7 days at the two higher doses. The rates of DNA synthesis had returned to control values by 14 days in rats, whereas this effect was sustained throughout the 90 days of treatment in mouse liver (Kolaja *et al.*, 1995a, 1996b).

Loss of normal cellular regulation of apoptosis has been shown to be an important component of the carcinogenesis process. Cell growth may increase due to an increase in cell proliferation and/or a decrease in cell death (apoptosis). Less apoptosis was seen in non-focal hepatocytes of fasted rats given phenobarbital (0.05% in drinking-water) than in those of untreated fasted or non-fasted rats, all rats having been initiated with NDEA (10 mg/kg bw) after a 70% hepatectomy. Fasting increased apoptosis and decreased DNA synthesis in rat hepatic focal lesions, and phenobarbital treatment during fasting partially reversed these effects (Hikita *et al.*, 1998).

Phenobarbital (2 mmol/L) inhibited both transforming growth factor (TGF)β- and bleomycin-induced apoptosis in male mouse hepatocytes. In addition, the regulation of *p53*, *BCL-2* and *BAX* was modified in hepatocytes from male mice treated with phenobarbital (Christensen *et al.*, 1998).

BCL-2 plays an important role in the regulation of apoptosis. Treatment of male B6C3F_1 mice with phenobarbital (0.05% in the drinking-water for up to 30 days) increased the amount of BCL-2 protein and decreased that of the BAX protein. Greater expression of BCL-2 protein was observed in the 85% of basophilic hepatic foci and adenomas resulting from long-term treatment with phenobarbital than in surrounding normal cells. BCL-2 protein was expressed in only 12–14% of eosinophilic focal lesions and adenomas (Christensen *et al.*, 1999).

Phenobarbital increased DNA synthesis — measured by bromodeoxyuridine immunocytochemistry — in male Wistar rats treated orally at a dose of 80 mg/kg bw per day for 7 days, with significant increases in the labelling index in the liver, proximal tubule of the kidney and thyroid. The increased DNA synthesis reached a maximum in the liver after 3 days of treatment and increased steadily in the proximal tubule and thyroid over the 7 days of treatment. The labelling indexes in the testis, adrenal cortex and medulla, distal tubule of the kidney and exocrine pancreas were no different from those of controls. Significant decreases were observed in the labelling indexes in the pituitary and the endocrine pancreas (Jones & Clarke, 1993).

(ii) *Administration after a carcinogen*

Mouse: B6C3F_1 mice, 15 days of age, were given a single intraperitoneal dose of NDEA at 5 mg/kg bw; at 24 weeks, some of the mice received drinking-water

containing 0.05% (w/v) phenobarbital for 7 days. An osmotic minipump containing radiolabelled thymidine was implanted in all animals at 24 weeks of age. The labelling index in hepatocytes of altered foci in the NDEA-treated males and females was sixfold higher than in the surrounding hepatocytes. After exposure to phenobarbital, significantly increased labelling indexes were found in hepatocytes in foci and in surrounding hepatocytes in females, but in males the increase was observed only in the surrounding hepatocytes. The authors concluded that this difference explained the finding that phenobarbital promoted NDEA-initiated tumours in immature female mice and inhibited tumour formation in immature males (Weghorst & Klaunig, 1989).

The responsiveness of liver cells to mitogenic stimulation by phenobarbital in NDEA-initiated $B6C3F_1$ mice was investigated further. The mice were given feed containing NDEA at a concentration providing a dose of 5 mg/kg bw per day at 15 days or 24 weeks of age, after which they were given drinking-water containing 0.05% (w/v) phenobarbital for 2 weeks. Other groups received only NDEA or only phenobarbital. At days 7 and 14, but not at day 3, the DNA labelling indices were significantly increased by about fourfold in males and by about twofold in females given only phenobarbital. In males, phenobarbital given after initiation with NDEA produced a statistically significant decrease in the labelling of liver foci cells at 14 days but no change at 3 or 7 days. In females, increases of 1.5- and 1.3-fold were found at 7 and 14 days (Siglin *et al.*, 1991).

Male $B6C3F_1$ mice, 30 days of age, were given a single intraperitoneal dose of 90 mg/kg bw of NDEA; after 36 weeks, they received drinking-water containing phenobarbital at 0.002, 0.01 or 0.05% (w/v) for up to 45 days. Phenobarbital increased the DNA labelling index in normal hepatocytes at concentrations of 0.01 and 0.05% but not at 0.002%, at 7 and 14 days. The values had returned to control levels by 28 and 45 days. Hepatocytes in liver foci showed increased DNA labelling indexes at 0.01 and 0.05% but not at 0.002%; the increased level persisted for at least 45 days. In contrast, adenomas were unresponsive to phenobarbital (Klaunig, 1973).

Female C3H and $C3B6F_1$ mice, 15 days of age, received a single dose of 16 mg/kg bw NDEA intraperitoneally; at 21 days of age, some groups were given drinking-water containing 0.05% phenobarbital for 140 days. C3H mice had 54.1 adenomas per mouse, while $C3B6F_1$ mice had 0.57 per animal, and in both strains the number was similar with and without phenobarbital. However, phenobarbital doubled the number of altered hepatic foci in C3H, but not in $C3B6F_1$ mice. Phenobarbital increased the labelling index of eosinophilic, but not basophilic, foci in C3H mice. The labelling index of cells in foci was similar in basophilic and eosinophilic adenomas in mice treated with NDEA with and without phenobarbital. The greatest effect of phenobarbital was to decrease the labelling index in normal hepatocytes compared with cells in altered foci in C3H mice, in which the ratio of the labelling index in cells in foci and in normal hepatocytes was 214 for basophilic and 82 for eosinophilic foci without phenobarbital, and 545 for basophilic and 455 for eosinophilic foci with phenobarbital. For $C3B6F_1$ mice, the ratios were 13.1 for basophilic and 11.4 for eosinophilic cells

without phenobarbital, and 19.9 and 19.1 with phenobarbital. The author concluded that the relative effect of phenobarbital on proliferation in cells of altered foci and normal hepatocytes was the main determinant of strain sensitivity to carcinogenesis (Pereira, 1993).

Rat: Changes in cell proliferation were investigated in altered foci in the liver after initiation with AAF and promotion with phenobarbital in male Buffalo rats from 30 days of age. The rats were fed a diet containing 0.02% AAF for 4 weeks and then a diet containing 0.05% phenobarbital for 39 weeks, at which time the animals were killed. The labelling index and size were greatest for liver foci that were γ-GT-positive, ATPase-negative and glucose-6-phosphatase-negative. The average labelling index was 4.5 times that in representative 'background' areas which were γ-GT-negative, ATPase-positive and glucose-6-phosphatase-positive. All types of altered foci with γ-GT-positive phenotypic markers had an average labelling index ≥ 2.2 times background, whereas this was not the case for the other markers. Two solid hepatocellular carcinomas were found to have higher labelling indexes than the hyperplastic nodules from which they appeared to arise (Pugh & Goldfarb, 1978).

Altered foci were induced in female Wistar rats, 4–8 weeks old, by administration of a single dose of 75 or 150 mg/kg bw NDEA or 150 or 250 mg/kg bw NMOR. Other groups of animals were given drinking-water containing NDEA at a concentration providing a dose of 5 mg/kg bw per day for 40 days. Altered foci were identified by the presence of γ-GT-positive cells, and cell proliferation was measured by uptake of [^{3}H]thymidine. The percentage of DNA-synthesizing cells 2–7.5 months after administration of the carcinogen was four- to 30-fold higher in hepatocytes of altered foci than in surrounding tissue. Administration of phenobarbital at 50 mg/kg bw by gavage 24–30 h before sacrifice stimulated DNA synthesis in γ-GT-positive foci to a greater extent than in surrounding normal hepatocytes (Schulte-Hermann *et al.*, 1981).

In NDEA-initiated male Wistar rats, phenobarbital increased the proliferation of preneoplastic cells over that of normal cells. Young rats were given drinking-water containing NDEA at a concentration providing a dose of 10 mg/kg bw per day for 2 or 4 weeks. One week after cessation of treatment with NDEA, the initiated animals were given drinking-water containing 0.07% (w/v) phenobarbital for various times; other groups were exposed to phenobarbital only. Labelling indexes were determined by the uptake of radiolabelled thymidine given as seven injections at 6-h intervals; this procedure ensured labelling of all cells entering DNA synthesis during the 36 h after the first injection. Administration of phenobarbital for 10 days increased the labelling index several-fold, but this effect was no longer evident after exposure for 1 month. Administration of NDEA alone for 2 weeks increased the labelling 4.5-fold in normal areas and 5.5-fold in foci, when measured after 12 months. Exposure to NDEA followed by 12 months of exposure to phenobarbital caused a 45% increase in the labelling index in normal hepatocytes as compared with exposure to NDEA alone and nearly a threefold increase in the number of altered foci. Similar findings were reported after 4 weeks of NDEA and 4 months of phenobarbital in the drinking-water, except that the labelling

index in altered foci was increased by only 1.5-fold with phenobarbital as compared to NDEA only (Barbason *et al.*, 1983).

Seven-week-old female Wistar rats received NMOR at a single oral dose of 250 mg/kg bw and then received a diet containing phenobarbital providing a dose of 50 mg/kg bw per day for 10 or 28 weeks. The size of the liver and the DNA content increased during exposure to phenobarbital and returned to normal within a few weeks after cessation of exposure. The incidence of apoptotic bodies was very low in normal liver tissue but increased steeply when phenobarbital was withdrawn; resumption of phenobarbital somewhat inhibited the formation of apoptotic bodies. In eosinophilic altered foci, the number of apoptotic bodies was greater than in the surrounding liver tissue during exposure to phenobarbital and increased to much higher levels than in the surrounding liver after phenobarbital withdrawal. The number was significantly suppressed by re-administration of phenobarbital (Bursch *et al.*, 1984).

Female Wistar rats were given a single dose of 250 mg/kg bw NMOR by gavage at 5 weeks of age and 10 weeks later received phenobarbital in the diet at a concentration providing a dose of 50 mg/kg bw per day, or control diet. [^{3}H]Thymidine was infused continuously by means of osmotic minipumps during exposure to phenobarbital. Hepatocytes in altered foci were identified by histochemical staining for γ-GT or by reaction with anti-CYP2B antibodies which could be located by immunohistochemistry. After 14 days of exposure to phenobarbital, the liver mass had increased by 35%. The DNA labelling index increased steadily over the 14-day period, resulting in labelling of 12% of the cells compared with 3% in the controls. In normal hepatocytes, the phenobarbital-induced enhancement of DNA labelling was confined to the CYP2B-containing cells in the pericentral lobular region. In the absence of phenobarbital, the labelling index was approximately 10-fold higher in γ-GT-positive foci than in surrounding hepatocytes. Phenobarbital caused a further increase in the labelling index in foci, but only during the first 2 days of exposure (Schulte-Hermann *et al.*, 1986).

In a similar protocol, animals were fed a diet containing phenobarbital providing a dose of 50 mg/kg bw per day for 10, 28 or 49 weeks. The group treated for 10 weeks was not further treated for the subsequent 18 weeks, and that treated for 28 weeks was allowed an 11-week recovery period. Exposure to phenobarbital increased the liver weight by 20% during the first weeks of exposure and by 35% at the end of the experiment; the DNA content was also increased. The increase in liver weight was reversible within 2 weeks of phenobarbital withdrawal, while the increase in DNA content reversed more slowly. NMOR produced mostly basophilic foci, 50% of which were γ-GT-positive; after exposure to phenobarbital, however, most of the foci consisted of eosinophilic and diffusely basophilic hepatocytes, of which approximately 90% were γ-GT-positive. Phenobarbital increased the number of altered foci by fivefold. Throughout the experiment, the rate of cell proliferation, measured as the labelling index, was 10-fold higher within the foci than in surrounding hepatic tissue. On withdrawal of phenobarbital after 10 and 28 weeks of exposure, the number of foci declined rapidly, and the size remained constant rather than increasing progressively.

Phenobarbital increased the labelling index in the foci during the first 2 days of exposure, but this effect was no longer present after 2 weeks. No decrease in the labelling index was found with prolonged exposure to phenobarbital. Withdrawal of phenobarbital did not result in a significant decrease in the labelling index in normal hepatocytes or in the foci. The authors concluded that phenobarbital did not cause development of foci from single initiated cells. Instead, the γ-GT-positive altered foci appeared to derive from pre-existent foci of initiated cells. As had been seen in previous experiments, much less apoptosis was seen in liver foci during exposure to phenobarbital than in foci of controls, and more apoptosis occurred in the foci after cessation of phenobarbital exposure (Schulte-Hermann *et al.*, 1990).

