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Liver Tumors in Rodents: Extrapolation to Man

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I. Introduction

Man is not the species of choice for studies in the field of experimental pathology. He's too big. He costs too much to feed. He is expensive to house and maintain. And he seems bent on actively defying any attempt to carry out a controlled experiment on him as a member of his species. However, the greatest drawback to the use of man as an experimental model is that, although men kill other men freely for political purposes, humans as a species have evolved a thought process known as "ethics" which proscribes interim sacrifice at planned time points in long-term observational studies. Consequently, it is very difficult to trace the origins and pathogenesis of any eventually neoplastic lesion in man and equally difficult to chart the occurrence, persistence, progression, or regression of putatively precancerous lesions.

In the light of these serious limitations, had the second part of the title of this chapter had been "extrapolation *from* man," I would have had to say that man is an extremely inappropriate model for the prediction of liver tumor risk in rats and mice. In fact, there are only a few examples of agents (e.g., steroids, vinyl chloride) which give rise to liver tumors of similar kinds in rodents and humans. Otherwise there is seemingly little overlap between the spectrum of factors which contribute importantly to the causation of liver neoplasia in man and that of factors which do so in rodents. Moreover, whereas liver neoplasia is a rare disease in westernized man, it is relatively common in rodents generally, and actually reaches an incidence of 100% "spontaneously" in some strains of mice.

In this chapter, I propose to start by distinguishing between various types of hepatic neoplasia. Next I will discuss the factors known to be associated with increased risk of liver neoplasia as a human disease, and the extent to which it is known that the same factors have a similar effect in laboratory animals. Finally, against this background, I will consider situations where there are data indicating that a substance possesses hepatocarcinogenic activity for laboratory rodents, but where there are no comparable data for humans.

II. Different Kinds of Liver Tumors

The liver consists mainly of five kinds of cells: (1) parenchymal (also called liver cell), (2) bile duct, (3) blood vessel, (4) reticuloendothelial (called Kupffer cell), and (5) connective tissue.

Each of these cell types may be the origin of benign or malignant tumors. The criteria for distinguishing between benign and malignant neoplasia are considered below. Hepatocellular adenomas (benign) and hepatocellular carcinomas (malignant) arise in parenchymal cells; cholangiomas (benign) and cholangiocarcinomas (malignant) arise in bile duct cells; angiomas or hemangiomas (benign) and angiosarcomas or hemangioendotheliomas (malignant) arise from blood vessel cells; Kupffer cell sarcomas (malignant) arise in reticuloendothelial cells, and sarcomas (malignant) arise in connective tissue cells.

In this chapter we are mainly concerned with tumors arising from parenchymal cells, bile duct cells, and blood vessel cells.

III. Etiological Factors for Hepatic Neoplasia in Man and the Availability of Animal Models

A. Hepatitis B Virus

Although primary liver cancer is relatively rare in Europe and North America, it occurs commonly in certain parts of Asia and Africa. In the areas of high incidence there is also a high incidence of viral hepatitis B infection. There is good evidence that chronic carriers of the hepatitis B virus are prone to develop macronodular cirrhosis and that this tends to progress to hepatocellular carcinoma. The detection of the hepatitis B virus genome both in the DNA of liver cells of carriers and in the DNA of hepatocellular carcinomas (Shafritz and Kew, 1981; Prince, 1981) provides strong evidence of the involvement

of the virus in the etiology of the neoplasia. If the virus is vertically transmitted from a mother who is a chronic carrier to her child, then the risk of the child developing a hepatocellular carcinoma is especially high. Furthermore, if the father is negative for surface antibodies to the virus (indicating that he is immunologically defective), then the risk of the child developing a liver cancer is even higher (Larouze *et al.*, 1976).

There is no known parallel for this form of viral hepatocarcinogenesis in rats or mice. However, a form of viral hepatocarcinogenesis in the woodchuck may be a good model. In this species, the introduction of a virus closely resembling the hepatitis B virus leads to the development of chronic viral hepatitis which progresses to macronodular cirrhosis and eventually to hepatocellular carcinoma (Summers, 1981; Johnson and Williams, 1981).

B. Steroids

The occurrence of liver cell tumors, mostly benign and amenable to surgical excision, but occasionally fatal because of intraperitoneal hemorrhage or inoperable malignancy, is well documented for women taking various forms of contraceptive pills (Baum *et al.*, 1973; Neuberger *et al.*, 1980). Similar hepatic neoplasms have been reported in humans exposed to androgens such as oxymethalone and methyltestosterone (Johnson *et al.*, 1972; Farrell *et al.*, 1975; Sweeney and Evans, 1976).

In mice, several contraceptive pill formulations have been found to enhance the incidence of liver cell tumors (Committee on Safety of Medicines, 1972). In most strains or mice, liver tumors arise spontaneously more frequently in males than in females. Castration and the administration of estrogens reduces liver tumor incidence in males while ovariectomy and the administration of androgens increases liver tumor incidence in females (Agnew and Gardner, 1952; Andervont, 1950).

C. Aflatoxin B_1 and Other Aflatoxins

Aflatoxin B_1 and related aflatoxins derived from the mold *Aspergillus flavus* are potent hepatotoxins for many different species (Lancaster *et al.*, 1961; Kraybill and Shimkin, 1964). Even at a level of only 1 μg/kg diet aflatoxin B_1 has been reported to give rise to liver tumors in the rat (Wogan *et al.*, 1974). Aflatoxin B_1 has also been found to cause liver tumors in the rainbow trout (Sinnhuber *et al.*,

1968), in salmon (Wales and Sinnhuber, 1972), and in a few primates (Adamson *et al.*, 1973; Reddy and Svoboda, 1975). Against this background of response in various species, it is, a priori, to be expected that aflatoxin B_1 is a liver carcinogen for man. However, the evidence that this is so is fundamentally no more than circumstantial. Thus, although high levels of aflatoxin have been found in food in geographical areas where liver cancer in humans is common (Linsell, 1978), there is no compelling supportive evidence, as there is in the case of the hepatitis B virus, that the association is causal.

