STUDIES ON DRUG-INDUCED CONGENITAL MALFORMATIONS IN EXPERIMENTAL ANIMALS

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A B S T R A C T

A study has been made of the relationship between chemical structure and salicylate-induced embryopathic effects.

The subject of teratology is introduced from a historical viewpoint, and the more important pharmacological and therapeutic effects of salicylates are considered. Some emphasis is placed on the administration of these drugs during pregnancy, particularly when a teratogenic effect has been evoked. The distinctions between maternal toxicity and the different embryopathic effects are emphasised.

The experimental section shows that AH A rats are sensitive to salicylate teratogenesis but that Dutch rabbits are not. The magnitude of the effect, in the case of aspirin, is related to its solubility. Investigations with a number of compounds with only minor structural differences from salicylate have yielded no malformation. The teratogenic effect is produced only in the presence of salicylic acid.

The detailed examination of the rat foetuses has revealed a number of birth defects not previously associated with salicylates. Further, this report constitutes one of the first attempts to study the histopathology of many of these defects.

In the General Discussion, these findings are related to the present knowledge of teratological principles. Finally, consideration is given to the variation in teratogenic response which is so evident in most studies of this nature. The only conclusion to be drawn from this discourse is that, in addition to the specific teratogenic agent, there are many other factors working simultaneously

which are necessary to induce a malformation. This is encouraging because, in clinical terms, it is possible that the elimination of just one of these factors may prevent birth defects from occurring in a child.

C O N T E N T S

S E C T I O N A

INTRODUCTION

CHAPTER 1

A HISTORY OF TERATOLOGY

> There shall be confusion also in many places,
> and the fire shall be oft sent out again, and
> the wild beasts shall change their places, and
> menstruous women shall bring forth monsters:
>
> Apocrypha - 2 Esdras, 5^8

Interest in human congenital malformations reaches far back into mythology where satyrs, centaurs and the rather more authentic cyclops did abound. Further, fairy tales from many nations tell of monsters with two heads or double bodies. The ancient Egyptians appreciated the existence of terata, which were often depicted on tombs; and the inscrutable sphinx appeared as the body of a lion and the head of a man.

In the times of Sargon I, the ancient astrologers of Ninevah and Babylon attached prophetic meanings to the birth of monsters. Thompson (1930) refers to clay tablets dated 2800 B.C. which carried evidence of these beliefs.

The Chaldeans in the 6th century B.C. recognised at least 62 types of congenital malformations and attempted a form of classification. They too regarded the monsters as prophetic manifestations of divine will. For example, if a boy was born without a right testis, the king of that country would surely perish. Some magi also attributed the origin of the monsters to other effects such as maternal impressions during pregnancy, the mixing of semens and the position of the stars. Incredibly, some of these fantasies remain

adherent today in a few remote regions and, indeed, among some uninformed people in far more civilised places.

One hundred years later, the Hippocratic school recorded double monsters which gravely interfered with labour. From this period until the 2nd century A.D. monsters stimulated considerable interest. They were described in extravagant detail by many of the great philosophers, among them Empedocles, Aristotle, Pliny and Galen.

After the time of Galen, teratologists resorted to fantasy to explain the birth of monsters. This period, which offered poorer explanations than the pre-Galen era, reached its acme in the Middle Ages when a combination of ignorance and superstition ran amok. The original Chaldean explanation - that monsters were the results of the wrath of God or His glorifications - was revived. Medieval medical books contained imaginary, semi-human creatures which were attributed to sodomy, bestiality and allied sexual perversions. Among these figments were a few reports which had an air of authenticity about them. Lycosthenes (1557), for example, described quite plausible double monsters.

The presence of congenital malformations did not escape the pen of Shakespeare. Othello, the Moor of Venice, in Act 1, scene 3 recalls seeing

".....men whose heads
Do grow beneath their shoulders."

At the end of the 16th century, Ambroise Paré began the revival of the scientific approach to teratology which had been lacking since the time of Galen. Paré's writings contained evidence of the superstitious Medieval period but, more significantly, showed a

commendable approach towards reporting authentic cases and adducing more scientific explanations for them. The ridiculously incredible monsters described included the angelic 'bird-boy', and a monster of which the upper half resembled its human mother and the lower half its canine father. These supposedly resulted from acts of bestiality, for it was not known then that the interspecific mixing of gametes was almost invariably infertile. The creditable side of Paré's work offered some ideas towards a classification of teratogenic effects, the first of note for some 2000 years. The factors he considered to be causative were not entirely accurate but an effect such as a deficiency in the semen was obviously an important consideration to have made.

The so-called 'scientific epoch of teratology' started in the 18th century. Reputable workers such as Morgagni (1706, 1719, 1761) and Duverney (1761) wrote accurate descriptions of monsters and attacked the incredible theories postulated in former times. Later, Étienne and Isidore Geoffroy-Saint-Hilaire (1822, 1829, 1837) and Meckel (1826) were among those who became interested in the subject and adduced quite reasonable explanations for terata.

Many new classification systems were described. The first of note came from Isidore Geoffroy-Saint-Hilaire (1837) and this was elaborated at the end of the century by Dareste (1891). His contemparies Blanc (1893), Guinard (1893) and Gould and Pyle (1901) also classified congenital malformations. However, the majority of these systems were restricted to monsters surviving after birth, therefore omitting the gross lethal types.

Towards the end of the 19th century came the publication of two outstanding histories on teratology. The comprehensive work of the

Italian Taruffi (1881) filled eight volumes and was followed in 1894 by the concise, classical text of Ballantyne.

Experimental teratology began early in the 19th century in amphibians and birds. It was generally considered that mammals were safe from the factors which affected these lower animals and that adverse external conditions would either kill the developing mammalian embryo or leave it unscathed. Such conditions as trauma, haemorrhage and amniotic anomalies were believed to deform the unborn mammal but, where no comparable physical effect was implicated, the reason for the abnormality was imputed to genetic factors.

The advent of experimental mammalian teratology was in the 1930's. The early work was concerned with the effects of certain diets, with particular emphasis on deficiency of some components, on the unborn mammal. Hale (1933, 1935, 1937) demonstrated that diets lacking certain vitamins were teratogenic to pigs. This work was continued later by Warkany who, with different co-workers (1941, 1943, 1944, 1946, 1948, 1953, 1954) found that a vitamin-deficient diet could be fed to rats for a considerable period before mating but only exerted its teratogenic effect during a precise stage in pregnancy. An interesting feature in vitamin-induced teratogenicity relates to vitamin A which is teratogenic when administered either in excess or in deficit. The tremendous contribution made by Warkany during the last 30 years towards understanding the nature of birth defects cannot be overemphasised.

In 1948, the cytotoxic nitrogen mustard (Haskin) and trypan blue (Gillman, Gilbert, Gillman and Spence) were shown to be teratogenic in rats. Gillman's team was working on reproductive failure among Africans, a condition which appeared to be related to circulating

particles. These were attributed to abnormal metabolism or increased permeability of the gut which were sequels to malnutrition. Trypan blue was selected to produce this condition experimentally because of its particulate nature.

During this period, the effect of atmospheric pressure on embryonic development was receiving some attention from Ingalls, Curley and Prindle (1950, 1952), who demonstrated the teratogenicity of anoxia in mice. It appears that the effect observed with vitamin A, which is teratogenic when deficient or in excess, is also true of oxygen. Ferm (1964) followed the discovery that anoxia is teratogenic by demonstrating that hyperbaric oxygen had a similar effect.

Cortisone was the first of many hormones shown to be teratogenic in mice (Baxter and Fraser, 1950). Later, Fraser, Fainstat and Kalter (1953) demonstrated that susceptibility to cortisone-induced teratogenicity may be an inherited factor. This implicated additional factors, besides the known genetic and exogenous ones, which had to be considered when assessing teratogenicity.

The disastrous radiation effects which followed the atomic explosions of the second World War stimulated the interest in radiobiology in relation to teratogenicity. Since 1950 many detailed accounts of the use of x-rays as tools in experimental teratology have appeared in the literature (e.g. Wilson and Karr, 1951; Wilson, Jordan and Brent, 1953).

Nelson's (1955) investigations on deficient diets led to the use of antimetabolites to eliminate specific constituents from those diets. Her work soon made it clear that these antimetabolites were strongly teratogenic.

Rubella contracted early in gestation is an example of a virus which is teratogenic in man (Gregg, 1941). Viruses in other animals are also known to adversely affect embryonic development. The more recent observations that both herpes simplex virus (Schaffer, 1960) and cytomegalovirus (Weller and Hanshaw, 1962) may be teratogenic in humans have stimulated more interest in this field.

Recent studies have shown that a number of compounds having therapeutic properties are teratogenic to experimental animals. Among these may be included tumour-inhibiting drugs, some antibiotics, many hormones and the salicylates. Further, antimitotic and anti-thyroid drugs, some antibiotics, certain sex steroids, abortifacients and thalidomide are teratogenic in man.

Thalidomide has sedative and hypnotic properties and was shown to be remarkably free from acute toxicity. However, prolonged treatment can cause peripheral neuritis (Florence, 1960) in certain sensitive individuals. The most dramatic toxic effect of this compound became apparent after it had been in clinical use for several years. Lenz (1961) and McBride (1961) independently associated its administration during early pregnancy with the appearance of a number of congenital limb deformities in babies. Although thalidomide was withdrawn from the market, there was an aftermath of many reports of malformed children whose mothers had already taken the drug during pregnancy. As is the case with most teratogens, thalidomide did not affect all the children whose mothers took it during the critical stage in pregnancy. This concept will be discussed in Chapter 14.

Subsequent investigations with thalidomide showed that it was teratogenic in non-human primates at doses comparable with the human therapeutic level. It induced congenital malformations in most

commonly-used laboratory animals but appreciably higher doses were required. In this context, it is interesting that the rat, which is susceptible to most known teratogens, has shown little sensitivity to thalidomide and no characteristic malformations have been demonstrated consistently.

Although thalidomide was the most severe human teratogen among chemical therapeutic agents, it was by no means the first to be discovered. The abortifacient property of aminopterin necessitated its use in early pregnancy. Unfortunately, its action was not always successful and malformations were sometimes evoked in the developing embryo if it survived the treatment (Thiersch, 1952).

During the 1950's, it became apparent that therapeutic doses of certain androgens and progestins caused masculinisation in the human female foetus. That testosterone propionate had this effect in the rhesus monkey had been known since the work of Van Wagenen and Hamilton (1943). Nevertheless, such compounds may be used therapeutically in human pregnancy where there is a history of miscarriage. The correlation between some of these steroids and human congenital malformation was made by Wilkins, Jones, Holman and Stempfel (1958), but the development of new hormones with little or no teratogenic activity has probably justified their continued use. Further, any malformation occurring is restricted to the external genitalia; all internal organs are female. An accurate determination of sex at birth followed by minor plastic surgery allows the normal sexual development of the girl.

The malformations caused by thalidomide in children have instigated a closer scrutiny of drugs to be given to women of childbearing

potential and, to meet this problem, the pharmaceutical industry has adopted the tools of the experimental teratologist and has modified them to achieve a different end.

CHAPTER 2

SOME ASPECTS OF THE PHARMACOLOGY AND THERAPEUTICS OF SALICYLATES

Introduction

The salicylates are a group of compounds having a common 2-hydroxybenzoate radical. They were initially derived from the glycoside salicin, which was obtained from the bark of Salix alba. In the latter half of the 19th century, Gerland (1852) discovered a process for synthesising salicylic acid by the action of nitrous acid on o-aminobenzoic acid. This relatively less expensive form soon replaced those salicylates derived from natural sources. About this time, their use in the treatment of certain inflammatory conditions was recognised. MacLagan (1876) and Stricker (1876) found independently that sodium salicylate was useful in treating rheumatic fever. Later, Campbell (1879), knowing that salicylates increased the urinary excretion of uric acid, used them to treat gout.

Since then, a range of therapeutic uses has been adopted. Salicylic acid has keratolytic, bacteriostatic and fungicidal properties and is used as a corn and wart remover and in the treatment of certain types of eczema. Methyl salicylate is also used topically but as a counter-irritant. There is a wider range of systemic uses following oral salicylate administration. They are effective anti-pyretics in febrile patients and are non-narcotic analgesics. A combination of these two effects helps relieve colds and minor respiratory infections. Salicylates are used in the treatment of gout, acute rheumatic fever and rheumatoid arthritis and may be of

use in cases of sub-acute thyroiditis and of renal calculi (Woodbury, 1941). The more important therapeutic properties are discussed below in greater detail.

For many years, salicylates have been one of the most important groups of therapeutic agents. Aspirin (acetylsalicylic acid), which was synthesised in 1853 by Von Gerhardt but not introduced into medical practice until 1899 (Dreser; Wohlgemut), almost certainly is the most widely used drug in the world. The number of aspirin tablets taken each year was estimated to be 12,500 million - an equivalent of 3000-4000 tons of drug (Dyson and May, 1959). These compounds are used in the treatment of a wide range of minor ailments and are freely available to the public without prescription. Therefore, they are often taken in uncertain doses and without medical direction. Because of this, a thorough knowledge of their possible harmful effects is essential.

Chemistry

The gastric irritation caused by salicylic acid has necessitated the synthesis of various derivatives for systemic use. These constitute three classes: (a) esters of salicylic acid, having a substitution made in the carboxyl group; (b) salicylate esters of organic acids which are obtained by substitution in the hydroxyl group (aspirin is an ester of acetic acid); and (c) salts of salicylic acid, for example sodium and ammonium salts.

The predominant pharmacological effects of salicylates are exerted by their salicylic acid content and the intensity of these effects can be altered by substitutions in the carboxyl or hydroxyl groups.

The Fate of Salicylates in the Body

Truitt and Morgan (1960) reported that the absorption rate of orally administered salicylates is controlled by their dissolution characteristics and by the gastric emptying time. Two years previously Schanker, Tocco, Brodie and Hogben (1958) had found that absorption occurred by passive diffusion of undissociated molecules from the stomach and upper small intestine. It is more rapid if the preparation is given in solution than in tablet form, the latter having to dissolve before absorption (Nelson and Schaldemose, 1959). Woodbury (1941) reported that the buffered forms are absorbed fairly rapidly because the increased solubility of the drug allows better distribution over the gastric mucosa, thereby affording a larger surface area for absorption.

Salicylate ingestion may cause nausea, gastritis, gastric ulceration and haemorrhage. In turn, gastric bleeding may induce iron-deficiency anaemia. Soluble forms are more rapidly absorbed and, it is believed, consequently cause less gastric irritation. Salicylates also inhibit stomach motility, thus delaying emptying and affording more time for the irritation to be exerted (Woodbury, 1941).

The biochemical basis of the gastric lesions remains unknown but various factors have been examined. Levy and Hayes (1960) suggested that the insolubility of the salicylate crystals caused a physical attrition, while Weiss, Pitman and Graham (1961) suggested that the lesions were due to an accumulation of salicylate anions in the mucosal and capillary endothelia. Capillary fragility has also been suggested as a possible contributary factor.

The peak blood level of salicylate in man was found by Milne

(1963) to be reached approximately two hours after ingestion and it is eliminated from the blood within 48 hours. Salicylates are rapidly distributed throughout all body tissues and most intercellular fluids, and readily cross the placenta. The main factor controlling the plasma-salicylate level is dose but there is no direct relationship (Woodbury, 1941).

After absorption, aspirin is rapidly hydrolysed to salicylate. This may then conjugate with glycine and glucuronic acid in variable proportions. These metabolites include salicyluric acid (Bertagnini, 1856), salicylic phenolic glucuronide, salicylic acyl glucuronide (Alpen, Mandel and Smith, 1951), 2,5-dihydroxybenzoic acid (Roseman and Dorfman, 1951), 2,3-dihydroxybenzoic acid (Bray, Thorpe and White, 1950) and 2,3,5-trihydroxybenzoic acid (Dumazart and Ouachi, 1954). The structural formulae and percentage excreted in man of salicylic acid and its metabolites are presented in Table 2.1.

Excretion of salicylic acid and salicylate ions is almost entirely renal and involves three basic stages (Levy and Leonards, 1966). These are glomerular filtration in which free salicylate readily diffuses across the glomerulus; the active process of tubular secretion; and the partial reabsorption of salicylate by passive diffusion of non-ionised salicylic acid from the tubules.

The rate of the clearance of the drug from the body is determined by the urine pH. At pH levels below 6, clearance is low apparently because of considerable reabsorption of the drug. However, when the pH of the urine exceeds that of the blood (i.e. above 7.4) there is a much higher clearance, even after small doses have been given. There is still some reabsorption at high urinary pH levels. This is

effected when water is reabsorbed and a concentration of salicylic acid is left in the tubular fluid, establishing a concentration gradient. An increase in urine flow decreases the gradient and, therefore, decreases the reabsorption of salicylic acid from the tubules.

Salicyluric acid and the acyl and phenolic glucuronides are also excreted by the kidney. The rate is limited by the formation of the metabolite because such small amounts are formed, but urine pH appreciably affects the clearance of exogenously administered salicyluric acid (Schachter and Manis, 1958). Gentisic acid is exceptional among the metabolites in that its excretion, like that of salicylate itself, is naturally pH dependent.

Metabolic Effects

Salicylates induce various changes in body metabolism. Factors affected include oxygen consumption, blood sugar concentration, nitrogen balance and mucopolysaccharide synthesis in connective tissue.

There are many sites of action and the mechanisms are extremely complex, and their effects on carbohydrate metabolism emphasise this complexity. Large doses cause hyperglycaemia; by stimulating (via the hypothalamus) adrenaline release; by reducing aerobic glucose metabolism; and by increasing glucose-6-phosphatase activity. Salicylates may also lower the blood sugar level in diabetic patients by increasing glucose utilisation by peripheral tissues and by blocking gluconeogenesis (Woodbury, 1941).

Much attention has been directed towards Brody's (1956) discovery of the salicylate-induced uncoupling of oxidative phosphorylation;

that is, it inhibits the phosphorylation of adenosinediphosphate. It particularly affects the terminal stage of the oxidation of cytochrome C and stimulates adenosinetriphosphatase activity. The uncoupling inhibits some adenosinetriphosphate-dependent enzyme systems, including muscle phosphorylase and brain glutamate synthetase. It may also explain the increased oxygen consumption and hypothermia induced by salicylates. However, other agents which uncouple oxidative phosphorylation, such as 2,4-dinitrophenol, can induce hyperthermia in experimental animals. It has also been suggested that the uncoupling may contribute towards hypoglycaemia, hypocholesterolaemia and glycogenolysis and to the impairment of mucopolysaccharide biosynthesis. Blackman, Parke and Garton (1955) indicated that the mechanism of uncoupling was linked with the pK values, demonstrating a correlation between them and the effectiveness of the uncoupling agents. Later, Gladtke and Liss (1958) showed that uncoupling may be related to the lipid solubility of the reagents. More recently, Weinbach and Garbus (1969) presented evidence which suggested that uncoupling agents may also exert their effect by interacting with mitrochondrial proteins, thereby reorganising their structure. This could alter the structure and, therefore, the function of the enzymes coupling phosphorylation to electron transport.

Salicylates inhibit the activity of enzyme systems other than those involved in oxidative phosphorylation. These include pyridine nucleotide-dependent dehydrogenases (Bryant, Smith and Hines, 1963), transaminases (Huggins, Smith and Moses, 1961; Yoshida, Metcoff and Kaiser, 1961), glutamate decarboxylase (Gould, Huggins and Smith, 1963) and xanthine oxidase (Mitidieri and Affonso, 1959) and they

may interfere with many reactions in amino acid metabolism (e.g. Simoes and de Barros, 1955). They inhibit the dehydrogenases by competing with the pyridine nucleotide and thereby decrease oxygen consumption. The transaminase inhibition is effected by competition with the substrate and may affect the sizes and patterns of the amino acid pool. The interference with brain glutamate decarboxylase activity may be concerned in the convulsions in cases of salicylate intoxication. Xanthine oxidase interference may alleviate excess uric acid production in gout.

Salicylates are known to interact with certain endocrine tissues. Very large doses stimulate the hypothalamus which, in turn, stimulates an increased secretion of adrenal catecholamines and, through the release of adrenocorticotrophic hormone, adrenocorticosteroids (Calesnick and Buetner, 1949). It is equivocal whether the production of the latter is important in salicylate-induced anti-inflammatory activity, but Smith (1966), who had worked extensively on the subject, concluded that it was not. The work of Wolff and Austen (1958) showed that salicylates also inhibit thyroid function by depressing the production of thyrotrophic hormone.

Therapeutic Effects

Salicylates are weak, non-narcotic, peripheral analgesics and their clinical effectiveness appears to be related to their multiple pharmacological actions (Palazzo and Strani, 1965). They are only effective where the pain-evoking stimulus is chemical (such as in rheumatic pain), the early phases of certain malignant diseases, headaches originating from vascular or muscle contraction, and acute inflammation following trauma but, generally, are ineffective

against visceral or severe traumatic pain. The possible mechanisms of action were reviewed concisely by Randall (1963). Depression of the pain-induced impulses through the thalamus may be one mechanism for relieving some types of pain, but much of the evidence available indicates that the primary action is at the peripheral sites. Salicylates appear to modify the physiological cause of the pain (e.g. bradykinin) by selectively blocking it at the site of origin - a theory upheld by the work of Lim (1966). The simultaneous relief of inflammatory pain with the reduction of oedema by salicylates had led to the idea that the analgesic effect may result from the action on the water balance mechanism of tissues which reduces the oedema. This is supported by the fact that many of the symptoms relieved by salicylates - namely muscular aches, many types of headache and toothache - are associated with oedema.

Salicylates are widely used for their antipyretic activity. Therapeutic levels lower the body temperature of febrile patients but do not produce hypothermia in non-febrile ones. The site of the antipyretic action is probably central, in the hypothalamus. This area controls body temperature, the level of which is raised in fever. Salicylates depress this rise by increasing heat loss, causing vasodilation in the skin, coupled with dilution of the blood and an increase in its flow, and sweating (Randall, 1963). The major response of febrile patients to salicylates is sweating but the antipyresis is maintained even after atropine-induced prevention of sweating. Higher doses of salicylate increase oxygen consumption and raise the metabolic rate, thereby having a pyretic effect. This promotes sweating and enhances dehydration which is seen in

some cases of salicylate intoxication (Woodbury, 1941).

Salicylates also diminish some of the characteristics of experimentally-induced acute inflammation, especially increased capillary permeability, and suppress many symptoms attributed to inflammatory reactions of rheumatic disease (Smith, 1966). The mechanism of action has yet to be elucidated but there appears to be no single site. Most attention seems to have been focused on the effects on the chemical mediators of inflammation such as the kinin system and histamine and on the uncoupling of oxidative phosphorylation. Whitehouse (1963, 1964a) postulated that this uncoupling action inhibits mucopolysaccharide synthesis by preventing the necessary energy from reaching the cells. These polysaccharides, being polyanions, may affect the retention of cations and their associated water through the connective tissues. Hence, the movement of fluids and tissue swelling seen in inflammation may be impaired by salicylate treatment. Other workers, notably Austen (1963), have examined the action of salicylates on antigen-antibody reactions because of the relationship between immunological processes and rheumatoid arthritis. He concluded that no single site of action explains the suppressive effect of salicylate on the various factors of immunological processes.

A correlation between drugs with in vivo anti-inflammatory activity, among them salicylate, and certain immunosuppressant ones was reported by Forbes and Smith (1967). They were working on the hypothesis that the lymphocytes found in particular inflammatory lesions synthesise proteins which damage tissue. Their investigations showed that both groups of compounds, irrespective of their mode of action in vivo, inhibited protein synthesis in lymphocytes in vitro.

Toxicity

The accessibility of salicylates increases the likelihood of overdosage, causing intoxication. Therefore, it is imperative to acquire as detailed a knowledge as possible of their harmful effects at all stages of life.

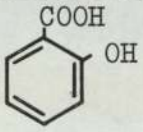
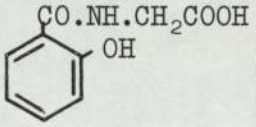
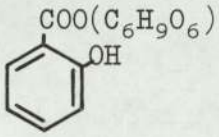
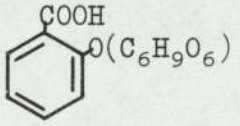
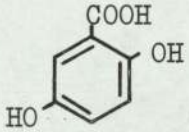
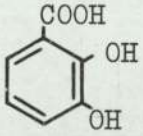
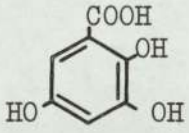
Acute salicylate poisoning is generally due to either accidental ingestion by children or attempted suicide in adults. The commonest toxic symptom is a disturbance of the acid-base equilibrium which results from the numerous simultaneous reactions. It is manifested by metabolic acidosis in the very young and by respiratory alkalosis in older children and adults. In very severe cases, the respiratory failure may be followed by depression, stupor, coma and, ultimately, death (Woodbury, 1941).

Phenyl salicylate poisoning does not conform to this pattern, and its most conspicuous symptoms are due to the phenol which is liberated by hydrolysis (Woodbury, 1941).

Salicylates are known to possess embryopathic activity. Newborn babies have shown congenital salicylate intoxication, usually because of attempted suicide by the mother just before term (e.g. Earle, 1961), but no definite teratogenic effect has been demonstrated in humans. The elegant experiments of Warkany and Takacs (1959) showed that these compounds have such an action in certain laboratory animals, and this concept will be discussed in the following chapter.

TABLE 2.1

STRUCTURAL FORMULAE AND PERCENTAGE EXCRETED IN MAN OF SALICYLIC ACID AND ITS METABOLITES

Name of metabolite	Structural formula	Mean percentage in urine *
Salicylic acid		61
Salicyluric acid		8
Salicylic acyl glucuronide		5
Salicylic phenolic glucuronide		22
2,5-Dihydroxybenzoic acid		1
2,3-Dihydroxybenzoic acid		
2,3,5-Trihydroxybenzoic acid		

* From Alpen, Mandel, Rodwell and Smith (1951).