In a study of the longevity of hepatocytes during promotion by phenobarbital, measured as the loss of thymidine label, adult female Fischer 344 rats were given NDEA at a dose of 200 mg/kg bw intraperitoneally, followed 4 weeks later by partial hepatectomy and then 4 days later by administration of drinking-water containing 0.05% (w/v) phenobarbital for up to 28 weeks. Labelled thymidine (four doses over 18 h) was given after partial hepatectomy but before phenobarbital administration. The comparison group did not receive phenobarbital. There was no significant difference in the decrease in thymidine label in the liver measured at 9 and 28 weeks. It was concluded that the growth advantage of foci induced by phenobarbital was not due to enhanced destruction of normal hepatocytes (Hayes *et al.*, 1987).

Comparison of mouse and rat: In NDEA-initiated mice and rats, phenobarbital at 100 or 500 mg/kg of diet increased DNA synthesis in hepatic foci in both species, but treatment with 10 mg/kg of diet failed to increase the growth of focal lesions. In addition, a significant decrease in apoptosis was observed in focal hepatocytes of both species. DNA synthesis in hepatic adenomas was unaffected by treatment with phenobarbital. The normal surrounding liver showed increased DNA synthesis at 7 days, which had returned to control levels by 28 and 45 days of treatment (Klaunig, 1993; Kolaja *et al.*, 1995b, 1996c).

(*b*) *Biochemical events related to cell cycle growth factors and cell cycle regulation*

Administration of a diet containing 0.05% (w/w) phenobarbital to female Sprague-Dawley rats for 16 days decreased epidermal growth factor (EGF) receptor RNA by 65% and rat leukaemia virus RNA by 35% (Hsieh *et al.*, 1988). No effects were found on the expression of c-*myc*, H-*ras* or 30S (an endogenous retrovirus-like sequence) RNAs. Gupta *et al*. (1988) found similar effects in male Sprague-Dawley rats given a semi-synthetic diet supplemented with choline and containing 0.06% (w/w) phenobarbital. EGF receptors were decreased by 12, 66 and 60% at 3, 10 and 28 days after the beginning of exposure to phenobarbital. Phenobarbital also decreased the EGF receptor–EGF dissociation constant. In another study, provision of drinking-water containing 0.1% (w/v) phenobarbital to female Fischer 344 rats decreased the EGF

receptors by 33 and 73% at 2 and 8 weeks. Furthermore, phenobarbital diminished the inhibition of hepatocyte proliferation by extracellular Ca^{2+} (Eckl *et al.*, 1988).

In female $B6C3F_1$ mice treated with NDEA followed by phenobarbital (500 mg/kg of diet for 4 months), the expression of TGFα, measured by immunohistochemistry, differed according to the type of foci. Treatment with phenobarbital modulated the relative number of basophilic and eosinophilic foci, but did not affect the staining pattern of the growth factor. In basophilic foci, TGFα staining was absent, in contrast to that seen in eosinophilic foci. Staining was also found in 20% of basophilic hepatocellular adenomas and 60% of hepatocellular carcinomas. The presence of the EGF receptor followed the pattern of staining for TGFα (Moser *et al.*, 1997).

Phenobarbital reduced the ability of normal hepatocytes (in contrast to NDEA-initiated hepatocytes) to respond to mitogenic stimuli. This decreased proliferative response was attributed to an increase in the concentrations of TGFβ1 and insulin-like growth factor receptor in the phenobarbital-treated hepatocytes. As this receptor activates the growth factor through proteolytic processes, the reduced mitogenic response may be due in part to the increased capacity of phenobarbital-treated hepatocytes to activate TGFβ1 (Jirtle *et al.*, 1994). In male Fischer 344 rats, phenobarbital selectively promoted a subpopulation of NDEA-induced preneoplastic cells that expressed reduced concentrations of TGFβ receptor types I, II and III. The cells therefore showed less ability to respond to negative growth signals mediated by this growth factor (Mansbach *et al.*, 1996).

The expression of the *WAF1/CIP1* gene product p21 was reduced by phenobarbital in NDEA-initiated altered hepatic foci. Glutathione *S*-transferase (placental form)-positive eosinophilic altered foci showed decreased *p21* expression, suggesting a role for altered signalling in the G_1–S phase check-point in liver carcinogenesis induced by phenobarbital (Martens *et al.*, 1996).

Oncogene mutation and expression have been associated with several stages of carcinogenesis. Lafarge-Frayssinet and Frayssinet (1989) examined the expression of the protooncogenes ki-*ras*, *fos* and *myc* in one spontaneously transformed and one untransformed rat liver cell line derived from 10-day-old rats after treatment with phenobarbital. In the transformed cell line, strong expression of the three oncogenes was observed in the presence or absence of phenobarbital, but in the untransformed cells, phenobarbital caused overexpression of all three oncogenes. In primary rat hepatocyte cultures, phenobarbital induced a slight increase in c-*fos* expression but had no effect on c-*myc* expression. The authors suggested that this indicated a decrease in the G_0–G_1 cell-cycle shift (Duivenvoorden & Maier, 1994). Jenke *et al.* (1994) observed an increase in c-*raf* expression in NDEA-induced hepatic nodules and foci in female Sprague-Dawley rats after treatment with phenobarbital.

(*c*) *Effects related to oxidative damage*

The possible involvement of arachidonic acid metabolism in promotion of tumours by phenobarbital was investigated in male Fischer 344 rats, 6–7 weeks of age, which

were given a single intraperitoneal injection of 200 mg/kg bw NDEA; after 2 weeks, some animals were given a diet containing 0.05% (w/w) phenobarbital with varying amounts of inhibitors or antioxidants, for 10 weeks. Phenobarbital increased the number of γ-GT-positive foci by three- to 10-fold, depending on the experiment. Quercitin (an inhibitor of lipoxygenase), morin (a dual inhibitor of lipoxygenase and cyclooxygenase), acetylsalicylic acid (an inhibitor of cyclooxygenase) and *para*-bromophenacyl bromide (an inhibitor of phospholipase A_2) all partially inhibited this effect of phenobarbital, thereby indicating a role of lipoxygenase, cyclooxygenase and phospholipase A_2. The antioxidants *n*-propyl gallate and ethoxyquin also exerted dose-dependent inhibition of phenobarbital promotion (Denda *et al.*, 1989). Phenobarbital enhanced the formation of reactive oxygen in neoplastic rat liver nodules. Newborn Wistar rats were given a single injection of 0.15 μmol/kg bw [15.3 μg/kg bw] NDEA and, after weaning, were given a diet containing 0.05% (w/w) phenobarbital for 8–12 months. Some groups were removed from the phenobarbital diet 3–6 weeks before being killed. Reactive oxygen was measured by lucigenin chemiluminescence in liver microsomes isolated from nodules or from surrounding normal tissue. The level of reactive oxygen was about twofold greater in nodules than in surrounding tissue. Animals exposed to phenobarbital up to the time of death had twofold more reactive oxygen in both nodules and in surrounding tissue than did animals that had been withdrawn from the phenobarbital diet 3–6 weeks before. The generation of reactive oxygen was inhibited by 80–90% by SKF 525A, a specific inhibitor of CYP activity, indicating the involvement of the CYP enzyme systems. Accordingly, CYP content and CYP activity, measured as benzoxyresorufin dealkylation rates, were greater in animals given phenobarbital up to time of death than in those withdrawn from phenobarbital 3–6 weeks before death, and there were no differences between nodules and surrounding tissue (Scholz *et al.*, 1990).

As prostaglandins have been implicated in tumour promotion, the expression of cyclooxygenase, an enzyme involved in prostaglandin synthesis, was examined in Kupffer cells of male Wistar rats *in vivo* and *in vitro* after treatment with phenobarbital. Kupffer cells from rats given drinking-water containing phenobarbital at 0.075% (w/v) for 56 days or incubated *in vitro* with 1 mmol/L phenobarbital for 8 or 24 h showed an increase in cyclooxygenase-2 mRNA and protein and total cyclooxygenase-2 activity, suggesting a role for paracrine activity involving Kupffer cells in the proliferative response of hepatocytes to phenobarbital (Kroll *et al.*, 1999).

Hepatocyte cell cultures have been used to investigate the possible mechanisms of the increased or inhibited cell proliferation found *in vivo*. Some investigators have studied the effects of various agents on cultured hepatocytes from phenobarbital-exposed animals. Phenobarbital increased the incorporation of radiolabelled thymidine into the DNA of hepatocytes derived from adult male Wistar rats and maintained in a medium containing 30 nmol/L dexamethasone and 30 ng/mL EGF. The effect was related to the concentration of phenobarbital in the medium, 2 mmol/L producing a maximum increase of 30% (Edwards & Lucas, 1985). The continuous presence of 3 mmol/L phenobarbital permitted survival of hepatocytes isolated from the

livers of 3-month-old Donryu male rats for at least 49 days. Phenobarbital also inhibited the proliferation of fibroblasts in the cultures. The hepatocytes continued to secrete relatively large amounts of albumin and maintained a high basal level of tyrosine aminotransferase activity, indicating that they had retained differentiated phenotypes (Miyazaki *et al.*, 1985).

Stimulation of DNA synthesis in hepatocytes isolated from male Sprague-Dawley rats by EGF was enhanced by 60–80% by the addition of 1 mmol/L phenobarbital to the medium 2 h after plating. EGF was added from the 12th to the 24th h. DNA synthesis was maximally stimulated between 44 and 46 h. EGF binding was increased by phenobarbital after 12 h as compared with controls. Phenobarbital was most effective in increasing DNA synthesis when added to hepatocytes in G_o or G_1 phase (Sawada *et al.*, 1987). In contrast, phenobarbital had no effect on EGF binding to hepatocytes derived from female Fischer 344 rats, unless the hepatocytes were preincubated with phenobarbital for 1 h before addition of EGF, in which case, EGF binding was inhibited at phenobarbital concentrations in the millimolar range (Meyer *et al.*, 1989). Preincubation with phenobarbital inhibited the phorbol ester-induced redistribution of calcium and phospholipid-dependent protein kinase C in primary rat hepatocytes but reversibly inhibited phorbol ester-induced protein kinase C activation, suggesting that it alters a component of the signalling pathway other than protein kinase C isoenzymes (Brockenbrough *et al.*, 1991).

Hepatocytes isolated from animals given drinking-water containing 0.1% phenobarbital for up to 2 weeks showed greater rates of EGF-stimulated proliferation at physiological calcium concentrations (1.2–1.8 mmol/L) than with 0.2–0.4 mmol/L calcium, which is the optimal concentration for EGF-stimulated proliferation of hepatocytes from unexposed animals. Hepatocytes from animals exposed to phenobarbital for 3–28 days proliferated at two- to threefold greater rates than hepatocytes from unexposed animals. Hepatocytes from animals exposed to phenobarbital for more than 1 month proliferated slowly at all concentrations of calcium. The authors concluded that phenobarbital altered the cellular growth response to calcium, and that its effects on hepatocyte proliferation could therefore not be explained solely by changes in EGF receptors (Eckl *et al.*, 1988).

The effects of phenobarbital on EGF-induced DNA synthesis in normal hepatocytes was compared with those in hepatocytes derived from putative preneoplastic cells isolated from nodules induced in rats given drinking-water containing 0.005% NDEA for 2 months. Phenobarbital at up to 50 mmol/L was not mitogenic to normal hepatocytes cultured *in vitro*; at concentrations of 0.001–1.5 mmol/L, it stimulated EGF-induced DNA synthesis, as measured by [^{3}H]thymidine incorporation. The greatest stimulation occurred with 0.001 and 0.01 mmol/L, whereas higher concentrations inhibited EGF-induced DNA synthesis. Less inhibition was seen in hepatocytes from foci induced by NDEA. Hepatocytes from animals given drinking-water containing 0.1% phenobarbital for 2 months required only 0.1 mmol/L phenobarbital to show reduced binding of EGF to its receptor, whereas > 1 mmol/L was required to produce a

similar effect in control hepatocytes. Furthermore, EGF-stimulated DNA synthesis in hepatocytes from phenobarbital-exposed animals was more readily inhibited by TGFβ1. The authors concluded that phenobarbital down-regulated EGF receptors and that long-term exposure of rats to phenobarbital further sensitized the hepatocytes to down-regulation, by increasing the intracellular concentration of TGFβ1 (Jirtle & Meyer, 1991). Incubation of hepatocytes from normal male Fischer 344 rats with 3, 4, 5 or 6 mmol/L phenobarbital inhibited TGFα-induced DNA synthesis in a dose-dependent manner. Phenobarbital also inhibited DNA synthesis in hepatocytes derived from persistent hepatic nodules from rats initiated with 1,2-dimethylhydrazine and promoted with orotic acid (Manjeshwar *et al.*, 1992).