Curiously, although it is easy to produce liver tumors in rats by administering aflatoxins to them by the oral route, mice are resistant to liver tumor induction in this way. Wogan (1969) failed to produce liver tumors either in random-bred or inbred mouse strains by feeding aflatoxin B_1 at a level of 1 mg/kg in the diet. However, Vesselinovitch *et al.* (1972) produced liver tumors in 80% of (C57BL × C3H)F_1 hybrid mice by administering aflatoxin B_1 by the intraperitoneal route during the first 7 days of life.

D. Alcoholic Cirrhosis

An obsession with sin and its consequences has long had the effect of making the theory that cirrhosis due to an excessive intake of alcohol predisposes to primary liver cancer appealing to puritans. Nevertheless, the evidence that this is a common sequence of events is not robust. If all forms of cirrhosis predispose equally to cancer, one might expect there to be a similar relationship between the incidences of cirrhosis and liver cancer in geographically different areas. But this is not so. In certain areas of South Africa, non-Caucasians who develop one or other form of cirrhosis have a 40–50% risk of developing a liver cancer (Thompson, 1961), whereas the comparable figure for Chicago in the United States is only 5% (Stuart, 1965).

There are, in fact, several different varieties of cirrhosis. The form most associated with increased liver cancer risk is the *postnecrotic* or *macronodular* type, whereas the type most commonly associated with alcoholism is the *hobnail* or *finely nodular* type, sometimes referred to as nutritional cirrhosis (Lee, 1966).

Historically, confusion has arisen because alcoholic beverages, particularly when prepared by primitive methods from diseased crops in hot and humid climates, are apt to be contaminated with true carcinogens (e.g., mold toxins, nitrosamines).

Overall, it seems that the risk of liver cancer development in persons who develop nutritional cirrhosis because of an excessive intake

of alcohol per se is not very high, unless these persons are also chronic carriers of hepatitis B virus and/or are additionally exposed to some liver toxin, such as aflatoxin. The variation in distributions of cirrhosis and primary liver cancer between the different social classes in England and Wales (see below) are consistent with this conclusion. On the other hand, according to Arrigoni *et al.* (1985), in Italy, hepatocellular carcinoma occurs as commonly in patients with alcoholic cirrhosis who are not infected with hepatitis B virus as in those that are.

In view of the fact that the association between exposure to alcohol and increased liver cancer risk is no more than weak in man, it is not perhaps surprising that cirrhosis and liver tumors are most definitely not responses that are seen in laboratory animals as a consequence of exposure to ethyl alcohol.

Numerous investigators have exposed laboratory animals to high daily doses of ethanol over long periods with very little evidence of adverse effect as far as the liver is concerned. Thus, Ketcham *et al.* (1963) exposed CDBA/2F_1 female mice for up to 15 months to 20% (v/v) ethanol instead of drinking water. This treatment had no effect on longevity, primary tumor incidence at any site, or the growth or spread of tumor implants. Moderate fatty infiltration of liver parenchymal cells was seen in the livers of animals killed after 1 year's treatment, but this change partly regressed during a subsequent alcohol-free period. Cirrhosis was not seen. Kuratsune *et al.* (1971) saw no liver tumors among 108 male and 42 female CF_1 strain mice provided intermittently with 43% aqueous solution of ethanol instead of drinking water and observed for up to 34 months. The same investigators also saw no liver tumors in 100 male ddN strain mice given a 19.5% aqueous solution of ethanol intermittently instead of drinking water and observed for up to 22 months.

Schmähl (1976) maintained Sprague–Dawley rats on 30 ml/kg 25% aqueous ethanol in drinking water on 5 days per week for up to 780 days without producing any evidence of hepatotoxic activity or any liver tumors. Earlier, Gibel (1967) exposed 40 Sprague–Dawley rats to 0.5 ml 30% (w/v) ethanol once daily for up to 20 months. Apart from slight liver changes in 10% of the animals after 6 months of treatment, no adverse effects on the liver were encountered.

Herrold (1969) gave 0.5 ml 50% ethanol twice weekly by mouth to five male and five female hamsters for a period of 10–11 months. She then followed the animals for life (average 21 months of age) but observed no adverse effects on the liver.

Hollander and Higginson (1971) gave 10% aqueous ethanol instead of drinking water to 19 male and 33 female mastomys for 2 months

and then increased the concentration to 20% for the remainder of their life span (up to 30 months). The incidence of malignant carcinoid tumors of the stomach, to which this species is prone, was not adversely affected by the exposure to ethanol and none of the treated animals developed primary tumors of the liver.

In reviewing the weakness of the association between alcohol consumption and liver cancer risk in man and the lack of any evidence for such an association in laboratory animals, I should make it clear that my comments do not necessarily apply to the more substantial evidence for a causal association between alcohol consumption and risk of cancer development at other sites (e.g., head and neck) in man (Maclure and MacMahon, 1980; Tuyns, 1979). However, in relation to these forms of cancer, also, the question of whether the association between these cancers and alcohol consumption is indicative of carcinogenesis by alcohol *per se,* or by contamination of alcoholic beverages by carcinogens, or by some other mechanism, needs to be seriously addressed.

E. Vinyl Chloride

Heavy exposure to vinyl chloride is associated with an increased risk of angiosarcoma of the liver in humans, rats, and mice (Creech and Johnson, 1974; Maltoni, 1977). Vinyl chloride is a genotoxic agent, and the assumption is that the liver tumors are a direct consequence of this activity.

F. Thorium Dioxide

Thorotrast (thorium-232 dioxide) was at one time used as a radiographic contrast medium to outline body cavities such as the renal pelvis or for the visualization of blood vessels. Once introduced into the tissues, thorium is taken up by reticuloendothelial cells throughout the body, including the Kupffer cells of the liver. These cells thereafter become sources of radiation with which they bombard surrounding cells and thereby increase the risk of their mutation to cancerous cells. Thus, patients who have received thorotrast are at increased risk for developing various kinds of primary liver cancer. Boyd *et al.* (1968) reported 3 cases of cholangiocarcinoma arising in intrahepatic bile ducts and 1 case of hepatic hemangioendothelioma among 109 patients who survived for at least 1 year after receiving thorotrast. Numerous other anecdotal cases of primary liver cancer arising following thorotrast administration are to be found in the literature (e.g., MacMahon *et al.,* 1947; Nettleship and Fink, 1961; Stemmermann, 1960).