CHAPTER 3

THE ADMINISTRATION OF SALICYLATES DURING PREGNANCY

Miscellaneous Effects of Salicylates in Pregnancy

In the last quarter of the 19th century it became apparent that salicylates produced adverse effects in mammals during pregnancy, Both Furbringer (1875) and Binz (1893) found that the use of salicylates to cure experimentally induced fever in rabbits frequently resulted in abortion. Binz (1893), however, pointed out that the effect of the fever could not be eliminated, and emphasised that salicylates should be used with extreme care during gestation. In spite of these findings, this abortifacient action of salicylates could not be demonstrated in the guinea pig (Balette, 1883; Schuchardt, 1886; Binz, 1893). These reports were upheld by the observations of Gunn and Goldberg (1922) and Hanzlik (1927) who expressed the opinion that salicylates are not important abortifacients in humans.

The facility of salicylates to cross the placenta was also discovered in this era. Zweifel (1887) showed that salicylate administered to the mother crossed the placenta to the foetus. This he did by giving 3g of salicylic acid to women prior to partuition and subsequently recovering some from the maternal urine, the foetal urine and the placental blood. The transplacental passage of salicylates from foetus to dam was reported later by Lannois and Briau (1898). They injected rabbit and guinea pig foetuses with sodium salicylate and subsequently recovered some of the compound from the maternal urine and tissues.

The possible harmful effects of salicylates administered during pregnancy received little more attention until 1948 when Jackson, using rabbits, confirmed the observations of Zweifel (1887) and Lannois and Briau (1898) that sodium salicylate crosses the placenta. Jackson (1948) further showed that this salicylate was no more toxic to the foetus than to the adult. He found that sublethal doses given near the end of gestation to rats and rabbits had no more effect upon the offspring than upon the adult.

The facility of salicylates to traverse the placenta means that the blood salicylate levels of mother and foetus are similar. Consequently, if a large dose of salicylate is taken immediately before parturition, the progeny are born with the same high blood salicylate level as the mothers, and show obvious symptoms of salicylate poisoning. A case exemplifying this was described by Earle (1961).

In 1962, Lemaire and Grosjean carried out an original investigation on the effects of sodium salicylate on the adrenals of pregnant rats and their progeny. They administered 500 mg/kg intraperitoneally on the 20th day of gestation to one group, another group was injected subcutaneously with 200 mg/kg from days 10 to 19 and a third group received two daily subcutaneous injections of 250 mg/kg over the same period.

The acute administration of 500 mg/kg and the chronic administration of 200 mg/kg/day depleted the ascorbic acid content of the maternal and foetal adrenals. This was consistent with the findings of Daniels and Everson (1936) who reported that aspirin stimulated ascorbic acid excretion in the urine. The 200 mg/kg treatment also caused a reduction in the mean weight of the foetal adrenals.

Further, the chronic, twice daily administration of 250 mg/kg depleted the ascorbic acid content and lowered the mean weight of the maternal adrenals, but did not affect those of the foetus.

This work confirms the common effects of salicylates upon the dam and her developing foetus. It was rather surprising, however, that these workers reported no trace of teratogenicity, having given a potentially teratogenic dose to a susceptible species on critical days (days 10-12).

The tuberculostatic drug p-aminosalicylic acid was the subject of an investigation undertaken in 1953 by Follmer and Mayer. They injected rats subcutaneously with up to five times the human oral therapeutic level of 250 mg/kg/day. The treatment started when the females were placed with the males and was continued daily until the end of the weaning period. Three and five times the human therapeutic level caused most of the rats to abort. However, those given 250 mg/kg/day littered normally but their progeny failed to grow to the same extent during weaning as the controls. These results must be viewed with some reservation because the human therapeutic level referred to the oral route but the rats were treated subcutaneously. Further, my own investigations, not presented in this thesis, showed that oral doses of up to 3500 mg/kg/day given from days 8-12 of pregnancy did not adversely affect the rat conceptus.

Potential Teratogenicity of Salicylates

It is proposed to discuss in some detail many of the publications referring to the potential teratogenicity of salicylates. In order to clarify the results, a summary of the investigations is presented in Table 3.1.

(a) Rats

The teratogenic effect of salicylates was first demonstrated in the classical work of Warkany and Takacs (1959). They injected rats subcutaneously with sodium salicylate or methyl salicylate on the 9th, 10th or 11th day of gestation. The dosage of methyl salicylate given was 100-500 mg/kg and that of sodium salicylate was 60-180 mg/kg.

The highest doses of both compounds killed some of the dams and other rats showed only intrauterine resorption. However, of the surviving foetuses examined, 47% from the methyl salicylate groups and 32% from sodium salicylate groups were malformed. The abnormalities observed were generally common to both drugs. They included eventration of the abdominal viscera, hepatic hypertrophy, subdivision of the liver lobes, shortening of the posterior half of the body, exencephalia, diverse rib, sternal and vertebral malformations, central nervous system malformations including hydrocephalia, hare lip, facial clefts, palatoschisis, protruding tongue, bottle-jaw oedema, ablepharia and eye malformations. One type of gross malformation, namely craniorrhachischisis, was restricted to the methyl salicylate treatment. This malformation was later produced with sodium salicylate by Gulienetti, Kalter and Davis (1962) who estimated that such foetuses were surrounded by about twice the normal volume of amniotic fluid.

From their results, Warkany and Takacs (1959) concluded that the salicylates were not efficient teratogens as the dose necessary to induce birth defects was so near to the lethal one. Further, the level which was teratogenic in rats was higher than the therapeutic one in man. In Takacs and Warkany's (1968) publication,

this difference was estimated to be four to five times higher than the maximum dose for treating acute rheumatic fever in humans. This later paper was a continuation of their 1959 study and demonstrated foetal cardiovascular malformations following salicylate treatment of the dams. Most of these malformations were found in foetuses with other abnormalities. Transposition of the aorta to the right was the commonest malformation but ventricular septal defects and dextrocardia were also seen. Heart defects had been produced previously in foetuses from rats injected subcutaneously with 0.1 or 0.2 ml of methyl salicylate on the 9th or 10th day of pregnancy (Monie, 1964). However, no detailed histological report was presented until Takacs and Warkany's (1968) publication. They also examined the pituitary glands of foetal rats following salicylate treatment of their dams. They found two cases of pituitary agenesis and other cases in which the anterior lobes varied in their position and in their connection with the posterior lobes and the brain.

The 1959 paper by Warkany and Takacs obviously stimulated interest in salicylate teratogenicity. This was evident from the series of publications which appeared in the literature in the middle 1960's. This may have been related as much to the rapidly increasing interest in the science of experimental teratology, following the thalidomide tragedy (Lenz, 1961; McBride, 1961), as to the extensive therapeutic usage of the salicylate group of compounds. It was hardly surprising that the work which followed involved acetylsalicylic acid (aspirin), perhaps the most widely used of all drugs.

Obbink and Dalderup (1964a, 1964b) fed 600 mg/kg/day of aspirin

to rats in their diet from the 6th day of gestation until term. The dams lost weight and, by the end of pregnancy, showed only intrauterine resorption.

However, Loosli, Loustalot, Schalch, Sievers and Stenger (1964) were successful in their attempt to demonstrate the teratogenicity of aspirin in Wistar rats. They administered the compound by oral gavage at the dose levels of 100, 200, 300 or 400 mg/kg/day from the 7th to the 16th days of gestation. The 400 mg/kg dose reduced the maternal weight and, in common with 300 mg/kg, increased the incidence of intrauterine resorption. The dose level producing the greatest number of congenital malformations was 200 mg/kg. The foetuses from the dams receiving this treatment showed gross defects consistent with the type described by Warkany and Takacs (1959). Schardein, Blatz, Woosley and Kaump (1969) later produced minor skeletal anomalies after treating Holtzman rats throughout organogenesis with the comparatively low dose of 99 mg/kg of aspirin.

Experiments undertaken by McColl, Globus and Robinson (1965) involved the administration of aspirin to male and female Sprague-Dawley (CD) rats for at least three days before mating. The females were subsequently treated daily throughout pregnancy with 250 mg/kg/day of the compound given in the diet. This treatment decreased the number of does conceiving and reduced the body weight and litter size of those which became pregnant. There was also an increase in the incidence of offspring mortality and of intrauterine resorption. Further, the foetuses showed multiple defects which, by that time, had come to be associated with salicylate teratogenicity. They also observed two previously undescribed anomalies, namely enlarged

right auricle and inhibited ossification.

The teratogenicity of aspirin in Wistar rats was confirmed by Baba, Nagahama, Akiyama and Miki (1966) who recorded carpal flexure, oligodactylia, radial hypoplasia, renal hypoplasia, hydronephrosis and hydroureter, as well as other malformations previously ascribed to aspirin teratogenicity in rats. In a separate paper, Baba and Nagahama (1966) described hydrops universalis in foetuses whose dams received massive doses of aspirin from days 14 to 19 or 17 to 19 of pregnancy. The former publication contained the first report of rat foetuses malformed by the administration of phenyl salicylate to their dams. Doses of 200 or 400 mg/kg of the body weight of the dam at mating were teratogenic when the former regimen was administered from the 7th to the 9th day of gestation and the latter from the 7th to the 12th day. Most of the foetuses had been resorbed by term but a few of those surviving showed exencephalia and kinked and fused ribs.

Baba et al (1966) attempted to equate the dose of aspirin and the time of its administration with the type of malformation produced. Although they drew some conclusions, evaluation of their results was obviously complicated by their procedure of treating the dams for at least three consecutive days.

Brown and West (1964) and, later that year, West alone, adopted a somewhat different approach to the problem. The initial results of Brown and West (1964) agreed with those of other workers (e.g. Obbink and Dalderup, 1964a) that 500 mg/kg of aspirin, which is approximately ten times the maximum human therapeutic dose, induces only intrauterine resorption in rats. Further, 50 mg/kg/day of the

compound still resulted in a relatively large number of dead foetuses and resorption sites.

These workers went on to investigate the influence of different dietary constituents on the effects of aspirin in pregnant hooded Lister rats. They fed the rats with either a high carbohydrate diet (65% sucrose and 24% casein) or a high protein one (89% casein). In addition, they administered aspirin, apparently throughout pregnancy, at different dose levels in the diet. The rats on the high carbohydrate diet received 50, 250 or 500 mg/kg/day and those on the high protein one were given 500 mg/kg/day. The rats receiving the high carbohydrate diet showed a dose-related response in the incidence of intrauterine death and subsequent resorption. There was also a dose-related response in maternal weight change.

An interesting observation related to the rats given 500 mg/kg/day of aspirin; those on the carbohydrate diet lost weight during pregnancy while those on the protein diet gained weight. This indicated that a high protein diet reduced the toxicity of aspirin. Further, although both groups showed only intrauterine death, the greater percentage of the embryos from the former group died earlier than those from the latter one. This was apparent by the ratios of death foetuses to resorption sites, there being a higher incidence of dead foetuses in the casein group.

West (1964) continued this line of investigation by further modifying the diet with (or without) magnesium sulphate. The full diet contained about 600p.p.m. magnesium and the deficient diet contained about 60p.p.m. The effect of magnesium was studied because its deficiency elevates the urinary free histamine levels in rats

(Bois, Gascon and Beaulnes, 1963), as does pregnancy (West, 1960). Further, it affects some agents, for example cholesterol, which produce coronary lesions (Olsen and Parker, 1964), and is essential for the growth and maintenance of the soft tissues (Martindale and Heaton, 1964). The rats were given aspirin at levels between 12.5 and 500 mg/kg/day apparently throughout pregnancy. West (1964) reiterated his earlier results when, with Brown, he had shown that aspirin was more toxic when given in a high carbohydrate diet than in a high protein one. Moreover, he showed that this difference was greatly increased in the absence of adequate magnesium. The pregnant rats on the high protein diet tolerated a dose corresponding to a high therapeutic level in man (50 mg/kg) while one quarter of this amount induced foetal death and resorption in rats fed a high carbohydrate diet. West (1964) observed no foetal malformation despite administering a potentially teratogenic regimen to a susceptible species. A most important feature emphasised in this work, however, was the necessity to record the dietary constituents fed to animals in toxicity tests. It is possible that variations in the diet may explain the differences in the dose levels of aspirin reported to be teratogenic by different authors.

The series of reports of work carried out by Goldman and Yakovac (1963, 1964a, 1964b, 1965) indicate a very practical approach to the problem of salicylate teratogenicity. They attempted in 1964 (a) to determine which, if any, of the major effects of salicylate poisoning was important in inducing the teratogenic effect, and went on to investigate whether the effect was directly on the embryo or mediated through the mother.

Positive control work was carried out in Sprague-Dawley rats which were injected subcutaneously with a single dose of 500 mg/kg of sodium salicylate on the 9th or 10th day of gestation. This treatment induced late resorption, retarded foetal growth and increased the incidence of abnormalities. These abnormalities were generally consistent with those described by Warkany and Takacs (1959) in relation to salicylate teratogenicity in the rat. However, previously undescribed malformations included cranioschisis, encephalocele, spina bifida, omphalocele, anophthalmia, microphthalmia and exophthalmia.

Goldman and Yakovac (1964a) investigated an effect of salicylate intoxication which could act directly on the embryo, namely the uncoupling of oxidative phosphorylation. This was produced experimentally by the specific uncoupling agent 2,4-dinitrophenolate and results in the inhibition of adenosinetriphosphate biosynthesis. This compound was given by a single subcutaneous injection on the 9th, 10th or 11th day of gestation at dose levels between 8 and 50 mg/kg. Maternal death and some intrauterine resorption was induced at the higher levels and the mean weight of the live foetuses was reduced. However, no malformed foetus was observed. It appeared unlikely, therefore, that the uncoupling of oxidative phosphorylation was responsible for the teratogenicity. These results agree with those described by Obbink and Dalderup (1964b). These workers also investigated the effect of uncoupling oxidative phosphorylation in pregnant rats by using the maximum tolerated dose of dinitrophenol.

Unfortunately, 2,4-dinitrophenol has a very short biological

half life and is rapidly reduced to aminonitrophenols and diamino-nitrophenols - compounds which are inactive as uncoupling agents (Williams, 1959). Therefore, the lack of teratogenicity observed with dinitrophenol did not necessarily dissociate salicylate-induced uncoupling of oxidative phosphorylation from the teratogenic effect. However, other investigations indicate that at least 10 non-steroidal anti-inflammatory agents can uncouple oxidative phosphorylation (Whitehouse, 1965). That none of these has teratogenic activity similar to the salicylates confirms that no direct link exists between the uncoupling action and the induction of birth defects.

Salicylates stimulate the maternal hypothalamus, leading to the release of adrenocortical hormones. However, Goldman and Yakovac (1964a) showed that this effect was not related to the teratogenic one as maternal adrenalectomy prior to a teratogenic dose of sodium salicylate did not influence the number of malformations produced. Further, the exogenous administration of one adrenocortical hormone - namely cortisone - had no supplementary effect.

These workers also found that the interaction of an effect exerted directly on the embryo (the uncoupling of oxidative phosphorylation) and one mediated through the dam (glucocorticosteroid release following salicylate-induced stimulation of the maternal hypothalamus) did not produce anomalies. These conditions were reproduced by administering 2,4-dinitrophenolate and exogenous cortisone. Further, they were given together with an acidic salt load which causes metabolic acidosis, thereby potentiating salicylate toxicity.

The electrolyte balance appeared to play an important role in salicylate teratogenicity. The incidence of malformations induced by

sodium salicylate was increased with an ammonium chloride load to the mother. However, a saline or sodium bicarbonate load to the dam protected against salicylate teratogenicity. The synergistic effect of ammonium chloride was considered to be related to its action in inducing maternal stress or by maintaining a high blood-salicylate level through acidification of the urine, or both (Goldman and Yakovac, 1964a).

It was the factor relating to maternal stress which Goldman and Yakovac (1963) decided to pursue. They adopted the technique of immobilisation to produce maternal stress. Immobilisation was known to induce systemic stress in mice and also to enhance vitamin A teratogenesis in rats (Härtel and Härtel, 1960). Sodium salicylate was administered in single subcutaneous injections at the dose levels of 200, 300, 400 or 500 mg/kg during immobilisation on the 10th day of gestation. Control animals received either sodium salicylate alone or were immobilised and given no salicylate.

Immobilisation alone was not teratogenic but markedly retarded foetal growth. However, immobilisation greatly increased the total number of abnormal foetuses produced by sodium salicylate in proportion to the dose of salicylate and the duration of immobilisation, and also increased the occurrence of foetuses with multiple gross defects. Immobilisation caused non-teratogenic doses of sodium salicylate (e.g. 300 mg/kg) to become teratogenic.

Graham and Parker (1948) determined that 200 mg/kg of sodium salicylate was the equivalent of a therapeutic dose in man for certain rheumatic diseases. At one and a half times this dosage, Goldman and Yakovac (1963) found that as little as 4½ hours of

immobilisation rendered this treatment teratogenic. The mechanism of this action was not clear.

In their 1964 (b) paper, Goldman and Yakovac reported the effects of central nervous system depressants upon the teratogenic effects of sodium salicylate enhanced by immobilisation in rats. They injected 40 mg/kg of sodium pentobarbitone or 2 mg/kg of chlorpromazine, intramuscularly, 1 hour before immobilisation and subsequent treatment with the salicylate. The period of immobilisation lasted $3^1/_4$ or $4^1/_2$ hours and sodium salicylate was injected subcutaneously at 300, 400 or 500 mg/kg.

The results showed that pretreatment with these central nervous system depressants prevented the toxic and the teratogenic action of sodium salicylate from being enhanced by immobilisation. Therefore, 300 mg/kg of sodium salicylate was not teratogenic; 300 mg/kg plus immobilisation was teratogenic; but 300 mg/kg plus immobilisation after treatment with sodium pentobarbitone or chlorpromazine was not teratogenic.

Goldman and Yakovac (1965) later investigated the effects of reserpine on salicylate teratogenicity and immobilisation. Reserpine, like chlorpromazine, is a central nervous system depressant. However, it differs from chorpromazine in that the former causes systemic responses common to teratogenic doses of salicylates and to immobilisation.

They injected 1 mg/kg of reserpine intramuscularly into rats 18 hours before immobilisation on the 10th day of gestation. The does were subsequently given 400 mg/kg or 500 mg/kg of sodium salicylate subcutaneously. Reserpine was found to potentiate maternal morbidity

and the teratogenicity induced by salicylate alone or augmented by immobilisation. Reserpine alone was also teratogenic, producing foetal malformations generally similar to those described in relation to salicylate teratogenicity.

The overall conclusion drawn from these studies by Goldman and Yakovac was that the mechanism of action of salicylate teratogenicity was probably not a direct effect upon the embryo. Instead, there seemed to be a primary effect on the dam which subsequently induced the malformation in the progeny.

Another approach to determining the mechanism of teratogenic action of salicylates came from Smith. His initial finding with Dawkins and Gould (Dawkins, Gould and Smith, 1966) was that salicylates impair protein synthesis. He extended these investigations with Janakidevi by demonstrating that salicylates inhibit the activity of nucleic acid polymerases prepared from rat liver (Janakidevi and Smith, 1969), and interfere with nucleic acid synthesis in mice (Janakidevi and Smith, 1970a). Attention was then focused on rat foetuses at 13 or 16 days post coitum (Janakidevi and Smith, 1970b). This in vitro study showed that salicylate tissue levels of 2 and 10 mM (equivalent to the subcutaneous injections of about 600 and 3000 mg/kg of sodium salicylate) inhibited RNA polymerase activity in the 16 day foetuses, but that the 13 day foetuses were affected only by the 10 mM concentration. These results may be of significance in the induction of foetal death, but the dose level required to induce the 10 mM concentration would almost certainly kill the dam and, therefore, would not be relevant to effects in the embryos. Their findings also indicate that the sensitivity to the inhibitory effect

increases with gestational age. At present, it is not possible to equate this effect with the teratogenic one because few defects are produced after the 12th day post coitum.

Some of the experiments undertaken by Goldman and Yakovac were repeated by Chebotar (1967) using a larger number of rats. He investigated the effects of alkalosis (using sodium bicarbonate), acidosis (using ammonium chloride), maternal stress (induced by immobilisation) and a major tranquillizer (chlorpromazine) on salicylate teratogenicity. His results confirmed the conclusions published earlier by Goldman and Yakovac (1963, 1964a, 1964b).

In addition, Chebotar (1967) found that sodium salicylate was teratogenic in his rats at dose levels between 300 and 600 mg/kg and was lethal to the pregnant does at higher doses. Further, the compound killed the greatest percentage of embryos when given on the 9th day of gestation. Malformations were induced when the dams were dosed between the 7th and 13th days of pregnancy, but the highest incidence occurred after treatment on the 10th day. This is one day later than that found to have the greatest sensitivity by most other workers (e.g. Warkany and Takacs, 1959). Chebotar (1967) also described foetuses with microcaudia and pelvic underdevelopment - malformations previously undescribed in relation to salicylate teratogenicity.

Chebotar's other contribution towards the study of salicylate teratogenicity was also in 1967, as the co-worker of Barilyak. They found that a combination of the minimum teratogenic doses in rats of sodium salicylate (300 mg/kg) and the antidiabetic drug carbutamide (800 mg/kg) lowered the blood sugar level. Glucose was used to induce hyperglycaemia in some of the treated rats, presumably to

try to assess the importance of drug-induced hypoglycaemia in relation to the teratogenicity. However, this could not be evaluated because no group of rats given the salicylate and carbutamide together with the glucose, received equivalent doses of the drugs without supplementary glucose.

Barilyak and Chebotar (1967) also demonstrated a synergistic effect between these two drugs in producing birth defects in rats. A non-teratogenic dose of each compound (25-200 mg/kg of sodium salicylate with 100-600 mg/kg of carbutamide), when administered together in different amounts, induced a teratogenic effect.

The interaction of salicylates and other agents in relation to teratogenicity had received some attention before Barilyak and Chebotar's (1967) publication. Bertone and Monie (1965) reported that treatment of Long-Evans rats with the potentially teratogenic regimens of subcutaneously administered methyl salicylate together with artificially produced hypoxia (equivalent to an altitude of 25000 feet) for six hours, yielded malformations similar in numbers and types to an additive result of the two agents. However, a response greater than this additive one was seen in the case of intrauterine resorption. These results led Bertone and Monie (1965) to suggest that hypoxia aggravated the salicylate-induced increase in oxygen utilisation by the tissues.

A fascinating set of results was presented by Selz and Goldsmith (1966) following their interaction studies in Wistar rats with aspirin and the antihistamine cyclizine. They found, quite predictably, that cyclizine enhanced the teratogenicity of potentially-teratogenic doses of aspirin. However, non-teratogenic doses of

aspirin actually reduced the teratogenicity of potentially-teratogenic doses of cyclizine. It appears from the report, that these results were based only on external morphological examinations of the foetuses. Subsequent skeletal and visceral examinations may illuminate these findings.

A possible misinterpretation of results was shown recently by Lansdown (1970) who examined histologically rat foetal vertebrae damaged by salicylate treatment of the dams. In the introduction to the paper, he wrote that skeletal defects such as, among others, micromelia are 'invariably' induced by high doses of salicylates. An extensive search of the literature showed that congenital limb defects are not induced commonly by these drugs. Further, the only cases of micromelia associated with them were produced in quails by himself and Grasso (1969), but hemimelia was produced in rats by Davis (1969).

Subsequently, in his description of foetuses with craniorrhachischisis, he stated that the nervous tissue appeared normal macroscopically. Such a condition is incompatible with craniorrhachischisis, as it is well known that this defect arises from a primary failure of the neural tube to close (Sternberg, 1929). Unfortunately, the photomicrographs of the defects show only skeletal elements, but the splayed neural arches resemble those described in Chapter 13 of this thesis. It is most unlikely that such a defect could have been associated with normal nervous tissue.

Lansdown (1970) did make one interesting observation. His examination of salicylate-affected foetal cartilage revealed histological changes not observed in the present study. He found

anomalies in chondrogenesis which were followed by a premature hypertrophy and degeneration of the chondrocytes, leading to an irregular onset of ossification. This is contrary to the results of skeletal examinations presented in Chapter 13 where the histological appearance of the bones was normal but was retarded for the stage of development, rather than advanced.

In discussing a possible mechanism of the salicylate-induced defects in the skeleton, however, he appears to have failed to realise that central nervous tissue induces the development of its associated skeletal structures. Possibly as a result of this, he did not consider the action of the salicylate in terms of malforming the central nervous tissue which, in turn, would induce abnormal skeletal development.

(b) Mice

The potential teratogenicity of salicylates has been investigated by fewer workers using other species. Both Loosli et al (1964) and Obbink and Dalderup (1964a) were unsuccessful in attempts to induce congenital malformations in mice with aspirin. The former team administered between 100 and 500 mg/kg from the 7th to the 16th days of gestation and the latter workers gave 1200. mg/kg in the diet from the 6th day of pregnancy until term. The massive dosage given by Obbink and Dalderup (1964a) greatly increased the incidence of foetal death and intrauterine resorption, but no malformation was recorded.

However, in the following year, Trasler (1965) produced malformations in foetal mice whose dams received about 15 or 25 mg/kg of aspirin two or three times daily on the 8th and 9th or 9th and 10th days of gestation. In addition to malformations previously

ascribed to salicylate, she observed short snout, microcephalia and polydactylia. Further, there was a strain difference in susceptibility to these abnormalities and a potentiation of the incidence of spontaneous cleft lip in the more sensitive A/Jax strain.

Larsson and his co-workers at the Karolinska Institute in Stockholm, undertook in mice to investigate more deeply the particular action of salicylates which caused the teratogenic effect. Mammalian embryos show a very rapid rate of acid mucopolysaccharide synthesis under normal conditions. Consequently, Larsson, Boström and Ericson (1963) decided to study the effect on acid mucopolysaccharides, the rate of synthesis of which is inhibited in the adult by salicylates. Initially, A/Jax strain mice were given a single treatment of 10 mg of sodium salicylate on different days of pregnancy. Among the malformations seen were characteristic rib and vertebral anomalies, syndactylia, adactylia and, in addition, certain reddish-brown spots in the skin. Histological examination of these spots showed them to be large masses of blood in 'thin-walled capsules.'