Hepatocytes were isolated from both humans and rats and tested for the effects of phenobarbital on EGF-induced DNA synthesis. The labelling index of the rat hepatocytes was five- to 10-fold higher than that of the human hepatocytes. Whereas incubation with phenobarbital was found to increase EGF-induced DNA synthesis in rat hepatocytes, phenobarbital had no effect in human cells. However, initial differences between the human and rat hepatocytes with respect to viability and the relative biological ages of the donors may limit the significance of this comparison (Parzefall *et al.*, 1991).

The mitogenic effects of phenobarbital were also examined in hepatocyte cultures from rats treated with *N*-nitrosomethyl(acetoxymethyl)amine. These initiated hepatocytes proliferated and formed colonies under conditions that induced senescence and death in hepatocytes from untreated rats. The colony-forming efficiency of the initiated cells isolated from liver 5 weeks after initiation was approximately 10% in the presence of 2 mmol/L phenobarbital in the medium and less than 0.2% in the absence of phenobarbital. A low rate of DNA synthesis was found in hepatocytes in the absence of phenobarbital; thus, proliferation of initiated hepatocytes *in vivo* appeared to depend on sustained exposure of the cells to phenobarbital (Kaufmann *et al.*, 1988). Similarly, the propagation of a phenobarbital-dependent hepatocyte cell line (6/27/C1) was shown to be promoter-dependent, in that clonal expansion occurred only when phenobarbital was replaced by another liver tumour promoter in the culture medium (Kaufmann *et al.*, 1997).

Treatment of rat and mouse hepatocytes with phenobarbital also resulted in induction of DNA synthesis and suppression of apoptosis. However, these effects appear to be species-specific, as hamster and guinea-pig cells showed no increase in DNA synthesis and no suppression of either spontaneous or TGFβ1-induced apoptosis (James & Roberts, 1996). Human hepatocytes were also refractory to these effects (Hasmall & Roberts, 1999).

In cultured rat hepatocytes, phenobarbital diminished spontaneous apoptosis and slightly suppressed TGFβ1-induced apoptosis. It increased endogenous and TGFβ-induced peroxide formation, significantly decreased superoxide dismutase formation and increased catalase by twofold (Díez-Fernández *et al.*, 1998). Treatment of HepG2 cells with phenobarbital resulted in an increase in erythropoietin synthesis, which was

related to a decrease in the intracellular concentration of hydroxyl free radicals involved in oxygen-regulated gene expression (Ehleben *et al*., 1998).

(*d*) *Effects on the thyroid*

Reduced concentrations of T3 and T4 and resulting increases in the concentration of TSH have been shown to mediate the thyroid tumour promoting effects of hepatic microsomal enzyme inducers such as phenobarbital (McClain *et al*., 1989). Male Sprague-Dawley rats fed a diet containing 1200 mg/kg phenobarbital for 3, 7, 14, 21, 30, 45, 60 or 90 days showed a 25% reduction in T4 concentration and an 80% increase in thyroid weight. Thyroid cell proliferation was increased 2.6-fold after 7 days of treatment and returned to control levels only by day 45 of treatment (Hood *et al*., 1999). In another study, a mitogenic response was found in rat thyroid only after 8 weeks of treatment with phenobarbital at 0.1% (w/w) in drinking-water (Zbinden, 1988).

The effect of phenobarbital on thyroid function and biliary excretion of T4 was examined by giving male and female rats a diet containing phenobarbital to provide a target dose of 100 mg/kg bw per day for 2 weeks. Increased liver and thyroid gland weights, decreased circulating concentrations of T4 and T3 and increased TSH concentrations were found in both male and female rats. The effects on the hormones decreased after 3 months of treatment. Treatment of thyroidectomized rats with phenobarbital increased the plasma clearance of T4. Bile-duct cannulated phenobarbital-treated male rats showed a marked increase in hepatic uptake of [^{125}I]T4 and a 42% increase in its biliary excretion, due mainly to increased excretion of T4 glucuronide. This corresponded to an increase in the total hepatic activity of T4–UDP-glucuronosyl transferase (UGT). These effects were observed in both male and female rats, but the response was greater in males (McClain *et al*., 1989).

The effects of phenobarbital on hepatic microsomal T4–UGT and T3–UGT activities and thyroid function were examined in OF-1 male mice after 14 days' oral exposure to 80 mg/kg bw per day. Phenobarbital induced liver hypertrophy and increases in liver weight and microsomal protein and CYP content, but no significant change in T4–UGT or T3–UGT activity. No significant changes in serum T4 and T3 concentrations were observed, and the histological appearance of the thyroid was not affected. Thus, phenobarbital did not affect thyroid hormone metabolism or thyroid function in mice (Viollon-Abadie *et al*., 1999).

Treatment of male albino rats with a daily oral dose of 100 mg/kg bw phenobarbital for 2 weeks resulted in induction of CYP enzymes, had no effect on T4 concentrations but significantly decreased those of T3 on day 4, and increased TSH concentrations by 60% on day 16, although this increase was not statistically significant. The weight of the thyroid was significantly increased on day 16 and that of the liver on days 4 and 16. Mild to moderate thyroid follicular hypertrophy and moderate hepatocellular hypertrophy occurred in all phenobarbital-treated animals (Johnson *et al*., 1993).

The effect of phenobarbital on thyroid function and metabolism was also studied in thyroidectomized male Sprague-Dawley rats. The animals received thyroid hormone replacement via implanted osmotic minipumps, which resulted in T4 and T3 serum concentrations similar to those in controls, and were then given phenobarbital in the diet at 1200 mg/kg for 10 days. Phenobarbital reduced the total T4 concentration on days 3–10 and that of free T4 on days 7–10 after minipump implantation, and decreased the total T3 concentration on days 7–10. UGT activity towards T4 was increased 2.7-fold by phenobarbital and correlated with serum T4 concentrations (Barter & Klaassen, 1992). In a study in intact male Sprague-Dawley rats, administration of a diet containing phenobarbital at 1200 mg/kg for 21 days resulted in a 1.9-fold increase in UGT activity towards T4, a 30–40% reduction in T4 concentration and a concomitant increase (50%) in TSH concentration (Barter & Klaassen, 1994). Similar effects on thyroid hormone concentrations were observed in rats treated with an oral dose of 50–100 mg/kg bw phenobarbital daily for 7 or 14 days (de Sandro *et al*., 1991) and in epileptic dogs treated with phenobarbital at a daily dose of 1.0–16.4 mg/kg bw for 1.3 weeks–8 years (Gaskill *et al*., 1999). In contrast, treatment of euthyroid dogs with an oral dose of 1.8–3 mg/kg bw phenobarbital every 12 h for 1 week followed by 2.7–4.5 mg/kg bw every 12 h for 2 weeks did not change the serum concentrations of T4 (total and free) or TSH (Daminet *et al*., 1999).

Feeding male Sprague-Dawley rats a diet containing 600–2400 mg/kg phenobarbital for 15 days resulted in dose-dependent induction of UGT activity towards T4 and a reduction in serum T4 concentrations (Liu *et al*., 1995).

4.3 Reproductive and developmental effects

The potential teratogenic effect of phenobarbital has been reviewed (Friedman & Polifka, 1994). A classic pattern of minor dysmorphologies has been described in children born to mothers treated with phenobarbital for epilepsy during pregnancy. This syndrome includes nail hypoplasia and a typical appearance produced by midfacial hypoplasia, depressed nasal bridge, epicanthal folds and ocular hypertelorism. In an earlier review, Dansky and Finnell (1991) found evidence that phenobarbital monotherapy was associated with malformations similar to those reported with hydantoins, suggesting a common biochemical pathway. They also noted that the risks appeared to be greater after treatment of women with epilepsy than treatment of women without seizure disorders.

4.3.1 *Humans*

As reported in an abstract, 57 women receiving phenobarbital monotherapy during pregnancy were identified in a population of 61 090. When compared with a matched control group, the exposed infants showed significant increases in the frequency of major malformations or growth retardation (15.8% versus 6.7%), ‘anticonvulsant

face' (12.9% versus 2.5%) and fingernail hypoplasia (25.8% versus 4.5%) (Holmes *et al*., 1990).

As reported in an abstract, pregnancy outcome was evaluated prospectively in 84 women given phenobarbital monotherapy who were identified through the California Teratogen Information Service (USA). Forty-six of 63 ascertained liveborn infants (seven cases were lost to follow-up and there were 12 spontaneous and one therapeutic abortions) were evaluated by a dysmorphologist. Of these, seven (15%) had facial features characteristic of anti-epileptic therapy and 11 (24%) had hypoplastic fingernails (Jones *et al*., 1992a).

In a prospective cohort study of the pregnancy outcomes of women being treated for epilepsy with anti-convulsant therapy, 72 infants were born to mothers who had received phenobarbital monotherapy during the first trimester (Dravet *et al*., 1992). This group comprised 12 infants with microcephaly, 44 who were not microcephalic and 16 unrecorded outcomes [odds ratio apparently not significant].

In a study of the risk of intrauterine growth delay in the offspring of mothers with epilepsy, prospective data on 870 newborn infants in Canada, Italy and Japan were pooled and analysed. A total of 88 infants were born to mothers who had received phenobarbital monotherapy. By logistic regression, the risk for small head circumference was shown to be higher (relative risk, 3.6; 95% CI, 1.4–9.4) among the infants of phenobarbital-exposed mothers (Battino *et al*., 1999). Subsequent analysis showed statistically significant dose- and concentration-dependent effects of phenobarbital on small head circumference.

A study of the development of sexual identity was carried out among the offspring of mothers with epilepsy who had taken phenobarbital during the index pregnancy in the Amsterdam Academic Medical Centre between 1957 and 1972. Of 243 exposed subjects, 147 participated in the follow-up [age not indicated]. The controls were an equal number of persons from the original pool of 222, matched for birth date, sex and maternal age. Three tests of psychosexual development were used: the Gender Role Assessment Schedule, the Klein Sexual Orientation Grid and the Psychosexual Milestones in Puberty questionnaire. Exposed and control subjects did not differ with respect to 'gender' role behaviour, although greater numbers of persons exposed prenatally to anticonvulsants reported past or present cross-sexual behaviour and/or sexual dysphoria (Dessens *et al*., 1999).

The intelligence scores of adult men whose mothers had received phenobarbital during pregnancy and who had no history of a central nervous system disorder were measured. The population was drawn from the Danish Perinatal Cohort that was assembled in 1959–61 (Reinisch *et al*., 1995). A total of 114 exposed offspring and 153 controls were matched for a number of variables. Exposure to phenobarbital, especially during the last trimester, was associated with significantly lower verbal intelligence scores.

In a preliminary note on a prospective randomized double-blind trial on the efficacy of prenatal phenobarbital therapy in the prevention of neonatal hyperbilirubinaemia,

1522 exposed and 1553 control infants were studied (Yaffe & Dorn, 1991). The phenobarbital treatment consisted of administration of a 100-mg tablet daily during weeks 34–36 of gestation. The frequency of neonatal hyperbilirubinaemia was reduced in those exposed to phenobarbital. In a follow-up of 36% (719) of the 2003 children in one of the two geographical study areas at the age of 5.1–6.8 years in three intelligence tests, the 415 children who had been exposed to phenobarbital tended to score higher than the controls, but the difference was significant only on the Visuo-Motor Integration Test. The observations were continued on 341 adolescents. Pubertal development appeared to be affected, in that there was a trend for the pubertal stage to be delayed in the treated group. The boys showed significantly higher cognitive function as assessed by the Wechler Intelligence Scale. [The Working Group could not locate a subsequent publication on this cohort that contained a complete report.]

In a clinical intervention trial for infantile intracranial haemorrhage, 110 women in preterm labour were assigned to receive either placebo (n = 60) or phenobarbital (10 mg/kg bw intravenously followed by 100 mg/kg bw per day orally) until delivery (Shankaran *et al*., 1996a). There was a trend towards a decreased incidence of intracranial haemorrhage of any grade in the group given phenobarbital (22% versus 35%). The authors noted that the mode of action of this response is not clear but may be related to hypertensive peaks in the neonate. In a follow-up study, no adverse consequences on growth or in the McCarthy General Cognitive Index was seen in the phenobarbital-treated offspring up to 3 years of age (Shankaran *et al*., 1996b), but the study has been criticized for a low and potentially biased follow-up rate (Doyle, 1996).

