Specificity of Effects of Various 'Indirect' Teratogens and Carcinogens Dependent on the Development of Hydroxylases in Rat Foetus

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Scheme 1. Mechanism of biochemical action of dialkynitrosamines, azoxyalkanes and aryl-dialkyltriazenes

Molecular structure, enzymic α hydroxylation and subsequent dealkylation by concerted reaction of dialkynitrosamines, azoxyalkanes and aryl-dialkyltriazenes, directly yielding alkylazonium as proximate alkylating (or diazotating) carcinogen. Methyl derivatives are used as examples.

N–CH₂–R, hydroxylation necessarily leads to an asymmetric carbon atom. In this case specific effects were to be expected and have been observed which did not occur with the dimethyl compounds.

The symmetrical 1,2-dialkylhydrazines are rapidly dehydrogenated, in the presence of metal traces, to form the corresponding azoalkanes. Oxidation in vivo yields the azoxyalkanes, which proved to be more potent than the hydrazines. Therefore in Scheme 1 only azoxymethane is formulated, which is isomeric with dimethylnitrosamine. A more detailed report on the chemistry and the possible biochemical mechanisms of action is given by Druckrey (1973a,b).

Many compounds of the three groups, although likewise dealkylated by the liver microsomal fraction, did not induce liver cancer but, depending on their chemical structure, produced, specifically, cancer of certain other organs, such as the oesophagus,
colon, bladder and brain, as demonstrated in systematic studies on adult rats by Druckrey et al. (1967), Magee & Barnes (1967) and Preussmann et al. (1969). This striking 'organotropy' suggested that the activating 'drug-metabolizing enzymes' are not unspecific, but are rather a group of highly specific hydroxylases with respect both to the organ in which they are active and the substrate attacked. In the experiments to be reported here it will be shown that the time of their occurrence during prenatal development is also significantly different, especially as far as demethylation and de-ethylation are concerned.

Methods

The compounds tested were synthesized in our institute by R. Preussmann and G. F. Kolar or commercially obtained from Schuchardt, Munich, Germany. After tests of identity, purity and stability, all substances were tested for acute toxicity to adult, newborn and foetal rats. The experiments were performed with syngenic rats of the strains BD IX (CPAH, agouti) or BD VI (CPaH, black) described by Druckrey (1971). The duration of pregnancy after observed coitus is 22 days ± 6 h. Although the rate of spontaneous tumour formation till the age of two years is below 2%, almost every organ proved to be susceptible to chemical carcinogens.

As demonstrated by Druckrey et al. (1967) on numerous examples, carcinogenesis can be regularly induced by a single dose even of very short-lived substances. The same applies to teratogenesis. This makes it possible to study systematically the efficacy of substances at well-defined stages of prenatal and postnatal development. In preceding experiments with ethyl nitrosourea, which is highly reactive and decomposes to ethyl diazonium without enzymic activation, it has been shown by Ivancovic & Druckrey (1968) and confirmed by Koestner et al. (1971) and Swenberg et al. (1972) that a single dose, when given to pregnant rats after the 11th day of gestation, induced malignant tumours exclusively of the brain and nervous system in the whole progeny. Even a dose of 2 mg/kg, corresponding to 1% of the acute LD50, was significantly effective. Consequently the sensitivity of the foetal brain and nervous system must be exceedingly high, about 100 times higher than that of adult rats. At high doses malformations were also observed. With reference to these results, three periods were selected for the treatment, namely the 10th, the 15th and the 22nd (last) days of gestation. The days are defined as the number of 24 h periods after observed coitus. In all experiments the offspring were carefully reared. If malformations incompatible with survival occurred the foetuses were killed and the skeleton stained by Alizarin.