The malformations related to salicylate teratogenicity appeared to involve either the vascular or skeletal systems, both of which contain mucopolysaccharides. This led Larsson, Boström and Ericson (1963) to suggest that vascular changes in the limbs may have caused the malformations in the extremities. They further postulated a possible sequence of events with the reddish-brown spots leading ultimately to limb malformations.

Larsson, Ericson and Boström (1963) followed this work with a more detailed investigation. Using similar techniques, they observed previously undescribed malformations affecting the lens and an

overlapping of the palatine processes of the maxillae, as well as malformations described by other workers in relation to salicylate teratogenicity. The reddish-brown spots discussed in their previous paper received further study. As well as the type described in that paper, dilated blood vessels and diffuse haemorrhages were also observed. The authors equated these spots with the dilated blood vessels described by Greene and Saxton (1939) and Inman (1941) which preceded spontaneous amputation of the ear pinnae of rabbits. This amputation was followed by haemorrhage and necrosis.

Larsson and Boström (1965) went on to inject A/Jax and C.B.A. strain mice intramuscularly with 10 mg (about 500 mg/kg) of sodium salicylate or the sodium salt of its therapeutically inert isomer p-hydroxybenzoic acid, or the sodium salt of acetylsalicylic acid on the 9th or 12th day of pregnancy. Their results showed a good correlation between teratogenic action and the ability to depress acid mucopolysaccharide synthesis. p-Hydroxybenzoic acid does not inhibit this synthesis and induced very few anomalies, while sodium aspirin, which has only a slight inhibitory effect upon the synthesis, yielded a slightly higher incidence of malformed foetuses. Finally, sodium salicylate, which substantially inhibits acid mucopolysaccharide synthesis, was markedly teratogenic.

Larsson and Boström (1965) further related this effect to the uncoupling action by salicylates on oxidative phosphorylation. This would decrease adenosinetriphosphate production and, therefore, 'active sulphate' would not be available for sulphurylation of a mucopolysaccharide precursor. Recent data indicated that salicylates inhibit the enzyme L-glutamine-D-fructose-6-phosphate transaminase

which synthesises glucosamine-6-phosphate. This is a key intermediate in mucopolysaccharide synthesis, and this inhibition supports the evidence of these workers.

These experiments also showed a strain difference in susceptibility to the teratogenicity which was consistent with that found with cortisone, a steroidal anti-inflammatory agent which similarly inhibits mucopolysaccharide synthesis.

At this stage, the importance of the work of Larsson and his team was recognised in an editorial in the British Medical Journal (1963) and by Blattner (1965). The evidence from their work supported their hypothesis that the action of salicylates in inhibiting mucopolysaccharide synthesis in combination, possibly, with their facility to uncouple oxidative phosphorylation may be responsible for the teratogenic effect. However, a number of anti-inflammatory agents inhibit mucopolysaccharide synthesis in vitro (Whitehouse, 1962, 1964b; Whitehouse and Haslam, 1962). Of these, only the salicylates and cortisone show teratogenic activity. This suggests that some other mechanism of action may be instrumental in inducing birth defects.

A technique for measuring the incorporation of the isotope S^{35}-sodium sulphate as an estimation of sulphomucopolysaccharide synthesis was used in rat and calf cartilage by Larsson, Boström and Jutheden (1968) and, later, in calf cartilage by Beaudoin, Boström, Friberg and Larsson (1969). This work added further evidence to the salicylate-induced inhibition of L-glutamine-D-fructose-6-phosphate transaminase.

Larsson was interested not only in the effect of salicylates on mucopolysaccharide synthesis. In 1966, he, Ivemark and Engfeldt reported that a microradiographic technique indicated that a potentially

teratogenic dose of sodium salicylate did not affect mineralisation in foetal mouse skeletons.

Larsson was also concerned with other effects of salicylates on pregnancy besides their teratogenic one. In 1966, with Eriksson, he investigated the influence of the time of administration of sodium salicylate on foetal death and resorption in two strains of mice. Their findings agreed with those of Trasler (1965), that A/Jax mice were particularly sensitive to salicylate intoxication. Reciprocal crossings of the two strains indicated that A/Jax dams and their progeny, irrespective of the sire's strain, were most susceptible. The mice were injected once with sodium salicylate on day 9, 11, 13, 15 or 17 of pregnancy. The incidence of intrauterine resorption increased the nearer to term that the animals were treated.

In this publication, Larsson and Eriksson (1966) discussed seven test foetuses with exencephalia and/or eventration of the abdominal viscera which they considered impossible to ascribe to the salicylate. This decision appears particularly surprising because, although the incidence was low, these types of malformations are frequently described in relation to salicylate teratogenicity (e.g. Warkany and Takacs, 1959).

Eriksson and Larsson (1968) followed up this study on salicylate-induced foetal death by demonstrating that sodium salicylate, but not other anti-inflammatory compounds (chloroquinone phosphate and cortisone acetate), initiate premature birth in their A/Jax mice.

A relationship between salicylate-induced mouse foetal death and superficial haemorrhage (previously investigated in 1963 - see Larsson, Boström and Ericson, and Larsson, Ericson and Boström) was

reported by Eriksson (1969). She found that all dead foetuses showed superficial haemorrhages and liver haemorrhage and necrosis. Superficial haemorrhages occurred in about 50% of the living foetuses and, in turn, about 50% of these also showed the liver changes.

Eriksson (1970) later showed that pretreatment with low doses of salicylate could reduce the effects of a subsequent high dose. Mice were injected intramuscularly with 150 mg/kg/day of sodium salicylate on days 15 and 16 of pregnancy. This was followed by an injection of 500 mg/kg on day 17. The pretreatment significantly reduced the incidence of foetal death below that induced with 500 mg/kg on day 17 alone. A similar reduction of the effect of the high salicylate dose was obtained by pretreatment with narcotic levels of sodium pentobarbitone (75 mg/kg/day intraperitoneally) administered on days 15 and 16.

This protection suggested a drug-induced enhancement of salicylate detoxification. However, while the barbiturate induced liver microsomal enzyme activity, this effect could not be demonstrated for the salicylate. Eriksson (1970) suggested that this may have been due to the study not including glucuronide or glycine formation. As these are major pathways in salicylate metabolism, valid appraisal of the lack of effect with salicylate cannot be made.

A form of aspirin having received very limited investigation is magnesium acetylsalicylate. Uhlenbroock and von Freier (1968) gave this compound to pregnant mice in amounts equivalent to the maximum human dose. It was not surprising that no toxicity or teratogenicity was observed because other salicylates are embryopathic in this species only at several times this dosage.

(c) Hamsters

The golden hamster, in common with the rat and mouse, appears susceptible to salicylate teratogenesis. Lapointe and Harvey (1964) administered two doses of about 320 mg/kg of salicylamide each day on the 6th and 7th or 9th and 10th days of gestation. Characteristic malformations as well as generalised oedema and albinism were observed in the foetuses. The dosage of salicylamide used was approaching the lethal one. Because of this the authors considered that, if this compound effectively traversed the placenta, the embryos appeared more resistant to it than the dam.

(d) Rabbits

Acetylsalicylic acid has been the only salicylate to be investigated for potential teratogenicity in the rabbit. The results have indicated that this compound does not cause reproducible congenital malformations in this species, and experiments reported later in this thesis will support this conclusion.

Earley and Hayden (1964) found that 250 mg/kg of aspirin given between the 6th and 12th days of gestation was lethal to all of the developing embryos, while doses in excess of this killed all of the does treated.

Loosli et al (1964) administered 100 or 300 mg/kg from the 7th to the 16th days of pregnancy, but failed to increase the incidence of malformed foetuses above the control level.

In the following year, Ikeda, Horiuchi, Yoshimoto, Suzuki, Furuya, Kawamata and Kaneko (1965) reported that 200 mg/kg/day of aspirin given from days 8 to 15 of pregnancy was without teratogenic activity in their rabbits.

McColl, Robinson and Globus (1967) met with little more success. They gave 200 mg/kg/day of aspirin to rabbits from days 8 to 16 of pregnancy. The anomalies described were small right auricles and an increase in the incidence of 13th ribs. These have not been described previously in relation to salicylate teratogenicity and no malformation typical of those indicative of salicylate teratogenicity was induced. Further, the auricles may have been in systole - a condition seen frequently in rabbit foetuses killed with barbiturate in the present study. Moreover, the incidence of 13th ribs is so variable in Stride Dutch rabbits that no significance is ever attached to their presence. It appears unlikely, therefore, that these anomalies were related to the aspirin.

The only report of birth defects in rabbits following salicylate treatment came from McColl (1966). He observed aortic arch anomalies in 18% of the foetuses whose dams were given 200 mg/kg of aspirin. Unfortunately, little more detail was given. This is regrettable because it constitutes the first report of salicylate teratogenicity in rabbits. Further, the production of cardiovascular defects following salicylate treatment of rats (Monie, 1964; Takacs and Warkany, 1968) and chicks (Gessner, 1970) indicates that these compounds are cardiac teratogens.

Another approach to the problem of the effects of salicylates on the unborn rabbit was followed by Schardein, Woosley, Hamilton and Kaump (1965). They studied the blastocysts after giving 150 mg/kg of aspirin to the dam on the first six days of gestation. The treatment produced microscopic alterations in the blastocysts. These included retardation in the growth of the embryonic disc,

abnormally shaped or degenerative disc cells, and excessive vacuolation, decreased mitotic activity and distorted nuclei of the trophoblast.

Blastocysts were also the focal point of the investigation by Lutwak-Mann, Hay and New (1969). They incubated 6-day old rabbit blastocysts in a sodium salicylate concentration equivalent to five times that recovered from blastocysts after maternal salicylate administration. After seven hours, they also found microscopic alterations in the blastocysts similar to those described by Schardein et al (1965).

These techniques with blastocysts are extremely elegant. However, in the light of the general lack of success experienced by most workers, among them Schardein et al (1969), who tried to produce malformed rabbit foetuses by treating their dams with salicylates, the relevance of the altered blastocyst to congenital malformations is not clear.

(e) Non-human primates

Many of the experimental teratological studies undertaken during the last decade, particularly those carried out by the pharmaceutical industry, have resulted from the birth defects produced in humans by thalidomide. The rats, mice and rabbits generally used in such investigations, usually of necessity, constitute the test species prior to treatment of women of childbearing potential. Unfortunately, they have many limitations, not the least of which is their teratogenic susceptibility to compounds previously used with comparative safety in humans. Such a problem is much less pronounced in the case of non-human primates which appear to be susceptible to most human teratogens studied in them at similar dose levels. Further,

they are generally not sensitive to many of the small animal teratogens which appear to be safe in humans. Consequently, when attempting to extrapolate the results of animal experiments in terms of potential effects in humans, more validity can be placed upon the work undertaken in monkeys.

Aspirin is the only salicylate to have been tested in pregnant non-human primates and the rhesus monkey is the only primate to have been used. Courtney and Valerio (1968) treated two pregnant monkeys. One was given two doses of 200 mg/kg/day from day 25 until term (day 165) and the other one was dosed identically between days 25 and 120, after which one of the daily doses was discontinued. Both delivered normal offspring.

Wilson and Fradkin (1969) gave their monkeys doses of aspirin larger than the equivalent level teratogenic in the rat. Their results indicated that the rhesus monkey was not susceptible to the teratogenic activity of aspirin. Wilson (1968) went on to treat macaques with six 250 mg/kg doses of aspirin during days 19-22 of pregnancy. His results indicated that the embryopathic activity of aspirin in this species is more likely to result in abortion than in congenital malformation.

(f) Humans

The teratogenicity of salicylates has been demonstrated in the rat, the mouse and the hamster. However, to put the matter into perspective, such an effect has never been conclusively demonstrated in man.

When examining results obtained from the use of p-aminosalicylic acid, the possible effects produced by the patients' tuberculosis

should be considered. This opinion was expressed by Varpela (1964) who reported from Helsinki an increase of two to three times the incidence of malformed babies born to mothers treated for tuberculosis with p-aminosalicylic acid, streptomycin and isoniazid. However, Lowe (1964) found no evidence of p-aminosalicylic acid teratogenicity in Cardiff; neither did Marcus (1967) in the Transvaal.

Reports of malformed children born after maternal salicylate ingestion during pregnancy have been speculative to say the least. Harley (1964) discussed an achondroplastic twin whose mother had occasionally taken aspirin (as well as phenacetin, chloral hydrate and senna pod extract) during pregnancy. The other twin showed jaundice and slight lethargy but no evidence of any other abnormality. It seems unlikely that this occurrence was caused by the aspirin since the same mother subsequently had another achondroplastic child following a pregnancy without a history of aspirin ingestion.

Another report was presented in 1968 when Petrucci and Brunetti described a girl born without a right eye. Her mother had received sodium salicylate therapy for diffuse arthromyalgia throughout pregnancy and had developed hypoprothrombinaemia near term. The authors attributed the latter condition to the salicylate and suggested a possible relation between this treatment and the malformation.

However, the most incriminating report to date was presented by Richards (1969). He made a retrospective survey of 833 consecutive cases of malformed children born in South Wales, and matched them with an equal number of controls. His results showed that 22.3% of mothers in the test group took some form of salicylate during the

first trimester, compared with 14.4% of the control mothers. The increase was highly significant ($P<0.001$).

Richards (1969) discussed the limitations of his enquiry; not the least of these being the large proportion of apparently significant differences which could have occurred by chance. Unfortunately, there was no indication in the paper of whether the salicylate was taken at a stage in pregnancy which would correlate with the type of malformation observed.

This important aspect was not discussed by Nelson and Forfar (1971), whose paper appears to support the findings of Richards (1969). Their retrospective survey involved 458 pregnancies terminating in children with birth defects and 911 controls. They showed that aspirin taken during the first 28 days - but not during the first 14 days - of pregnancy significantly increased the incidence of birth defects ($P<0.05$). This result was based on eight malformed children in the test group compared with three in the controls. Nelson and Forfar (1971) may not have done justice statistically to their results. It appears that the Chi-square test was used to obtain the probability of $P<0.05$. This is a two-tailed test and would allow for aspirin increasing or decreasing the incidence of birth defects. However, if considering only an increase, a one-tailed test involving comparing proportions may have been more pertinent. This would have given the higher level of significance of $P<0.01$.

Nevertheless, consideration of the stage of embryonic development during which the aspirin was taken indicates that only three of the eight defects (hydrocephalia, congenital heart disease and papilloma) could have been induced during this time. The remaining

malformations (achondroplasia, mongolism, congenital dislocation of the hip, hydrocele and talipes) must have arisen at a later stage of development or have been genetically determined. Although it is known that defects may be induced in experimental animals some time after the teratogen has been administered (Murphy, 1959; Wilson et al, 1953), such a condition rarely occurs. Further, it has never been associated with salicylates and was not observed in the present study. With the omission of only ~~two~~ three of these defects from the aspirin group, the difference from the controls is no longer significant. Consequently, the association between aspirin and these defects must remain equivocal.

The most extensive survey to date was undertaken by the Royal College of General Practitioners (Crombie, Pinsent, Slater, Fleming and Cross, 1970), and involved approximately 10,000 pregnant women. The results showed no indication that aspirin taken during pregnancy induced birth defects. As the number of cases studied in this survey greatly exceeded that in other reports, much more significance can be attached to its findings.

It appears that, if salicylates will ultimately be shown to be teratogenic in humans, they will exert this effect in only a very limited number of cases. In order to determine whether they have this property, surveys incorporating a much higher number of pregnancies terminating in babies with birth defects will have to be studied. Further, details of the critical time at which the drug was taken will have to be equated with the malformation of the child.

Conclusions

The salicylates are a group of compounds having embryopathic activity. The extent and manifestations of this vary with the type of salicylate, its dosage and the animal species and strain. A summary of investigations into the teratogenic activity of salicylates in mammals is presented in Table 3.1. These drugs appear to be toxic to all unborn animals including man, and abortifacient in monkeys, rabbits and mice. Some preparations are unquestionably teratogenic in rats, mice and hamsters, and are possibly so in rabbits. However, such activity has never been demonstrated conclusively in primates.

TABLE 3.1

SUMMARY OF TERATOLOGICAL INVESTIGATIONS WITH SALICYLATES IN MAMMALS

Compound	Species	Teratogenicity	Authors
Acetylsalicylic acid	Man	-	Crombie et al (1970)
		-	Harley (1964)
		+	Nelson and Forfar (1971)
	Rhesus monkey	-	Courtney and Valerio (1968)
		-	Wilson (1968)
		-	Wilson and Fradkin (1969)
	Rat	+	Baba and Nagahama (1966)
		+	Baba et al (1966)
		-	Brown and West (1964)
		+	Loosli et al (1964)
		+	McColl et al (1965)
		+	Monie (1964)
		-	Obbink and Dalderup (1964a, 1964b)
		+	Schardein et al (1969)
		+	Selz and Goldsmith (1966)
		-	West (1964)
	Mouse	-	Loosli et al (1964)
		-	Obbink and Dalderup (1964a, 1964b)
		+	Trasler (1965)
	Rabbit	-	Earley and Hayden (1964)
		-	Ikeda et al (1965)
		-	Loosli et al (1964)
		+	McColl (1966)
		-	McColl et al (1967)
		-	Schardein et al (1969)
Magnesium acetylsalicylic acid	Mouse	-	Uhlenbroock and von Freier (1968)
Sodium acetylsalicylic acid	Mouse	+	Larsson and Boström (1965)
Methyl salicylate	Rat	+	Bertone and Monie (1965)
		+	Takacs and Warkany (1968)
		+	Warkany and Takacs (1959, 1968)

TABLE 3.1 contd/

Compound	Species	Teratogenicity	Authors
Phenyl salicylate	Rat	+	Baba et al(1966)
Sodium salicylate	Man	?	Petrucci and Brunetti (1968)
	Rat	+	Barilyak and Chebotar (1967)
		+	Chebotar (1967)
		+	Goldman and Yakovac (1963, 1964a, 1964b, 1965)
		+	Gulienetti et al (1962)
		-	Lemaire and Grosjean (1962)
		+	Takacs and Warkany (1968)
		+	Warkany and Takacs (1959, 1968)
	Mouse	+	Eriksson (1969)
		+	Larsson and Boström (1965)
		+	Larsson, Boström and Ericson (1963)
		±[1]	Larsson and Eriksson (1966)
		+	Larsson, Ericson and Boström (1963)
p-Aminosalicylic acid	Man	-	Lowe (1964)
		-	Marcus (1967)
		+[2]	Varpela (1964)
	Rat	-	Follmer and Mayer (1953)
Salicylamide	Hamster	+	Lapointe and Harvey (1964)
Any salicylate	Man	+	Richards (1969)

[1]Larsson and Eriksson (1966) described certain malformations which they could not relate to the salicylate. Although only a small number was observed, they were of a type associated with the teratogenicity of these compounds. For this reason, attention has been drawn to them in this table.

[2]Patients also received streptomycin and/or isoniazid.

CHAPTER 4

BASIS OF PROJECT

The salicylates are used therapeutically more extensively than any other group of drugs. Their principal value is as anti-inflammatories, analgesics and antipyretics, and their availability to the public is unrestricted. Consequently, a large percentage of the population take them indiscriminately to combat a wide range of ailments.

The history of their adverse effects on pregnancy and their teratogenicity in certain species discussed above stimulated this investigation. It was decided to try to relate the chemical structure of salicylates to their effects upon the developing embryos of some experimental animals. Further, considerable attention was to be directed towards the detailed examination of foetuses with salicylate-induced birth defects. This aspect of the work was recognised as being an integral part of attempting to understand the aetiology of the defects.

Initial experiments entailed confirmation of results reported by other workers. In contrast, the investigations into the effects on the embryo of different chemical groups related to the salicylate molecule and many of the observations made on the malformed foetuses are entirely original.

It is hoped that this work will contribute towards understanding the nature of salicylate embryopathies and of other birth defects.

S E C T I O N B

TECHNIQUES

CHAPTER 5

TECHNIQUES

Experimental Animals

The investigations were carried out in AH A (Allen and Hanburys Albino) rats and Dutch rabbits. The rats, which were derived from Charles River Sprague-Dawley stock and Manor Farm Wistar stock, were primiparous and were supplied by Allen and Hanburys rat breeding unit. They weighed between 220g and 270g at mating. The rabbits were supplied by an outside breeder. They were multiparous, having had one to three normal litters before being used in the experiments. Their weight at mating ranged from 2kg to 2.5 kg.

Mating

Only natural mating was used.

Eight female rats were introduced into a breeding unit containing three males. The breeding units were made to specification. Different combinations of numbers of males and females have been run together, but these numbers proved the most productive. Vaginal smears, utilising physiological saline introduced by a Pasteur pipette, were examined daily. The presence of sperm in the smear signified that copulation had occurred and was taken as an indication of the start of pregnancy. The day on which sperm first appeared was called day 1.

One doe rabbit was introduced into a cage containing one buck, and two mating acts were allowed before she was removed. The day of mating was called day 1.

Rabbits were allocated into groups on a weight basis which resulted in total weight of each group being approximately the same.

It was deemed unnecessary to use this procedure for rats because all those used for any given experiment were of remarkably uniform weight. Consequently, allocation into groups was purely random.

Husbandry

The room temperature of the animal houses was 17.5°C ± 2°C and there were 8-10 air changes per hour.

Rats were housed, generally in groups of four, in M.R.C. cages and fed Oxoid breeding diet and water ad libitum. Rabbits were housed individually in galvanised iron cages made to specification, and were fed BOCM coney rabbit pellets and water ad libitum. The available details of the constituents of the diets are presented in Appendix 1.

Treatment

The following B.P. compounds were used:- acetylsalicylic acid, benzoic acid, salicylic acid and sodium salicylate. The calcium carbonate and phenol were Analar reagents. o-Methoxybenzoic acid was obtained from The British Drug Houses Limited and salicylamide came from Bush Boke Allen Limited. Koch-Light Laboratories Limited supplied the various dihydroxybenzoic acids. The structural formulae of all the compounds used are presented in Fig. 5.1. All insoluble drugs were passed through a British Standard Number 200 sieve before use, ensuring that the maximum particle size was 75 μ. Most of the particles in the micronised aspirin were between 1.66 μ and 3.31 μ. This was determined microscopically according to the method described by the British Standards Institution (B.S. 3406: Part 4: 1963). More detailed information on the particle sizes is

presented in Appendix 2. Soluble aspirin consisted of a 3:1 by weight ratio of acetylsalicylic acid and calcium carbonate.

The preparations were administered orally during the gestation period. The vehicle varied according to the solubility of the drug and to concurrent experiments. Generally, soluble compounds were administered in distilled water and insoluble ones were suspended in a 0.25% aqueous solution of sodium hydroxyethyl cellulose (Natrosol). In one investigation the surfactant sodium dioctylsulphosuccinate (Mannoxol OT/P) was used in a 0.001% aqueous solution.

Rats were restrained manually and the preparation was introduced into the oesophagus by means of a metal dosing needle. A standard volume of 10 ml per kg body weight was administered.

Excessive movement of the rabbits was restricted by wrapping them in a cloth and a Meredith plastic catheter (10 ch.) was used to introduce the preparation into the oesophagus. This was facilitated by the use of a perspex gag made to specification. A standard volume of 2 ml per kg body weight was administered.

Post-Mortem Procedures

The pregnant animals were usually killed two days before anticipated parturition.

Rats were killed by chloroform inhalation 20 days post coitum and their viscera exposed from a ventral aspect. The genitalia were removed and both uterine horns were opened. The foetuses were removed and dissected from their extra-embryonic membranes, and the placenta removed by breaking the umbilical cord at a point of weakness close to the foetus - a point at which minimal bleeding occurs. The foetuses and placentae were weighed and the foetuses

were examined microscopically (general conformation, buccal cavity, limbs, urinogenital opening and anus). They were labelled according to their position in the uterus and fixed in 70% alcohol (I.M.S. 74 O.P.). Resorption sites were weighed and resorption scars were counted. Some of the foetuses were eviscerated and their skeletons were stained according to a variation of Williams' (1941) modification of Dawson's (1926) technique. This clears the muscles and connective tissue and stains the ossified bone red and the cartilage blue. The techinique is given in Appendix 3. Their bone conformation was examined microscopically. The remaining foetuses were sectioned either transversely, by a technique similar to that described by Wilson (1965), or longitudinally, and examined microscopically.

Rabbits were killed by cervical dislocation 30 days post coitum and their viscera exposed from a ventral aspect. Their dissection was similar to that described for the rat but the foetuses were fixed in 10% formol saline. Some of the foetuses were subsequently eviscerated and x-rayed. The radio-opacity of the skeletons was increased by subjecting them to a technique based on that described by O'Rahilly and Meyer (1956). Briefly, the skinned and eviscerated foetuses were fixed in 10% formol saline and then immersied in 0.5% aqueous silver nitrate for 5-8 days. Details of the radiographic technique appear in Appendix 4. The bone conformation was subsequently examined on the radiographs. The remaining foetuses were sliced in a similar way to the rats.

Histological Procedures

Tissues intended for histological examination were embedded in paraffin wax. Any bony material was initially decalcified in 8% aqueous formic acid. The processing of the tissue involved the removal of all the air and water therefrom and their subsequent replacement with paraffin wax. This was achieved automatically by using an Elliott Tissue Processor, and took 18 hours. During this period, the formol saline-fixed tissue was passed through 70%, 90% and 96% alcohol, four changes of absolute alcohol, three of xylol and three of molten paraffin wax, the last of which was under vacuum. It was finally cast in blocks of paraffin wax which were supercooled to prevent crystallisation.

Sections were cut to a thickness of 4μ on a base sledge microtome, floated onto slides and dried. They were subsequently stained according to the Mayer's haematoxylin and eosin technique. The mechanism of the haematoxylin staining is not fully understood, but it is used here as a nuclear stain which appears to be effected largely by virtue of its potassium alum mordant. Eosin is an acid dye which is negatively charged, this conferring anionic activity. It therefore attaches to positively charged, acidophilic tissues such as connective tissues. The technique is presented in Appendix 5.