The influence of prenatal exposure to phenobarbital on childhood IQ (measured by the Bayley Mental Development Index) at 2 years of age was studied in a double-blind, placebo-controlled trial in which the drug was given *in utero* for the prevention of intracranial haemorrhage to patients with an imminent risk of premature delivery (Thorp *et al*., 1999). The dose of phenobarbital was targeted to yield serum phenobarbital concentrations in the mother and infant of 15–17 μg/mL. [The Working Group noted that the study group also received 10 mg vitamin K every 4 days until delivery.] The incidence of antenatal fetal or postnatal death did not differ between the groups. In the 121 (32%) of 375 children who participated in the 2-year follow-up, a significantly lower Bayley mental developmental index was found in the treated group compared with the controls (104 versus 113). Backward regression analysis indicated the presence of five other covariates that were statistically significant, including maternal education and patent ductus arteriosis.

A prospective study of 983 infants born to women with epilepsy in Canada, Italy and Japan indicated that the incidence of malformations in the infants of women who had not taken an anti-epileptic drug (n = 98) was 3.1%, whereas that in the infants of women on phenobarbital monotherapy (n = 79) was 5.1%; the resulting odds ratio was 1.7 [no CI provided]. No specific pattern of malformations was identified after phenobarbital monotherapy. For any anti-epileptic therapy, the incidence of malformations was 9.0% (Kaneko *et al*., 1999).

In a review of exposure to anti-epileptic drugs and pregnancy outcome, it was noted that phenobarbital lowers folate concentrations in both humans and rats, which may contribute to its teratogenic potential (Lewis *et al*., 1998).

4.3.2 *Experimental systems*

The teratogenic effects of phenobarbital in experimental animals have been reviewed (Finnell & Dansky, 1991). A paucity of work on the effects of exposure to phenobarbital *in utero* on pregnancy outcome was noted, the available evidence suggesting that the potency was less than that of other anti-convulsants, such as phenytoin and valproic acid.

In a review of the literature, the influences of route of administration and dose on the nature of the adverse pregnancy outcome were emphasized (Middaugh, 1986). Dietary exposure resulted in high blood concentrations (40–200 μg/mL), reduced food consumption and maternal weight gain and low birth weights and anatomical and biochemical abnormalities in the offspring. Studies in which injections were given that tended to result in plasma concentrations similar to those for therapeutic uses (5–20 μg/mL) showed smaller effects on pregnancy outcome, although effects on behavioural and biochemical end-points were sometimes apparent.

Long-Evans rats were given phenobarbital by oral gavage at a dose of 40 mg/kg bw per day during the first 7 days of lactation to investigate whether neonatal exposure altered the sensitivity to carcinogens later in life. Twenty-four hours after injection of 37-week-old male offspring with [^{3}H]aflatoxin B_1 at a dose of 1 mg/kg bw, more aflatoxin B_1–DNA adducts were found in the liver than in untreated controls. In other male offspring of the same age, neonatal exposure to phenobarbital caused a 2.6-fold increase in the activity of ethylmorphine-*N*-demethylase (Faris & Campbell, 1981).

(*a*) *Developmental toxicity*

(i) *Morphology*

Cleft palate was observed after exposure of groups of 2–28 A/J, C3H and CD1 mice to doses of 90–175 mg/kg bw phenobarbital by intramuscular injection at various developmental periods between days 11 and 14 of gestation. The A/J and CD1 strains were more sensitive than the C3H strain (Walker & Patterson, 1974).

The teratogenic effect of phenobarbital was studied in groups of 15–22 ICI mice given diets containing 0, 50 or 150 mg/kg on days 6–16 of gestation. One of 171 fetuses at the low dose, 6/155 at the high dose and none of the controls had cleft palate [not noted as significant unless pooled over all phenobarbital-treated litters]. No dose-related effects on maternal or fetal body weights or on fetal viability were observed (Sullivan & McElhatton, 1975).

The incidence of cleft palate after oral administration of phenobarbital at 0, 7.5, 20 or 40 mg/kg bw to NMRI-derived albino mice on days 6–15 of gestation was significantly elevated at the highest dose. The two higher doses caused sedation and

dyspnoea in the treated females that lasted 1–3 h, but food intake and body-weight gain were not affected. There were no effects on fetal growth, viability or other malformations (Fritz *et al*., 1976).

Thinning of the cerebral cortex was noted in the brains of neonates of does that received phenobarbital at 18.5 mg/kg bw per day by gavage during the last 10 days of gestation (Dydyk & Rutczynski, 1977).

Sodium phenobarbital was administered in the drinking-water to mice of three inbred strains (SWV, LM/Bc and C57BL/6J) for at least 20 days before mating and throughout gestation. The targeted doses were 0, 60, 120 and 240 mg/kg bw per day, but the actual plasma concentrations of phenobarbital were used to sort the dams into one of four groups (0, 1–10, 10.1–18 and >18.1 μg/mL). There were 5–11 females per group. Dose-related increases in the incidence of malformations were seen in all strains. While defects of the palate, heart, urogenital and skeletal systems were prominent in the SWV and Lm/Bc strains, no palate and only few heart defects were seen in the C57 strain (Finnell *et al.*, 1987a,b).

Concentrations of up to 800 μg/mL phenobarbital did not affect the ability of explants of day-12.5 ICR mouse secondary palates to grow medially and fuse in an organ culture system. The findings were in contrast to the inhibitory effects seen with two other anti-epileptic drugs (Mino *et al*., 1994).

Phenobarbital was among seven anti-epileptic drugs evaluated for its effects on embryonic cardiac function in C57BL/6J mouse embryos. Day-10 embryos were exposed in whole-embryo culture to concentrations of up to 20 times the human therapeutic plasma concentration. Phenobarbital ranked third highest in potency to cause embryonic bradycardia, suggesting that the pharmacological effect of altered ion channels contributes to the teratogenic effects by affecting blood flow and pressure and subsequently contributing to hypoxia. It was postulated that the reoxygenation process also contributes to tissue damage (Azarbayjani & Danielsson, 1998).

Bradycardia and cardiovascular defects were also reported in 4-day-old white Leghorn chick embryos exposed *in situ* to phenobarbital at 1.75×10^{-5} mol (4.45 mg) by topical administration on eggs (Nishikawa *et al*., 1987).

(ii) *Perinatal effects on hepatic enzymes*

Behavioural effects were studied in the offspring of Sprague-Dawley rats given phenobarbital at a dose of 0, 5, 50 or 80 mg/kg bw per day on days 7–18 of gestation or 80 mg/kg bw per day on days 7–10, 11–14 or 15–18 of gestation by oral gavage. With the longer duration, the highest dose of phenobarbital increased the incidence of malformations and mortality in offspring, reduced fetal body weight, delayed the development of the mature swimming angle and induced trends towards delayed startle and reduced alternation behaviour. With the shorter durations, phenobarbital increased the mortality rate of offspring at all doses, but impaired growth only in those exposed on days 11–14. Swimming ability was delayed in those treated on days 7–10 and 11–14.

The author concluded that phenobarbital was a behavioural teratogen at high doses (Vorhees, 1983).

Sprague-Dawley rats [group size not specified] received a subcutaneous injection of 0 or 40 mg/kg bw per day phenobarbital on days 12–19 of gestation. At birth, the male offspring had a reduced anogenital distance, a marker of androgen action in the fetus, in the absence of an effect on body weight. [The Working Group noted that the individual and not the litter was used as the statistical unit.] In adulthood, the males showed a significant decrease in fertility, enlarged livers and reduced seminal vesicle weights (Gupta *et al*., 1980a). Exposure to phenobarbital at 40 mg/kg bw per day, beginning on gestation day 17, reduced the testosterone concentrations in fetal brain and in the serum of male offspring perinatally (Gupta & Yaffe, 1982). Evaluation of female offspring exposed to 40 mg/kg bw per day phenobarbital on days 12–19 of gestation (six dams per group) showed reduced body growth, a 2-day delay in puberty [not adjusted for reduced growth], altered estrous cycles and reduced fertility in adulthood (Gupta *et al*., 1980b). In a subsequent study, the same dose was given daily on gestation days 12–20, 14–20 and 17–20, and another group received 20 mg/kg bw per day on postnatal days 1–8 [group size not specified for any treatment] (Gupta & Yaffe, 1981). Litter size and birth weight were unaffected, but the growth of females was reduced between days 20 and 50 and the age at vaginal opening was delayed by 2–3 days in all phenobarbital-exposed groups [not adjusted for body weight]. Estrous cycles and fertility were also altered in all exposed groups. The researchers attributed the effects to androgen deficiency during a critical developmental period (Yaffe & Dom, 1991).

Administration of phenobarbital at a concentration of 500 mg/L in the drinking-water of Mongolian gerbils during gestation (intake, 60 mg/kg bw per day) and lactation (intake, 136 mg/kg bw per day) [group size appears to be 11, with four controls] reduced the proportion of animals bearing litters, decreased the pup weights at birth and delayed the development of early reflexes (Chapman & Cutler, 1988).

A series of experiments on brain development, behaviour and neurochemistry were conducted in HS/Ibg mice that received a diet containing phenobarbital (acid form) at 3 g/kg on days 9–18 of gestation. Postnatal growth and brain weights were reduced on day 22 but not on day 8, 15 or 50. Histological analysis of the brains from 50-day-old offspring indicated that, although the cerebellar and hippocampal layers were not affected, there were 30% fewer Purkinje cells and 15% fewer hippocampal pyramidal cells in treated offspring (Yanai *et al*., 1979). Beginning at 50 days of age, the offspring were tested in a radial-arm maze; significant decrements in performance were noted in the exposed offspring. No effects were found on brain acetylcholinesterase activity at this age (Kleinberger & Yanai, 1985). Impaired performance in the Morris water maze and greater calculated maximal binding of muscarinic receptors in the hippocampus were noted at 22 and 50 days of age (Yanai *et al.*, 1989). In another study, basal protein kinase C activity was increased in the hippocampi of 50-day-old mice [sex not specified] that had been exposed prenatally to phenobarbital. In addition, the protein

kinase C response to carbachol (a cholinergic agonist) was impaired, and there was increased hemicholinium binding, an indicator of the amount of choline transporters. There were no effects on maternal health, and the viability and growth of the offspring were not impaired. The results indicated behavioural effects related to learning and memory deficits (Steingart *et al.*, 1998).

Electroencephalograms were recorded in 90-day-old Sprague-Dawley rats born to dams that had received phenobarbital at 0, 20, 40 or 60 mg/kg bw per day by subcutaneous injection from 28 days before until the end of gestation. There were no statistically significant effects on the growth, viability or development of the offspring, although the average litter size was reduced from 11 pups in the control group to 7 pups per litter at the high dose. Because this reduction suggested that this dose was near the toxic level, electroencephalograms were not recorded for this group. The electroencephalographic spectra were averaged over a 24-h period before analysis as a percentage of the total spectral power. The results indicated suppression of phasic synchronization frequencies associated with learning and attention focus, particularly in female offspring (Livezey *et al*., 1992).

Groups of 4–12 pregnant Sprague-Dawley rats received phenobarbital at 0, 20, 40 or 80 mg/kg bw per day by subcutaneous injection on days 5–20 of gestation. The offspring were evaluated for external abnormalities, growth, reproductive function and binding of spiroperidol (a dopamine agonist) in the hypothalamus. No malformations were seen, but early postnatal growth was reduced at the high dose. A significant decrease in spiroperidol-binding was noted in females at 22 days of age, but not at 2 months; no effects were noted in male offspring. Benzodiazepine-, muscarinic- and serotonin-binding sites in the frontal cortex were not altered in animals of either sex at either age. There was a dose-related reduction in the percentage of females with normal reproductive cycles at 8–9 weeks of age, but fertility and the litter size after successful mating were normal (Takagi *et al*., 1986; Seth *et al*., 1987).

Neonatal male Sprague-Dawley rats received phenobarbital at 30 mg/kg bw per day by subcutaneous injection on postnatal days 1, 3 and 5, and the controls received saline. When the animals were 24 weeks of age, testosterone metabolism was studied in microsomal preparations. Total CYP activity was not altered by exposure. In adult males, but not females, neonatal treatment with phenobarbital increased testosterone 16α- and 2α-hydroxylation and androstenedione formation. These products are formed primarily by the action of CYP2C11. Immunoblot analysis of hepatic protein kinase Cα activity indicated a 63% reduction in the livers of treated males. There were no effects on serum testosterone concentrations (Zangar *et al.*, 1995). A previous study by this group with the same protocol but western blot analysis indicated that phenobarbital increased the activity of CYP2B (Zangar *et al*., 1993); however, the expected metabolites of testosterone resulting from the activity of this isozyme were not detected in the subsequent study, perhaps owing to low activity.

