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METHODS USED IN TERATOGENIC TESTING

by

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Teratogenic testing as we know it today has only come into being since the thalidomide tragedy of 1961. It was already well known at that time that certain chemical compounds produce teratogenic effects in experimental animals (Murphy, Dagg & Karnofsky, 1957; Sokal & Lessman, 1960; Barker, 1960): it had also been well established that the time, route, dose and duration of exposure of a substance could be important in determining its teratogenic effect (Jackson, 1959). However, before 1961 no-one appears to have screened new chemical compounds routinely as a part of safety evaluation prior to their clinical use, although techniques available at that time were probably adequate for screening procedures to have been carried out. In fact, the procedures used today are, in many instances, modifications of techniques used non-routinely before 1961.

It is commonly recommended that drugs should be examined for teratogenic activity in two species of animal: in most cases the species used are the rat or mouse, and the rabbit. The miniature pig and the monkey might well figure more prominently in the future (*Principles for the testing of drugs for teratogenicity*, 1967). Although the chick embryo has been used extensively in embryological work, it is not considered to be suitable for the routine screening of drugs for teratogenicity. Whatever the species used, the choice of strain is important. In our view, random-bred, rather than inbred stock should be used, and the strain should, as far as possible, be free from spontaneous malformations. A strict record should be kept of the malformations and anomalies that occur in control animals. Because of the need for as much information as possible on the incidence of various abnormalities in untreated animals, it is highly advantageous to use the same strain of animal for all teratogenic work within a laboratory. We do not consider it necessary to use specified-pathogen-free animals, but they should be housed under good environmental conditions which should be standard for all tests. Animal accommodation should provide controlled heating and lighting and noise should be kept to a minimum. All animals should receive the same, nutritionally adequate, diet.

In Britain, three dose-levels are usually used in a teratogenic screen. In a three dose-level test, the high dose should be toxic but not lethal to the mother, the low dose should produce a clinically relevant effect and the third dose should be intermediate between the other two. In our experience, optimal dose-levels can best be determined through close co-operation between pharmacology and toxicology departments. Drugs should be given to test animals by the same route as they are to be administered clinically. If the drug is to be given orally, the best method of animal dosing is by gavage. There are disadvantages of administering drugs in the diet: firstly, it is difficult to estimate the exact amount of drug taken by the animal, and secondly, if the drug has an unpleasant taste, the animal will not eat the food.

Drug treatment should be started early enough, and continued long enough, to cover the period of organ formation in the species used for the test. Thus, in the rat and mouse treatment should extend from the 6th to the 15th days of pregnancy, inclusively, and in the rabbit it should extend from the 6th to the 18th days, inclusively. Some authorities consider that dosing should continue daily throughout pregnancy and not just through the period of organogenesis: however, it has been suggested that such prolonged dosing may seriously alter the mother's metabolism and therefore mask a teratogenic effect (*Principles for the testing of drugs for teratogenicity*, 1967).

The number of animals used should be large enough to satisfy statistical requirements. In Britain, it is recommended that at least 20 pregnant female rodents and 8 pregnant female non-rodents be used per group. In the United States, the recommended minimum numbers are 20 pregnant female rodents and 10 pregnant female non-rodents. If another species which is more closely related to man or which has similar metabolic features is used, then the higher the number studied, the more likely it is that statistically analysable and reproducible results will be obtained.

The time of mating should be estimated as closely as possible in order to determine the beginning of pregnancy. In the case of rodents, the males should only be caged with the females for a limited time: at the end of the mating period, successful copulation should be verified by the examination of a vaginal smear for spermatozoa. In the case of rabbits, we always mate the doe with two bucks and then inject the doe intravenously with 25 iu chorionic gonadotrophin. As far as possible, females of comparable weight and age range should be used in each test. In some instances, it may be advisable to use virgin females, but, in others, females of known fertility are to be preferred. It is advisable to cage animals singly in order to avoid effects due to overcrowding. Females should be weighed regularly throughout pregnancy and daily during the dosing period. All animals should be examined daily. Careful handling should be practiced throughout and all clinical observations

should be recorded. Vaginal bleeding should be noted, as this may be associated with resorption or abortion.