Statistical Analyses

If the results obtained from test animals were appreciably different from the controls, they were subjected to the 'Student's' *t* test to determine whether the difference could have been due to chance. The value of *t* was determined from the expression:-

$$t = \frac{\text{difference between means}}{\text{standard error of difference between means}}$$

and the percentage points of the t distribution were obtained from the table presented by Bernstein and Weatherall (1952).

On occasions, the assumptions of the t test were shown to be incorrect by results obtained from related experiments. In these cases, the Mann-Whitney U test was applied. This is a useful alternative to the t test and assesses whether two independent groups could have come from the same population. It is based on ranking, and U is the number of times that a score from the group of n_1 cases is preceded by one from the group of n_2. The probability of the values associated with U was determined from the tables reproduced by Siegel (1956).

Where results from the two tests differed, that difference was generally small. The level of significance was taken as $P<0.05$ and, when the probability approximated closely to this value, both tests were applied. Where the results were conflicting, interpretation was based on related findings and discussion of all such cases is presented in the text.

Determination of Serum-Salicylate Levels

The method employed was based on that described by Keller (1947) in which the recovery of salicylate is up to 100%. It involves the use of a colorimeter and determines the total of salicylic acid plus its salicyl metabolites.

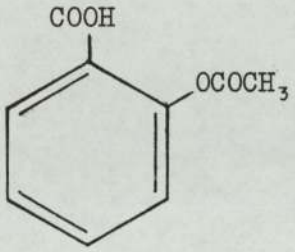
The technique involved the use of non-pregnant female rats which were dosed orally with the salicylate preparation and killed at intervals thereafter. A quantity of approximately 10 ml of blood

was collected from the posterior vena cava immediately after death, and a volume of approximately 5 ml of serum was obtained from it. It was necessary to kill the rats because regular withdrawal of blood from one rat would not yield sufficient serum.

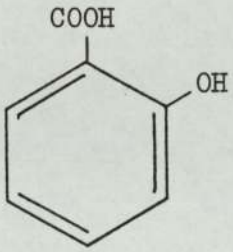
Details of the technique are presented in Appendix 6.

FIG. 5.1

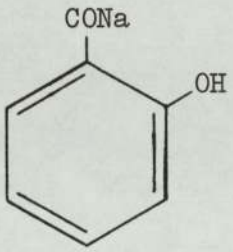
STRUCTURAL FORMULAE OF COMPOUNDS STUDIED IN THE THESIS



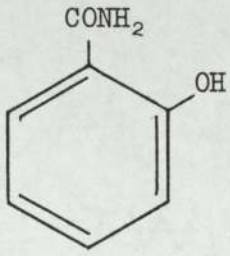
(a) Acetylsalicylic acid



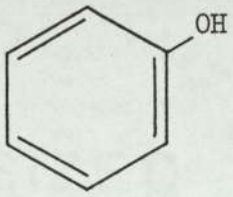
(b) Salicylic acid



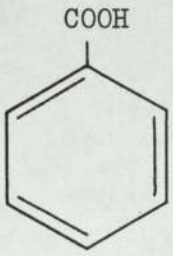
(c) Sodium salicylate



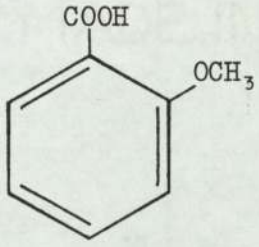
(d) Salicylamide



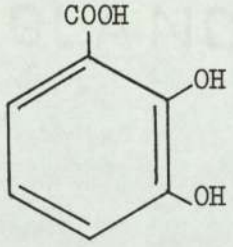
(e) Phenol



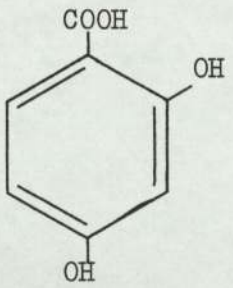
(f) Benzoic acid



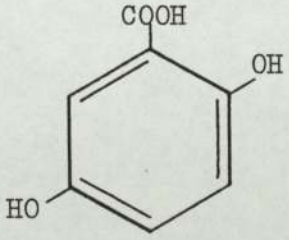
(g) o-Methoxybenzoic acid



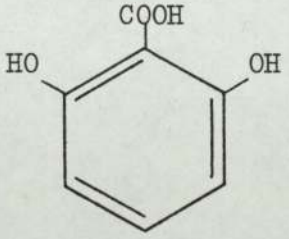
(h) 2,3-Dihydroxybenzoic acid



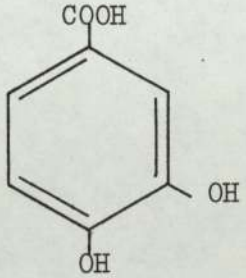
(i) 2,4-Dihydroxybenzoic acid



(j) 2,5-Dihydroxybenzoic acid



(k) 2,6-Dihydroxybenzoic acid



(l) 3,4-Dihydroxybenzoic acid

S E C T I O N C

TOXICITY, TERATOGENICITY AND INTRAUTERINE DEATH

CHAPTER 6

TOXICITY, TERATOGENICITY AND INTRAUTERINE DEATH

The administration of potentially teratogenic regimens to pregnant animals often involves high doses of the teratogen. In such cases, maternal toxicity may be induced and this is most easily recognisable by a loss of weight. In the experiments described in this thesis, treatment was restricted to a specific time in early pregnancy. Any reduction in maternal weight during this period could be interpreted as a direct toxic effect of the preparation on the dam. However, in some cases, the retardation in weight gain may continue after the drug has been withdrawn, and is maintained until term. The explanation of this is rather more complicated. It may be caused by a prolonged effect of the drug on the dam, even after dosing has been discontinued. Alternatively, the drug may be toxic to the embryos - either directly or mediated through some effect on the dam - and delay the growth rate of the foetuses. At term, such foetuses would appear smaller, having lower weights and a lesser degree of ossification than controls. Further, malformed foetuses are generally smaller than normal ones. If this embryotoxic effect is more severe, some of the conceptuses may die in utero. Either of these events would retard maternal weight gain, particularly during late pregnancy. Further, it is likely that effects on the weight gain of the dam would have arisen from a combination of two or more of these possibilities. A graph illustrating maternal weight during pregnancy in relation to some of these considerations is presented in Fig. 6.1.

In the following chapters on the experimental work, all of the details known in relation to these factors will be presented in discussion.

When teratogens are administered, they may result in the production of biological malformations which are usually congenital. Such malformations may be caused by a variety of factors, but this thesis is primarily concerned with those induced by the action of drugs given to the dam during pregnancy. Birth defects produced by a specific teratogen may occur in all species or may be restricted to one strain within a species. These defects must be of characteristic types which would relate to the amount of teratogen administered and the stage of gestation during which it had been given. A teratogen may be considered efficient if it can reproducibly malform a high percentage of conceptuses and, in so doing, exert a minimum level of maternal toxicity and intrauterine death. The malformations may represent an increase in the incidence of 'naturally-occurring' or 'spontaneous' ones, or an induction of completely novel ones.

The so-called spontaneous malformations appear when the dam has not been subjected to any known exogenous teratogen. Their cause may be inheritance, mutation or some other unknown factor. Before studying specifically-induced defects, it is essential to be aware of the incidence and types of spontaneous ones which occur in the experimental animals to be used. These are summarised in Table 6.1, and represent observations made between 1964 and 1970 on rabbits and between 1967 and 1970 in rats and dogs. Detailed analyses of the numbers and types of malformations are presented in Appendices 7, 8 and 9.

In addition to the rats and rabbits studied later in this thesis, the incidence of spontaneous malformations in Beagle dogs produced in the Allen and Hanburys breeding colony has been included.

On occasions, malformations may be so severe that they result in the death of the developing embryo or foetus. Alternatively, death may be caused by other factors, such as (1) a direct lethal effect on the conceptus of an exogenous substance administered to the dam; or (2) indirectly on the conceptus, but mediated through an effect on the dam; or (3) maternal cachexia induced by an exogenous substance or other mechanism. Intrauterine death in rodents and lagomorphs is followed by a phenomenon termed 'resorption'. This is a most important process which has evolved in this group of animals as one factor in their specialisation of reproduction, which has contributed greatly to their undoubted success.

Resorption involves the selective reabsorption of the dead conceptus and its placenta back into maternal circulation, and is rarely completed by the termination of the pregnancy. The conceptus is resorbed first and is followed by its placenta. The condition of the resorbing conceptus at term has been the subject of different terminology but, in this thesis, the following will be used. The term dead foetus will be applied where any remnant of the foetus is attached to the placenta; resorption site will be used when only the resorbing placenta remains; and resorption scar will refer to the scar of attachment of the totally resorbed placenta to the uterine wall. The size of the dead foetus or resorption site at term is an indication of the time at which the death of the conceptus occurred.

Since this system was adopted, Jacobsen (1970) classified resorptions into four 'types' but, in so doing, made no mention of resorption scars. The term resorption site used in this thesis occupies two 'types' differentiated on a size basis in Jacobsen's (1970) classification. His histological examinations confirmed my observations that all resorption sites are placentae having undergone variable degrees of resorption. My own studies have revealed many intergrades between the two extremes described by Jacobsen (1970). His other two 'types' constitute dead foetuses, but he divides them on the basis of the macroscopic appearance of the macerated foetus. This is not entirely satisfactory as it has always been difficult to determine the point at which the foetal remnant is no longer recognisable as such.

Any system attempting to classify intrauterine resorption is fraught with the difficulty that the process may be initiated at any stage in pregnancy, and that it is continuous. Therefore, there are no clearly defined stages, necessitating a very general approach to the problem.

In other animal groups, intrauterine death may result in the loss of any other developing sibling. The process of resorption in rodents and lagomorphs allows the survival of one or more conceptuses after the death of others in the litter.

The incidence of spontaneous intrauterine death in the rats and rabbits is presented in Table 6.2. These data represent results obtained over six years of working with the rabbits (1964-1970) and three years with the rats (1967-1970) and show a remarkable similarity in the incidence in both species.

FIG. 6.1

INFLUENCE OF DIFFERENT FACTORS ON

MATERNAL WEIGHT CHANGE DURING PREGNANCY

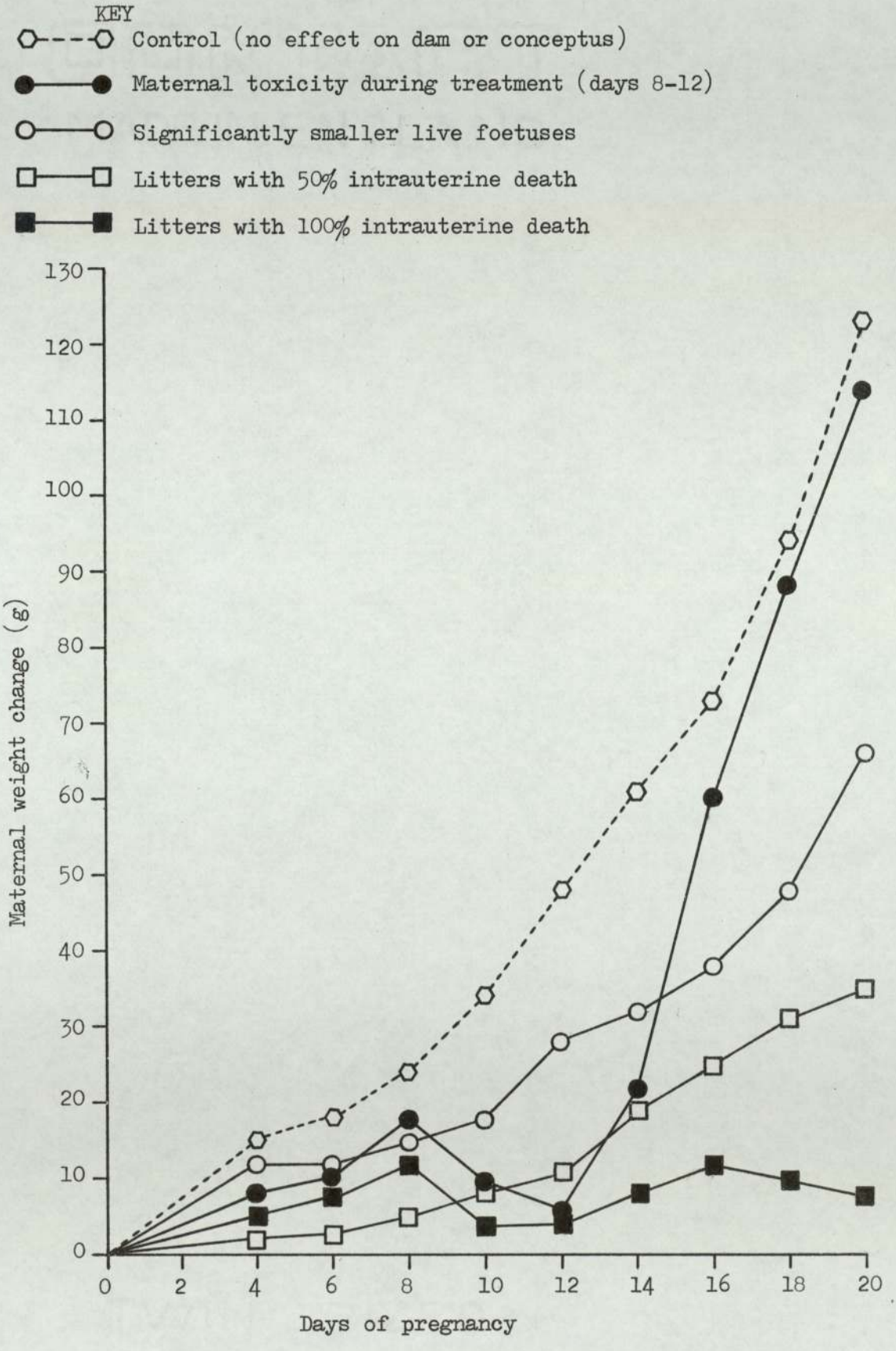


TABLE 6.1

INCIDENCE OF SPONTANEOUS CONGENITAL MALFORMATIONS IN EXPERIMENTAL ANIMALS

Test animal	Total No. of foetuses	Total No. of spontaneously malformed foetuses	Incidence of spontaneously malformed foetuses
AH Wistar rats	19193	359	1.87%
Stride Dutch rabbits	7720	119	1.54%
AH Beagle dogs	3109	21	0.68%

TABLE 6.2

INCIDENCE OF SPONTANEOUS INTRAUTERINE DEATH IN EXPERIMENTAL ANIMALS

Species	Total No. of litters	Total No. of implantation sites	Total No. of live foetuses	Mean No. of live foetuses per litter	Total No. of intrauterine deaths	Mean No. of intrauterine deaths per litter	Incidence of intrauterine deaths
AH Wistar rats	1831	21628	19193	10.48	2435	1.33	11.27%
Stride Dutch rabbits	1241	8713	7720	6.22	993	0.80	11.40%

S E C T I O N D

EXPERIMENTAL RESULTS

CHAPTER 7

INVESTIGATIONS IN RABBITS

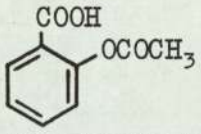
Introduction

Knowing that aspirin, sodium salicylate and salicylic acid are teratogenic in the rat, and salicylamide is so in the hamster, it was decided to extend these observations by investigating the susceptibility of the rabbit. This species was studied because of its extensive use in reproductive and teratogenic experiments.

Experimental Work

For convenience, each drug used will be considered separately. The standard techniques described in Chapter 5 were employed throughout.

a) Acetylsalicylic acid



The teratogenic effect of aspirin in rats and mice was discussed in Chapter 3. The failure to produce such an effect in the rabbit was described by Earley and Hayden (1964), Loosli et al (1964) and McColl et al (1967). Although Schardein et al (1964) disrupted blastocysts by incubating them with aspirin, this team subsequently reported no teratogenicity after treating the pregnant dams (Schardein et al, 1969). McColl (1966) produced aortic arch anomalies in 18% of the foetuses from dams given 200 mg/kg of aspirin, but gave no information on actual numbers affected, and provided no detail of the nature or site of the malformations. Further, in a later paper on the same subject (McColl et al, 1967), no mention was made of this.

It seemed reasonable to start the investigations with the dose level reported to be teratogenic by McColl (1966) - i.e. 200 mg/kg - but, in the experiments described here, only soluble aspirin was used. Two higher doses were also tested in the first experiment (7.A). The highest level in this experiment (250 mg/kg/day) was that with which Earley and Hayden (1964) killed all the developing embryos. The failure to reproduce malformed foetuses regularly led to the establishment of three subsequent experiments in which progressively higher dose levels were administered. The protocol for these is given in Table 7.1. Although Earley and Hayden (1964) killed all their dams with doses in excess of 250 mg/kg/day, Stride Dutch rabbits survived treatment with doses up to 600 mg/kg/day.

The condition of all does given 625 or 650 mg/kg/day of soluble aspirin deteriorated rapidly and they died or were killed within five days after the first dose. They became anorexic and showed fatty livers and gastric haemorrhages. However, other rabbits were able to tolerate nine consecutive daily doses of up to 600 mg/kg.

All rabbits dosed with aspirin lost weight appreciably during the treatment period. The loss was most marked after the first dose, where it varied from 87g to 398g, whereafter it became much less severe. There was no relationship between weight loss and the dose level of aspirin or the initial weight of the dam. Further, four of the 17 control dams lost a small amount of weight (23g — 42g) after the onset of dosing. When the aspirin was withdrawn, most rabbits proceeded to gain weight but at no stage did their weights compare faviourably with those of the controls. In summary, the toxicity as

indicated by weight loss in the test rabbits was obviously due to the aspirin treatment but was not dose-related.

Four test dams did not recover from the effects of the aspirin even after treatment was discontinued. One such animal was observed in each of the 200, 250, 275 and 400 mg/kg groups. Their condition did not improve and they lost weight until they started to abort. This occurred between 19 and 21 days post coitum, and these does were then killed and examined. All four were emaciated, had fatty livers and showed gastric ulceration. They contained resorption sites and dead foetuses, none of which showed any malformation. Again, the frequency and intensity of effects bore no relation to dose level.

One dam given 200 mg/kg/day of aspirin and one control dam from a different experiment (7.C) littered on the 29th day of gestation. Both had normal, healthy progeny but they were omitted from the results presented in Table 7.2. It was considered that the aspirin was not implicated in the case of the test animal. Fourteen rabbits of the 82 mated did not become pregnant.

Table 7.2 summarises the results obtained with soluble aspirin. The most pronounced effect was seen in the mean weight of the test foetuses. The following doses significantly reduced the mean foetal weight below the control value:- 200 mg/kg by 26% ($P<0.001$), 250 mg/kg by 11% ($P<0.02$), 300 mg/kg by 22% ($P<0.01$), 500 mg/kg by 12% ($P<0.01$), 550 mg/kg by 23% ($P<0.05$) and 600 mg/kg by 20% ($P<0.05$). The affected foetuses showed retarded ossification. However, there was no significant effect in the animals treated at 225, 275, 350, 400 or 450 mg/kg. It is difficult to evaluate such results, but it is

conceivable that the effect of aspirin varied with different rabbits and was not dose-related above a certain level.

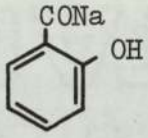
There were 346 foetuses alive at term and all were examined in detail. Four of these showed abnormalities. Limb flexures (Figs. 7.1B and 7.2), which were seen in one foetus whose dam was treated with 500 mg/kg/day, are known to occur spontaneously in these rabbits. Although no such defect was observed in the controls in this study, it was considered that the aspirin was not implicated.

Two siblings from another doe in this group showed ablepharia (Fig. 7.3), a malformation which occurs spontaneously in 0.13% of these rabbits. Its incidence in Experiment 7.C was obviously higher than this (1.75%) but the lack of effect at higher dose levels indicated that it was not drug-induced.

Similarly, the isolated case of microcaudia (Fig. 7.4) in the 250 mg/kg group appears to have no relation to the aspirin. However, the recurrence of this defect after treatment with other salicylates (described below) will be discussed later in this chapter.

This section of the investigation demonstrated that soluble aspirin had no reproducible teratogenic effect in Stride Dutch rabbits. The remarkably high dose of 625 mg/kg/day killed all treated does, but the marginally lower level of 600 mg/kg administered daily throughout organogenesis produced considerable maternal toxicity without inducing any detectable congenital malformation.

b) Sodium salicylate



Having demonstrated that it is most unlikely that soluble aspirin is teratogenic in the rabbit, attention was directed towards sodium salicylate which produces congenital malformations in rats and mice. However, there is no report of its administration to pregnant rabbits during organogenesis at dose levels likely to produce birth defects. Since the work described in this chapter was undertaken, Lutwak-Mann et al (1969) demonstrated adverse effects in rabbit blastocysts incubated with the drug. Unfortunately, in the light of the reports by Schardein et al (1965, 1969), who failed to produce birth defects with doses of aspirin which were known to disrupt blastocysts, these results cannot be evaluated as an indication of a teratogenic effect.

As no other author had administered sodium salicylate orally, the lowest dose level in the first experiment (7.E) was that selected initially for aspirin. Failure to demonstrate a teratogenic effect led to the establishment of two successive experiments (7.F and 7.G) and the protocol for all these is given in Table 7.3.

There were two intercurrent deaths in this series of experiments. These occurred in Experiment 7.F, where the condition of one doe given 400 mg/kg/day markedly deteriorated and the animal died on the 22nd day of pregnancy; also, a control doe was killed after starting to abort on the 27th day. Both dams had a large bolus of matted hair obstructing the pyloric region of the stomach. They also had scant intestinal contents, fatty livers, and emaciated muscles, and their

uteri contained only resorption sites. The condition of both animals was considered to have been caused by the fur balls. Eleven of the rabbits mated did not become pregnant.

As was the case with aspirin, all test animals lost weight during the treatment period. This was not quite so pronounced with sodium salicylate but there was a clearer correlation with the dose level. While the control dams gained a mean of 146g during the nine consecutive daily treatments, those given 200 mg/kg/day of the drug lost 203g, those given 300 mg/kg/day lost 210g, those given 400 mg/kg/day lost 201g, those given 450 mg/kg/day lost 236g, those given 475 mg/kg/day lost 284g, those given 500 mg/kg/day lost 298g, and those given the highest dose of 525 mg/kg/day were the most severely affected, losing 304g. When the treatment was discontinued, the condition of the test dams improved and, by term, all except those given 475 mg/kg/day and over were gaining weight to the same extent as the controls.

The results of this study are presented in Table 7.4. Rabbits dosed with 450 mg/kg/day showed an increase in the number of resorption sites by a factor of 12 compared with the controls. Although this difference is considerable, it is not significant statistically. Neither does it appear to be so biologically, because there was no increase in intrauterine death in the does given the larger doses of 475 or 500 mg/kg/day.

Only in Experiment 7.G was there any effect upon foetal number or size. No foetus survived 525 mg/kg/day where, by term, only resorption sites and scars remained. In this group, the incidence of resorption was more than twice the control value but this difference was not

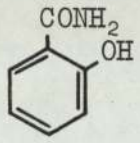
significant statistically. However, it appeared to be a drug effect because the test dams showed only resorption. That the difference was not significant was due to the small number of control does which became pregnant. It was decided not to repeat the experiment to clarify this point because the purpose of the investigation was to attempt to produce birth defects and there had been no indication of drug-induced teratogenicity. At the two lower levels there was a drug-induced retardation of foetal growth. At term, the foetuses from the dams given 475 mg/kg/day were 19% smaller than the controls ($P<0.01$) and those from dams given 500 mg/kg/day were smaller by 17% ($P<0.02$). The effect on the smaller foetuses, which showed retarded ossification, was reflected in the weight gain of their dams which was less than that of the controls.

The most important information derived from this series of experiments relates to the malformations, where there were three cases of microcaudia (Fig. 7.4) among the 196 foetuses examined at term. One occurred at each of the two highest dose levels where foetuses survived and, in view of the findings with aspirin, may suggest an effect of the salicylate. However, a similar abnormality was observed in the control group of Experiment 7.E, indicating that such defects can occur spontaneously in these rabbits. Limb flexures (Figs. 7.1B and 7.2) were also observed in these investigations and discussion of such defects is presented below (page 92).

The tests with sodium salicylate showed that, like aspirin, this compound had no reproducible teratogenic effect in Stride Dutch rabbits. While aspirin killed the dam before the conceptus, sodium

salicylate killed all the conceptuses but not the dams at 525 mg/kg/day, while foetuses survived the slightly lower level of 500 mg/kg/day. This latter dose produced considerable maternal and foetal toxicity but did not induce birth defects.

c) Salicylamide



Since Lapointe and Harvey (1964) reported that large doses of salicylamide were teratogenic in golden hamsters, it was decided to investigate the susceptibility of rabbits to this compound. The study involved four experiments, the protocol for which is given in Table 7.5.

Salicylamide was by far the most innocuous drug tested in rabbits. Even the highest dose administered (1000 mg/kg/day) had no effect upon the weight gain of the pregnant rabbits. There were three intercurrent deaths, involving one doe in the 500 mg/kg group and two controls. All died from the the effects of fur balls obstructing the pyloric region of the stomach. Fourteen rabbits did not become pregnant.

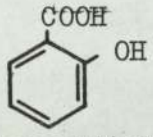
The results of this study are summarised in Table 7.6. Both test groups in Experiment 7.I had significantly fewer foetuses per litter than the controls. The number was reduced by 29% ($P<0.01$) in the 300 mg/kg group, and by 32% ($P<0.002$) in the 400 mg/kg group. The incidence of intrauterine resorption was increased in these groups, there being no case in the controls. However, such values are within the normal limits for these rabbits. In view of the absence of an effect on litter number in the groups given much larger doses, it was

considered that the statistically significant differences seen in Experiment 7.I had no biological significance and, therefore, were not related to salicylamide.