Experiments

Dimethylnitrosamine and some methylalkynitrosamines proved to be highly toxic to the foetus. However, even the highest-tolerated dose of 10 mg/kg was neither teratogenic nor carcinogenic. Only when administered on the 22nd day, were a few nephroblastosomas observed in the offspring. The results are identical with those of Alexandrov (1968). In his experiments only 3/94 of the descendants died with nephroblastosomas, whereas the incidence in the dams was about 80%. Diethylnitrosamine has already been tested by Wrba et al. (1967) and more thoroughly by Thomas & Bollmann (1968). The occurrence of malignant tumours in the progeny was rather sporadic, but only low dosages were used. Since diethylnitrosamine proved to be not particularly foetotoxic, it was tested at higher doses, because the diethyl compounds are of special interest as will be shown below. Intravenous injection of 70 mg/kg at the 15th day did not produce any malformation, and no tumours were observed in an offspring of 42 rats. Only after treatment on the 22nd day of gestation, when a dose of 150 mg/kg was tolerated, were unequivocal positive results obtained, which were dependent on the route of administration. On intravenous injection of 150 mg/kg diethylnitrosamine induced olfactory neuroblastomas in 3/15 descendants. After oral administration by gavage 14 liver carcinomas, four nephroblastosomas and four neurogenic tumours were observed in an offspring of 23 rats as reported by Ivankovic (1973). The average induction time of 800 days, however, was significantly longer than in corresponding experiments on adult rats.
The complete lack of any teratogenic or carcinogenic effects on the foetus at the 15th day is of special interest. Since the foetal nervous system is highly sensitive to chemical carcinogens at this stage of development, the negative results lead to the conclusion that the reactive intermediates of the dialkynitrosamines, though formed in the organism of the dams, are too short-lived to reach the foetus. Therefore the activation process must occur in the foetus itself, which is not possible at the 15th day. The enzyme system relevant to oxidative dealkylation of both dimethylnitrosamine and diethylnitrosamine apparently only appears at the end of foetal development, as positive results were only observed after exposure at the 22nd day. This is in agreement with biochemical studies of Lee & Spencer (1964) and Parke & Williams (1969) on drug-metabolizing enzymes. However, the high foetotoxicity of dimethylnitrosamine remains an open problem.

In the next group, 1,2-dimethylhydrazine and azoxymethane, in contrast with dimethylnitrosamine, proved to be non-toxic to the foetus. Although both compounds are potent carcinogens in adult rats particularly to the colon (Druckrey, 1970), even the highest-tolerated dose of 66% of the LD 50 (for the predominantly used azoxymethane it was 20 mg/kg), administered intravenously on the 15th day of gestation, did not induce any malformations or tumours in an offspring of more than 200 reared rats. Only when given on the 22nd day, 5 nephroblastomas and 3 malignant neurinomas were observed among 44 descendants. Therefore the results clearly correspond to those obtained with the nitrosamines; the capacity for enzymic activation occurs only at the end of prenatal development. In newborn rats azoxymethane, at a single dose of 20 mg/kg, was considerably more effective and produced malignant tumours in almost all treated animals (Druckrey & Lange, 1972).

The view that the activation and dealkylation of azoxymethane is initiated by enzymic hydroxylation is strongly supported by the results of Spatz & Laqueur (1968), who demonstrated that methylazoxymethanol acetate is carcinogenic to rat foetuses before the 15th day. Further, the production of severe malformations of the brain, the cerebellum and the retina, which were dependent on the time of exposure, has been reported by Spatz (1969), Hirono (1972) and Jones et al. (1972). In these experiments the stable acetate was used and at high dosage. The free methylazoxymethanol decomposes rapidly corresponding to first-order kinetics, as shown by Nagasawa et al. (1972). This explains the negative outcome of the transplacental experiments with azoxymethane. However, it should not be overlooked that there are two methyl groups which may be in the cis or trans position and therefore three hydroxylated products are theoretically possible.

In contrast with the methyl compounds 1,2-diethylhydrazine, azoethane and azoxyethane proved to be highly carcinogenic to rat foetuses at the 15th day, as reported by Druckrey et al. (1968) and Druckrey (1973a,b). A single dose of 12–33% of the LD 50 induced malignant tumours of the brain and the cranial and/or peripheral nervous system in 236 out of 252 descendants. Even with 2–6% of the LD50 positive results were still observed in 30–40% of the progeny. The effect of the three ethyl derivatives was practically identical and independent of the route of administration. For azoethane, which is volatile, the pregnant rats were exposed to the vapours only once for 1 h. The lowest effective concentration was 120 p.p.m. This is the first example of transplacental carcinogenesis by inhalation. Despite the high carcinogenicity of the three ethyl compounds, almost no teratogenic effect was observed after treatment on the 15th day, although rat foetuses at this stage are particularly susceptible to chemical production of malformations.

Subsequently, the experiments were repeated at earlier stages to find out at which day the foetus becomes susceptible. 1,2-Diethylhydrazine and azoxyethane were used. Administration on the 12th day still induced a high yield of neurogenic tumours in the offspring, but after exposure on the 11th day only sporadic cases were observed. However, when the pregnant rats were treated on the 10th day, no tumours developed in the progeny of about 150 rats. On the other hand, most of them showed microphthalmia or anophthalmia, which never occurred spontaneously in BD VI and BD IX rats. The lifespan was normal. Autopsy revealed hypoplasia or aplasia of one or both optic nerves, often associated with hydrocephalus, as described by Griesbach (1973). The sensitive
period proved to be rather short and strictly limited to the time between 8 days 22 h and 9 days 20 h after observed coitus.