Pregnancy should be terminated not more than one day before the expected time of delivery—for this purpose, a knowledge of the strain used is obviously essential. In the strains of animals we use, the days of choice for caesarian section are: mouse, 19 days; rat, 21 days; New Zealand White rabbit, 29 days. The method of killing the mother will vary from laboratory to laboratory: we kill mice by cervical fracture, rats by cervical fracture or ether and rabbits by an intravenous injection of saturated magnesium sulphate. Corpora lutea should be counted and recorded, and the uterus then examined for the number and position of live foetuses and resorptions. After weighing the entire uterus, we remove the foetuses and weigh them individually. They are numbered in the following way: starting from the distal end of the left horn of the uterus to the cervix and then from the distal end of the right horn to the cervix (Fig. 1). Details of weights etc. are entered on a record sheet,

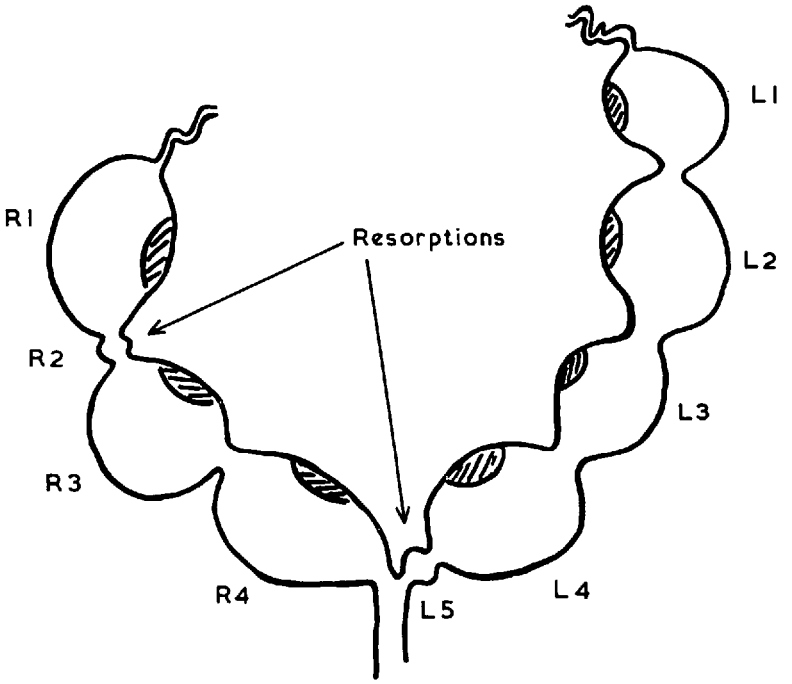
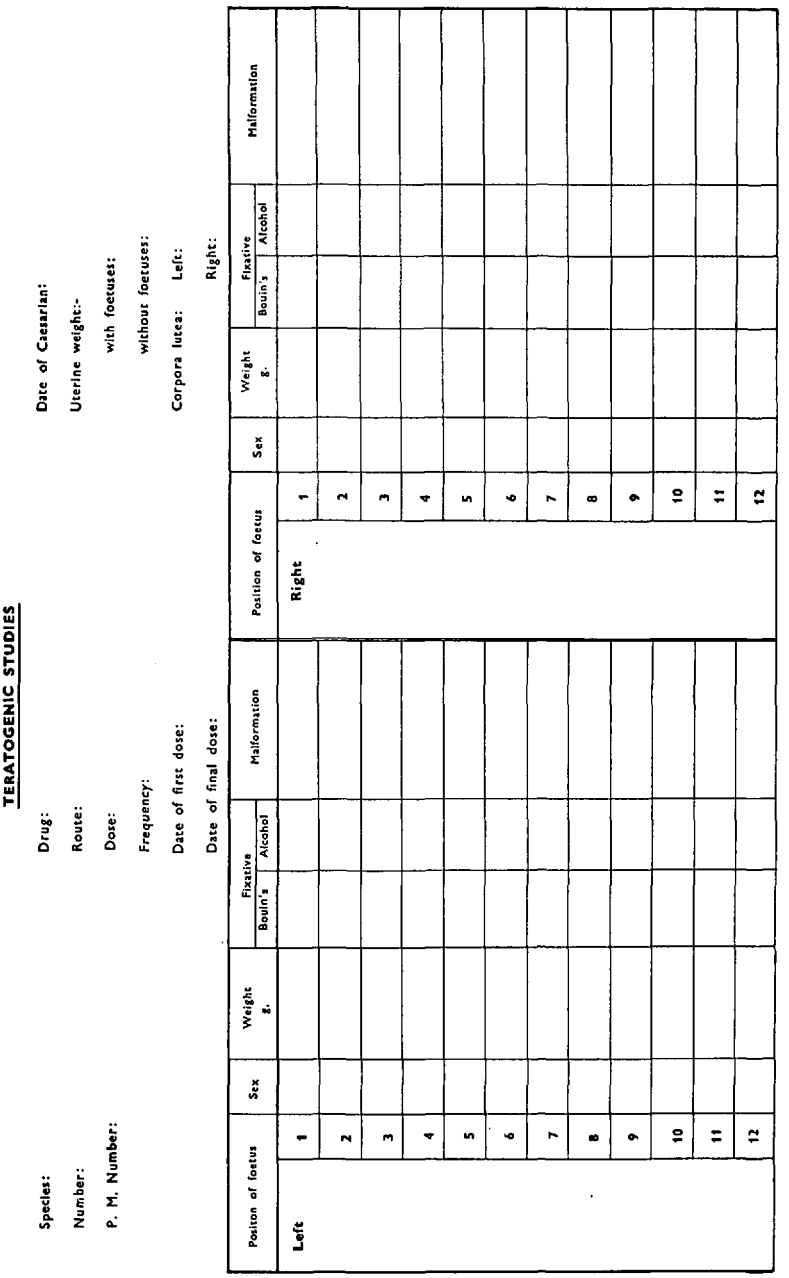