Examination of the 173 foetuses in this investigation revealed three with defects. One test and one control foetus showed only limb flexures (Figs. 7.1B and 7.2) but the other was grossly malformed (Fig. 7.1). It occurred in the 400 mg/kg group and showed cranioschisis, hydrocephalia, scoliosis, hemivertebrae, limb flexures, talipes equinovarus and arthrogryposis. This overall syndrome has never been observed previously in these rabbits but each defect has occurred individually before. As there was no other comparable malformation at this or any higher dose level, this case was considered to be not drug-induced.

No subsequent investigation was undertaken with salicylamide. This was because it was considered impracticable to increase the dose beyond 1 g/kg as there was no indication of any toxic effect on the dam or conceptus at this level.

d) Salicylic acid



The production of congenital malformations with salicylic acid in rats, reported in Chapter 12 of this thesis, initiated the testing of this substance in rabbits. This involved three experiments and the protocol for these is given in Table 7.7.

Thirteen rabbits showed no evidence of pregnancy. All dose levels of the compound (150 - 450 mg/kg/day) were toxic and reduced

maternal weight during the treatment period. The amount of weight lost was clearly dose-related. The mean weight lost throughout the dosing period was as follows:- 74g in the 150 mg/kg group, 97g in the 200 mg/kg group, 119g in the 250 mg/kg group, 201g in the 300 mg/kg group, 264g in the 350 mg/kg group, and 409g in the 400 mg/kg group. Those rabbits given the extremely toxic dose of 450 mg/kg/day lost a mean of 426g before they were killed. The dams treated with doses of up to 350 mg/kg/day recovered after the drug was withdrawn and, except for one in the 250 mg/kg group which subsequently aborted, showed an overall weight gain throughout pregnancy similar to that of their respective controls. Only one test rabbit in Experiment 7.N survived until term. She was given 400 mg/kg/day and recovered well when the treatment was discontinued, but never gained weight to an extent comparable with the control.

There were ten intercurrent deaths in these investigations. One doe in the 250 mg/kg group and two controls (one from each of Experiments 7.L and 7.M) were killed after starting to abort. This had resulted from their poor condition which deteriorated because they had fur balls. These does contained only resorption sites. The other seven deaths were attributed to the toxicity of salicylic acid. Three of the four rabbits given 400 mg/kg/day did not recover from the effects of the drug and were killed between the 17th and 23rd days of pregnancy because of their poor condition. Similar cachexia necessitated the killing, after between five and eight doses, of all four dams given 450 mg/kg/day. The condition of these dams was similar to that described above (page 82) in association with toxic doses of salicylates.

The results of the study are presented in Table 7.8. Dose levels of up to 350 mg/kg/day did not affect the number of foetuses in each litter or their weights. Only one doe survived a higher dose and this animal showed only intrauterine resorption. This was reflected in her lower weight gain towards term.

Detailed examinations were carried out on the 179 foetuses coming to term. One foetus in the 300 mg/kg group and one control from the previous experiment (7.L) showed limb flexures (Figs. 7.1B and 7.2) of the type which occurs spontaneously in these rabbits. What appears more important, however, is a further case of microcaudia (Fig. 7.4) seen in this investigation. It occurred in the 350 mg/kg group, the highest level at which foetuses survived. Discussion of this defect in conjunction with similar ones also observed is presented below.

The experiments with salicylic acid demonstrated that this compound was lethal to the embryos at about the same level as it was to the dams. Lower doses, which induced maternal toxicity, did not affect the development of the conceptus.

Discussion

While these experiments did not produce definite evidence of teratogenicity, they did produce some topics worthy of discussion. For instance, salicylamide had no effect even when given in extraordinarily high doses, and no further discussion of this drug will be made here. In fact, the rabbits tolerated very large doses of the other drugs. The sublethal dose of aspirin, for example, was over double that with which Earley and Hayden (1964) killed all their rabbits.

An interesting observation relates to the highest doses of the different drugs. Sodium salicylate killed the foetuses before killing the dams but, with aspirin, the reverse was the case. However, salicylic acid killed both dams and foetuses at about the same level.

Maternal toxicity as indicated by reduction in body weight was induced by all regimens. This effect was dose-related with sodium salicylate and salicylic acid but not so with aspirin. Whether this latter case was a true effect is equivocal for, although it is reasonable to expect a drug effect to be dose-related, it appeared that the effect varied with the individual rabbits rather than with the different groups.

Many rabbits recovered their weight when the drug was withdrawn but those which did not typically showed smaller foetuses and/or an increase in the incidence of intrauterine resorption. This was particularly relevant with high doses of sodium salicylate which retarded foetal growth and, like salicylic acid, induced resorption. Aspirin also affected foetal size but not in relation to dose level.

The intercurrent deaths of some rabbits were directly related to the drugs. These does became anorexic and, later, lethargic and emaciated and their post-mortem appearance was quite characteristic. Their livers were friable and fatty and the perivisceral fat was very sparse, these changes resulting probably from anorexia. Further, there were numerous ulcerations in the gastric mucosa, particularly in the fundic region, and the intestinal contents were scant and liquefied.

The most important feature of the work presented in this chapter was the failure to produce drug-induced birth defects. The highest

dose of aspirin tolerated by the rabbits was three times that found by McColl (1966) to induce malformations, and yet no teratogenic effect was observed. The limb flexures recorded occur spontaneously in 0.83% of Stride Dutch rabbits. There is no associated bony malformation, and pups born with this anomaly have been observed to assume normal conformation and movement within five days after parturition. Eight such defects occurred in the 648 test foetuses (1.23%) and two in the 255 controls (0.78%). However, these anomalies showed no relationship to dose level. Although there was an increase in the incidence of such defects in the test groups, it was considered to be not a drug effect.

A similar numerical 4:1 ratio was seen in the cases of microcaudia where the percentages were 0.62% in the test groups and 0.39% in the controls. This malformation has been observed on only one previous occasion in a control foetus, having occurred spontaneously in 0.014% of the rabbits examined to date. Its incidence in these experiments was appreciably higher than hitherto observed and it is known to be associated with aspirin teratogenicity in rats (Chapter 8). However, with such a low number of foetuses affected by this malformation and no indication of a dose-related effect, the only reasonable conclusion to draw is that it was not specifically induced by the test compounds. A similar conclusion must be drawn about the single grossly malformed foetus seen in Experiment 7.I, and the two cases of ablepharia.

That salicylate teratogenicity has been reported in the rat but could not be induced in the rabbit suggests some fundamental difference in the fate of the drug in the two species. One possible explanation is that they metabolise the drug differently (Bray, Ryman and Thorpe,

1948). However, if a similarity in the metabolism of the drug is a criterion for teratogenicity, it would be reasonable to expect that man would show similar sensitivity to the rat, because both show common metabolic pathways for salicylates. Since to date man has shown no clear susceptibility to salicylate teratogenesis, some other explanation must be sought for the observed differences between rats and rabbits.

It is possible that the degree of plasma protein binding of salicylate may be of importance as it is the unbound drug which evokes the toxic effects and would be available to traverse the placenta. In this respect, the rat and rabbit do differ. The percentage of salicylate bound to plasma proteins in the rabbit is similar to that in the guinea pig - an animal in which salicylate teratogenicity has not been reported. Appreciably less is bound in rats and dogs (Kucera and Bullock, 1969). Unfortunately, as was the case with guinea pigs, there is no report of any investigation into the sensitivity of dogs to salicylate teratogenicity. It is interesting to note that both man and the green monkey show a higher degree of protein binding than the rabbit. If this criterion is important in the induction of birth defects by salicylates, it may explain the susceptibility of the rat and the resistance of the rabbit and man.

This situation would be consistent with the findings of Brown and West (1964), that a high protein diet reduced the toxic effects of aspirin. Such a diet would increase the protein available for binding to salicylate, both in the gut and in the plasma. Consequently, there would be less unbound salicylate ready for absorption from the

gut. The amount could be reduced further by the higher level of proteins in the plasma which could also bind to salicylate. The unbound salicylate is the fraction absorbed and that which imparts the activity. Therefore, with less of this available, the toxic effects would be less severe.

The relationship between protein binding and salicylate toxicity could be investigated further by administering the salicylate together with a non-teratogenic compound which normally becomes protein bound (e.g. almost any of the fatty acids, such as palmitic acid). Such a compound would compete with the salicylate for binding on to the proteins in the gut and in the plasma. If this procedure increased the magnitude of the salicylate teratogenicity, it would suggest a possible relationship between the degree of protein binding and salicylate-induced birth defects.

The experiments in rabbits have shown that birth defects could not be induced by the salicylates tested. All except salicylamide were administered in progressively higher doses until they killed either the dams or the foetuses. At marginally sublethal levels, no drug-induced malformation was observed. These results confirmed the findings of other workers that the rabbit is not sensitive to the teratogenic action of salicylates. It was concluded, therefore, that the rabbit was not suitable for such investigations and it was not used in other experiments reported in this thesis.

Conclusions

(1) Marginally sublethal doses of aspirin, sodium salicylate and salicylic acid were not teratogenic in Stride Dutch rabbits.

(2) Doses of salicylamide up to 1000 mg/kg/day induced no toxic effect in the rabbits.

FIG. 7.1

MULTIPLE BIRTH DEFECTS IN THE RABBIT FOETUS

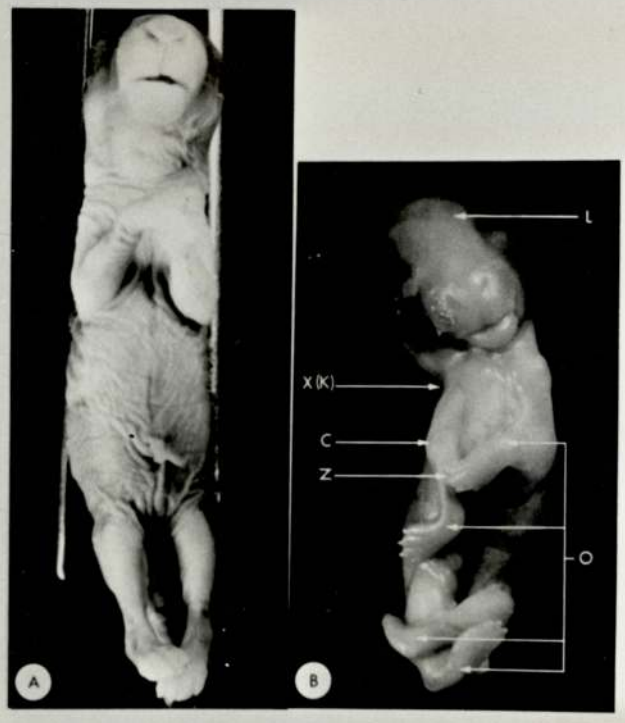


Fig. 7.1A

Macroscopic appearance of the ventral aspect of a normal foetus.

Fig. 7.1B

Corresponding appearance of a foetus showing a domed head typical of hydrocephalia (L). It also shows scoliosis (X), arthrogryposis (C), talipes equinovarus (Z) and limb flexures (O). Radiographs of this pup showed that it also had cervical hemivertebrae (K).

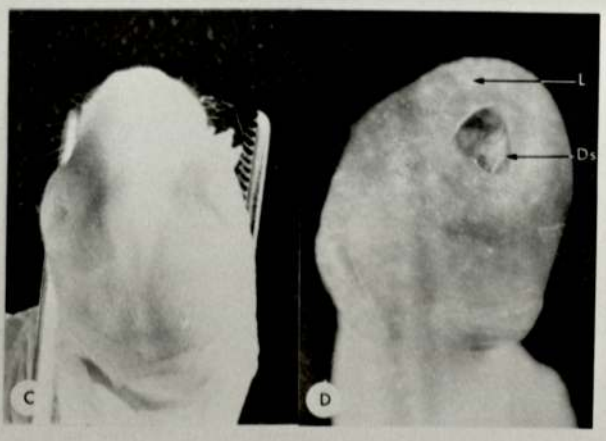


Fig. 7.1C

Macroscopic appearance of the dorsal aspect of the head of a normal foetus.

Fig. 7.1D

Corresponding appearance of the malformed foetus showing hydrocephalia (L) and cranioschisis (Ds).

FIG. 7.2

CONTACT PRINTS FROM RADIOGRAPHS OF LIMB FLEXURES IN RABBIT FOETUSES

The macroscopic appearance is presented in Fig. 7.1B.

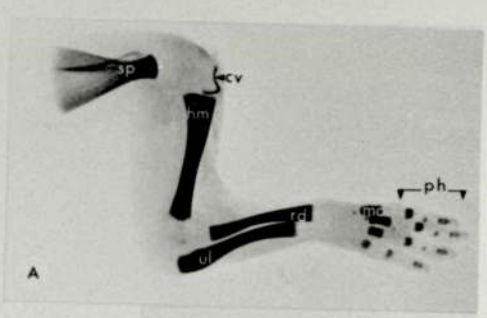


Fig. 7.2A

Forelimb of a normal foetus showing the scapula (sp), the clavicle (cv), the humerus (hm), the metacarpals (mc) and the phalanges (ph).

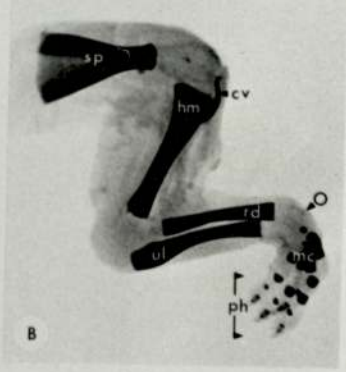


Fig. 7.2B

Flexed carpus (O) in a foetus. The skeletal elements all appear normal. The abbreviations are presented in the caption for Fig. 7.2A.

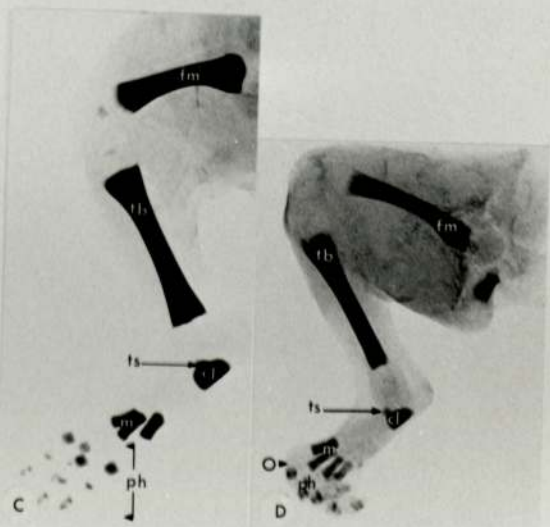


Fig. 7.2C

Hindlimb of a normal foetus showing the femur (fm), the tibia (tb), the calcaneus (cl), the talus (ts), the metatarsals (m) and the phalanges (ph).

Fig. 7.2D

Limb flexure (O) at the articulation of the metatarsals (m) and phalanges (ph). The abbreviations are presented in the caption for Fig. 7.2C.

FIG. 7.3

ABLEPHARIA IN THE RABBIT FOETUS

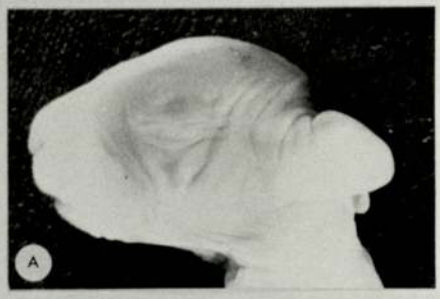


Fig. 7.3A

Macroscopic appearance of the lateral surface of the head of a normal foetus.

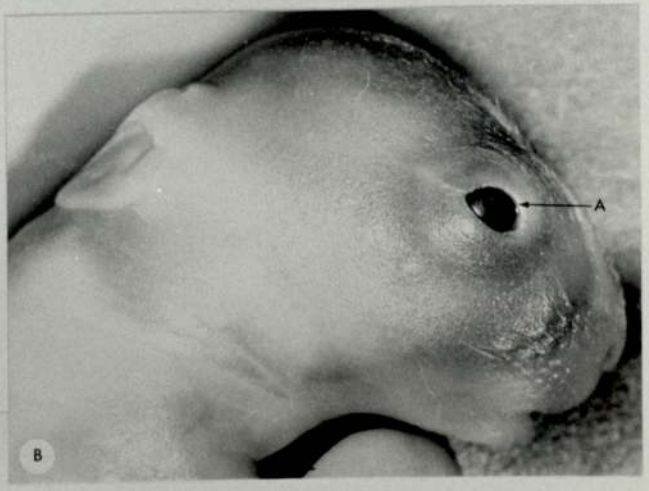


Fig. 7.3B

Corresponding appearance of a foetus with ablepharia (A).

FIG. 7.4

MICROCAUDIA IN THE RABBIT FOETUS

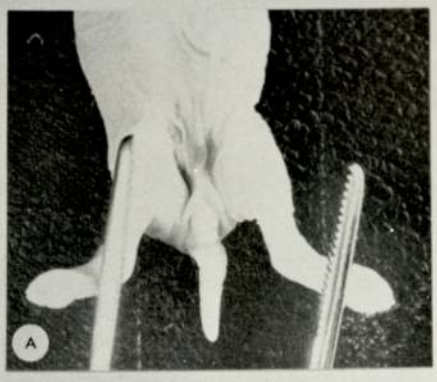


Fig. 7.4A

Macroscopic appearance of the tail of a normal foetus.

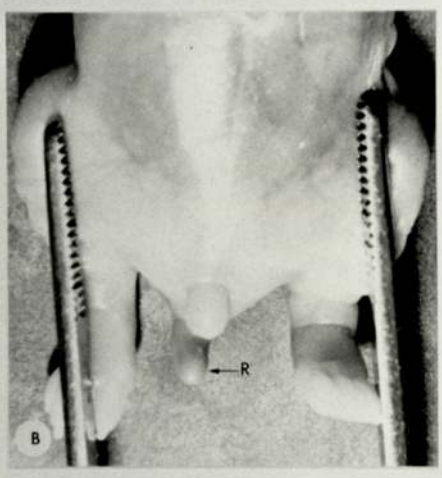


Fig. 7.4B

Corresponding aspect of a foetus with microcaudia (R).

Fig. 7.4C

Contact print of a radiograph showing the sacral and caudal region of a normal foetus. The 1st sacral (1.s) and 1st caudal (1.c) vertebrae are located. Only 13 of the 15 caudal vertebrae appear in the photograph.

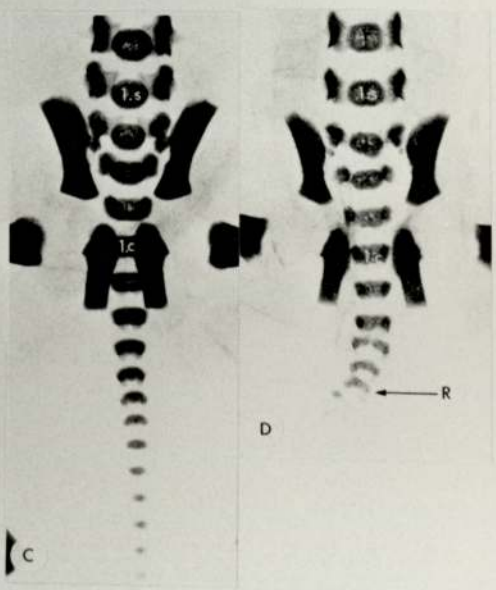


Fig 7.4D

Corresponding aspect of a foetus with microcaudia (R), in which the 1st sacral (1.s) and 1st caudal (1.c) vertebrae are located. The first three caudal vertebrae appear normal, the following two are displaced laterally, and the remaining three are reminiscent of hemivertebrae. There is complete agenesis of the distal seven vertebrae.

TABLE 7.1

PROTOCOL FOR THE INVESTIGATIONS INTO

THE TERATOGENICITY OF SOLUBLE ASPIRIN

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rabbits mated
7.A	Control	8-16	8
	200	8-16	7
	225	8-16	4
	250	8-16	4
7.B	Control	8-16	5
	275	8-16	5
	300	8-16	6
	350	8-16	6
7.C	Control	8-16	5
	400	8-16	6
	450	8-16	5
	500	8-16	5
7.D	Control	8-16	4
	550	8-16	4
	600	8-16	4
	625	8-16	4

TABLE 7.2

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF SOLUBLE ASPIRIN

Experiment code	Dose level (mg/kg/day)	No. of rabbits	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
7.A	Control	7	43	2	6.1	39.7	5	0.71	1.28	0	0	0
	200	5	28	2	5.6	29.3*	6	1.20	2.61	0	0	0
	225	3	12	0	4.0	42.6	0	0	0	0	0	0
	250	3	16	0	5.3	35.3*	0	0	0	0	0	1[1]
7.B	Control	4	22	0	5.5	36.5	3	0.75	0.96	0	0	0
	275	1	6	0	6.0	32.3	1	1.0	0.70	0	0	0
	300	5	32	0	6.4	28.4*	0	0	0	0	0	0
	350	5	25	2	5.0	34.6	0	0	0	0	0	0
7.C	Control	4	24	0	6.0	34.5	1	0.25	1.84	0	0	0
	400	3	20	0	6.7	35.0	0	0	0	0	0	0
	450	5	38	3	7.6	35.3	1	0.20	1.84	0	0	0
	500	4	32	1	8.0	29.3*	1	0.25	0.41	0	0	1[2]+2[3]
7.D	Control	2	5	0	2.5	43.6	0	0	0	5	2.5	0
	550	3	20	0	6.7	33.6*	3	1.0	1.0	0	0	0
	600	3	22	1	7.3	34.8*	4	1.33	1.34	0	0	0
	625	0										

* Statistically significantly different from the controls.

1 Microcaudia.

2 Limb flexures.

3 Ablepharia.

TABLE 7.3

PROTOCOL FOR THE INVESTIGATIONS INTO

THE TERATOGENICITY OF SODIUM SALICYLATE

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rabbits mated
7.E	Control	8-16	6
	200	8-16	5
	300	8-16	6
7.F	Control	8-16	5
	400	8-16	5
	450	8-16	5
7.G	Control	8-16	4
	475	8-16	4
	500	8-16	4
	525	8-16	4

TABLE 7.4

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF SODIUM SALICYLATE

Experiment code	Dose level (mg/kg/day)	No. of rabbits	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
7.E	Control	3	21	0	7.0	30.2	0	0	0	0	0	1[1]
	200	4	31	2	7.8	33.4	1	0.25	1.99	0	0	0
	300	5	36	0	7.2	33.1	4	0.80	1.43	0	0	1[2]
7.F	Control	4	24	0	6.0	34.5	1	0.25	1.84	0	0	0
	400	2	13	1	6.5	32.0	1	0.50	0.14	0	0	0
	450	5	25	1	5.0	33.8	12	2.40	0.71	0	0	4[2]
7.G	Control	2	5	0	2.5	43.6	0	0	0	5	2.50	0
	475	4	24	0	6.0	35.2*	8	2.0	0.62	0	0	1[1]
	500	3	17	0	5.7	36.4*	3	1.0	0.72	0	0	1[1]
	525	3	0	0	0	0	15	5.0	0.01	3	1.0	0

* Statistically significantly different from the controls.

1 Microcaudia.

2 Limb flexures.

TABLE 7.5

PROTOCOL FOR THE INVESTIGATIONS INTO

THE TERATOGENICITY OF SALICYLAMIDE

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rabbits mated
7.H	Control	8-16	4
	250	8-16	4
7.I	Control	8-16	6
	300	8-16	5
	400	8-16	5
7.J	Control	8-16	5
	500	8-16	5
	600	8-16	5
7.K	Control	8-16	4
	1000	8-16	4

TABLE 7.6

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF SALICYLAMIDE

Experiment code	Dose level (mg/kg/day)	No. of rabbits	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
7.H	Control	1	8	0	8.0	31.9	1	1.0	0.59	0	0	0
	250	4	22	0	5.5	35.8	0	0	0	0	0	0
7.I	Control	3	21	0	7.0	30.2	0	0	0	0	0	1[1]
	300	3	15	1	5.0*	28.5	2	0.67	1.34	0	0	0
	400	4	19	4	4.8*	32.9	5	1.25	1.46	0	0	1[2]
7.J	Control	4	24	0	6.0	34.5	1	0.25	1.84	0	0	0
	500	2	13	0	6.5	37.0	3	1.50	0.93	0	0	1[1]
	600	5	28	0	5.6	35.0	1	0.20	1.23	0	0	0
7.K	Control	2	5	0	2.5	43.6	0	0	0	5	2.50	0
	1000	2	18	0	9.0	32.9	1	0.50	0.80	1	0.50	0

* Statistically significantly different from the controls.

1 Limb flexures.

2 Cranioschisis, hydrocephalia, scoliosis, hemivertebrae, limb flexures, talipes equinovarus, arthrogryposis.

TABLE 7.7

PROTOCOL FOR THE INVESTIGATIONS INTO

THE TERATOGENICITY OF SALICYLIC ACID

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rabbits mated
7.L	Control	8-16	6
	150	8-16	6
	200	8-16	6
	250	8-16	6
7.M	Control	8-16	5
	300	8-16	5
	350	8-16	5
7.N	Control	8-16	4
	400	8-16	4
	450	8-15[1]	4

[1]These rabbits were scheduled to be dosed until day 16 but none survived beyond day 15.

TABLE 7.8

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF SALICYLIC AGID

Experiment code	Dose level (mg/kg/day)	No. of rabbits	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
7.L	Control	3	21	0	7.0	30.2	0	0	0	0	0	1[1]
	150	4	29	0	7.25	34.8	0	0	0	0	0	0
	200	6	45	2	7.5	35.9	2	0.33	1.74	0	0	0
	250	1	6	0	6.0	35.2	2	2.0	1.92	0	0	0
7.M	Control	4	24	0	6.0	34.5	1	0.25	1.84	0	0	0
	300	4	28	1	7.0	32.1	0	0	0	0	0	1[1]
	350	4	28	0	7.0	34.4	0	0	0	0	0	1[2]
7.N	Control	1	8	0	8.0	31.9	1	1.0	0.59	0	0	0
	400	1	0	0	0	0	1	1.0	0.01	4	4.0	0
	450	0										

1 Limb flexures.

2 Microcaudia.

CHAPTER 8

POSITIVE CONTROL EXPERIMENTS IN RATS

Introduction

In the previous chapter it was shown that the rabbit is not sensitive to the teratogenic action of acetylsalicylic acid. Therefore, it was decided to pursue investigations in the rat, an animal known to be susceptible. Loosli et al (1964) were the first to report that aspirin causes congenital malformations in Wistar rats. The teratogenic regimen was 200 mg/kg/day administered between days 7 and 16 of pregnancy. Their observations were confirmed by Baba et al (1966) who produced malformed progeny of Wistar rats by treating the dams with aspirin at doses of 200 or 400 mg/kg/day between days 7 and 9 of pregnancy. Aspirin is also teratogenic in Sprague-Dawley (CD) rats (McColl et al, 1965) but Brown and West (1964) and West (1964) found no malformation after giving pregnant hooded Lister rats up to 500 mg/kg/day.