Doubtless the liver would be among the sites for the development of neoplasms in rats and mice were a properly designed study which mimicked human exposure to thorotrast undertaken. Unfortunately, no such study has been reported in the literature in either of these species. On the other hand, Swarm *et al.* (1962) reported hepatic hemangioendotheliomas in two of three female rabbits given intravenous thorotrast.

IV. Mortality from Primary Liver Cancer in England and Wales

In 1984 there were 479 deaths in males and 231 deaths in females from primary cancer of the liver [International Classification of Diseases, ninth revision (ICD, No. 155.0)]. Corresponding figures in 1974 (311 deaths in males, 196 in females) and in 1964 (265 deaths in males, 164 in females) showed that there seems to have been some increase in both sexes. There were also a further 148 deaths in each sex from cancer of intrahepatic bile ducts in 1984 (ICD 155.1), a figure markedly higher than the 21 male and 34 female cases recorded in 1974. Earlier figures for cancer of this site are not available. In 1984 there were also 86 deaths in males and 87 deaths in females where the cancer was not specified as primary or secondary (ICD 155.2). According to Case (1956), the age-standardized mortality from cancers of the liver and gallbladder in England and Wales fell in both men and women belonging to successive quinary–quinquennial cohorts with birth dates centered on 1871, 1881, 1891, and 1901. The contrast with the more recent trends may reflect the changing pattern of alcohol consumption over this century with a sharp decline from the high levels at the beginning of the century, followed by a marked rise over the last two or three decades.

In addition to deaths diagnosed as primary liver, there were also in 1984 a further 1210 deaths in males and 1070 in females classified as of chronic liver disease of cirrhosis (ICD 571). These also showed an increase over the corresponding figures for 1974 (901 deaths in males and 853 in females) and in 1964 (657 deaths in males and 652 in females). Of the 1984 deaths, the major contributions were from alcoholic cirrhosis of the liver (ICD 571.2, 435 male and 242 female deaths), cirrhosis with no mention of liver (ICD 571.5, 517 male and and 409 female deaths), alcoholic liver damage unspecified (ICD 571.3, 101 male and 70 female deaths), and biliary cirrhosis (ICD 571.6, 28 male and 182 female deaths), but it is not possible to study trends in

these due to changes in the ICD classifications used in compiling the mortality data. Unfortunately, the death certificate data from which these totals were compiled are not detailed or reliable enough to throw useful light on the relationship between liver cancer and cirrhosis. In any event, it is clear that the liver is the primary site of only a very small proportion of fatal neoplasms in humans in England and Wales: deaths in ICD 155.0, 155.1, and 155.2 combined formed only 1179 of a total of 140;101, about 0.8%.

According to the Registrar General's decennial supplement on Occupational Mortality in England and Wales for 1970–1972 (Registrar General, 1978) for men aged 15–64, there is little difference between social classes I–III in risk of death from primary liver cancer, but for men in semiskilled occupations (social class IV), the standardized mortality ratio (SMR) is slightly increased, while for men in unskilled occupations (social class V), the 61 deaths that were observed were over 50% higher than the 39 expected for men as a whole. By comparison, the SMR for men in the same age group for cirrhosis of the liver is highest in social class II (almost 150), second highest in social class V (120), and lowest in social classes IIIB and IV. Obviously, there is a very poor relationship between the distribution of deaths for, on the one hand, liver cancer and, on the other hand, cirrhosis between the social classes. These data are consistent with the conclusion reached earlier that the association between alcoholic cirrhosis and risk of developing primary liver cancer is relatively weak.

V. Factors Other Than Test Chemicals Which Influence the Risk of Hepatic Neoplasia in Laboratory Rodents

Tumors originating in the various kinds of liver cells are commonly found in laboratory rats and mice which have not been deliberately exposed to any potentially carcinogenic agent. The causation of these apparently "spontaneously arising" tumors in rats and mice is no less a mystery than the causation of many kinds of cancer in man. However, a number of factors which influence the incidence of these "spontaneous" tumors have been identified, and it is important to consider and discuss these factors for two reasons. First, it is well recognized that in laboratory animals it is easier to increase the incidence of tumors of kinds that occur "spontaneously" in high incidence than that of tumors that occur "spontaneously" only in low incidence. This suggests that the primary and most important causal factor of high-incidence tumors may already be present in the test system. This being

so, a variety of additional relatively weak stimuli may, perhaps nonspecifically, promote the "germination" of tumors, the seeds of which are already present in the test system. Second, if it is known that certain nonspecific factors (e.g., calorie intake, fat intake, sex hormone status; see below) influence liver tumor risk, then one must expect that test materials which bring about changes in the status of animals with respect to these factors will also, indirectly, affect liver tumor risk.

Before we consider nonspecific environmental factors, however, we need briefly to mention genetic influences including male–female differences in incidence.

A. Genetic Constitution

Different inbred strains of mice have remarkably different incidences of "spontaneous" liver tumors, with some strains (e.g., C3H) exhibiting a lifetime expectation of developing one or more parenchymal cell tumors of up to 100% in both sexes and other strains exhibiting an almost zero lifetime incidence (Andervont, 1950; Grasso and Hardy, 1975). Some of the exceptionally high liver tumor-susceptible strains were, in fact, purposely developed by selective inbreeding. However, even wild house mice bred in captivity are not free from liver tumor risk. Andervont and Dunn (1962) reported a 3.5% incidence of hepatomas in female house mice living to a mean age of 30 months and a 9% incidence of males living to a mean of 23 months. These incidences are much higher than for humans living in Europe or North America.