Fig. 1. Foetal numbering system.

one sheet per female (Fig. 2). All foetuses are examined for external malformations and cleft palate and full details are recorded. Alternate foetuses are then placed in 70 per cent alcohol for skeletal examination, and Bouin's fluid for visceral examination. Each bottle is clearly marked with the number

Fig. 2. Teratogenic studies: record sheet.



of the mother and the position of the foetus in the uterus. Rat foetuses are usually left for about a fortnight in Bouin's fluid before examination. Free-hand, razor-blade sections are then made using the technique described by Wilson (1965), after the foetuses have been re-examined for external malformations. The ears and fore-limbs are first removed and then the upper part of the head, a cut being made through the jaws and just above the ears. After examination of the palate, the cut surface of the head is placed on a cork board and 1 mm slices are then made through the head. The slices are then placed (cephalic surface upwards) in 70 per cent alcohol in white plastic spot trays. Sectioning is continued from the region of the shoulder joint through the thoracic and abdominal regions to the kidney region. The urinogenital organs are examined in situ. The sections are then examined under a dissecting microscope for any malformations. A systematic record is made on a specially designed form of all malformations and anomalies.

Foetal examination: viscera

Dam no.:

Position of foetus in uterus:

Sex:

Head	Abdomen
Palate	Liver
Nasal cavities	Alimentary canal
Olfactory lobes	Adrenals
Eyes	Kidneys
Brain	Gonads
Thorax	Bladder
Oesophagus	
Thymus	
Heart and vessels	
Lungs	
Diaphragm	

If further examination is considered necessary, histological serial sections may then be prepared.

For the skeletal examination of rat and rabbit foetuses, we use a modification of the technique of Staples & Schnell (1964). In the case of rats, a small incision is made in the abdominal wall after 2 days in 70 per cent alcohol and the foetuses are then returned to alcohol for a further period of 3 days: the foetuses are then eviscerated and any visceral abnormalities noted. The eviscerated foetuses spend a further day in alcohol and overnight in acetone. They are then placed in a potassium hydroxide/alizarin solution for 2 days

after which they are rinsed in cold water and cleared in a mixture of ethanol, glycerol and benzyl alcohol for 2 days. They are then transferred to 70 per cent ethanol for examination. In the case of rabbits, the foetuses are skinned and eviscerated and are then air-dried for 24 hours instead of being transferred to acetone. All foetuses are thoroughly examined for malformations which are recorded on a form designed for this purpose.