Initially, it was considered essential to find a strain of rat which was regularly susceptible. Having achieved this, it was then necessary to find the dose level at which malformations could be induced regularly and to determine the susceptible time during the gestation period. This preliminary work was conducted with aspirin.

Experimental Work

The standard techniques (Chapter 5) were applied and details of the individual experiments will be given as they arise. Discussion of the results will be presented in this section.

(a) The establishment of aspirin teratogenicity in AH A rats

One experiment was necessary to demonstrate that aspirin is teratogenic in AH A rats, and the results of this are presented in Table 8.1. The dose level selected was based on that shown to be teratogenic by Loosli et al (1964). One rat died from faulty administration of the preparation and two others did not become pregnant.

Aspirin significantly reduced the mean weight of the live foetuses by 27% ($P<0.001$), a feature of which was their retarded ossification. However, the most relevant observation made in this study was in relation to the birth defects. Areas of subcutaneous haemorrhage were found in one test and one control foetus; although there was apparently no relation to the aspirin in this experiment, this observation merits some discussion. Such lesions occur spontaneously in 1.48% of AH A rat foetuses. However, Larsson and his co-workers in 1963 described lesions of this nature in foetal mice after treating their dams with salicylate. Therefore, any increase in the incidence of these haemorrhages in aspirin-treated rats must be considered in the light of Larsson's findings. This aspect will be discussed fully in Chapter 13.

This experiment (8.A) involved the detailed examination of 87 foetuses of which five test ones showed axial skeletal defects. These included scoliosis, kyphosis, hemivertebrae, inhibited central ossification in the thoracic and lumbar regions, fused ribs and sternal anomalies. They were consistent with malformations described for salicylate teratogenicity in rats (e.g. Warkany and Takacs, 1959; Loosli et al, 1964; McColl et al, 1965). Although they were not so

severe as some described by Loosli et al (1964), they were considered to demonstrate the susceptibility of AH A rats to aspirin teratogenicity. This conclusion was supported by the results of subsequent experiments in which gross malformations were induced.

(b) The determination of the stage in pregnancy during which the rats are susceptible to aspirin teratogenicity

An attempt to relate the dose of aspirin and the time of its administration to the type of gross malformation produced was made by Baba et al (1966). Unfortunately, their experiments lacked precision as all the rats at each dose level were treated for a minimum of three consecutive days. It will be shown later in this chapter that certain malformations can be induced by treating the dams on one particular day of pregnancy.

The present investigation necessitated an initial experiment to establish the level of soluble aspirin which would induce birth defects after one dose, and the protocol for this is given in Table 8.2. It was decided to administer the drug on the 9th day of pregnancy as this was found by other workers to be the day of greatest susceptibility (e.g. Warkany and Takacs, 1959). There was a comparatively high number of control animals because they were being used in a concurrent experiment with an AH compound.

There were six intercurrent deaths among the rats given 600 mg/kg or more of soluble aspirin. Their deaths were attributed to the high dose levels they received but, as is the general case when death follows acute treatment, no pathological change was observed. One control rat did not become pregnant.

All dams dosed with 400 mg/kg or more lost weight after dosing, and this loss persisted in those rats which subsequently died during the experiment. The others recovered within two days and regained some weight, but at no stage after dosing was their weight gain comparable to that of the controls.

The results of the investigation are presented in Table 8.3, and the effects of the different dose levels upon resorption, malformation and foetal weight are illustrated in Fig. 8.1. Aspirin significantly decreased the mean number of live foetuses per litter in the 500 mg/kg group by 56% ($P<0.02$), in the 600 mg/kg group by 100% ($P<0.001$) and in the 700 mg/kg group by 96% ($P<0.001$) compared with the controls. It correspondingly increased the incidence of intrauterine resorption in these groups by over 10, 18 and 19 times respectively ($P<0.001$ in all three cases). Furthermore, the foetuses were significantly smaller by 18% in the 400 mg/kg group ($P<0.01$), by 30% in the 500 mg/kg group ($P<0.001$) and by 63% in the 700 mg/kg group ($P<0.001$) compared with the controls, and showed less advanced skeletal ossification.

Congenital malformations were induced with doses of 300 mg/kg and above, where the dams and their progeny survived the aspirin treatment. With the exception of the three cases of microcaudia, the malformations were those associated with salicylate treatment of rats on the 9th day of pregnancy. Because of the axial grade of embryonic development, it would be reasonable to expect tail abnormalities to be induced at a later stage of organogenesis. Indeed, experiments described later in this chapter demonstrate clearly that caudal hemivertebrae (producing a curly tail) can be

induced by treating the dam on the 12th day of pregnancy (Table 8.6.). However, it appears that some factor essential for normal tail development was affected by the aspirin on day 9.

Experiment 8.B demonstrated that severe malformations could be induced with 500 or 700 mg/kg of aspirin. Therefore, 500 mg/kg was the obvious choice for the dose to be used in an experiment to delineate the stage in pregnancy susceptible to aspirin teratogenicity. An additional dose level of550 mg/kg was used because it was considered that some days of organogenesis may be less sensitive than day 9. The rats were treated on a single day between the 7th and 13th days of gestation because no malformation had been attributed to aspirin administered outside these limits. The experimental protocol is given in Table 8.4. The results of this investigation (8.C) prompted the establishment of Experiment 8.D, in which the 7th and 13th days of pregnancy were investigated at a higher dose level - i.e. 750 mg/kg. In these two experiments three rats died from faulty administration of the preparation and nine others did not become pregnant.

With the exception of rats treated on day 11, all test dams lost weight between the day of dosing and the following day. The amount of weight lost varied with the day of treatment and was not affected by the difference in dose levels within this range. It was not surprising that day 9, which has been shown to be the day of pregnancy with the highest degree of sensitivity to salicylates (e.g. Warkany and Takacs, 1959), was associated with the greatest weight loss (a mean of 17g per rat). This was followed in descending order by days: 8, 13, 7, 12 and 10, but those treated on day 11 showed no weight change. All affected dams recovered within two days of the treatment

and subsequently gained weight to the same extent as the controls.

The results of the investigation are presented in Tables 8.5 and 8.6, and are illustrated as histograms in Fig. 8.2. The rats in Experiment 8.C were killed 21 days post coitum and this accounts for the larger size of the foetuses.

As was the case with the maternal weight change, any effect of the aspirin on litter size was related to the day of pregnancy rather than to the dose levels. In Experiment 8.C, the mean number of live foetuses per litter in all test groups was lower than that in the controls. This difference was significant only when the aspirin was given on day 9 at 500 mg/kg when there were 75% fewer foetuses ($P<0.01$) or at 550 mg/kg when there were 91% fewer ($P<0.002$), or on day 10 at 500 mg/kg when no foetus survived.

Although there was no statistical significance in the lower mean number of live foetuses per litter in does treated on days 8, 10 (at 550 mg/kg), 11 or 12, there is evidence that there may have been drug effect. This is indicated by statistically significant increases in the incidence of intrauterine resorption in these groups. The three groups with statistically significantly fewer foetuses showed resorption increased above the controls by multiples of 30, 36 and nine respectively ($P<0.001$ in all three cases). However, significant differences were also found in rats treated on day 8 where the increase was by over 20 times at 500 mg/kg ($P<0.002$) and by over six times at 550 mg/kg ($P<0.01$); on day 10 where it was by over 18 times at 550 mg/kg ($P<0.002$); on day 11 where it was by over 12 times at 500 mg/kg ($P<0.01$); or on day 12 where it was by nearly 17 times at 550 mg/kg ($P<0.01$). Therefore, it

seems reasonable to conclude that the aspirin reduced the mean number of live foetuses per litter at at least one dose level on days: 8, 9, 10, 11 or 12, and caused a corresponding increase in the incidence of intrauterine resorption.

The mean weight of the live foetuses was significantly reduced below the control value by some regimens. There was no consistent relationship with the day of administration but it occurred only at the higher dose levels. After treatment of the dams with 550 mg/kg on day 8 the foetuses, which showed retarded ossification, were smaller by 15% ($P<0.01$); after a similar dose on day 9 they were smaller by 27% ($P<0.002$); and after 750 mg/kg on day 13 they were smaller by 8% ($P<0.02$).

In Experiment 8.C, 415 foetuses were examined and 29 of these showed gross malformations. These are listed in Table 8.5, and the malformations are correlated with the days on which they were instigated in Table 8.6. The majority of these malformations were induced on one specific day but some recurred after treatment on different days. These included aglossia, microstomia, eventration of the abdominal viscera, craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, arthrogryposis and palatoschisis. The defects showed no consistent association with the different dose levels within the range studied and were induced when the dams were treated on any day from the 7th to the 12th days of pregnancy. No malformation was induced on day 13. Day 9 was confirmed as being the day of highest susceptibility with 22% of the foetuses showing gross malformations. This was followed in descending order by days: 12, 10, 11, 8 and 7. Day 9 also had the highest number of different malformations with 17 of the 35 observed over all of the days.

Some of the malformations recorded have been observed previously by other workers after treating pregnant rats with salicylate. However, it seems that polydactylia has been recorded only in mice (Trasler, 1965). This leaves certain anomalies which appear to be novel in relation to salicylate teratogenicity. These include agnathia, micrognathia, hypoplasia of the premaxillae, maxillae and nasal bones, hypoplasia of the upper incisors, agenesis of the lower incisors, microstomia, aglossia, buccal atresia, agenesis of the submaxillary salivary glands, cardiac hypoplasia, cardiac angioma, agenesis of the pericardium, agenesis of the diaphragm, arthrogryposis and curly tail.

It is interesting to note how the malformations produced on each day generally reflected the axial grade of embryonic development. Anterior defects occurred when salicylate was given at the start of organogenesis, and were followed by those affecting the midline. Malformations of the extremities were induced when it was given near the end of the susceptible period.

The results of Experiment 8.C needed to be qualified by further work on days 7 and 13 which had so far yielded only one abnormal pup. This work was undertaken in Experiment 8.D when higher doses of aspirin were used to determine whether the limits of the susceptible period, delineated in Experiment 8.C, should be extended. The results, presented in Table 8.5, showed a significant reduction in mean foetal weight in the rats treated on day 13 (discussed above) but no gross malformation among the 115 foetuses examined. They indicated that the rats are not generally sensitive to aspirin teratogenicity when treated on the 7th or 13th days of pregnancy.

These investigations demonstrated the susceptibility of AH A rats to the teratogenic effects of aspirin, and showed that a broad spectrum of malformations could be produced when the dams were treated from the 8th to the 12th days of pregnancy. Consequently, it was decided that the rats used in all subsequent investigations should be treated only during this period.

Conclusions

(1) AH A rats were susceptible to the teratogenic activity of aspirin.

(2) Thirty-five types of gross congenital malformations were produced following treatment of the dams on the 7th, 8th, 9th, 10th, 11th or 12th days post coitum.

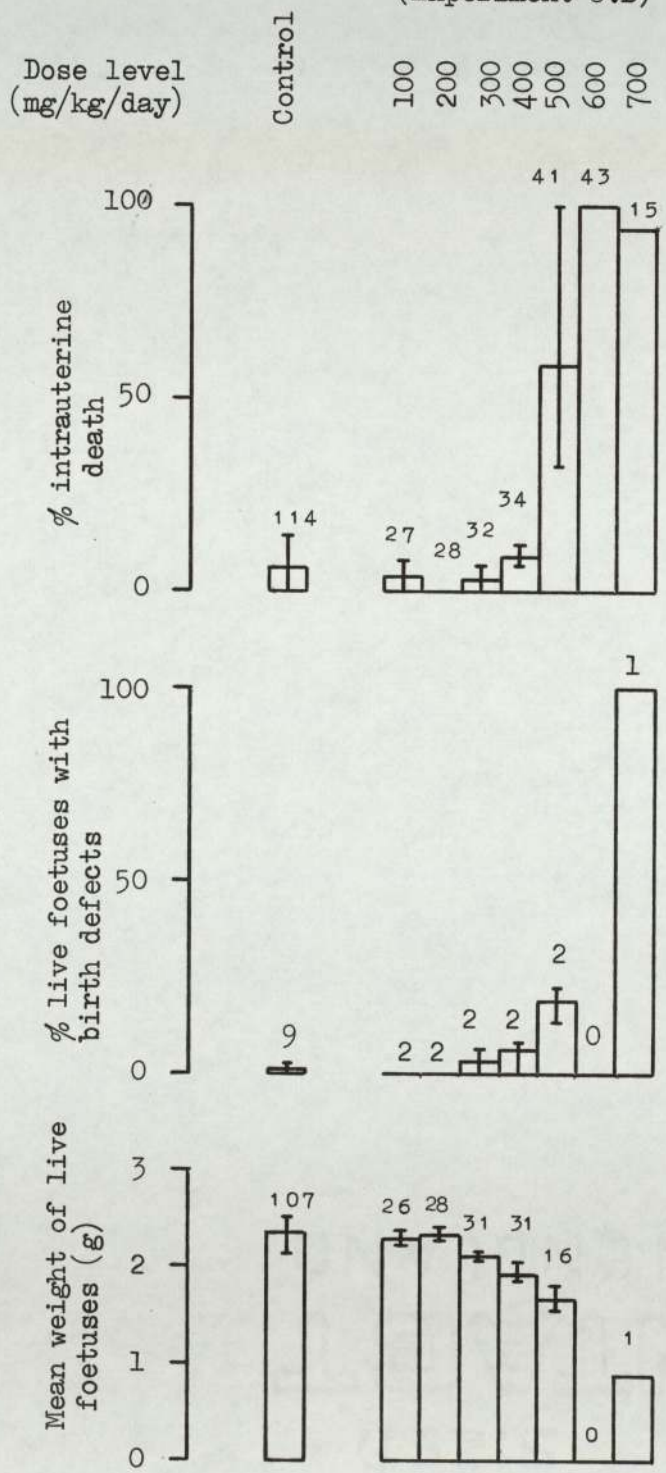
(3) Although the majority of these malformations were induced on one specific day, a number recurred after treatment on different days.

(4) Many of the malformations had been observed previously in association with salicylate teratogenicity, but seventeen defects novel in this capacity were also described.

FIG. 8.1

COMPARISON OF THE INCIDENCE OF INTRAUTERINE DEATH AND BIRTH DEFECTS, AND OF THE MEAN FOETAL WEIGHT IN RATS TREATED WITH ASPIRIN ON THE 9TH DAY OF PREGNANCY - WITH RANGE OF EXPERIMENTAL VARIATION

(Experiment 8.B)



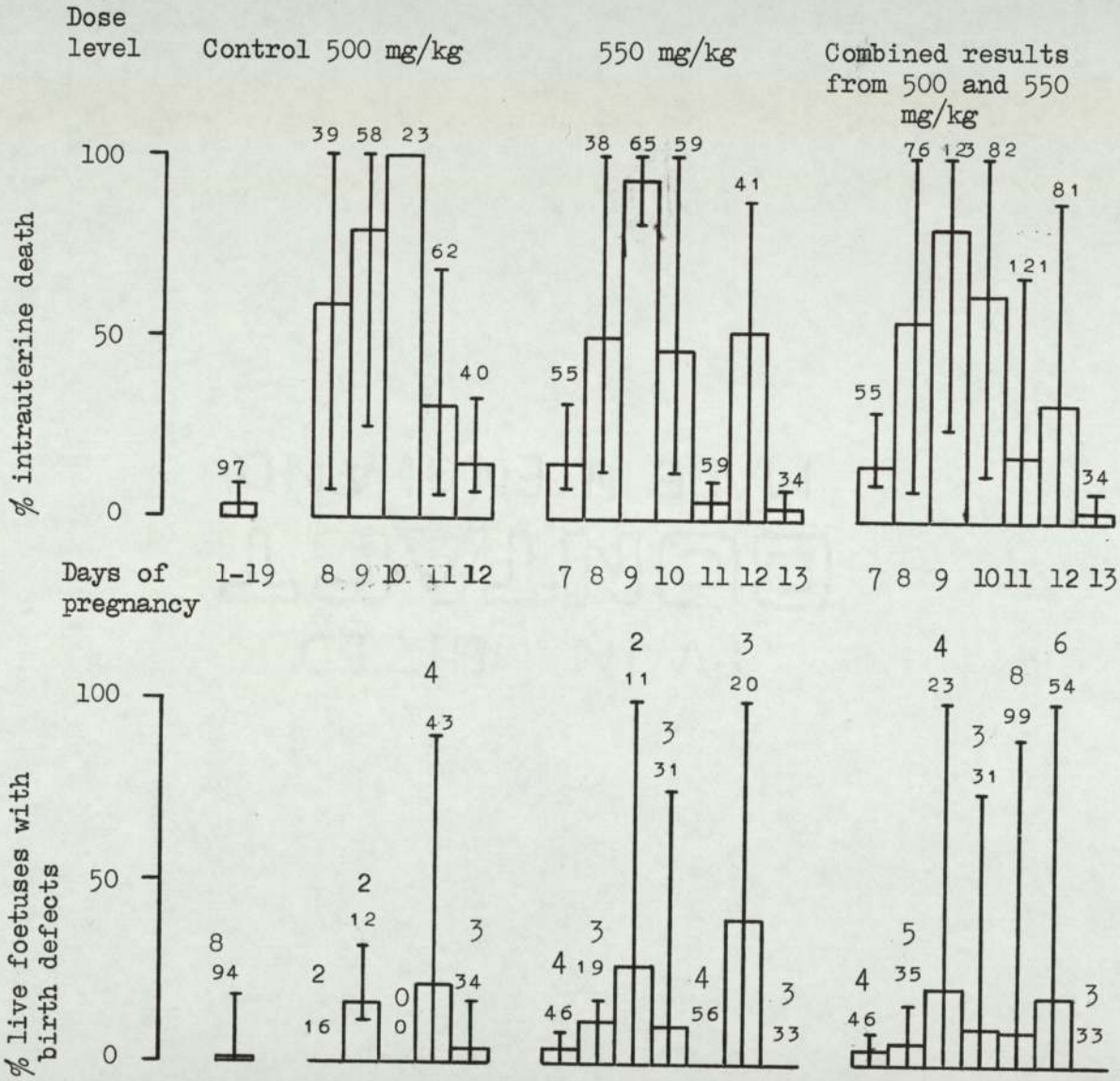
The numbers above the columns indicate the total numbers of implantations.

The numbers above the columns indicate the total numbers of litters containing live foetuses.

The numbers above the columns indicate the total numbers of live foetuses.

FIG. 8.2

COMPARISON OF THE INCIDENCE OF INTRAUTERINE DEATH AND BIRTH DEFECTS IN RATS TREATED WITH ASPIRIN ON DIFFERENT DAYS OF PREGNANCY - WITH THE RANGE OF EXPERIMENTAL VARIATION (Experiment 8.C)



N.B. The numbers above the columns relating to intrauterine death indicate the total numbers of implantations.

The large numbers above the columns relating to birth defects indicate the total numbers of litters containing live foetuses. The smaller ones indicate the total numbers of foetuses.

TABLE 8.1

RESULTS OF THE INVESTIGATION UNDERTAKEN TO ESTABLISH THE TERATOGENICITY OF ASPIRIN IN AH A RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated	No. of pregnant rats studied	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
					Alive	Dead								
8.A	Control	1-19	8	6	59	0	9.83	2.11	4	0.67	0.125	0	0	1[1]
	225	1-19	4	3	28	0	9.33	1.55*	6	2	0.180	1	0.33	1[1] + 5[2]

* Statistically significantly different from the controls.

1 Subcutaneous haemorrhages.

2 Scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, inhibited central ossification.

TABLE 8.2

PROTOCOL FOR THE EXPERIMENT UNDERTAKEN TO ESTABLISH THE LEVEL OF A SINGLE TERATOGENIC DOSE OF ASPIRIN IN THE RAT

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
8.B	Control	1-19	10
	100	9	2
	200	9	2
	300	9	2
	400	9	2
	500	9	3
	600	9	4
	700	9	4
	800	9	1
	900	9	1
	1000	9	1

TABLE 8.3

RESULTS OF THE INVESTIGATION UNDERTAKEN TO ESTABLISH THE LEVEL OF A SINGLE TERATOGENIC DOSE OF ASPIRIN IN THE RAT

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
8.B	Control	9	107	0	11.89	2.36	7	0.78	0.090	0	0	1[1]
	100	2	26	0	13.0	2.32	1	0.50	0.025	0	0	0
	200	2	28	0	14.0	2.33	0	0	0	0	0	0
	300	2	31	0	15.50	2.13	1	0.50	0.142	0	0	1[2]
	400	2	31	0	15.50	1.93*	3	1.50	0.172	0	0	2[2]
	500	3	16	1	5.33*	1.65*	24	8.0*	0.194	0	0	2[3]+1[4]
	600	3	0	0	0*	0	43	14.33*	0.140	0	0	0
	700	2	1	0	0.50*	0.87*	30	15.0*	0.218	0	0	1[4]
	800	0										
	900	0										
	1000	0										

* Statistically significantly different from the controls.

1 Subcutaneous haemorrhages.

2 Microcaudia.

3 Exencephalia, macroglossia.

4 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera.

TABLE 8.4

PROTOCOL FOR THE INVESTIGATIONS UNDERTAKEN TO DETERMINE THE STAGE IN PREGNANCY DURING WHICH RATS ARE SUSCEPTIBLE TO ASPIRIN TERATOGENICITY

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
8.C	Control	1-19	8
	550	7	4
	500	8	4
	550	8	4
	500	9	4
	550	9	4
	500	10	4
	550	10	7
	500	11	4
	550	11	4
	500	12	4
	550	12	4
	550	13	3
8.D	Control	7 and 13	4
	750	7	4
	750	13	4

TABLE 8.5

RESULTS OF THE INVESTIGATIONS UNDERTAKEN TO DETERMINE THE STAGE IN PREGNANCY DURING WHICH RATS ARE SUSCEPTIBLE TO ASPIRIN TERATOGENICITY

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
				Alive	Dead								
8.C	Control	1-19	8	94	0	11.75	3.33	3	0.38	0.056	0	0	1^{1}
	550	7	4	46	0	11.50	2.90	9	2.25	0.037	0	0	$1^{1}+1^{2}$
	500	8	3	15	1	5.0	3.17	23	7.67*	0.052	0	0	0
	550	8	3	19	0	6.33	2.83*	7	2.33*	0.021	12	4.0*	$1^{3}+1^{5}$
	500	9	4	12	0	3.0*	3.35	46	11.50*	0.156	0	0	$1^{1}+1^{4}$
	550	9	4	4	7	1.0*	2.43*	54	13.50*	0.101	0	0	$2^{5}+1^{6}$
	500	10	2	0	0	0*	0	7	3.50*	0.071	16	8.0*	0
	550	10	4	31	0	7.75	3.17	28	7.0*	0.080	0	0	3^{7}
	500	11	4	42	1	10.50	3.08	19	4.75*	0.026	0	0	9^{8}
	550	11	4	56	0	14.0	3.16	3	0.75	0.098	0	0	0
	500	12	3	34	0	11.33	3.23	6	2.0	0.022	0	0	1^{9}
	550	12	3	20	0	6.67	3.17	19	6.33*	0.128	2	0.67*	$4^{9}+1^{10}+1^{11}+1^{12}+1^{13}$
	550	13	3	33	0	11.0	3.08	1	0.33	0.027	0	0	0
8.D	Control	7+13	3	39	0	13.0	2.44	7	2.33	0.249	0	0	0
	750	7	3	45	0	15.0	2.54	0	0	0	0	0	0
	750	13	3	31	0	10.33	1.79*	17	5.67	0.188	0	0	2^{1}

* Statistically significantly different from the controls.

1 Subcutaneous haemorrhages.

2 Micrognathia, microstomia, aglossia, agenesis of the lower incisors, hypoplasia of the nasals, maxillae and premaxillae, hydrocephalia, eventration of the abdominal viscera.

3 Agnathia, microstomia, aglossia, buccal atresia, hypoplasia of the upper incisors, agenesis of the submaxillary salivary glands, omphalocele, subcutaneous haemorrhages.

4 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, oedema, cardiac hypoplasia, cardiac angioma, agenesis of the pericardium, agenesis of the diaphragm, eventration of the abdominal viscera, arthrogryposis.

5 Eventration of the abdominal viscera.

6 Exencephalia, exophthalmia, ablepharia, macroglossia.

7 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera.