Neonatal male Sprague-Dawley rats received phenobarbital at 0 or 40 mg/kg bw per day by subcutaneous injection during the first 7 days of life. Body-weight gain was

reduced throughout life. Serum testosterone concentrations were lowered between day 4 and 24 and were elevated in adulthood. Closer analysis indicated peaks of testosterone secretion in the adults (Wani *et al*., 1996).

Growth hormone and monooxygenase activities were studied in adult Sprague-Dawley rats that received seven daily subcutaneous injections of phenobarbital at 0 or 40 mg/kg bw beginning on the first postnatal day. Neonatal exposure resulted in a long-term decrease in peak concentrations of growth hormone at 65 and 150 days of age in males and at 65 days in females. The body-weight gain of males was reduced by about 10% between days 5 and 30 and by 7% through 175 days of age. There was a 15-fold increase in microsomal hydroxylase activity in the livers of neonatal males and females. This effect was no longer observed at 25 or 45 days of age, but reappeared between 65 and 150 days of age, when the increase averaged 15–20% in males and 30–35% in females (Agrawal *et al*., 1995).

Hepatic drug-metabolizing enzymes were studied in the offspring of Sprague-Dawley rats given phenobarbital by intraperitoneal injection at a dose of 80 mg/kg bw per day for 7 consecutive days before delivery. Pentoxyresorufin-*O*-deethylase activity and CYP2B expression were induced in 5-day-old, but not fetal or 10-day-old pups. Testosterone 6β hydroxylase was not affected. In groups that received drinking-water containing 0.1% phenobarbital from day 13 of gestation to 3 weeks after parturition (end of lactation), the activities of pentoxyresorufin-*O*-deethylase and CYP2B were increased at 3 but not 4 weeks of age in animals of each sex; no effects on testosterone 6β hydroxylase were observed. No effects on CYP3A expression were noted in any treated group (Asoh *et al*., 1999).

(*b*) *Reproductive toxicity*

Daily injections of 140 mg/kg bw phenobarbital to female hamsters slightly before, but not after, the period of pituitary gonadotropin (luteinizing hormone) release (14:00–15:00 h) blocked ovulation for up to 8 days. The authors speculated that the resistance arose from an alteration of the central mechanism controlling gonadotropin release (Alleva *et al*., 1975).

4.4 Effects on enzyme induction or inhibition and gene expression

4.4.1 *Humans*

In healthy volunteers, oral administration of phenobarbital at 2–3 mg/kg bw per day for 3 weeks increased antipyrine clearance but not the liver size (Roberts *et al.*, 1976). Biopsy samples from the livers of patients treated for epilepsy with phenobarbital and phenytoin were found to have elevated total CYP activity, and the livers of these patients were enlarged (Pirttiaho *et al.*, 1978).

4.4.2 *Experimental systems*

The ability of phenobarbital to induce the expression of CYP genes has been investigated and reviewed extensively (Conney, 1967; Okey, 1990; Waxman & Azaroff, 1992). The CYP types induced most effectively by phenobarbital in rat liver are CYP2B1 and CYP2B2. Other members of the superfamily induced by phenobarbital include CYP2A1, CYP2C6, CYP2C7, CYP2C11, CYP3A1 and CYP3A2. Relatively high concentrations of phenobarbital (typically 80 mg/kg bw per day given by intraperitoneal injection for 4 days) are required for this type of induction. Other enzymes induced by phenobarbital include aldehyde dehydrogenase, epoxide hydrolase, NADPH:cytochrome *P*450 reductase, UGT and several glutathione *S*-transferases. In addition, increased activities of γ-GT and catalase, but no change in glutathione peroxidase, have been reported (Furukawa *et al.*, 1985). Phenobarbital at 0.05% (w/v) in drinking-water induced the activity of hepatic O^6-methyltransferase in rats that had received a single dose of NDMA (O'Connor *et al.*, 1988). The induction of detoxication enzymes may contribute to the observed inhibition of tumorigenesis by phenobarbital administered before or concurrently with DNA-reactive carcinogens.

The levels of 4-aminobiphenyl adducts in bladder DNA of rats given this carcinogen were decreased by phenobarbital at 0.1% (w/v) in drinking-water for 8 days (Olsen *et al.*, 1993). This suggests that phenobarbital may induce drug-metabolizing enzymes that deactivate carcinogens.

The dose–response relationship for the effects of phenobarbital on total CYP, glucose-6-phosphatase and UGT activities was examined in male Sprague-Dawley rats (age not specified) given intraperitoneal injections of phenobarbital at doses ranging from 1 to 125 mg/kg bw per day on 6 consecutive days. The total CYP activity was determined spectrophotometrically in microsomal preparations. Dose-related increases in CYP activity were found: phenobarbital at 1 mg/kg bw per day had no effect, at 3 mg/kg bw per day it caused a significant increase, and a maximal increase (~ two-fold) was observed at 75 mg/kg bw per day. For UGT, a similar pattern was seen: at 3 mg/kg bw per day, phenobarbital caused a significant increase, and a 2.8-fold increase in activity was seen at 125 mg/kg bw per day. In contrast, the latter dose caused a 50% decrease in the activity of glucose-6-phosphatase in liver microsomes of these rats (Tavoloni *et al.*, 1983).

CYP and related enzymes were induced for up to 90 weeks in C3H mice by exposing them to phenobarbital at 85 mg/kg bw per day. The concentration in the diet was adjusted between 0.048% and 0.083% in order to maintain the dose. After 4 weeks of exposure to phenobarbital, slight cellular and nuclear hypertrophy was seen in the immediate vicinity of hepatic veins. After 4 weeks of age, hypertrophy of centrilobular cells was evident. Between 25 and 40 weeks, focal proliferative lesions developed, followed by large eosinophilic nodules. The activities of microsomal ethylmorphine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase were increased 8–11-fold and three- to sixfold, respectively, during the exposure period. The activities of CYP and

NADPH-cytochrome *c* reductase were increased about twofold. Additionally, the activity of benzo[*a*]pyrene hydroxylase, which is associated with CYP1A1, was increased fivefold, and that of glutathione *S*-transferase was increased two-to-three fold (Collins *et al.*, 1984).

C57BL, C3H, B6C3F$_1$ (C57BL × C3H) and C3B6F$_1$ (C3H × C57BL) mice were compared with respect to their susceptibility to induction of liver enzyme activities by phenobarbital, as they differ markedly in their susceptibility to phenobarbital-induced carcinogenesis. Four-week-old mice were given drinking-water containing 0.05% phenobarbital for 4 or 28 days. The ratio of liver:body weight was increased in all strains and at both times. Similarly, increased activities of CYP isozymes, aminopyrine-*N*-demethylase and 7-ethoxyresorufin-*O*-deethylase and an increased extent of testosterone oxidation were found in all strains. The authors concluded that the strain specificity of the cancer-promoting activity of phenobarbital was not due to any of these effects (Lin *et al.*, 1989).

The phenobarbital dose–response relationships for CYP2B-mediated enzymatic activities were investigated in male and female Fischer 344/Ncr rats. Rats aged 8 weeks were given diets containing phenobarbital at concentrations ranging from 0 to 0.15%. After 14 days, the animals were killed, and the enzyme activities were determined in liver homogenates. The maximal increases over control were: benzyloxyresorufin *O*-dealkylation, 265-fold (female rats) and 54-fold (males); pentoxyresorufin *O*-dealkylation, 197-fold and 58-fold; and testosterone 16β-hydroxylation, 1320-fold (females) and 118-fold (males) (Nims *et al.*, 1993).

The expression, inducibility and regulation of four CYP isozymes (PB$_1$, PB$_2$, MC$_1$ and MC$_2$) [CYP2C6, CYP2C11/12, CYP1A1, CYP1A2], glutathione transferases and microsomal epoxide hydrolase were studied in young female Wistar rats giving drinking-water containing 0.01% (w/v) NDEA for 10 days. One group of animals was not further exposed; a second group was given 3-methylcholanthrene by intraperitoneal injection at a dose of 40 mg/kg bw per day on 3 consecutive days before being killed, and a third group of animals was given NDEA and then a diet containing 0.05% (w/w) phenobarbital or an intraperitoneal injection at a dose of 80 mg/kg bw per day on 3 consecutive days before being killed. Frozen liver sections were analysed by immunohistochemistry for foci, nodules (adenomas) and carcinomas. Progressive loss of constitutive CYP expression was observed during tumorigenesis. Phenobarbital caused a heterogeneous pattern of response in preneoplastic and neoplastic lesions: some foci responded to the same degree as the surrounding normal liver, some were less, or not at all, inducible, and others were more inducible than the surrounding normal hepatocytes, particularly with regard to CYP2C11/12. The pattern of induction of this enzyme was closely linked to that of NADPH:cytochrome *P*450 reductase, suggesting that the regulation of these two enzymes is coordinated (Kunz *et al.*, 1987).

Incubation with 1.5 mmol/L phenobarbital caused a 2.5-fold increase in the activity of 7-ethoxycoumarin *O*-deethylase in cultured human hepatocytes, but had no effect on the activity of aryl hydrocarbon hydroxylase. The median effective dose (ED$_{50}$) for this

increase was about 0.5 mmol/L (Donato *et al.*, 1990). In comparison, P450b, P450e and P450p [CYP2B1, CYP2B2 and CYP3A1] mRNA levels increased in primary cultures of adult rat hepatocytes exposed to various concentrations of phenobarbital. The concentrations that resulted in half-maximum increases in the activities of these three enzymes were 0.015, 0.0057 and 0.3 mmol/L, respectively, with maximal induction of 193-fold, 11-fold and 12.6-fold. Thus, there was only a threefold difference in ED_{50} between CYP2B1 and CYP2B2, whereas there was a 20-fold difference between CYP2B1 and CYP3A1. The authors concluded from the different ED_{50}s that the classes of CYP gene were induced by different pathways (Kocarek *et al.*, 1990).

Modification of gene expression is an important response to xenobiotic compounds, as changes in gene expression frequently modulate toxic response. In differential display approaches, the expression of over 7500 mRNAs was examined in the liver of chick embryos treated with phenobarbital *in vivo*. Twenty-nine cDNA fragments were significantly changed 48 h after treatment with phenobarbital *in ovo*. Of these, 18 were increased and 11 were decreased. The subcloning and sequencing of 20 of these fragments showed that CYP2H1, glutathione *S*-transferase, UGT, fibrinogen, glutamine synthetase and apolipoprotein B were up-regulated (Frueh *et al.*, 1997).

The aryl hydrocarbon (Ah) receptor mediates the transcriptional response to a number of hydrocarbons, resulting in the induction of CYP1A enzymes. Phenobarbital did not appear to bind strongly to the Ah receptor but did induce CYP1A in liver cells. In wild-type and Ah-receptor-knockout C57BL/6J mice, phenobarbital induced CYP1A2, but not CYP1A1, even in the absence of the Ah receptor (Corcos *et al.*, 1998).

A specific nuclear receptor (CAR) has now been identified that mediates induction by activation of the phenobarbital response element located in the 5′ flanking region of inducible CYP genes (Trottier *et al.*, 1995; Zelko & Negishi, 2000). Activation of nuclear factor κB modifies the expression of many genes, and Mejdoubi *et al*. (1999) reported that it participates in the expression of phenobarbital-responsive genes.

Altered hepatocyte foci in the livers of male rats given phenobarbital overexpressed two forms of CYP, CYP2B1,2 and CYP2C7 (Decloître *et al.*, 1990). In a study of the effects of phenobarbital on CYP2B1 and CYP2B2 in cultured rat hepatocytes, phenobarbital induced a concentration-dependent increase in benzyloxyresorufin *O*-deethylase activity up to 25-fold that of controls. Co-incubation with interleukin-6 or addition of interleukin-6 up to 12 h after phenobarbital inhibited this induction. The authors suggested that the inhibition is mediated by the early molecular events of the induction process (Clark *et al.*, 1996). In a study in C57BL/6 mice and in mouse hepatocytes in culture exposed to phenobarbital, the overall constitutive expression of CYP2B10 was greater in male than in female mice. Phenobarbital induced the expression of both CYP2B9 and CYP2B10 (Jarukamjorn *et al.*, 1999). The relationship between phenobarbital treatment, CYP gene expression and growth patterns in hepatic hyperplastic nodules induced by NDEA was studied in nodules from rats treated with phenobarbital. Phenobarbital increased the number of γ-GT-positive nodules, but it did not change the pattern of labelling indexes or the average labelling index. A slight but uniform increase

in CYP1A2 expression was also seen. CYP2B1/2 was underexpressed in 53% of the γ-GT-positive nodules (Chen *et al.*, 1992).