In the third group, 1-phenyl-3,3-dimethyltriazene, when administered on the 15th day of gestation, was not carcinogenic to the foetus. However, severe malformations of the skeleton were observed, which were dependent on the dose. After low doses, the surviving rats showed microencephalia of the same type as that described by Spatz & Laqueur (1968) and Jones et al. (1972) in experiments with methylazoxymethanol acetate. The induction of malignant tumours in the offspring by 1-phenyl-3,3-dimethyltriazene proved to be possible only after treatment on the last day of gestation. Since positive results were already obtained with the monomethyl compound by the 15th day, oxidative demethylation is probably relevant to the carcinogenicity of 1-phenyl-3,3-dimethyltriazene. The mechanism of its high teratogenic effects, however, must be different.

The 3,3-diethyl derivatives of 1-phenyltriazene, the metapyridyl homologue and its N-oxide, had the same effects as observed in the foregoing experiments with 1,2-diethyl-hydrazine, azoethane and azoxyethane. Administration on the 10th day of gestation produced malformations in the optic sphere, often associated with hydrocephalus, but did not induce any tumours in the progeny. On the 15th day, however, all these diethyl compounds were highly carcinogenic to the foetus as reported by Druckrey (1973a,b). After a single dose of $33\%$ of the LD$_{50}$ 29/32 of the offspring died between 265 and 430 days of age with neurogenic tumours, most of them in the brain.

Discussion

The results demonstrate that the ethyl compounds, in contrast with their methyl homologues, are already metabolically activated in the foetal organism by the 10th day of development. The only exception is diethylnitrosamine. The dialkylnitrosamines, however, are in fact amides of nitrous acid, and only the two other groups are dialkylamines in the proper sense. The findings strongly support the view that the hydroxylases involved are different and specific. This is not only with regard to the substrate attacked, but also to the time at which they occur during foetal development.

The significant differences observed in both teratogenicity and transplacental carcinogenicity, further, indicate that the kind of biological response depends on the stage of differentiation rather than on proliferation. The exceedingly high sensitivity of the foetal brain and nervous system to 'genotoxic' substances (Druckrey, 1973b) requires serious consideration. This the more since alkylation of DNA not only of glial but also of neuronal cells in vivo has been demonstrated by Kleihues et al. (1973).

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Differential Sensitivity of the Developing Mouse Embryo to Mortality, Malformation and Neoplasm Induced by Urethane

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If a toxic chemical penetrates the placenta of an animal, it could be very hazardous to the embryo, because the embryonic stage is extremely sensitive to various toxic agents. It has been previously reported (Larsen, 1947; Klein, 1952; Nishimura & Kuginuki, 1958; Vesselinovitch et al., 1967; Nomura & Okamoto, 1972; Nomura, 1973, 1974) that urethane possesses the typical characteristics of transplacental toxicity; when administered to pregnant mice, it causes embryonic deaths, malformations and neoplasms in their embryos. The present paper deals with a quantitative analysis of these three types of toxicity induced by urethane in the developing embryo. I hope that these quantitative data will contribute to our knowledge of toxicology thereby serving as fundamental data for assessing the human hazards from transplacental chemicals. The details of these results have been published previously (Nomura & Okamoto, 1972; Nomura et al., 1973; Nomura, 1973, 1974).

Fig. 1 summarizes the major part of the experimental results obtained after administration of urethane to pregnant mice at various stages of the developing embryo. In a similar way to the case of X-irradiation of pregnant mice (Russel & Russel, 1954), interrupted pregnancy (preimplantation deaths) and embryonic deaths occurred with high frequency after exposure to urethane during the preimplantation stage and organogenesis, whereas occurrence of malformation of an organ was confined to exposure at a very early stage of its formation. Fig. 1 shows the case of lung and liver malformations. The similar action of urethane to X-rays may be attributed, at least partly, to the unique characteristics of urethane, namely that it almost freely penetrates the placental barrier and that its toxic activity is limited to a short period of about 24h after administration (Nomura et al., 1973).

The major difference between the urethane and radiation effects on the development of the mouse embryo is that tumours were detected in lung and liver with high frequency after the urethane treatments during organogenesis and foetal growth (Nomura & Okamoto, 1972).

For the assessment of the hazards of a chemical, it is very important to know whether its toxicity appears only above a threshold dose. The incidence of late embryonic deaths and malformations by urethane showed a sharp threshold dose; it dropped from about 90~25% at 1.5 mg/g body weight to virtually zero at 1.0 mg/g (see Fig. 1a) whereas the