8 Palatoschisis, cheiloschisis, folded nasals.

9 Curly tail.

10 Palatoschisis, polydactylia, arthrogryposis.

11 Palatoschisis, polydactylia.

12 Palatoschisis, curly tail.

13 Polydactylia.

TABLE 8.6

REPRESENTATION OF THE DAYS OF PREGNANCY ON WHICH GROSS CONGENITAL MALFORMATIONS WERE INDUCED IN RATS BY 500 AND 550 MG/KG OF ASPIRIN

Experiment code		Days of pregnancy						
		7	8	9	10	11	12	13
8.C	Number of foetuses examined	46	35	23	31	99	54	33
	Number of foetuses with gross congenital malformations	1	2	5	3	9	9	0
	Percentage of foetuses with gross congenital malformations	2.2	5.7	21.7	9.7	9.1	16.7	0
	GROSS CONGENITAL MALFORMATIONS							
	*Agenesis of the lower incisors	/						
	Hydrocephalia	/						
	*Hypoplasia of the nasals	/						
	*Hypoplasia of the premaxillae	/						
	*Hypoplasia of the maxillae	/						
	*Micrognathia	/						
	*Aglossia	/	/					
	*Microstomia	/	/					
	Eventration of the abdominal viscera	/	/	/	/			
	*Agenesis of the submaxillary glands		/					
	*Agnathia		/					
	*Buccal atresia		/					
	*Hypoplasia of the upper incisors		/					
	Omphalocele		/					
	Ablepharia			/				
	*Cardiac angioma			/				
	*Cardiac hypoplasia			/				
	*Agenesis of the diaphragm			/				
	Exencephalia			/				
	Exophthalmia			/				

TABLE 8.6 contd/

Experiment code		Days of pregnancy						
		7	8	9	10	11	12	13
8.C	GROSS CONGENITAL MALFORMATIONS							
	Macroglossia			/				
	Oedema			/				
	*Agenesis of the pericardium			/				
	Craniorrhachischisis			/	/			
	Fused ribs			/	/			
	Hemivertebrae			/	/			
	Kyphosis			/	/			
	Scoliosis			/	/			
	Sternal anomalies			/	/			
	*Arthrogryposis			/			/	
	Folded nasals					/		
	Cheiloschisis					/		
	Palatoschisis					/	/	
	*Curly tail						/	
	Polydactylia						/	

*Not previously associated with salicylate teratogenicity

CHAPTER 9

THE RELATIVE TERATOGENICITY OF DIFFERENT ASPIRIN PREPARATIONS

Introduction

These investigations were undertaken to compare the teratogenicity of different aspirin preparations. Soluble aspirin, which has been shown to induce congenital malformations in AH A rats (Chapter 8), was compared with micronised and 200# forms. The maximum size of the particles of the 200# aspirin was 75μ, and most of the micronised particles were between 1.66 and 3.31μ. These experiments were carried out to determine the form of aspirin most acceptable for instigating birth defects from the point of view of reproducibility and relative maternal and embryonic toxicity. Further, they afforded information on the range of doses which would induce foetal malformations after daily treatment throughout the sensitive period of development. The standard techniques (Chapter 5) were applied and details of the individual experiments will be given as they arise in discussion.

Experimental Work

This study involved three investigations which will be considered individually. The first of these was a comparison of the teratogenicity of various aspirin preparations. From this study, it appeared that particle flocculation affected the results obtained with micronised aspirin and this aspect was investigated further. Finally, the conclusions from this test were confirmed by estimating serum-salicylate levels.

(a) The teratogenicity of different aspirin preparations

The protocol for the first experiment (9.A) is presented in Table 9.1. The dose levels of 250 and 275 mg/kg/day were selected for all three forms, and other doses were used as it was anticipated that the preparations may have different absorption characteristics.

One rat died from faulty administration of the preparation, and eight others did not become pregnant. The pregnant rats in all the test groups lost weight during the dosing period. This was greatest in the dams given 500 mg/kg/day of 200# aspirin and averaged 6g per day. The only other correlation between the weight lost and the regimen was that the dams given 200# aspirin lost more weight than those given similar doses of the other forms. The dams recovered after the dosing was discontinued and proceeded to gain weight. However, only those rats given 225 mg/kg/day of soluble aspirin or 300 mg/kg/day of the micronised preparation increased weight to the same extent as the controls.

The results of this experiment are given in Table 9.2. At doses of 275 mg/kg/day and above, 200# aspirin was lethal to all the developing embryos. Consequently, at term, all the conceptuses appeared as resorption sites, the incidence of which was nearly three times as great in the 275 mg/kg group ($P<0.01$) and nearly ten times as great in the 500 mg/kg group ($P<0.001$) as in the controls. Furthermore, 250 mg/kg/day of this preparation reduced the mean number of live foetuses per litter by 90% ($P<0.001$) and correspondingly increased the incidence of intrauterine resorption by nearly five times ($P<0.002$). The lowest dose of 200# aspirin (225 mg/kg/day) did not affect the number of foetuses in each litter.

No effect was seen, either, with the lowest level of soluble aspirin (also 225 mg/kg/day), but 250 mg/kg/day reduced the litter number by 38% and increased resorption by over five times ($P<0.01$ in both instances). Although there was no statistically significant difference between the litter numbers of the 275 mg/kg group and the controls, there is evidence of a drug effect. It was considered so because there were appreciably fewer live foetuses per litter. Couple this with a significant fourfold increase in the incidence of resorption in this group ($P<0.02$) and a statistically significant difference at a lower dose level, and it seems reasonable to conclude that there was a drug-induced reduction in litter number and increase in resorption.

Micronised aspirin did not affect the mean number of live foetuses per litter and the incidence of intrauterine resorption.

The effects of the aspirin preparations on litter number allow certain conclusions to be drawn. Micronised aspirin had no adverse effect and, in this respect, was either better tolerated by the pregnant rats or less efficiently absorbed. There was no difference between 225 mg/kg of 200# aspirin and a similar dose of the soluble form. However, at higher levels, the 200# form was more toxic. This was shown in the mean number of live foetuses in each litter; there being 52% more with 250 mg/kg of the soluble preparation than with the same dose of 200# aspirin. Further, 275 mg/kg/day of 200# aspirin killed all the embryos whilst, with a similar dose of soluble aspirin, the litter number was reduced by less than one quarter - a margin not significantly different from the controls. Therefore, with reference to the mean number of live foetuses in each litter, 200# aspirin was more toxic than soluble aspirin which, itself, was more toxic than micronised aspirin. These

results are illustrated in Fig. 9.1.

The mean weight of the live foetuses followed this general trend. The 250 mg/kg/day regimen of 200# aspirin reduced the mean foetal weight by 52% ($P<0.001$) below the control value. This compares with a 16% reduction ($P<0.01$) produced by the same dose of the soluble preparation. The higher dose of 275 mg/kg/day of soluble aspirin also resulted in smaller foetuses, this time by 13% ($P<0.05$), while no foetus survived that dose of 200# aspirin.

One result in the micronised aspirin groups warrants some discussion. The rats treated with 275 mg/kg/day had foetuses significantly smaller than the controls by 12% ($P<0.02$). However, the dams given the higher dose of 300 mg/kg/day had foetuses which were larger even than the controls. It appears likely, therefore, that the statistically significant effect seen at 275 mg/kg was not related to the drug.

The conclusions drawn from the mean foetal weights support those made from the number of foetuses per litter - that the embryotoxicity of 200# aspirin was greater than that of the soluble preparation and this, in turn, was greater than that of the micronised form. All the smaller foetuses showed a lesser degree of ossification than the controls.

This experiment involved the detailed examination of 518 foetuses of which 53 showed congenital abnormalities. These are listed in Table 9.2. Malformations were produced by all doses of each preparation where foetuses survived until term. There was little difference between the number and severity of the defects induced by similar doses of each preparation, and the number generally increased with the dose level of each preparation. However, attention is drawn to two groups. Although only nine foetuses were examined at term after their dams had been

treated with 250 mg/kg/day of 200# aspirin, eight of these (89%) were malformed. This compares with 10% in the group given the same dose of soluble aspirin and 3% in those given that dose of the micronised preparation. The other group of note contained dams given 275 mg/kg/day of soluble aspirin. Most conceptuses survived until term when 22 of the 54 examined (38%) showed abnormalities. No foetus survived that dose of 200# aspirin, and the same dose of micronised aspirin resulted in only 3% of the foetuses being abnormal.

The malformations were of a nature that is associated with aspirin teratogenicity. However, there was one novel anomaly in the group given 225 mg/kg/day of soluble aspirin; this was a case of two adjacent foetuses showing fused placentae. Although no such defect appeared in the control group of this experiment, similar anomalies are known to occur spontaneously in 0.05% of AH A rats. Its incidence in this experiment was well below this (0.002%). However, any effects of the aspirin is precluded because treatment was not commenced until after implantation (and the development of the fused condition) had occurred.

These results followed the trends discussed above, with 200# aspirin being more toxic to the conceptus than the soluble form and this, in turn, being more toxic than the micronised preparation.

The results of Experiment 9.A demonstrate that soluble aspirin was the most efficient experimental teratogen among the preparations studied. It was less toxic to the dam and the conceptus than similar doses of 200# aspirin, and produced a higher number of malformed foetuses which showed a wider range of defects.

However, this experiment yielded certain results which obviously required further work in order to clarify them. One of these was the relatively mild effect evoked by the micronised aspirin. A possible explanation for this lay in the flocculation of the micronised particles in the Natrosol solution which would adversely interfere with their absorption.

(b) The effect of particle flocculation on the teratogenicity of micronised aspirin

This hypothesis was first tested in Experiment 9.B, the protocol for which is given in Table 9.3. The micronised aspirin was administered at the same dose levels as in Experiment 9.A, but the surfactant Mannoxol OT/P was added to the preparation. The effect of the surfactant was to reduce the surface tension of the vehicle and facilitate the wetting of the drug, thereby reducing flocculation of the micronised particles. In another group, the aspirin was administered without the Mannoxol OT/P to control the effect of the drug. The dose level of this group was that which produced the greatest effect in the previous experiment (9.A).

The results of Experiment 9.B are presented in Table 9.4. No regimen affected the weight gain of the dams during pregnancy. Further, there was no statistically significant difference in the mean number of live foetuses per litter. However, there may have been drug effect in the case of the group given micronised aspirin without the surfactant. The litter number was reduced by 22% but this was not significantly different from the controls. Perusal of the figures relating to the number of resorption sites will show a significant

thirtyfold increase in this group ($P<0.001$). It is possible, therefore, that 300 mg/kg/day of micronised aspirin decreased the mean number of live foetuses per litter and correspondingly increased the incidence of intrauterine resorption. However, in view of the conclusions drawn from Experiment 9.C, presented below, any influence of this regimen must remain equivocal.

All drug groups showed a significant reduction in mean foetal weight. In this respect, both 300 mg/kg groups had a remarkably similar effect. The reduction was by 16% in the micronised aspirin group and by 17% when the Mannoxol OT/P was added ($P<0.001$ in both cases). The two lower levels of the drug plus surfactant exerted an identical influence on foetal weight. Both reduced it by 8% ($P<0.05$) as compared with the controls. All smaller foetuses showed retarded ossification.

Three foetuses among the 472 examined showed areas of subcutaneous haemorrhage. The present cases occurred in the two 300 mg/kg groups. Discussion of this lesion will be presented in Chapter 13, and although there was no such anomaly in the control foetuses in this experiment, it cannot be unequivocally attributed to the aspirin.

This study also showed that the low degree of teratogenicity of micronised aspirin seen in Experiment 9.A may not have been attributable to particle flocculation. In the current investigation (9.B) the addition of the surfactant made no appreciable difference. Indeed, the dams given aspirin alone showed a slightly higher incidence of embryonic death than those given the preparation with Mannoxol OT/P. That no gross congenital malformation characteristic of salicylate

teratogenicity was induced indicated that the dose levels used may have been too low. Consequently, another experiment (9.C) was undertaken and the protocol for this is given in Table 9.3.

Unlike the lower doses used in Experiment 9.B, 350 mg/kg/day of micronised aspirin, irrespective of the surfactant, adversely affected the weight gain of the pregnant rats during treatment. The controls gained a mean of 19g during this period while the dams given aspirin alone lost 7g and those given the drug with Mannoxol OT/P lost 8g. However, the test dams recovered when the treatment was discontinued but did not gain as much weight as the controls. One rat did not become pregnant.

The results of this investigation are presented in Table 9.4 and Fig 9.2. They show quite conclusively that the wetting agent enhanced the embryotoxicity of the micronised aspirin. The mean number of live foetuses per litter was reduced by 27% by the aspirin and by nearly three times this (75%) by the wetted suspension. It is incredible that neither result is significant statistically when subjected to the 'Student's' *t* test. However, when referred to the Mann-Whitney *U* test, the latter case is significant to the level of $P<0.05$. This effect is confirmed by the infinite increase in resorption, there being no case in the controls. Further, the increase in resorption in the aspirin group, coupled with the reduction in litter number mentioned above, suggests that there was also a drug effect here. Hence, intrauterine death was induced by micronised aspirin and enhanced by the addition of the surfactant.

The mean weight of the live foetuses showed a similar trend.

The pups in the aspirin group were 36.5% smaller than the controls, and those in the aspirin/Mannoxol OT/P group were 44% smaller ($P<0.001$ in both cases). The small size of the foetuses, which in conjunction with increased intrauterine death was reflected in the lower maternal weights at term, was mainly due to the large percentage of grossly malformed pups which are invariably diminutive. Further, it was noticeable that the skeletal development of those not grossly malformed was markedly retarded.

The increased embryopathic effect of the wetted preparation was also reflected in the foetal abnormalities. Sixteen of the 39 foetuses (41%) surviving the aspirin treatment showed gross malformations, compared with six out of 10 (60%) in the other test group. The malformations observed are listed in Table 9.4 and were of a type associated with salicylate teratogenicity. The subcutaneous haemorrhages seen in one foetus in each test group occur spontaneously in these rats and were excluded from the above calculations.

It is difficult to explain why the wetting agent showed no obvious effect in Experiment 9.B but had such an influence in the following experiment. The failure of the regimen to show teratogenesis in the former test is also inconsistent with the results of Experiment 9.A. It can be only surmised that these discrepancies may have arisen because of unknown factors peculiar to Experiment 9.B but not operating in 9.A or 9.C.

The hypothesis tested in this investigation was proven. It appears that the highest dose of micronised aspirin used in Experiment 9.A was too low for reproducible teratogenic effects. This was

confirmed by the lack of malformation in Experiment 9.B for which reason the results of this experiment cannot be viewed with impunity. However, embryopathic effects were induced by the higher dose used in Experiment 9.C, and these clearly were enhanced by the addition of a surfactant to the preparation. In spite of this, there was no apparent increase in maternal toxicity. It is possible, therefore, that the flocculation of the micronised aspirin particles in the Natrosol solution adversely affected their absorption from the gut; the use of a wetting agent to prevent flocculation increased the absorption rate of the aspirin and thereby enhanced its embryotoxicity.

This conclusion was tested by estimating the serum-salicylate levels of female rats at intervals after a single dose of micronised aspirin, with or without Mannoxol OT/P.

(c) Estimation of the serum-salicylate levels of rats

The colorimetric method of Keller (1947) was used and the protocol for the study is given in Table 9.5. Details of the technique are presented in Chapter 5 and Appendix 6. It was impossible to withdraw sufficient blood from any one rat at all the time intervals investigated. Therefore, a number of rats were killed at different times after the treatment to facilitate the collection of an adequate volume, and to compensate for individual variation. The dose level used was the same as in Experiment 9.C - i.e. 350 mg/kg.

The salicylate concentration curves are presented in Fig. 9.3. The results demonstrate that the surfactant facilitated more rapid absorption of the aspirin, so that a higher blood level was attained

at a greater rate. Even after four hours, when the levels were falling, there was still a higher concentration of the salicylate given with Mannoxol OT/P. This information supports the conclusion drawn from Experiment 9.C. It appears that the flocculation of the micronised particles interfered with their absorption and resulted in a lower blood level. This, in turn, was responsible for the unexpectedly low degree of teratogenicity in Experiments 9.A and 9.B.

Discussion

These investigations have shown that soluble aspirin is a more efficient teratogen than micronised aspirin which, in turn, is more efficient than the 200# preparation. The criteria considered for efficient teratogenicity were discussed in Chapter 6 but, briefly, include low maternal toxicity, a minimal increase in intrauterine death and high numbers of malformed progeny. The highest degree of embryotoxicity was induced by 200# aspirin. However, doses marginally below those killing the embryos produced a low incidence of malformations. These effects may have been due to less efficient absorption resulting in a blood level with a lower peak but maintained over a longer period. This seems to be the situation in humans (Munzel, 1967). Soluble aspirin, which did not kill the embryos as did similar doses of the 200# form, produced a broad spectrum of gross malformations. This soluble preparation was better tolerated and its effect may have been related to the rapid induction of a higher blood level (Munzel, 1967). Such a teratogenic effect was also instigated by higher doses of micronised aspirin. Here, the enhanced embryopathic effects in the

presence of a wetting agent, which prevented the particles from flocculating, appeared to result from the higher serum-salicylate levels.

Conclusions

(1) Characteristic birth defects were induced by all the aspirin preparations tested, but the soluble form produced a higher number of more grossly malformed foetuses.

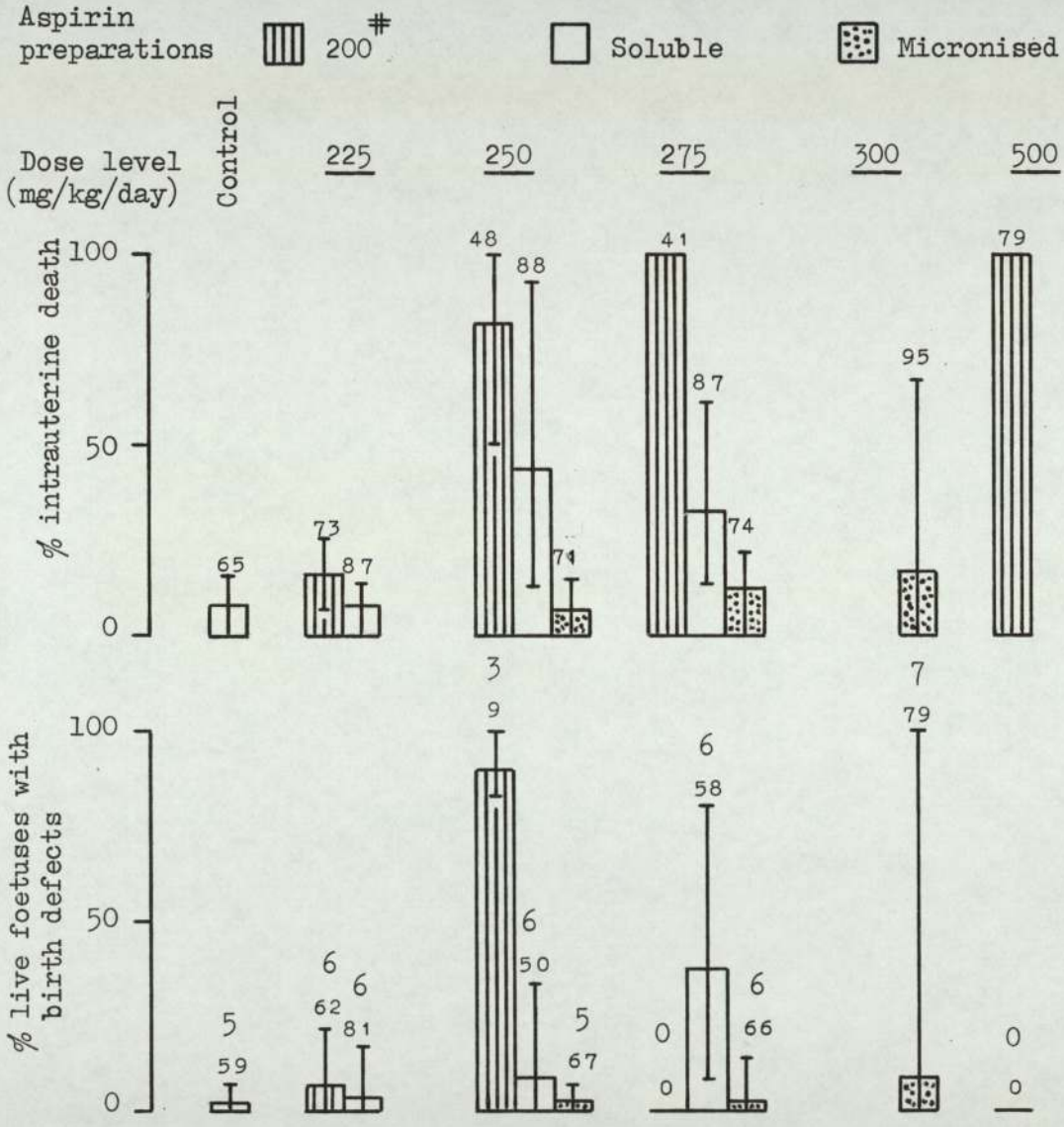
(2) Embryotoxicity in rats was induced by 200# aspirin at lower dose levels than either micronised or soluble forms.

(3) Flocculation of the micronised aspirin particles interfered with absorption. Therefore, larger doses were necessary to produce a teratogenic effect.

(4) Particle flocculation was reduced by the surfactant Mannoxol OT/P. Its addition to the micronised aspirin preparation raised the serum-salicylate level and enhanced the embryopathic effects.

FIG. 9.1.

COMPARISON OF THE INCIDENCE OF INTRAUTERINE DEATH AND BIRTH DEFECTS IN RATS TREATED WITH DIFFERENT ASPIRIN PREPARATIONS - WITH RANGE OF EXPERIMENTAL VARIATION (Experiment 9.A)



N.B. The numbers above the columns relating to intrauterine death indicate the total numbers of implantations.

The larger numbers above the columns relating to birth defects indicate the total numbers of litters containing live foetuses. The smaller numbers indicate the total numbers of foetuses.

FIG. 9.2.

COMPARISON OF THE INCIDENCE OF INTRAUTERINE DEATH AND BIRTH DEFECTS, AND OF THE MEAN FOETAL WEIGHT IN RATS TREATED WITH MICRONISED ASPIRIN, WITH OR WITHOUT MANNOXOL OT/P - WITH RANGE OF EXPERIMENTAL VARIATION (Experiment 9.C)

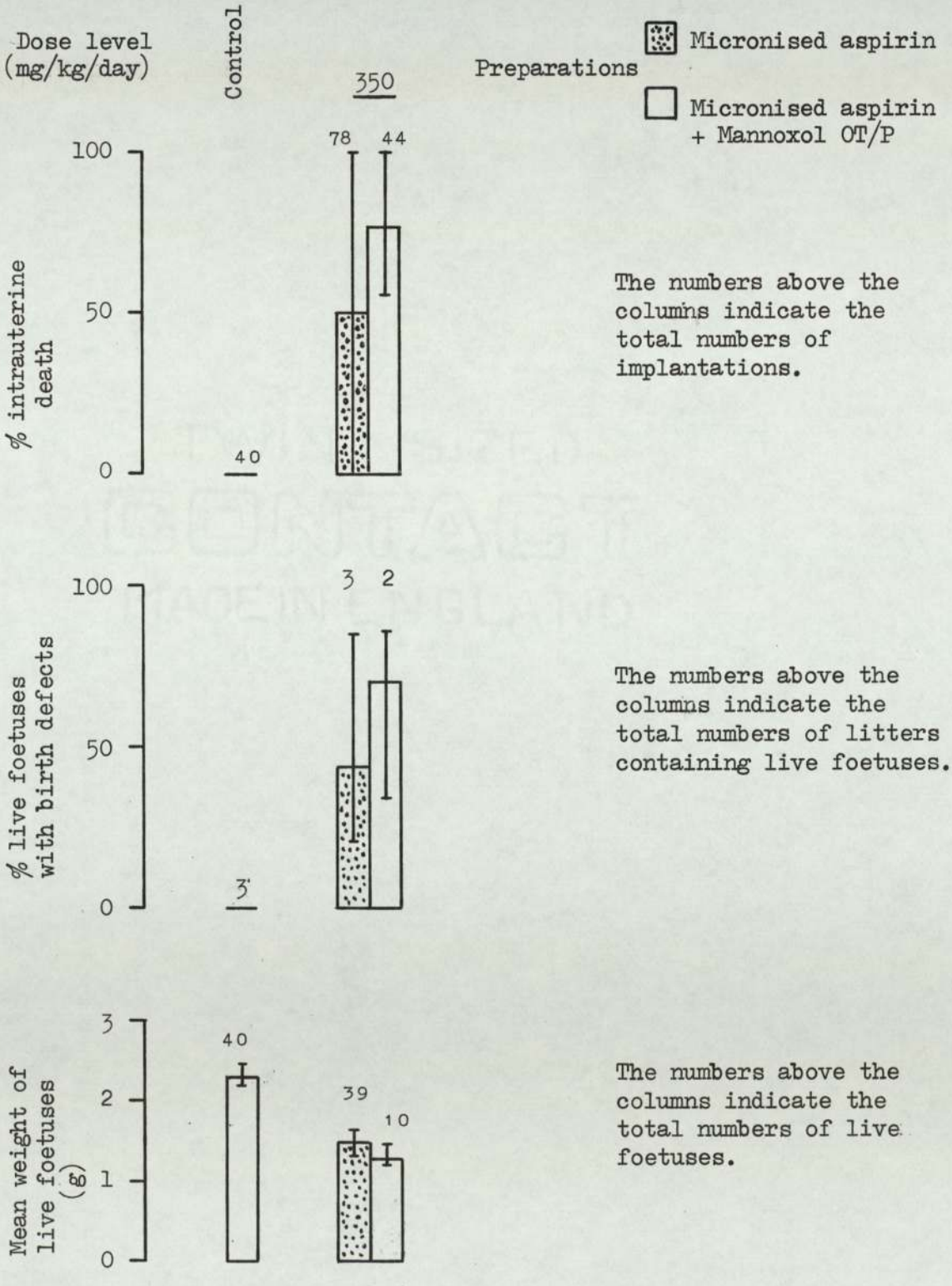


FIG. 9.3.