The induction of CYP2B, CYP2C and CYP3A by phenobarbital was examined in male and female patas (*Erythrocebus patas*) and cynomologus (*Macaca fascicularis*) monkeys. Hydroxylation of testosterone associated with CYP3A was increased two- to fivefold in phenobarbital-treated animals. Testosterone-16β hydroxylation activity was induced up to 15-fold, and benzyloxyresorufin *O*-dealkylation was induced 10-fold by phenobarbital in both species of monkey. Induction of CYP2C was observed with phenobarbital and was more pronounced in the cynomologous monkeys than in the patas monkeys (Jones *et al.*, 1992b).

4.5 Genetic and related effects

4.5.1 *Humans*

The potential for phenobarbital to induce sister chromatid exchange in the peripheral lymphocytes of epilepsy patients on phenobarbital monotherapy was examined. Nine male patients (six smokers) of mean age 38.8 ± 1.25 (SE) years and nine male controls (six smokers) of mean age 38.4 ± 1.27 (SE) years were compared. The same exclusion criteria were applied to the two groups: age > 50 years, recent illness, use of medication other than phenobarbital, use of illicit drugs, alcoholism, surgery, anaesthesia, blood or blood product transfusions, chemotherapy, exposure to ionizing radiation and unusual exposure to ultraviolet radiation within 1 year of examination. The daily coffee consumption was 0–20 cups among the patients and 0–12 cups among the controls. The patients' serum phenobarbital concentrations were consistently in the range 10–40 μg/mL, except for two patients on maintenance doses resulting in concentrations of 5 and 6 μg/mL. The mean numbers of sister chromatid exchanges/cell were 6.82 ± 0.54 (SE) in the exposed group and 6.14 ± 0.51 (SE) in the controls (Schaumann *et al.*, 1989).

4.5.2 *Experimental systems* (see Table 6 for references)

In some studies, phenobarbital has been shown to be weakly mutagenic in *Salmonella typhimurium* TA1535 or TA100 in the absence, but not in the presence, of an exogenous metabolic activation system. The effect was observed in two of two studies with TA1535 and in two of five studies with TA100. Direct addition of phenobarbital enhanced the mutagenic effect in *S. typhimurium* TA1535 of sodium azide or 2-aminoanthracene, but not that of 4-nitroquinoline *N*-oxide or 2-nitrofluorene (Albertini & Gocke, 1992).

Phenobarbital induced aneuploidy, but not mutation or recombinational events in fungi; it did not induce sex-linked recessive lethal mutations, somatic cell mutations or mitotic recombination in *Drosophila melanogaster*.

Table 6. Genetic and related effects of phenobarbital

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TM677, forward mutation, 8-azaguanine resistance	–	–	500	Liber (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA98, reverse mutation	NT	–	5000 µg/plate	McCann *et al*. (1975)
Salmonella typhimurium TA100, TA1535, TA1538, TA98, reverse mutation	NT	–	2500 µg/plate	Anderson & Styles (1978)
Salmonella typhimurium TA100, reverse mutation[c]	(+)	–	4000 µg/plate	Baker & Bonin (1985)
Salmonella typhimurium TA100, TA98, TA97, TA102, reverse mutation	–	–	5000 µg/plate	Matsushima *et al.* (1985)
Salmonella typhimurium TA100, reverse mutation	(+)	NR	2000 µg/plate	McGregor & Prentice (1985)
Salmonella typhimurium TA100, TA1537, TA1538, TA98, reverse mutation	–	–	5000 µg/plate	Rexroat & Probst (1985)
Salmonella typhimurium TA100, TA98, TA97, reverse mutation	–	–	3333 µg/plate	Zeiger & Haworth (1985)
Salmonella typhimurium TA1535, reverse mutation	(+)	–	500 µg/plate	Rexroat & Probst (1985)
Salmonella typhimurium TA1535, reverse mutation	(+)	–	500 µg/plate	Zeiger & Haworth (1985)
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	2000 µg/plate	Bruce & Heddle (1979)
Saccharomyces cerevisiae D7, gene conversion	–	–	10 000	Arni (1985)
Saccharomyces cerevisiae JD1, gene conversion	–	–	2000	Brooks *et al*. (1985)
Saccharomyces cerevisiae D7, mitotic recombination	–	–	10 000	Arni (1985)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Aspergillus nidulans P1, genetic crossing-over	–	NT	500	Carere *et al.* (1985)
Saccharomyces cerevisiae D7, reverse mutation	–	–	10 000	Arni (1985)
Schizosaccharomyces pombe P1, forward mutation	–	–	40	Loprieno *et al.* (1985)
Saccharomyces cerevisiae D61.M, aneuploidy	+	NT	100	Albertini *et al.* (1985)
Drosophila melanogaster, mitotic recombination	–		185 (feed)	Vogel (1985)
Drosophila melanogaster, mitotic recombination	–		4640 (feed)	Würgler *et al.* (1985)
Drosophila melanogaster, somatic mutation	–		5000 (feed)	Fujikawa *et al.* (1985)
Drosophila melanogaster, somatic mutation	–		185 (feed)	Vogel (1985)
Drosophila melanogaster, somatic mutation	–		4640 (feed)	Würgler *et al.* (1985)
Drosophila melanogaster, sex–linked recessive lethal mutations	–		10 000 (feed)	Donner *et al.* (1979)
DNA strand breaks, cross-links or alkali-labile sites, Chinese hamster V79 cells *in vitro*	–	–	2320	Swenberg *et al.* (1976)
DNA strand breaks, cross-links or alkali-labile sites, rat hepatocytes *in vitro*	–	NT	765	Sina *et al.* (1983)
DNA strand breaks, cross-links or alkali-labile sites, rat hepatocytes *in vitro*	–	NT	581	Bradley (1985)
DNA strand breaks, cross-links or alkali-labile sites, Chinese hamster ovary cells *in vitro*	–	–	11 600	Douglas *et al.* (1985)
Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	–	NT	254	Probst & Hill (1985)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Unscheduled DNA synthesis, rat primary hepatocytes *in vitro*	–	NT	10	Williams *et al*. (1985)
Gene mutation, Chinese hamster lung V79 cells, *Hprt* locus *in vitro*	(+)	NT	250	Kuroda *et al*. (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Hprt* locus and ouabain resistance *in vitro*	–	–	200	Garner & Campbell (1985)
Gene mutation, mouse lymphoma L5178Y cells, ouabain resistance *in vitro*	(+)	?	1000	Styles *et al*. (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	2500	Amacher & Turner (1980)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	NT	4126	Amacher *et al*. (1980)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	(+)	–	1000	Myhr *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	(+)	1500	Oberly *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	2000	Styles *et al*. (1985)
Gene mutation, BALB/c-3T3 cells *in vitro*, ouabain resistance *in vitro*	NT	–	6000	Matthews *et al*. (1985)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	4000[d]	Gulati *et al.* (1985)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	(+)	(+)	870	Natarajan *et al*. (1985)
Sister chromatid exchange, Chinese hamster V79 cells *in vitro*	+	NT	10	Ray-Chaudhuri *et al*. (1982)
Sister chromatid exchange, rat liver RL4 cells *in vitro*	–	NT	500	Priston & Dean (1985)
Micronucleus formation, Chinese hamster ovary cells *in vitro*	–	–	232	Douglas *et al.* (1985)
Chromosomal aberrations, Chinese hamster lung cells *in vitro*	–	–	2000	Ishidate *et al*. (1981)
Chromosomal aberrations, Chinese hamster liver cells *in vitro*	(+)	NT	100	Danford (1985)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	(+)	(+)	500	Gulati *et al.* (1985)
Chromosomal aberrations, Chinese hamster lung cells *in vitro*	–	NT	2000	Ishidate & Sofuni (1985)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	+	3480	Natarajan *et al*. (1985)
Chromosomal aberrations, rat liver RL4 cells *in vitro*	–	NT	1000	Priston & Dean (1985)
Aneuploidy, Chinese hamster liver cells *in vitro*	–	NT	1000	Danford (1985)
Cell transformation, BALB/c 3T3 mouse cells	–	+	667	Matthews *et al*. (1985)
Cell transformation, C3H10T1/2 mouse cells	–	–	2000	Lawrence & McGregor (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	–	NT	100	Barrett & Lamb (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	100	Sanner & Rivedal (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay (pH 6.7)	+	NT	700	LeBoeuf *et al*. (1996)
Cell transformation, RLV/Fischer rat embryo cells	–	NT	30	Traul *et al*. (1981)
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*	–	NT	500	Umeda *et al*. (1980)
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*	+	NT	23	Williams (1980)
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*	+	NT	150	Jone *et al*. (1985)
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*	–	NT	1160	Umeda *et al*. (1985)
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro* (dye transfer)	+	NT	100	Zeilmaker & Yamasaki (1986)
Inhibition of intercellular communication, Djungarian hamster fibroblasts *in vitro* (dye transfer)	–[e]	NT	1000	Budunova *et al*. (1989)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of intercellular communication, male B6C3F$_1$ mouse primary hepatocytes *in vitro* (labelled nucleotide transfer)	+	NT	20	Klaunig & Ruch (1987a)
Inhibition of intercellular communication, male C3H and BALB/c mouse and Fischer 344 rat primary hepatocytes *in vitro* (labelled nucleotide transfer)	+	NT	100	Klaunig & Ruch (1987a)
Inhibition of intercellular communication, male C57BL mouse primary hepatocytes *in vitro* (labelled nucleotide transfer)	–	NT	500	Klaunig & Ruch (1987a)
Inhibition of intercellular communication, male B6C3F$_1$ mouse primary hepatocytes *in vitro* (labelled nucleotide transfer)	+[f]	NT	20, 8 h	Klaunig & Ruch (1987b)
Inhibition of intercellular communication, male B6C3F$_1$ mouse primary hepatocytes *in vitro* (labelled nucleotide transfer)	+	NT	20, 12 h	Ruch *et al*. (1987)
Inhibition of intercellular communication, male B6C3F$_1$ mouse primary hepatocytes *in vitro* (dye transfer)	+	NT	116, 2 h	Klaunig *et al*. (1990)
Inhibition of intercellular communication, rat kidney epithelial NRK-52E cells *in vitro* (dye transfer)	–	NT	1860, 24 h	Konishi *et al*. (1990)
Inhibition of intercellular communication, Wistar rat primary hepatocytes *in vitro* (dye transfer)	+	NT	116, 5 h	Leibold & Schwarz (1993a)
Inhibition of intercellular communication, BD VI rat primary hepatocytes co-cultured with BALB/c 3T3 A31-1-8 cells *in vitro* (dye transfer)	+	NT	100, 2 h	Mesnil *et al*. (1993)
Inhibition of intercellular communication, Fischer 344 rat and B6C3F$_1$ mouse primary hepatocytes *in vitro* (dye transfer)	+	NT	232, 4 h	Baker *et al*. (1995)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of intercellular communication, male and female rhesus monkey and (sex not given) human primary hepatocytes *in vitro* (dye transfer)	–	NT	696, 4 h	Baker *et al*. (1995)
Inhibition of intercellular communication, male Wistar rat primary hepatocytes *in vitro* (dye transfer)	+	NT	464, 4 h	Guppy *et al*. (1994)
Inhibition of intercellular communication, human urothelial carcinoma cell ine JTC-30 cells *in vitro* (dye transfer)	+	NT	375, 2 days[g]	Morimoto (1996)
Inhibition of intercellular communication, rat primary hepatocytes co-cultured with WB-F344 rat liver epithelial cells *in vitro* (dye transfer)	+	NT	23.2, 1 h	Ren & Ruch (1996)
Inhibition of intercellular communication, rat primary hepatocytes, primarily expressing Cx3 *in vitro* (dye transfer)	+	NT	232, 2 h	Ren *et al*. (1998)
Inhibition of intercellular communication, WB-F344 rat liver epithelial cells, expressing Cx43 and WB-a/32-10 cells, expressing Cx32 *in vitro* (dye transfer)	–	NT	696, 2 h	Ren *et al*. (1998)
Inhibition of intercellular communication, human hepatoma cells *in vitro* (labelled nucleotide transfer)	+	NT	10, 4 h	Rolin-Limbosch *et al*. (1986)
Gene mutation, human lymphoblast TK6 cells *in vitro*	–	–	1000	Crespi *et al*. (1985)
Gene mutation, human lymphoblast AHH-1 cells *in vitro*	(+)	NT	1000	Crespi *et al*. (1985)
Sister chromatid exchange, human lymphocytes *in vitro*	–	–	500	Obe *et al*. (1985)
Chromosomal aberrations, human lymphocytes *in vitro*	(+)	NT	22.4–111	Foerst (1972)
Chromosomal aberrations, human lymphocytes *in vitro*	+	NT	5	Nandan & Rao (1982a)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks and alkali-labile damage, male CD-1 mouse liver cells *in vivo* (single-cell gel electrophoresis assay)	+		140	Sasaki *et al*. (1997)
DNA strand breaks and alkali-labile damage, male CD-1 mouse lung, spleen, kidney and bone-marrow cells *in vivo* (single-cell gel electrophoresis assay)	–		140	Sasaki *et al*. (1997)
Gene mutation, transgenic female C57BL/6 mouse, *lacI* locus in liver cells *in vivo*	–		500 ppm diet, 120 days	Gunz *et al*. (1993)
Sister chromatid exchange, DBA/2 mouse bone-marrow cells *in vivo*	–		50 ip × 6	Tice *et al*. (1980)
Micronucleus formation, (C57BL/6 × C3H/He)F_1 mouse bone-marrow cells *in vivo*	–		500 ip × 5	Bruce & Heddle (1979)
Chromosomal aberrations, DBA/2 mouse bone-marrow cells *in vivo*	–		50 ip × 6	Tice *et al*. (1980)
Chromosomal aberrations, male Swiss mouse spermatogonial germ cells	+		42 po given over 5 or 60 days	Nandan & Rao (1982b)
Binding (covalent) to DNA (^{32}P-postlabelling), B3C6F_1/CrlBR mouse liver cells *in vivo*	–		200 po × 1	Whysner *et al*. (1998)
Binding (covalent) to DNA (^{32}P-postlabelling), B3C6F_1/CrlBR mouse liver cells *in vivo*	–		1000 ppm in diet; 2 weeks	Whysner *et al.* (1998)
Inhibition of intercellular communication, male ACI/N rat liver *in vivo* (freeze–fracture analysis of area occupied by gap junctions)	(+)		25 diet, 4–8 weeks	Sugie *et al.* (1987)