Strain differences in spontaneous liver tumor risk are also evident in rats but are less well documented. In general, the very high incidences of "spontaneous" liver cell tumors found in some strains of mice are not encountered in rats, although I have seen incidences as high as 16% in untreated male and 29% in untreated female rats of a Wistar strain.

B. Sex

In most strains of mice, males are more susceptible to the spontaneous development of liver tumors than females. Furthermore, manipulation of hormonal status (e.g., by ovariectomy and/or androgen administration in females and by orchidectomy and/or estrogen administration in males) affects the risk of spontaneous liver tumor development in the direction of a higher risk being associated with increase in masculinity (Agnew and Gardner, 1952).

In the case of most strains of rats, the sexes are seemingly more or

less equal in their chances of developing liver cell tumors "spontaneously," with females tending to be slightly more at risk (e.g., Goodman *et al.*, 1979).

C. Dietary Intake: Effects of Overnutrition and Fat Intake in Mice

It has long been known from the classical studies of Tannenbaum and Silverstone that the risk of development of many kinds of neoplasms in mice is influenced by the composition of the diet and by caloric intake. Among the kinds of neoplasms influenced by dietary intake and the composition of the diet (e.g., levels of casein) is the liver cell tumor of mice. Tannenbaum (1940, 1947) suggested that diet restriction may act to reduce liver tumor incidence in mice via a hormonal mechanism. This theory was supported by the observation of Heston (1963) that the occurrence of hepatomas in the highly susceptible (C3H × YBR)F_1 male mouse was completely inhibited by hypophysectomy and also by the finding in the same strain of mice by Rowlatt *et al.* (1973) that diet restriction without endocrine ablation inhibited liver tumor risk.

Diets containing 18% or 45% casein have been found to lead to higher incidences of liver cell tumors in mice of either sex than a diet containing only 9% casein (Tannenbaum and Silverstone, 1949). The effect was observed irrespective of whether the animals were fed *ad libitum* or isocalorically.

The concentration of fat in the diet may have an even more dramatic effect on the "spontaneous" incidence of liver cell tumors in mice (Sokoloff *et al.*, 1960). In one study by Gellatly (1975), when the percentage of ground nut oil incorporated into a semisynthetic diet fed to C57BL mice was increased from 5% to 10%, survival to 80 weeks decreased but the percentage of survivors exhibiting benign or malignant liver cell tumors increased dramatically, particularly in females (see Table I).

More recently, Conybeare (1980), in a large study on random-bred Swiss mice, compared the effects of *ad libitum* feeding of two standard laboratory diets with those of restricting animals to only 75% of the food consumed by the *ad libitum*-fed animals. He recorded consistent beneficial effects of diet restriction on both survival and percentage of survivors which bore tumors. The reduction in tumor incidence was most clearly evident for sites in which the "spontaneous" tumor incidence is normally high in the strain of Swiss mice used for the study. The liver was one of these sites. Table II summarizes the data. Sur-

TABLE I

EFFECT OF CONCENTRATIONS OF GROUND NUT OIL ON INCIDENCE OF LIVER CELL TUMORS IN C57BL MICE[a]

Parameter	Sex			
	♂	♂	♀	♀
Ground nut oil in diet (%)	5	10	5	10
Number of mice	80	105	80	105
Survivors to 80 weeks (%)	80	67	93	78
Survivors with one or more histologically "type 2" liver nodules (%)	8	16	7	34
Survivors with one or more histologically malignant liver cell tumors[b] (%)	3	1	1	9

[a]Data from Gellatly (1975).

[b]Gellatly includes both hyperplastic and benign neoplastic lesions within his category of "type 2 nodules."

vival was consistently better in restricted compared with *ad libitum*-fed animals in both sexes and for both of the diets. When the tumor incidence data for the two sexes and for both the diets were combined, the effect of diet restriction in reducing tumor incidence in survivors was highly statistically significant (survivors with one or more neoplasm at any site, $p < .001$; survivors with malignant neoplasm at any site, $p < .01$; survivors with one or more benign or malignant liver cell tumors, $p < .0001$).

These impressive effects of dietary intake on the incidence of liver cell tumors in mice are, in fact, much larger than those of some test chemicals which have come to be labeled as carcinogens as a consequence of enhancement of liver tumor incidence in mice. The situation would, thus, seem to be wide open for the generation of both false-positive and false-negative results. A true liver carcinogen that reduces appetence might theoretically reduce overall tumor incidence, and even specifically liver tumor incidence, more by reducing food intake than it increases it because of its hepatocarcinogenic activity. Alternatively, a test chemical that increases appetence and food intake (e.g., sucrose—Hunter *et al.*, 1978) may increase liver tumor incidence nonspecifically. Another potential problem relates to the use of oily vehicles in carcinogenicity tests involving exposure via gavage. Oil given in this way may profoundly alter the nutritional status of

TABLE II

EFFECT OF DIET RESTRICTION ON SURVIVAL, INCIDENCE OF TUMORS AT ANY SITE, AND ON LIVER TUMOR INCIDENCE IN SWISS MICE[a]

	Diet 1 (PRD)[b]				Diet 2 (41B)[b]			
	♂		♀		♂		♀	
	AL	75%	AL	75%	AL	75%	AL	75%
Number of mice	80	80	80	80	80	80	80	80
Survival for 18 months (%)	60	69	60	80	56	64	64	74
Survivors with one or more neoplasms of any kind (%)	40**[c]	15	25**	6	62**	31	27	14
Survivors with one or more malignant neoplasms of any kind (%)	8	2	6	2	9	2	12	5
Survivors with one or more liver cell tumors (%)	23**	4	4	2	47***	12	6	0

[a]From Conybeare (1980).
[b]AL, *Ad libitum;* 75%, 75% of *ad libitum.*
[c]**AL > Restricted, $p < .01$; ***AL > Restricted, $p < .001$.

animals. It is very easy for experimentalists to forget that a dose of 0.1 ml oil to a 25-g mouse is equivalent, on a body weight basis, to a dose of over 250 ml oil to an adult human!