Foetal examination: skeleton

Dam no.:

Position of foetus in uterus:

Sex:

Skull	Fore limbs, *left, right*
Vertebral column	Humerus
Cervical vertebrae	Radius
Thoracic vertebrae	Ulna
Lumbar vertebrae	Metacarpals
Sacral vertebrae	Phalanges
Caudal vertebrae	Pelvic girdle
Clavicles and scapulae	Hind limbs, *left, right*
Ribs	Femur
Sternebrae	Patella
	Tibia
	Fibula
	Metatarsals
	Phalanges

In our laboratories, all sections and skeletal preparations are stored after examination by heat-sealing them (the transverse sections of the embryos serially) in lay-flat polythene tubing.

Although we consider that the Staples & Schnell alizarin method is suitable for routine preparation of skeletons, we have also used toluidine blue to stain the cartilage of some preparations (Burdi, 1965).

In some laboratories, all skeletons are examined routinely by the use of X-rays, and alizarin is only employed when a doubtful result is obtained.

It is recommended by the U.S.A. Food and Drug Administration that, in the case of rats, the foetuses should be randomised into groups, one-third for visceral examination (either by dissection or by the Wilson technique), and two-thirds for skeletal examination. It is further recommended that half the rabbit foetuses should be incubated for 24 hours after delivery for ascertainment of the survival-rate and that all rabbit foetuses should be subjected to external, visceral and skeletal examination. In this case, all foetuses are dissected before the alizarin technique is carried out.

If a compound is found to be teratogenic when given, either throughout pregnancy or throughout the period of organogenesis, it may well be considered worth-while to administer it, in a further test, on selected days during pregnancy to define more clearly when the teratogenic action takes place.

Table 1. Tests for teratogenicity as recommended in the United Kingdom and the United States of America.

U.K.	U.S.A.
Species	
(i) Mouse or rat (ii) Rabbit	At least 2 species: species used most frequently are mouse, rat, rabbit
Number of animals	
Preliminary studies: at least 5 pregnant female rodents per group; at least 3 pregnant female non-rodents per group	At least 20 pregnant female rodents per group At least 10 pregnant female non-rodents per group
Subsequent studies: at least 20 pregnant female rodents per group at least 8 pregnant female non-rodents per group	
Dose levels	
At least 3 dose levels: (i) high dose to be toxic but not lethal to mother (ii) low dose to produce a clinically relevant effect (iii) intermediate dose to be spaced logarithmically Negative control group essential	At least 2 dose levels: (i) high dose to be sub-toxic i.e. the maximum tolerated dose; (ii) low dose to be a multiple of the proposed therapeutic dose Negative control group essential Positive control group advantageous
Period of dosing	
From day 1 until day before term	Mouse and rat: day 6 through day 15 Rabbit: day 6 through day 18
Route	
To be given by the route intended to be used clinically; if given orally may be incorporated in the diet	Preferable to use route intended to be used clinically; for an oral preparation, to be given by oral intubation or capsule, not by incorporation in diet

Both the Food and Drug Administration (*Guide lines for reproduction studies for safety evaluation of drugs for human use*, 1966, circulated to U.S.A. pharmaceutical companies) and the Dunlop Committee (in communications to U.K. pharmaceutical companies) insist that a negative control group should be used in all teratogenic tests. They further suggest that it may be advantageous to use a positive control group. The Dunlop Committee suggest that evidence should be given to show that thalidomide causes foetal abnormalities in the strains of animals used. This would seem quite acceptable in the case of the rabbit where certain well-defined malformations are produced with the administration of thalidomide, but the recommendation is difficult to follow in the case of the rat for which thalidomide seems to be of doubtful teratogenic activity. Christie (1962), Seller (1962), Giroud, Tuchmann-Duplessis & Mercier-Parot (1962), and Brent (1964) have all failed to produce malformations in the rat, although they observed an increase in foetal resorptions.

The recommendations for the teratogenic testing of drugs in Britain and the United States are summarized in Tables 1, 2, and 3.

Table 2. Experimental procedure recommended for teratogenicity studies in the United Kingdom.