Table 6 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of intercellular communication, Wistar rat primary hepatocytes from γ-GT$^+$ and γ-GT$^-$ foci *in vitro* (dye transfer)	+		100 ip × 1, followed by 0.1% in drinking-water, 5 days	Leibold & Schwarz (1993b)
Inhibition of intercellular communication, male ACI/N rat liver *in vivo* (freeze–fracture analysis of area occupied by gap junctions)	–		0.05% diet, 2 weeks	Sugie *et al*. (1994)
Inhibition of intercellular communication, male Fischer 344 rat primary hepatocytes *in vivo/in vitro* (dye transfer)	+		400 po × 25	Krutovskikh *et al*. (1995)
Inhibition of intercellular communication, male Sprague-Dawley rat primary hepatocytes *in vivo/in vitro* (dye transfer)	+		50 po × 4 weeks	Ito *et al*. (1998)
Sperm morphology, (C57BL/6 × C3H/He)F_1 mouse *in vivo*	–		500 ip × 5	Bruce & Heddle (1979)
Sperm morphology, (CBA × BALB/c)F_1 mouse *in vivo*	–		100 ip × 5	Topham (1980)

γ-GT, γ-glutamyl transpeptidase

[a] +, positive; (+), weak positive; –, negative; NR, not reported; NT, not tested; ?, inconclusive

[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, μg/mL; in-vivo tests, mg/kg bw per day; po, oral gavage; ip, intraperitoneal injection

[c] Negative with TA97, TA98, TA102

[d] Without metabolic activation, 1000 μg/mL was cytostatic.

[e] Dye transfer enhanced 1.5–2.0-fold

[f] Abolished by 0.1 mmol/L dibutyryl cAMP

[g] Toxic dose; a non-toxic dose (250 μg/mL, 4 days) gave negative results.

In cultured, non-human mammalian cells, no DNA strand breakage or induction of unscheduled DNA synthesis was observed. Although mutations were induced in some studies, the inconsistent positive results were weak and not clearly associated with a particular locus; both positive and negative results were obtained in experiments at the *Tk* and *Hprt* loci and tests for ouabain resistance. Similarly, results for the induction of both sister chromatid exchange and chromosomal aberrations were equally divided between positive and negative in different laboratories. Single studies in cultured mammalian cells for the induction of micronuclei and aneuploidy did not show any effect of phenobarbital treatment. Variable results were also obtained in assays for cell transformation conducted in different laboratories, but the differences may have been due partly to the different transformation assays used. Assays for gap-junctional intercellular communication, however, also gave variable results in different laboratories even when the same Chinese hamster lung V79 cell system was used in five of six studies. In a single study with Djungarian hamster fibroblasts, cell-to-cell communication was actually enhanced. The negative result with NRK-52E cells, a rat kidney epithelial cell line, was interpreted by the authors as being consistent with the lack of effect of phenobarbital (which does not promote renal tumours) on the kidney, since barbital, a renal tumour promoter, did inhibit gap-junctional intercellular communication in these cells (Konishi *et al*., 1990).

In cultured human lymphoblastoid cells, no significant increase in the frequency of mutations was observed in one study, while, in cultured human lymphocytes, chromosomal aberrations were induced in two studies; sister chromatid exchange was not induced in another study.

In vivo, phenobarbital did not form adducts with DNA in mouse liver after either a single oral dose (200 mg/kg bw) or when given for 2 weeks in the diet (1000 mg/kg). A study of mutation induction in mice carrying the *Escherichia coli lacI* transgene as the target did not show any effect of phenobarbital. Studies of the bone-marrow cells of mice treated *in vivo* also did not show induction of sister chromatid exchange, micronuclei or chromosomal aberrations after treatment with phenobarbital alone. A significant response was observed in the single-cell gel electrophoresis assay with cells from the liver of mice given phenobarbital (140 mg/kg bw) by intraperitoneal injection 24 h before sacrifice, but not effect was seen with treatment 3 h before being killed; no effect was observed in a number of other organs. The frequency of morphologically abnormal sperm was not increased in mice treated with phenobarbital.

Phenobarbital was reported to induce chromosomal abnormalities, including translocations, in male mouse primary spermatocytes after oral administration for 5 or 60 days. [The Working Group noted that the level of the effects reported was little influenced by either the dose or the treatment regimen.]

A positive response was reported in a test for dominant lethal mutation in male Swiss mice (Nandan & Rao, 1983). [The Working Group noted that there was a significant response of all germ-cell stages, and a greater response in treated spermatogonial cells

than in post-meiotic cells; this result is highly improbable and has not been reported previously with any chemical.]

In another study on mutation induction in the *lacI* transgene, transgenic mice were first given a single intraperitoneal injection of 50 mg/kg bw NDEA; 7 days later they were started on a diet containing 500 mg/kg sodium phenobarbital for 14 days, followed by a normal diet for 7 days. Increased liver weights were observed in the phenobarbital-treated mice, and the mutation frequencies in the *lacZ* gene recovered from liver were consistently higher than the values obtained from mice treated only with NDEA. Feeding a diet containing sodium phenobarbital at 500 mg/kg for 21 days did not affect the mutant frequency in the *lacZ* transgene in groups of mice given 100 mg/kg bw NDEA. The authors noted that no statistical analysis was performed, because of the small group sizes, which also limited interpretation of the data (Okada *et al*., 1997). In female Sprague-Dawley rats that had undergone a 70% partial hepatectomy and then received NDEA by gavage (10 mg/kg bw), subsequent treatment with diets containing 0.05% (w/w) sodium phenobarbital for 12 months produced a significant increase in the number of hepatocytes containing chromosomal aberrations. Most of the damaged cells were seen in γ-GT-positive foci. Karyotypic analysis indicated that the most frequent aberrations in these foci were a trisomy of chromosome 1 or of its long arm and a monosomy of chromosome 3 or its short arm (Sargent *et al*., 1992).

Klaunig and Ruch (1987a) found that phenobarbital inhibited gap-junctional intercellular communication most effectively in hepatocytes from $B6C3F_1$ mice, less so in primary hepatocytes from C3H and BALB/c mice and Fischer 344 rats and not at all in primary hepatocytes from C57BL mice. This finding was confirmed in part, and extended, in another study that showed that phenobarbital inhibited gap-junctional intercellular communication in primary hepatocytes from male Fischer 344 rats and $B6C3F_1$ mice, but did not do so in primary hepatocytes from male and female rhesus monkeys or from a human [sex unspecified] donor (Baker *et al*., 1995).

In studies of the mechanism of inhibition of gap-junctional intercellular communication, it was shown that phenobarbital (20–500 μg/mL) reduced gap-junctional intercellular communication between $B6C3F_1$ mouse hepatocytes in culture. Phenobarbital (250 μg/mL) also reduced cAMP levels 1 h after treatment, but no effect was observed 2, 4 or 8 h after treatment. The addition of dibutyryl cAMP (0.1 mM) increased the cAMP levels approximately 50-fold in these mouse hepatocytes and completely abolished the inhibition of gap-junctional intercellular communication by phenobarbital (Klaunig & Ruch, 1987b). Inhibition of protein synthesis by cycloheximide had no effect on the inhibition of gap-junctional intercellular communication by phenobarbital in cultured rat hepatocytes, but inhibition was enhanced by treatment with diethylmaleate, to deplete intracellular glutathione, or by the addition of the CYP inhibitors, SKF 525A or metyrapone (Guppy *et al*., 1994). It was later shown that phenobarbital-induced inhibition of gap-junctional intercellular communication is a complex phenomenon. Treatment with 2 mmol/L phenobarbital of primary rat hepatocytes co-cultured with WB-F344 rat liver epithelial cells for 1 h sharply reduced

inter-hepatocyte dye-coupling from about 90% to 30%, but the cells fully recovered within 24 h, after which there was a more gradual reduction in dye-coupling to about 20% after 14 days. Dye-coupling between the WB-F344 cells was unaffected by phenobarbital over the same period and, in co-cultures, there was no dye transfer between the two cell types. The connexin (Cx32) steady-state mRNA levels were unaffected by treatment of the primary rat hepatocytes with phenobarbital for 14 days, and there was no change in Cx32 protein levels (Ren & Ruch, 1996). A similar transient decrease in gap-junctional intercellular communication after a 1-h exposure to phenobarbital was observed by Mesnil *et al*. (1993) in rat hepatocytes co-cultured with BALB/c 3T3 mouse-embryo cells, but the decrease was maintained at 50% of control values during a 3-week treatment period. Later studies from this group indicated that the inhibitory action of phenobarbital on gap-junctional intercellular communication is cell-specific rather than connexin-specific. Thus, phenobarbital inhibited gap-junctional intercellular communication in hepatocytes, which express primarily *Cx32*, but not in WB-F344 rat epithelial cells, a highly communicating line that expresses *Cx43*, or in WB-aB1 cells, a gap-junctional intercellular communication-incompetent line derived from WB-F344 that still expresses *Cx43*, or in WB-a/32-10 cells, which are derived from WB-aB1 by stable transduction with a *Cx32* retroviral expression vector.

Cx32-deficient mice are resistant to liver tumour promotion by phenobarbital. Phenobarbital treatment led to an approximate fivefold increase in the volume fraction occupied by glucose-6-phosphatase-deficient liver lesions in *Cx32*$^{Y/+}$ mice, whereas there was no such increase in *Cx32*$^{Y/-}$ mice. Even more pronounced differences were observed with respect to tumour response, phenobarbital clearly promoting the occurrence of large hepatomas in Cx32-proficient but not in Cx32-deficient mice. These results demonstrate that functional Cx32 protein is required for tumour promotion with phenobarbital (Moennikes *et al*., 2000).

After oral administration of phenobarbital (50 mg/kg bw per day for up to 6 weeks) to Sprague-Dawley rats, a direct microinjection dye-transfer assay was carried out on fresh liver slices (0.5–0.7 mm thick). The average area of dye spread decreased after 1 week and stayed at the same level up to week 6. The area and number of Cx32 spots per hepatocyte in the centrilobular zones of the liver also decreased between week 1 and week 6, whereas there was no change in the spots in the perilobular areas. No changes were observed in Cx26 (Ito *et al.*, 1998). Using a higher dose (400 mg/kg bw per day for 5 weeks), Krutovskikh *et al*. (1995) observed nearly total disappearance from the plasma membrane of cells in the centrilobular region of both the principal hepatocyte connexin Cx32 and Cx26, while Cx43 protein and the expression of its mRNA were stimulated.

DNA sequence analysis of the H-*ras* gene in liver tissue from male B6C3F$_1$ mice treated with phenobarbital (0.05% in the drinking-water for 1 year) revealed a point mutation (AAA) in codon 61 (normal sequence CAA) in one of nine hepatocellular adenomas and in none of five hepatocellular carcinomas. When 50 liver tumours found in the control group were analysed, an activated H-*ras* gene was found in 15/18 hepatocellular adenomas and 10/14 hepatocellular carcinomas. The most frequent mutation in

these tumours was a CG → AT transversion (59%), which is probably the result of a polymerase error (Fox *et al.*, 1990). During DNA synthesis on a non-instructional template, the most frequent polymerase error involves the preferential insertion of an adenine, the so-called 'A rule' (Strauss *et al.*, 1982).