It should not, however, be assumed that excessive caloric intake is the sole determinant of the effect of overnutrition in increasing liver cell tumor incidence in mice. In rats, overnutrition causes a wide spectrum of endocrine imbalances. It is possible, therefore, that the effect of overnutrition on liver tumor risk in mice is hormone-mediated. The fact that male mice are more susceptible than females to the "spontaneous development" of liver cell tumors is consistent with there being a hormonal influence on liver tumor risk. Also of possible importance is the fact that, under conditions of diet restriction, animals spend some part of each day with an empty, bacteriologically sterile stomach and small intestine, whereas under conditions of continuous

availability of food, there may be no period of the day in which upper gastrointestinal bacterial sterility exists.

At present, the precise explanation of how overnutrition enhances liver tumor risk in mice remains unclear and in urgent need of elucidation.

D. Partial Hepatectomy, Necrosis, and Regeneration

In rats, carefully timed partial hepatectomy enhances liver tumor incidence where there is concomitant exposure to known liver carcinogens (Craddock, 1977; Tatematsu *et al.*, 1977). Whether the same is true for mice and other species has not been adequately researched.

In mice, there is considerable circumstantial evidence for a threshold dose level for enhancement of liver cell tumor risk from exposure to nonmutagenic hepatotoxins (such as carbon tetrachloride, chloroform, and selenium), which relates to the dose required to cause repeated cycles of liver cell necrosis followed by regeneration (Edwards and Dalton, 1942; Eschenbrenner and Miller, 1945; Reitz *et al.*, 1980; FDA, 1974; Jorgenson *et al.*, 1985).

Other work suggests that liver cell injury which is not severe enough to result in necrosis may also contribute to liver cell tumor risk. This sequence of events has been described for nonmutagenic chemicals such as Ponceau MX and safrole (Crampton *et al.*, 1977; Grasso and Gray, 1977; Grasso, 1979).

E. Liver Enlargement Associated with Disturbed Lysosomal Pattern and Peroxisomal Proliferation

Some, but not all, hypolipidemic and porphyria-inducing agents and the phthalates cause enlargement of the liver and ultrastructural evidence of lysosomal disturbance and peroxisomal proliferation, but no overt evidence of liver cell damage in short-term tests. In the long term these same, nonmutagenic agents enhance liver tumor incidence in rats (Cohen and Grasso, 1981; De Matteis, 1978; NTP, 1982). More information with regard to the nongenotoxic mechanism involved in liver tumorigenesis by agents which cause these ultrastructural changes is needed. However, one fact seems clear: mere liver enlargement by itself is of limited value for the prediction of increased tumor risk. In the case of agents which stimulate peroxisomal proliferation,

liver enlargement is associated with increased risk of subsequent liver tumor development. In the absence of significant increased liver weight, liver tumor risk is not enhanced by such agents. On the other hand, the rodent liver increases two- or threefold during pregnancy as a physiological adaptive change (Wilson *et al.*, 1970). This enlargement is not associated with increased liver tumor risk.

F. The Role of Increased Metabolic Activity and/or Cell Turnover

The link between the several nongenotoxic factors which enhance liver cell tumorigenesis may simply be an increased rate of metabolic activity within liver cells and/or an increased rate of cell turnover. During ordinary metabolic processes numerous electrophilic metabolites capable of damaging cell proteins including DNA are formed. Overnutrition and various forms of metabolic stress may lead to increased cellular and nuclear damage as a result of increased production of endogenously generated electrophiles (Ames, 1983). Also, or alternatively, if there is a risk of genetic error during cell replication, then the rate of accumulation of such errors in liver cells is likely to depend on the rate of liver cell replication. In this way, nongenotoxins which nonspecifically enhance some aspect of cellular metabolic activity and/or the rate of cell turnover may indirectly predispose to the accumulation of genetic damage (i.e., because they increase free-radical production), leading to increased cancer risk. Relevant to this theory is the fact that increase in peroxisome numbers is associated with inceased hydrogen peroxide-generating oxidases and long-chain fatty acid oxidation enzymes (Reddy and Krishnakantha, 1975; Osumi and Hashimoto, 1979). High-fat diets also give rise to peroxisomal proliferation with increased peroxisomal β-oxidation (Ishii *et al.*, 1980; Neat *et al.*, 1980).

VI. The Significance of Enzyme-Altered Foci in the Pathogenesis of Hepatocellular Neoplasia

During recent years, there has been an increasing interest in the observation that agents which give rise to liver cell tumors in rodents in the long term often give rise to a wide variety of enzyme-altered liver cell foci in the short term (Gossner and Friedrich-Freksa, 1964; Friedrich-Freksa *et al.*, 1969a,b; Schauer and Kunze, 1968; Schiefer-

stein *et al.*, 1974; Scherer and Emmelot, 1976; Sirica *et al.*, 1978). Among the enzyme alterations most studied are (1) absent glucose-6-phosphatase, (2) absent ATPase, (3) diminished glycogen phosphorylase, (4) elevated arylesterase, (5) elevated β-glutamyl transpeptidase, (6) elevated epoxide hydrolase. A real insight into the nature of these localized liver changes might throw useful light not only on the pathogenesis of hepatocellular neoplasia but also, more generally, on the mechanisms of all forms of carcinogenesis. It is rightly or wrongly assumed that the foci represent clones of altered cells with each clone being derived from a single altered cell. The first question, therefore, concerns the nature of the cellular alteration. Is it a consequence of a change in the genetic information within the cell or merely a phenotypic expressional change (Pitot *et al.*, 1974)? The fact that liver cells look alike under the microscope does not necessarily mean that they are functionally identical. Do islands arise because a subset of the liver cell population respond to a particular metabolic requirement when other cells do not? Is the development of islands simply an adaptive change? There is plenty of evidence that most islands disappear after cessation of exposure to the agent which led to their appearance. However, this does not mean that individual cells returned to normal. More probably, enzyme-altered cells die off rather than divide after exposure ceases or when their purpose, if they have one, has been served. Sequential pathology studies prove that, at most, only a very small minority of islands persist and possibly progress to nodules or actual tumors. Alternatively, the observed facts are consistent with the possibility that no foci of altered hepatocytes progress to neoplasia and that tumors, when they arise, do so de novo from cells that have not passed through a stage of enzyme alteration.