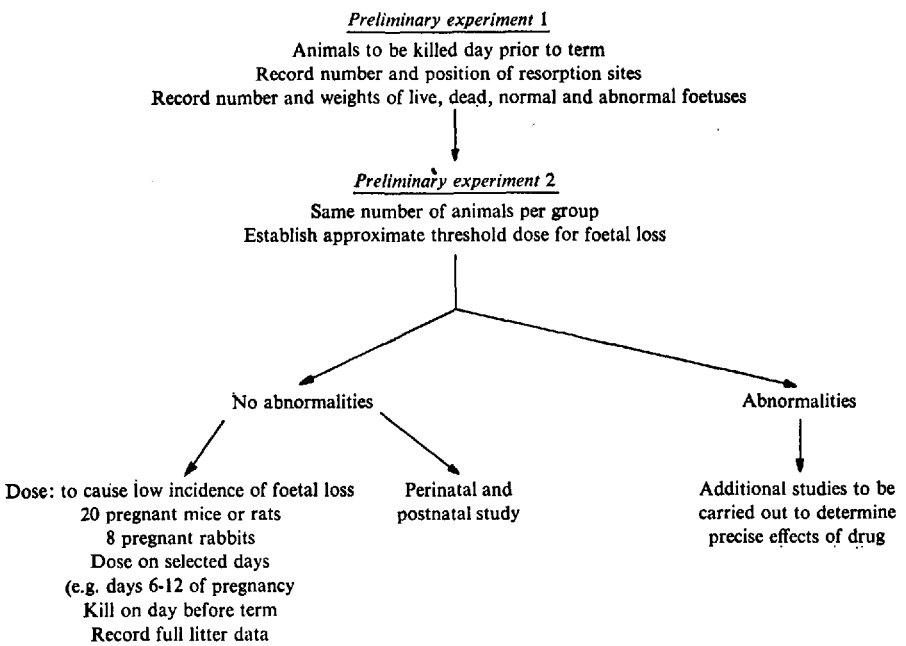
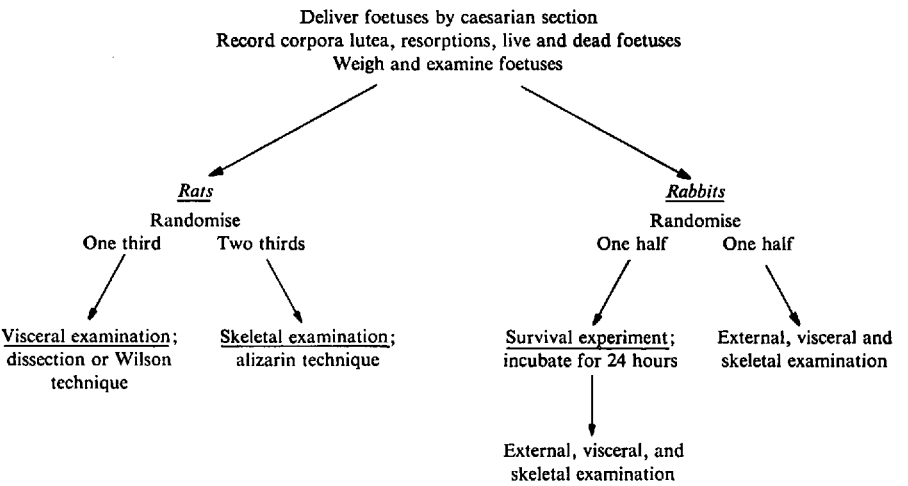


Table 3. Experimental procedure recommended for teratogenicity studies in the United States of America.



Reproduction studies required by drug safety committees include fertility, perinatal and postnatal studies in addition to teratology studies. In a fertility study, either both males and females, or females alone are dosed for about 60 days before mating. The number of pregnancies and number of living and dead progeny are recorded. Live pups are not examined internally. In a perinatal and postnatal study, the drug is either given daily throughout pregnancy and lactation (U.K.) or through only the final third of gestation and lactation (U.S.A.). Effects on lactation, nursing instinct etc. are recorded, and the progeny are examined externally and internally at weaning.

We have tried to show in this paper the methods in use at the present time in the teratogenic testing of drugs. We feel that certain advances have been made in the 7 years since the thalidomide tragedy, but obviously the development of even more precise methods could ensure that any potential teratogenic hazard is minimised.

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