In C3H/He mice, Ha-*ras* codon 61 mutations occurred in 6/21 (29%) liver tumours from untreated mice, but in 0/15 (0%) tumours from mice given a diet containing phenobarbital at a concentration of 0.04–0.07% providing a dose of 85 mg/kg bw per day. The absence of mutations in codon 61 (or in codon 12) in the phenobarbital-treated mice suggests a tumorigenic mechanism different from that of spontaneous tumours. In contrast, codon 61 mutations were found in 19/46 tumours from mice treated with NDEA (Rumsby *et al.*, 1991). In a similar study, two of eight CF1 mouse liver carcinomas that occurred after prolonged exposure to diets containing 1000 mg/kg phenobarbital were shown to carry a CG → AT transversion in the Ha-*ras* gene. One of eight tumours from control mice also carried a codon-61 mutation (Bauer-Hofmann *et al.*, 1990).

The same group analysed the pattern of codon 61 mutations in the Ha-*ras* gene of glucose-6-phosphatase-deficient hepatic lesions of male C3H/He mice given a diet containing 500 mg/kg sodium phenobarbital for 52 weeks. Ha-*ras* mutations were found in 12/21 lesions (57%) from untreated mice and in 4/16 (25%) in the phenobarbital-treated group ($p < 0.01$, Fisher's exact test). The commonest mutation was a CG → AT transversion (eight in untreated mice, three in treated mice). The wild-type sequence, CAA, was present in all the enzyme-deficient lesions, and, when a mutation was present, the signals for the wild-type and the mutant sequences were very similar. This result suggested that both normal and mutant alleles were present in each cell of the lesions (Bauer-Hofmann *et al.*, 1992).

Tumours from C3H/He mice were also screened for *p53* mutations in exons 5, 7 and 8, which contain nearly all the mutations so far described. No *p53* mutations were found in any of eight tumours recovered from mice treated with phenobarbital (Rumsby *et al.*, 1994).

4.6 Mechanistic considerations

4.6.1 *Liver tumours*

The available evidence indicates that phenobarbital is generally not genotoxic, and genotoxicity does not appear to play a role in its hepatocarcinogenicity. In particular, DNA adducts have not been detected with ^{32}P-postlabelling methods in animals given doses that produce liver tumours.

Phenobarbital is a microsomal enzyme inducer and has been studied extensively for its ability to promote hepatic tumours. There is evidence that the microsomal enzyme induction is correlated with hepatic tumour promotion by phenobarbital. Rats and mice that develop tumours after treatment with phenobarbital show expression of

CYP enzymes, CYP2B1 and CYP2B2 being the most important. The potency for CYP induction in mice and rats also correlates with the degree of tumour promotion. Phenobarbital does not induce these enzymes in hamsters and does not produce liver tumours in this species.

The enhancement of hepatic tumorigenesis by phenobarbital was shown to be due to tumour promotion rather than a syncarcinogenic effect. In an initiation–promotion model, phenobarbital administered before or with a carcinogen produced no tumours, whereas repeated exposure to phenobarbital after the carcinogen produced tumours.

Although the mechanisms of tumour promotion are not completely known, effects on the control of cell proliferation appear to play a role. Long-term exposure of rodents to phenobarbital produces hepatomegaly and hepatocellular hypertrophy and hyperplasia. Several studies have demonstrated a transient increase in DNA synthesis in normal hepatocytes. In initiation–promotion models, phenobarbital selectively increased the labelling index in foci as compared with normal surrounding liver. The foci progress to the stage of hepatocellular adenoma, in which cellular proliferation no longer depends on the presence of phenobarbital.

The mitogenic and tumour-promoting effects of phenobarbital appear to involve changes in growth factors, intracellular communication, gene expression and cell cycle signal transduction. These can mediate the effects of phenobarbital, including the transient increase in DNA synthesis, the selective mitogenic effects in foci and inhibition of apoptosis.

In addition, there are marked species and strain differences in susceptibility to hepatic tumour promotion by phenobarbital, which is genetically determined and heritable.

Overall, the experimental evidence supports the conclusion that the mode of action of phenobarbital in the production of hepatic tumours is non-genotoxic and involves tumour promotion.

4.6.2 *Thyroidal effects*

Although phenobarbital has not been shown to produce thyroid gland tumours in a bioassay for carcinogenicity, it has been shown to promote these tumours after administration of a carcinogen (NBHPA).

Phenobarbital is a microsomal enzyme inducer that increases the hepatic activity of thyroxin-UGT and alters thyroid function by enhancing the peripheral disposition of thyroid hormones. Phenobarbital-treated rats show decreased serum concentrations of thyroid hormones, increased concentrations of TSH, increased thyroid gland weights and follicular-cell hypertrophy and/or hyperplasia.

The tumour-promoting effects of phenobarbital have been shown to be mediated by increased pituitary secretion of TSH as a compensatory response to increased hepatic disposition of thyroid hormone, as opposed to a direct tumour-promoting or carcinogenic effect in the thyroid follicular epithelium.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

Phenobarbital and its sodium salt have been very widely used as a mild sedative or hypnotic in the treatment of neuroses and in pre- or post-operative sedation, and as an anticonvulsant in the treatment of epilepsy. Phenobarbital was introduced in 1912. Its use has decreased since the 1960s, but it is still produced worldwide and used extensively.

5.2 Human carcinogenicity data

Three large follow-up studies of cancer, two of incidence and one of mortality, from Denmark, England and the USA of patients treated primarily with phenobarbital for epilepsy showed an occurrence of brain cancer higher than expected. However, in the two incidence studies, the excess numbers of cases of brain cancer occurred within 10 years of hospitalization and decreased significantly over time. This inverse relationship between excess risk and time since hospitalization for epilepsy suggests that the brain tumours of some of the patients were the cause of their seizure disorder and that the association between use of phenobarbital and brain cancer is not causal. The finding in a small case–control study from the USA of an increased risk for brain tumours after prenatal exposure to phenobarbital was not confirmed in a larger case–control study, also from the USA, or in a cohort study from Denmark of transplacental exposure to phenobarbital and other anti-convulsants.

Of the three cohort studies of epilepsy patients, two showed a significant increase in the relative risk for lung cancer, with no clear pattern of risk with length of follow-up. One showed a non-significant increase. Dose–response analyses in a nested case–control study of lung cancer in the largest of the cohort studies (in Denmark) revealed no consistent relationship between lung cancer and cumulative exposure to phenobarbital. A survey among the controls indicated a higher-than-average prevalence of smoking.

After exclusion from the largest of the cohorts of epilepsy patients known to have received radioactive Thorotrast during cerebral angiography, a slight, non-significant increase in risk for primary liver cancer was seen. However, a nested case–control study of liver cancer with adjustment for other anti-convulsant therapy revealed no association with phenobarbital treatment. No cases of liver cancer were seen in the other two cohort studies, from England and the USA.

In the cohort study in Denmark, the observed number of cases of thyroid cancer was close to that expected in the general Danish population. In the same study, a statistically significant deficit of urinary bladder cancer was noted, which was shown in an analysis of the dose–response relationship to be inversely related to use of phenobarbital.

Use of phenobarbital, mostly as a sedative, was associated with moderately increased risks for cancers of the lung, ovary and gall-bladder in a cohort study based on a prepaid medical care programme in the USA.

5.3 Animal carcinogenicity data

The carcinogenicity of phenobarbital was investigated by oral administration in multiple studies in mice and several studies in rats. Phenobarbital consistently produced hepatocellular adenomas and carcinomas in mice. Hepatocellular adenomas were produced in rats after lifetime exposure in one study. Oral administration of phenobarbital in combination with known carcinogens resulted in the enhancement or inhibition of effects, depending on the carcinogen and the time of administration. In several experiments in mice and rats, sequential exposure to phenobarbital with known carcinogens enhanced the incidences of hepatocellular preneoplastic foci, adenomas and carcinomas. In two studies each, phenobarbital was found to promote liver carcinogenesis in patas monkeys but not in hamsters. Phenobarbital promoted thyroid follicular-cell tumours in one study in mice and in several studies in rats.

5.4 Other relevant data

Most of an administered dose of phenobarbital in humans was excreted in urine. The major urinary excretion products include unmodified phenobarbital, *para*-hydroxyphenobarbital, phenobarbital-*N*-glucoside and phenobarbital *para*-glucuronide. *para*-Hydroxyphenobarbital can be formed by direct hydroxylation of phenobarbital.

CYP2B1 and CYP2B2 are the primary members of the cytochrome P450 (CYP) superfamily of enzymes that are induced by phenobarbital *in vivo*. Although phenobarbital causes large increases in the activity of these enzymes in liver, the metabolism of phenobarbital itself is not increased. Phenobarbital has also been found to induce the activities of other CYP enzymes, including benzo[*a*]pyrene hydroxylase, UDP-glucuronosyl transferase and several glutathione-*S*-transferases. 'Phenobarbital-like induction' describes the effect on liver hepatocyte CYP enzymes of various compounds, including sedatives, pesticides and other compounds that induce a similar spectrum of isozymes.

Cell proliferation is initially stimulated by phenobarbital in normal hepatocytes and lasts a few days. It may even be inhibited by down-regulation of epidermal growth factor receptors. Phenobarbital exerts a selective and sustained mitogenic effect in cells of altered foci that progress to adenomas that are no longer dependent on the mitogenic effects of phenobarbital.

The biochemical mechanisms underlying enhancement of cell proliferation and tumour promotion by phenobarbital may involve alterations in gene regulation. The dose–response relationship for microsomal enzyme induction is similar to that for tumour promotion. Consequently, changes in gene regulation that presumably lead to mitogenesis and up-regulation of growth factors parallel the induction of CYPs.

Phenobarbital has also been shown to inhibit intercellular communication in hepatocytes, which could impede the transmission of growth control signals between normal and altered hepatocytes.

Owing to its effects on the induction of microsomal enzymes, phenobarbital enhances the hepatic disposition of thyroid hormone. The promotion of thyroid gland tumours in rats by phenobarbital has been shown to be mediated by increased secretion of pituitary thyroid-stimulating hormone as a compensatory response to increased thyroid hormone glucuronidation and biliary excretion.

Phenobarbital is a teratogen and developmental neurotoxicant in humans and experimental animals. Exposure of rats *in utero* induces long-term effects on hepatic drug-metabolizing enzymes. Neuroendocrine effects on reproductive function have been noted in exposed adult male rats and female hamsters.

Phenobarbital did not induce sister chromatid exchange in patients with epilepsy receiving only this drug.

In studies in which rodents were exposed to phenobarbital *in vivo*, no covalent binding to mouse liver DNA was observed, but the frequency of alkali-labile damage in mouse liver cells was increased. Gene mutation was not induced in a transgenic mouse strain, and sister chromatid exchange, micronuclei and chromosomal aberrations were not induced in mouse bone-marrow cells. Phenobarbital did not increase the frequency of sperm-head abnormalities in mice, but spermatogonial germ-cell chromosomal aberrations were reported in male mice in one laboratory. Further increases in the frequency of chromosomal aberrations were found in liver foci cells of mice treated with phenobarbital after previous treatment with a genotoxic agent.

Chromosomal aberrations but not gene mutations were induced in cultured human lymphocytes.

Tests for the genetic effects of phenobarbital *in vitro* are numerous and include assays for DNA damage, DNA repair induction, gene mutation and chromosomal aberrations in mammalian cells, tests for gene mutation and mitotic recombination in insects and fungi and tests for gene mutation in bacteria. Although the majority of the test results were negative, the numerous positive results cannot be ignored, even though they do not present a consistent pattern of genetic toxicity. The inconsistency of the results, the absence of any direct evidence of an interaction with DNA and the generally negative in-vivo data lead to the conclusion that phenobarbital is not genotoxic.

Phenobarbital transformed hamster embryo cells. It inhibited gap-junctional intercellular communication in hepatocytes of rats treated *in vivo* and in primary cultures of hepatocytes from rats and mice but not (in a single study) in primary cultures of human or rhesus monkey hepatocytes.

5.5 Evaluation

There is *inadequate evidence* in humans for the carcinogenicity of phenobarbital.

There is *sufficient evidence* in experimental animals for the carcinogenicity of phenobarbital.

Overall evaluation

Phenobarbital is *possibly carcinogenic to humans (Group 2B).*

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