The concept that there is a link between enzyme-island induction and hepatocarcinogenesis is enhanced by the fact that partial hepatectomy increases the incidence of enzyme-altered foci in rats exposed to hepatocarcinogens (Laib and Bolt, 1980; Pitot, 1979). The higher sensitivity of newborn rats, as compared with adult rats, to the induction of enzyme-altered foci in response to hepatocarcinogens ia also consistent with there being a link between island induction and hepatocarcinogenesis. Studies with tritiated thymidine carried out by Scherer and Hoffmann (1971) indicated a faster rate of cell turnover in enzyme-altered foci than in normal liver tissue in rats exposed to diethylnitrosamine.

Altered enzyme foci are not frequently found in untreated rats, particularly with increasing age (Ogawa *et al.*, 1981).

VII. The Prediction of Hepatocarcinogenic Risk for Man

In 1982, the European Chemical Industry Ecology and Toxicology Centre in Brussels assembled a group of scientists, including myself, to produce a monograph entitled "Hepatocarcinogenesis in Laboratory Rodents: Relevance for Man" (ECETOC, 1982). The task of the group was to review the available scientific evidence and to assess the relevance for man of the laboratory data. I have freely drawn on my experience as a member of that group of scientists in preparing the present chapter. I can now do no better than reproduce *Section G* of the report which we prepared. Section G is headed "Sequence of Steps Recommended for Establishing the Hepatocarcinogenic Potential of a Chemical to Laboratory Animals and Man." It is given in Table III (see also Figs. 1 and 2).

VIII. Summary

1. Man is a poor model for the prediction of agents that are hepatocarcinogenic for laboratory rodents. Relatively few agents are known to cause any form of primary liver cancer in man. The most important is hepatitis B virus, for which there is possibly a model in the woodchuck but not one in rats or mice. The only other agents known to cause primary liver cancer in man are certain steroid hormones, vinyl chloride, and thorium dioxide. There are animal models for the first two of these and a reasonable expectation that thorium dioxide would produce liver tumors in animals if the appropriate experiments were done. Aflatoxin, a potent hepatocarcinogen in rats and other species but not mice, is strongly suspected of being an important human hepatocarcinogen in certain geographical areas of the world, but the evidence is circumstantial. There is no more than a weak association between the nutritional type of cirrhosis secondary to excessive intake of alcohol and increased primary liver cancer in man, and no evidence at all that ethanol per se causes liver tumors in mice, rats, hamsters, or mastomys.

2. By contrast, a very large number of chemicals to which people in the West have been exposed for many decades have been found to be hepatocarcinogens in laboratory rodents. In most cases the levels of exposure required to produce liver tumors in rodents far exceed those to which man is normally exposed. The problem is to guess whether low-level exposure to such rodent hepatocarcinogens poses any real liver cancer threat to man?

TABLE III

SEQUENCE OF STEPS RECOMMENDED FOR ESTABLISHING THE HEPATOCARCINOGENIC POTENTIAL OF A CHEMICAL TO LABORATORY ANIMALS AND MAN[a]

The detection of hepatocarcinogenicity relies on long-term studies in animals. Although there are no reliable short-term tests that relate specifically to the detection of hepatocarcinogenicity, an early indication of possible hepatocarcinogenicity, or the lack of it, can be deduced from the results of other tests. Thus, a compound is unlikely to be a hepatocarcinogen if it gives negative results in mutation tests and no liver enlargement or disturbance of liver microarchitecture in an appropriate 14-day rat study. More substantial evidence is the failure to observe adverse hepatic changes in a 90-day rodent study. If liver changes are present in either a 14- or 90-day experiment, they may require further investigation.

1. *Stepwise Approach to the Investigation of Possible Hepatocarcinogenicity.* The sequence of steps is summarized in Figs. 1 and 2. It is emphasized that this guidance scheme should not be interpreted rigidly. The investigations carried out will vary from chemical to chemical, and many factors (e.g., physicochemical properties, potential routes of exposure) may influence the choice of experimental systems.

. . . The first three types of information concern chemical reactivity, genotoxicity, and short-term *in vivo* toxicity studies for identifying possible target tissues and demonstrating the presence or absence of cumulative toxicity. In the light of this information one of the following four positions may be reached (see also Fig. 1):

—Mutation negative, liver changes absent
—Mutation positive, liver changes absent
—Mutation positive, liver changes present
—Mutation negative, liver changes present

When mutagenicity tests are negative, and there is no evidence of hepatotoxicity in short-term tests, then no priority need be given to the investigation of hepatocarcinogenicity.

If mutation tests are positive but there is no evidence of liver toxicity, the next step is to seek confirmation of the mutagenic properties (ECETOC, 1980), possibly extending the search to an investigation of DNA-adduct formation and the nature and response of DNA repair processes. If these mutation tests confirm that the chemical has mutagenic potential, further studies (possibly including long-term animal studies) relevant to possible human exposure will usually be required to assess the carcinogenic potential for organs other than the liver.

If the mutation tests are positive and there is evidence of liver toxicity in short-term tests, corroborative tests for mutagenicity are required (ECETOC, 1980) and short-term toxicity studies extending to other animal species are advisable. These should include interspecies comparative studies to resolve possible variations in qualitative and/or quantitative metabolism and detoxification, including investigations in a nonrodent species. This would aid in the differentiation of species specificity regarding hepatic response. It should not be assumed at this stage that there is any relationship between positive findings in the mutagenicity tests and hepatotoxicity.

If the mutation tests are negative and liver toxicity positive, attempts should be made to confirm nongenotoxicity using a relevant *in vivo* procedure (ECETOC, 1980). The nature of the hepatotoxicity should also be characterized. Possible observations may include classical histopathological changes (e.g. zonal/focal degeneration) in the absence

(*continued*)

Table III (*Continued*)

of liver enlargement, in which instance the compound is a hepatotoxin and attempts at determining no-effect levels should be made. Alternatively, liver enlargement may be observed in the absence of histopathological changes, and in this situation attempts should be made to differentiate between liver cell enlargement *per se* and cell proliferation. Studies such as thymidine incorporation, estimation of ploidy, and counting of mitotic figures are useful in differentiating between the two processs. In many cases both cell enlargement and cell proliferation may be observed. Where cell proliferation is encountered, the use of other rodent or nonrodent species should be considered to give a better asessment of potential human hazard. Cell enlargement is frequently encountered and can be detected by microscopic or biochemical (DNA concentration) procedures. Factors often responsible for liver cell enlargement are increased intracellular lipid, or the proliferation of peroxisomes and smooth endoplasmic reticulum. Fatty infiltration may be determined histochemically, while proliferation of subcellular organelles may be measured either ultrastructurally or biochemically. Peroxisome proliferation may at times be preceded by fatty change of the liver, and hence if lipid accumulation is observed one might look carefully for peroxisome proliferation at a later stage. There seems to be little, if any, causal relationship between the proliferation of smooth endoplamic reticulum (SER) and hepatocarcinogenicity. Conversely, there seems to be a reasonably good correlation between the ability of a chemical to elicit peroxisome proliferation and the subsequent appearance of hepatic tumors in rodents. To assess the significance of such observations for man, short-term *in vivo* tests in a nonrodent species should be considered. 2. *The Importance of Comparative Studies of Metabolism and Pharmacokinetics.* Much of the above is relevant to the question of extrapolation to man. However, the most important information for genotoxic and nongenotoxic carcinogens alike comes from comparative studies of metabolism and pharmacokinetics. In the biotransformation of exogenous chemicals there can be important qualitative as well as quantitative differences between species and it is essential in assessing the possibility or extent of adverse effects in man, to look for such differences and take account of them.

[a]Extract from ECETOC (1982).

3. The mortality from primary liver cancer is very low in countries such as England and Wales where there is widespread exposure to low doses of both natural and synthetic agents which, in high dosage, cause liver tumors in rodents. This suggests that, if there is any risk, it can only be very small.

4. Death rate data collected in England and Wales by the Registrar General are consistent with there having been a small increase in the incidence of primary liver cancer in England and Wales during the past 20 years, but the apparent increase might well be a consequence of revisions in the International Classification of Diseases system and not real. During the first half of the present century the age-standard-

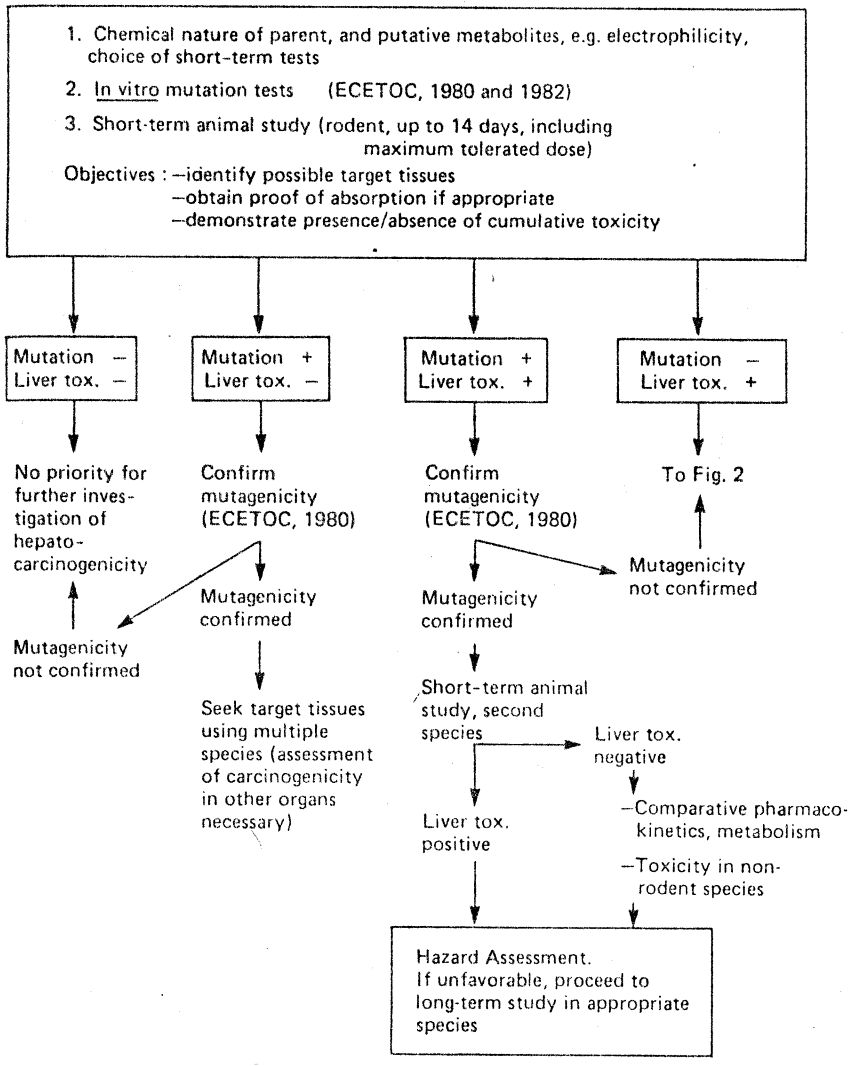

FIG. 1. Sequence of steps—I.

ized incidence of primary liver cancer in England and Wales was falling.

5. In all species, agents which cause liver necrosis with subsequent regeneration and the development of macronodular cirrhosis should probably be suspected of increasing the liver cancer risk, possibly by

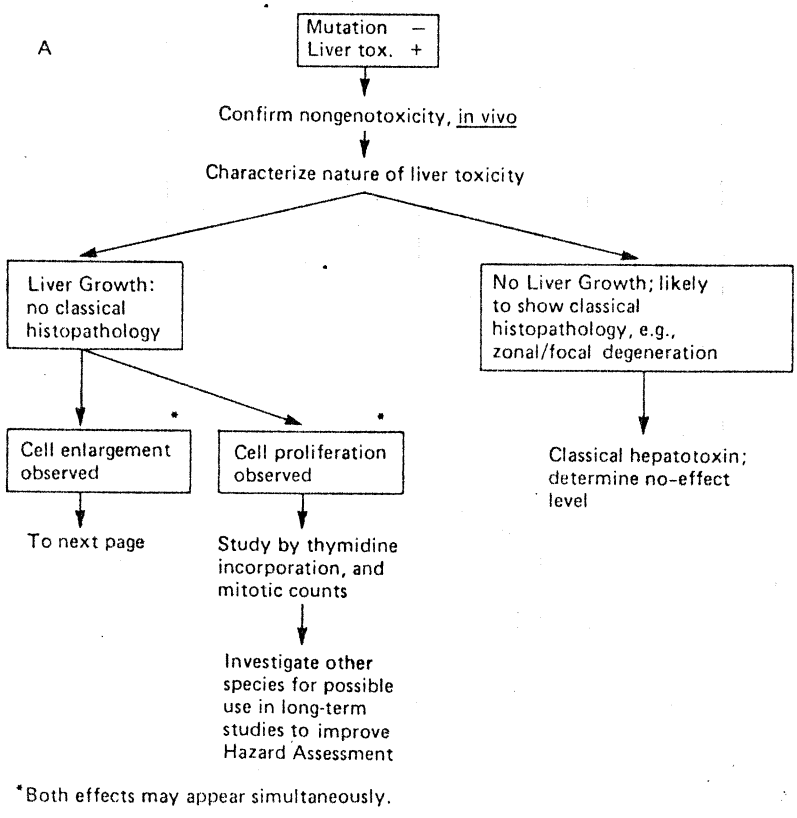

FIG. 2. Sequence of steps—II.

what has been referred to as a "tumor-promoting" process, although the use of this latter term is questioned.

6. The effect of partial hepatectomy in enhancing the hepatocarcinogenic effect of other agents is, perhaps, equivalent to the enhancing effect of regenerative hyperplasia.

7. Agents which in high dosages over long periods give rise to primary liver tumors in rodents give rise to a *variety* of changes after shorter periods of exposure to the same agents. This suggests that there may be many alternative pathways from normal to neoplasia.

8. Neither for man nor for rodents is it certain whether primary liver cancers can develop in the absence of preceding detectable liver damage. However, in tests in which rodents develop liver tumors following exposure to high doses of xenobiotic agents, it is rare for there to be no evidence of previous and/or contemporary liver damage.

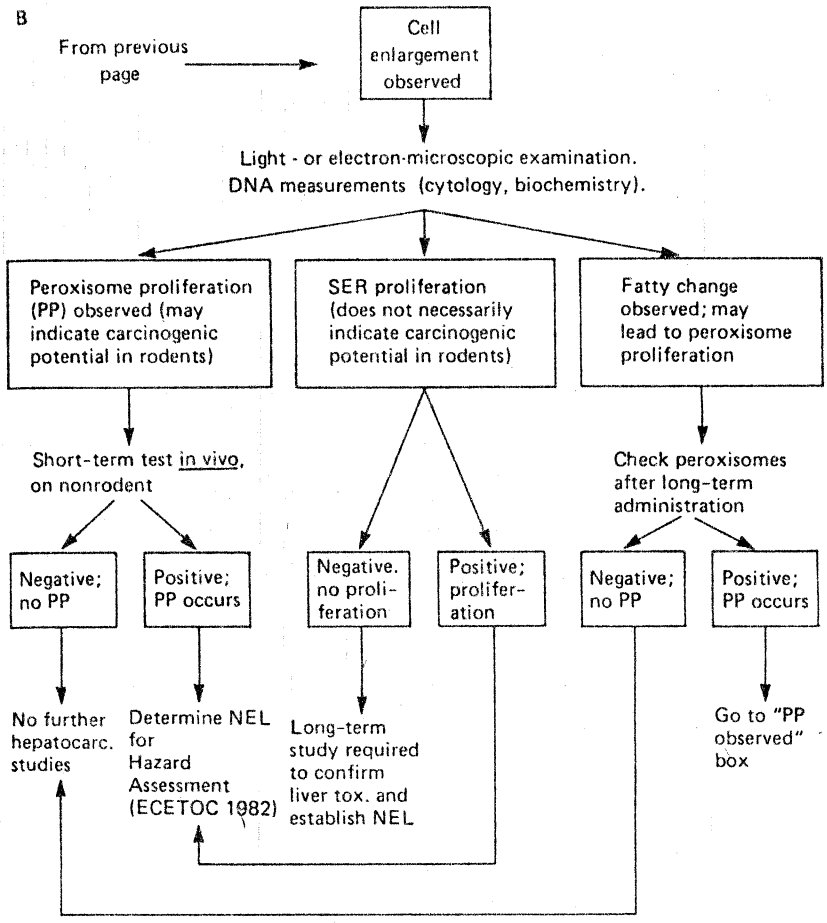

FIG. 2. (*Continued*)

Therefore, there is much to be said for attempting to obtain a better insight into the liver changes which precede liver cancer development in rodents and to develop sensitive, noninvasive, tests for ascertaining whether exposed humans show similar hepatic changes.

9. On the basis of present knowledge, agents which cause liver tumors in rodents by a genotoxic mechanism merit more concern than those that do so by a nongenotoxic mechanism. Features for distinguishing between these two kinds of mechanisms are discussed.

10. In the light of the data reviewed, mathematical calculations of liver tumor risk to humans based on tumor data derived from rodent

studies exposed to very high doses are wholly unreliable both from a qualitative and a quantitative viewpoint.

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