SERUM - SALICYLATE LEVELS OF FEMALE RATS AFTER ONE DOSE OF 350 MG/KG OF MICRONISED ASPIRIN, WITH OR WITHOUT MANNOXOL OT/P - WITH RANGE OF EXPERIMENTAL VARIATION (Experiment 9.D)

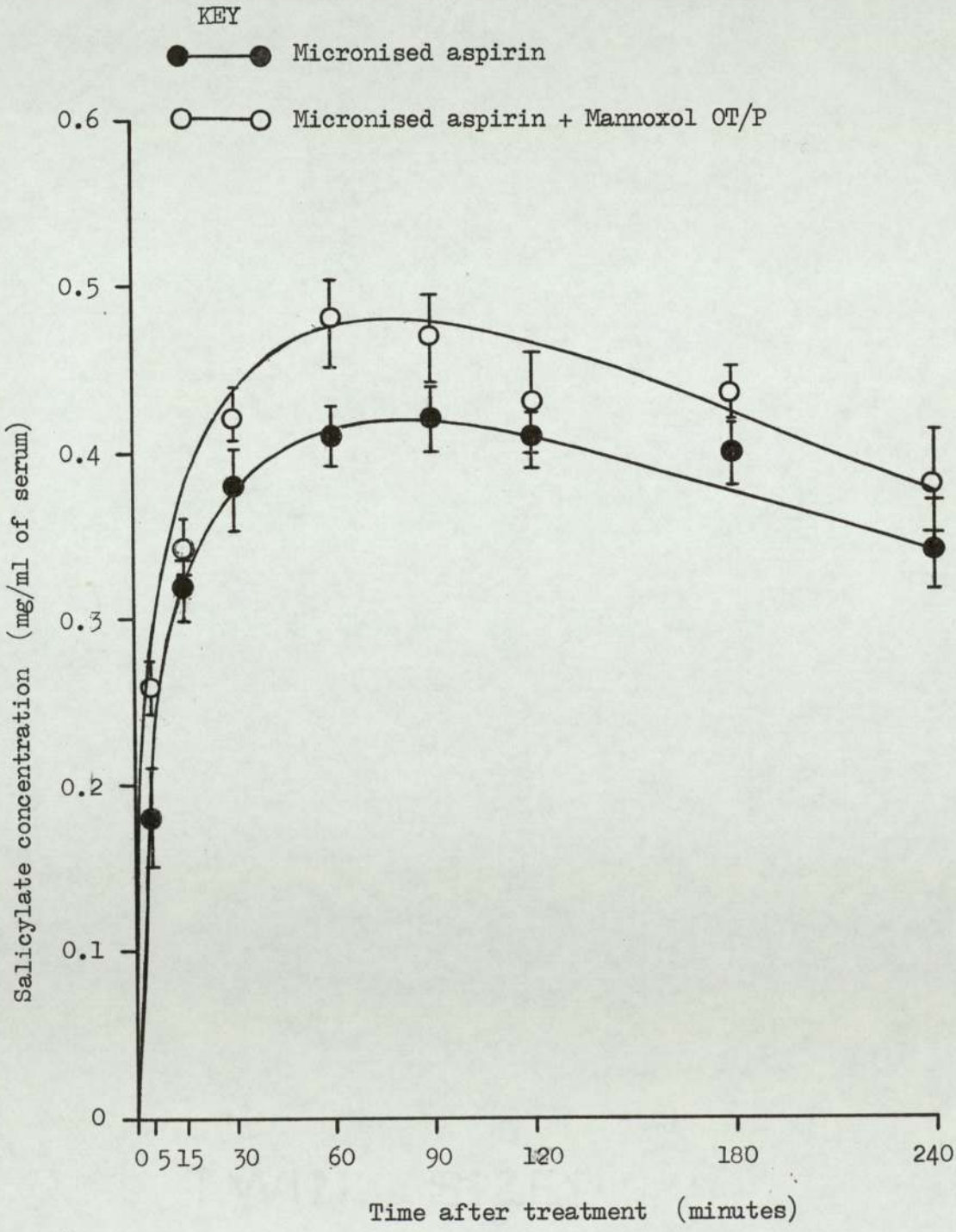


TABLE 9.1

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF DIFFERENT ASPIRIN PREPARATIONS IN RATS

Experiment code	Compound	Dose level (mg/kg/day)	Days of administration	No. of rats mated
9.A		Control	8-12	6
	200# Aspirin	225	8-12	6
		250	8-12	8
		275	8-12	8
		500	8-12	6
	Soluble aspirin	225	8-12	6
		250	8-12	7
		275	8-12	7
	Micronised aspirin	250	8-12	6
		275	8-12	6
		300	8-12	8

TABLE 9.2

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF DIFFERENT ASPIRIN PREPARATIONS IN RATS

Experiment code	Compound	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
				Alive	Dead								
9.A		Control	5	59	0	11.80	2.19	6	1.20	0.057	0	0	1^{21}
	200# Aspirin	225	6	62	0	10.33	2.07	11	1.83	0.309	0	0	$2^{1}+1^{2}+1^{3}$
		250	6	7	2	1.17*	0.95*	34	5.67*	0.253	5	0.83	$3^{4}+1^{5}+1^{6}+1^{7}+1^{8}+1^{9}$
		275	6	0	0	0	0	21	3.50*	0.222	20	3.33	0
		500	6	0	0	0	0	70	11.67*	0.322	9	1.50	0
	Soluble aspirin	225	6	80	1	13.33	2.27	6	1.0	0.479	0	0	$1^{10}+1^{20}+1^{21}$
		250	6	44	6	7.33*	1.73*	38	6.33*	0.269	0	0	$3^{1}+2^{21}$
		275	6	54	4	9.0	1.90*	29	4.83*	0.356	0	0	$4^{1}+1^{10}+1^{11}+4^{12}+1^{13}+1^{14}+1^{15}+1^{16}+1^{17}+1^{18}+6^{21}$
	Micronised aspirin	250	5	67	0	13.40	1.95	4	0.80	0.101	0	0	$1^{1}+1^{21}$
		275	6	66	0	11.0	1.82*	8	1.33	0.129	0	0	2^{1}
		300	7	79	0	11.29	2.41	16	2.29	0.165	0	0	$2^{2}+1^{3}+1^{10}+1^{19}+2^{21}$

* Statistically significantly different from the controls.

1 Exencephalia.

2 Exencephalia, exophthalmia, macroglossia.

3 Microcaudia.

4 Scoliosis, fused ribs, hemivertebrae, sternal anomalies.

5 Scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies.

6 Kinked ribs, inhibited central ossification.

7 Scoliosis, kinked ribs, fused ribs, hemivertebrae, sternal anomalies.

8 Scoliosis, fused ribs, hemivertebrae, sternal anomalies, inhibited central ossification.

9 Kinked ribs.

10 Omphalocele.

11 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera.

12 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies.

13 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, exophthalmia.

14 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, exophthalmia, eventration of the abdominal viscera.

15 Craniorrhachischisis, scoliosis, fused ribs, hemivertebrae, sternal anomalies, curly tail, kyphosis.

16 Craniorrhachischisis, scoliosis, fused ribs, hemivertebrae, sternal anomalies, kyphosis.

17 Exencephalia, myeloschisis.

18 Exencephalia, macroglossia.

19 Micrognathia, omphalocele.

20 Fused placentae.

21 Subcutaneous haemorrhages.

TABLE 9.3

PROTOCOL FOR THE INVESTIGATIONS INTO THE EFFECT OF PARTICLE FLOCCULATION ON THE TERATOGENICITY OF MICRONISED ASPIRIN IN RATS

Experiment code	Compound	Dose level (mg/kg/day)	Days of administration	No. of rats mated
9.B	Vehicle + Mannoxol OT/P	Control	8-12	8
	Micronised aspirin	300	8-12	8
	Micronised aspirin + Mannoxol OT/P	250 275 300	8-12 8-12 8-12	8 8 8
9.C	Vehicle + Mannoxol OT/P	Control	8-12	4
	Micronised aspirin	350	8-12	4
	Micronised aspirin + Mannoxol OT/P	350	8-12	3

TABLE 9.4

RESULTS OF THE INVESTIGATIONS INTO THE EFFECT OF PARTICLE FLOCCULATION ON THE TERATOGENICITY OF MICRONISED ASPIRIN IN RATS

Experiment code	Compound	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
				Alive	Dead								
9.B	Vehicle + Mannoxol OT/P	Control	8	109	0	13.63	2.30	1	0.13	0.10	0	0	0
	Micronised aspirin	300	7	74	0	10.57	1.93*	27	3.86*	0.057	0	0	1^{1}
	Micronised aspirin + Mannoxol OT/P	250	7	89	0	12.71	2.11*	3	0.43	0.025	0	0	0
		275	8	103	0	12.88	2.11*	11	1.38	0.041	0	0	0
		300	8	107	0	13.38	1.91*	8	1.0	0.207	0	0	2^{1}
9.C	Vehicle + Mannoxol OT/P	Control	3	40	0	13.33	2.33	0	0	0	0	0	0
	Micronised aspirin	350	4	39	0	9.75	1.48*	39	6.25*	0.083	0	0	$1^{1}+4^{2}+4^{3}+3^{4}+1^{5}+1^{6}+1^{7}+1^{8}+1^{9}$
	Micronised aspirin + Mannoxol OT/P	350	3	10	0	3.33*	1.31*	34	11.3*	0.117	0	0	$1^{1}+1^{2}+1^{3}+1^{10}+1+^{11}+1^{12}+1^{13}$

* Statistically significantly different from the controls.

1 Subcutaneous haemorrhages.

2 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera.

3 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera, arthrogryposis, exophthalmia, ablepharia.

4 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera, arthrogryposis.

5 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera, arthrogryposis, exophthalmia.

6 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera, exophthalmia.

7 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera, arthrogryposis, bottle-jaw oedema.

8 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, omphalocele, arthrogryposis.

9 Craniorrhachischisis, scoliosis, fused ribs, hemivertebrae, sternal anomalies.

10 Eventration of the abdominal viscera, arthrogryposis.

11 Craniorrhachischisis, scoliosis, kyphosis, fused ribs, hemivertebrae, sternal anomalies, eventration of the abdominal viscera, arthrogryposis, exophthalmia, ablepharia, macroglossia.

12 Scoliosis, fused ribs, hemivertebrae, sternal anomalies, generalised subcutaneous oedema.

13 Myeloschisis, scoliosis, fused ribs, hemivertebrae, sternal anomalies.

TABLE 9.5

PROTOCOL FOR THE DETERMINATION OF SERUM-SALICYLATE LEVELS IN RATS

Experiment code	Compound	Dose level (mg/kg)	Time after treatment (mins)	No. of rats
9.D		Control		5
	Micronised aspirin	350	5	3
			15	3
			30	3
			60	3
			90	3
			120	3
			180	3
			240	2
	Micronised aspirin + Mannoxol OT/P	350	5	3
			15	3
			30	3
			60	3
			90	3
			120	3
			180	3
			240	2

CHAPTER 10

THE ROLE OF THE CARBOXYL GROUP OF THE ASPIRIN MOLECULE IN RELATION TO ITS TERATOGENICITY

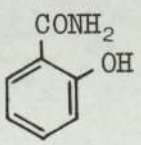
Introduction

Having considered the embryopathic effects of aspirin, attention was directed towards ascertaining the parts played by the various groups in the molecule. This chapter describes investigations undertaken to determine the importance of the carboxyl group. This group is protected in salicylamide by conversion to an amide, and is absent in phenol. Neither compound has the acetyl group in the ortho position, but this is hydrolysed to the phenol soon after entering the body. The possible importance of this group will be considered in the next chapter. The experiments described here were designed to establish whether salicylamide and phenol induce birth defects in the rat, a species sensitive to aspirin. A negative result would suggest that the carboxyl group is necessary for the teratogenicity and a positive result would show that it is not.

Experimental Work

The investigations with these two compounds will be discussed separately. The standard techniques described in Chapter 5 were employed throughout.

(a) Salicylamide



Salicylamide, like aspirin, has analgesic properties, but its

antipyretic and anti-inflammatory activity is lower than that of aspirin. It differs from other salicylates in that it does not act by virtue of hydrolysis to salicylic acid. Indeed, the only compound detected in the tissues after administration is free salicylamide.

Salicylamide produces congenital malformations in the golden hamster (Lapointe and Harvey, 1964). However, this does not indicate that the carboxyl group is unnecessary for aspirin teratogenicity in the rat because such activity has not been demonstrated in the hamster. Similarly, the failure of salicylamide to produce birth defects in rabbits is no criterion of its essential nature because this species is not susceptible to aspirin (Chapter 7).

The investigations with salicylamide in the rat involved three experiments and the protocol for these is given in Table 10.1. Successive tests with progressively higher doses were undertaken until maternal death was induced. A single dose of 2000 mg/kg killed two of the four dams, and treatment of the survivors was discontinued. In order to achieve even higher levels, large doses were administered twice daily in Experiment 10.C. Twice daily treatment with 1000 mg/kg was tolerated, but 2 x 1500 mg/kg/day was not and killed one of the five rats. Consequently, one of the doses was discontinued after two days, whereafter no further intercurrent death occurred. All three rats which died showed no obvious pathological change, a feature typical of animals dying soon after acute treatment. The rats which survived in these two groups were in poor condition for two days. They were lethargic, anorexic and their coats were staring. As a result of the anorexia, they lost weight after the onset of dosing and, although

they recovered, they gained less weight than the controls during the treatment period. However, by term, their overall weight change was comparable with that of the controls. This effect - an initial weight loss coinciding with the start of dosing, followed by a slight recovery as treatment continued and a full recovery after it was withdrawn - was typical of all test rats in Experiments 10.B and 10.C. In 10.A, however, the rats showed the characteristic weight changes during treatment but did not gain weight to the same extent as the controls during late pregnancy. Four rats did not become pregnant.

The results of the investigations are presented in Table 10.2. Only one group of rats showed significantly fewer live foetuses per litter than the controls. This occurred in the 1500 mg/kg/day group where there was a 74% reduction ($P<0.002$). However, there was no case of intrauterine death in these dams, and the results are drawn from only two animals. Further, rats treated at higher dose levels showed no such effect. Therefore, it is probable that this was not drug-related.

There are two other results concerning litter number which are worthy of comment. In the 1000 mg/kg/day group of Experiment 10.A there was a significant twelvefold increase in intrauterine death ($P<0.01$) coupled with a 45% reduction in the mean number of foetuses (which is not statistically significant). Within the limits of Experiment 10.A, this would appear to be a drug effect but the results obtained later with higher doses indicate that this was not so. Yet, there may have been a true drug effect in the rats given 1000 mg/kg twice daily; a 22% reduction in the mean number of live foetuses was associated with an increase in intrauterine death by a

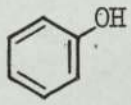
factor greater than four, but neither difference is statistically significant. Although one group was scheduled to receive a higher regimen, this was too toxic and could not be completed. Consequently, the lack of a comparable effect in this group is no criterion for dismissing the effect in the 1000 mg/kg b.i.d. group.

Experiment 10.A showed an apparent dose-related effect on mean foetal weight. The reduction was by 23% at 250 mg/kg, 28% at 500 mg/kg, 29% at 750 mg/kg and 33% at 1000 mg/kg ($P<0.001$ in all cases) compared with the controls. These smaller foetuses showed a lower degree of ossification, and their diminutive size was reflected in the retarded weight gain of their dams. However, the higher doses in the two subsequent experiments produced no effect on foetal weight, and it must be concluded that the small foetuses observed in Experiment 10.A were not induced by the salicylamide.

Only one of the 486 foetuses showed any anomaly; this was a case of subcutaneous haemorrhages which were seen in the 1000 mg/kg b.i.d. group. Such lesions occur spontaneously in these rats and this one instance was considered to be not drug-related.

These experiments involved doses of salicylamide which killed some of the adult rats. The quantities administered were very much higher than toxic doses of aspirin. However, marginally sublethal levels had no embryopathic effect. Such results indicated that the carboxyl group is essential for aspirin teratogenicity, and the investigation with phenol was undertaken to substantiate this conclusion.

(b) Phenol



Phenol has potent bactericidal activity which is effected by denaturing the protein (Esplin, 1941). In common with the salicylates, it has some antipyretic activity which involves a similar mechanism of action. Its use as a germicide has declined because of the availability of less toxic and more effective alternatives.

No report on the potential teratogenicity of phenol appears in the literature. My own unpublished investigations have shown that this compound produces birth defects in Stride Dutch rabbits. However, as was the case with salicylamide in the hamster, the embryopathic effects in the rabbit are not relevant, since this species is not susceptible to aspirin teratogenicity. Consequently, this is no indication that the carboxyl group is superfluous in the instigation of such an effect.

The study with phenol in the rat involved three experiments and the protocol for these is given in Table 10.3. As expected, orally administered phenol was toxic to the dams. One dose of 400 mg/kg killed all four rats treated but, as is usual with deaths following acute treatment, there was no apparent pathological change. Doses of up to 350 mg/kg/day retarded maternal weight gain but, when treatment was discontinued, the does recovered and, by term, were of a size comparable with the controls.

In order to increase the dose, the compound was given twice daily. The rats given 250 mg/kg b.i.d. became ill; they showed tremor, were anorexic, lethargic, and had staring coats and increased lacrimal

secretion. In spite of this, their weight loss was only slight; they lost a mean of only 1g throughout dosing, compared with a mean gain of 24g by the controls. By term, these test rats had gained appreciably less weight than the controls. Although the rats might have tolerated a higher dose, it was considered unethical to try this because of the maternal cachexia already induced. Four rats were not mated successfully.

The results of the experiments are presented in Table 10.4. There was no effect on the mean number of live foetuses per litter. Rats given 250 mg/kg once daily had a significant 22% reduction in that number ($P<0.02$), but there was no associated increase in resorption and no significant reduction in rats given larger doses. Therefore, this smaller litter number cannot be regarded as a drug effect.

There were two instances of significantly smaller foetuses in these experiments. They occurred in the 300 mg/kg group where they were 9% smaller than the controls ($P<0.05$), and in the 250 mg/kg b.i.d. group where they were 5% smaller ($P<0.02$). It is difficult to relate the former case to the drug, principally because rats treated at the higher dose of 350 mg/kg/day had foetuses of normal size. Further, the dams in the affected group gained weight to the same extent as the controls during late pregnancy. This is contrary to the condition typically found in association with this effect (see Chapter 6). In the case of the 250 mg/kg b.i.d. group, however, the dams gained less weight than the controls and this dose was approaching the maximum tolerated level. It was considered, therefore, that this was a drug-induced retardation of foetal growth.

Subcutaneous haemorrhages were the only anomaly observed. They occurred in six out of 409 foetuses and were present only in the controls. There was clearly no relation to the phenol.

The conclusion drawn from the experiments with salicylamide was verified by this study: the carboxyl group appears to be an integral part of the teratogenic moiety of aspirin.

Discussion

Unlike the study in rabbits (Chapter 7), the experiments with salicylamide in rats were persued until maternal death was induced. The drug had extremely low toxicity and very large doses were necessary to produce this effect. Marginally sublethal levels may have increased intrauterine death but did not induce birth defects. Phenol, on the other hand, killed the dams at a much lower dose level but, although retarding foetal growth, did not produce malformed progeny.

It is interesting that both compounds instigate congenital malformations in species which are not sensitive to aspirin, but do not have this effect in the rat - an animal in which aspirin is teratogenic. The essential nature of the carboxyl group in aspirin teratogenicity has been demonstrated. Its conversion to the amide in salicylamide and its absence in phenol appears to have prevented these compounds from inducing birth defects in rats. However, the possible importance of the acetyl group should not be overlooked as it is not present in the cases discussed here. This factor will be considered in the following chapter.

Conclusions

(1) Rats tolerated appreciably higher doses of salicylamide than of aspirin.

(2) Marginally sublethal doses of salicylamide and phenol did not induce birth defects in the rats.

(3) As both compounds differ from aspirin only in the carboxyl and acetyl groups, it was concluded that one or both groups are essential for aspirin teratogenicity in rats.

TABLE 10.1

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF SALICYLAMIDE IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
10.A	Control	8-12	4
	250	8-12	3
	500	8-12	3
	750	8-12	3
	1000	8-12	3
10.B	Control	8-12	3
	1000	8-12	3
	1250	8-12	4
	1500	8-12	4
	2000	8[1]	4
10.C	Control (b.i.d.)	8-12	4
	1000 (b.i.d.)	8-12	5
	1500 (b.i.d.)	8-9 [2]	5
	1500	10-12 [2]	

[1] It was intended to dose these rats from days 8-12 but the treatment was discontinued after the first dose had killed two of the four dams.

[2] It was intended to treat these rats twice daily from 8-12 but the toxicity induced necessitated the discontinuation of one daily dose from day 10.

TABLE 10.2

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF SALICYLAMIDE IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
10.A	Control	4	53	0	13.25	3.0	2	0.50	0.214	0	0	0
	250	3	38	0	12.67	2.32*	0	0	0	0	0	0
	500	3	34	0	11.33	2.15*	1	0.33	0.017	0	0	0
	750	3	40	0	13.33	2.13*	0	0	0	0	0	0
	1000	3	22	13*	7.33	2.01*	5	1.67*	0.112	0	0	0
10.B	Control	3	46	0	15.33	2.46	0	0	0	0	0	0
	1000	3	43	0	14.33	2.28	1	0.33	0.180	0	0	0
	1250	4	51	0	12.75	2.27	2	0.50	0.020	0	0	0
	1500	2	8	0	4.0*	2.38	0	0	0	0	0	0
	2000	2	29	0	14.50	2.24	2	1.0	0.205	0	0	0
10.C	Control (b.i.d.)	4	45	0	11.25	2.32	6	1.50	0.054	0	0	0
	1000 (b.i.d.)	5	44	9	8.80	2.04	16	3.20	0.016	1	0.2	1[1]
	1500 (b.i.d.) 1500	2	33	0	16.50	2.12	1	0.50	0.350	0	0	0

* Statistically significantly different from the controls.

[1] Subcutaneous haemorrhages.

TABLE 10.3

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF PHENOL IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
10.D	Control	8-12	10
	250	8-12	5
10.E	Control	8-12	3
	300	8-12	5
	350	8-12	5
	400	8[1]	4
10.F	Control (b.i.d.)	8-12	4
	250 (b.i.d.)	8-12	5

[1] It was intended to treat these rats from days 8-12 but all died after the first dose.

TABLE 10.4

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF PHENOL IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
10.D	Control	10	133	0	13.30	2.25	6	0.60	0.114	0	0	6[1]
	250	5	52	0	10.40*	2.12	6	1.20	0.020	0	0	0
10.E	Control	3	43	0	14.33	2.80	1	0.33	0.333	0	0	0
	300	3	43	0	14.33	2.55*	2	0.67	0.017	0	0	0
	350	4	48	0	12.0	2.79	2	0.50	0.555	0	0	0
	400	0										
10.F	Control (b.i.d.)	4	45	0	11.25	2.32	6	1.50	0.054	0	0	0
	250 (b.i.d.)	4	45	0	11.25	2.20*	3	0.75	0.012	0	0	0

* Statistically significantly different from the controls.

[1] Subcutaneous haemorrhages.

CHAPTER 11

THE ROLE OF THE ACETYL GROUP OF THE ASPIRIN MOLECULE IN RELATION TO ITS TERATOGENICITY

Introduction

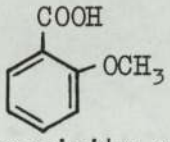
Having demonstrated that the carboxyl group plays an integral part in aspirin teratogenicity, attention was directed towards the acetyl group. Again the principle of replacing or omitting the group was employed, and o-methoxybenzoic acid, benzoic acid and salicylic acid were studied. The acetyl group of aspirin is replaced by the more stable methoxy group in o-methoxybenzoic acid, is absent altogether in benzoic acid, and is hydrolysed to ~~form~~ leave salicylic acid (which is the major metabolite of all the salicylates) and acetic acid. In man, this reaction occurs soon after entering the body, and salicylic acid constitutes up to 61% of the urinary metabolites of aspirin (Milne, 1963). The production of characteristic birth defects with any of these compounds would suggest that the acetyl group in aspirin is not essential for its teratogenicity.

The presence of the acetyl group in aspirin tends to retard the action of salicylic acid because the latter compound is the active moiety and exerts its physiological effect only after the hydrolysis of that group. The suitability of the acid group is related to the physical properties of the compound formed. The speed of hydrolysis depends largely on solubility, and acetylation is the most convenient process. Acetyl groups have advantages over the more soluble lactyl ones which are hydrolysed too rapidly, and over the less soluble benzoyl ones whose hydrolysis is so slow that they are excreted in an unchanged state (Dyson and May, 1911).

Experimental Work

The investigations with each compound will be considered separately. The standard techniques described in Chapter 5 were used throughout.

(a) o-Methoxybenzoic acid



Investigations into the teratogenicity of o-methoxybenzoic acid, which is the methyl ether of salicylic acid, involved two experiments and the protocol for these is given in Table 11.1. The compound was remarkably non-toxic and doses of up to 5000 mg/kg had to be administered before any appreciable effect was observed. Two doses of this extremely high level killed one of the four rats in the group, but all other test dams survived the full course of treatment. However, two others died after dosing was discontinued but there was no obvious pathological change associated with the rats' deaths.

All groups of test animals gained weight during the treatment period. In those rats given 250, 500 or 750 mg/kg of this agent, this increase was comparable with the controls - i.e. while the test animals gained a mean of 20g, 18g and 21g respectively, the controls gained 19g. However, the weight gain was rather less at higher dose levels, ranging from a mean of 7g in the 1000 mg/kg group to only 1g in the 5000 mg/kg group. After treatment was discontinued, the rats given 1000 mg/kg/day or more did not gain as much weight as those in the controls or the lower dose groups. Eight rats did not become pregnant.

The results of this investigation are presented in Table 11.2. There was no statistically significant increase in intrauterine death; nevertheless, the results warrant closer examination and discussion. In Experiment 11.B, for example, all test groups contained fewer live

foetuses per litter than did the controls. In the case of the rats treated at 3000 mg/kg, this difference was by the margin of 68% and was accompanied by an increase in resorption by nine times the control value. Although the number of foetuses was not significantly different from the controls in the 'Student's' t test, it was significant to the level of $P<0.05$ in the Mann-Whitney U test. However, the increase in embryonic death was not significant by either statistical method. Rats treated with 2000 or 4000 mg/kg/day of o-methoxybenzoic acid also had fewer foetuses and a higher incidence of resorption, but the difference from the controls was less pronounced than in the 3000 mg/kg group. These figures, although not dose-related, could be indicative of a drug effect; but the one rat surviving the higher dose of 5000 mg/kg/day contained all live foetuses. The only reasonable conclusion is that this latter rat was atypical because all of her companions died. That she survived may have been related to her inefficient absorption of the drug or to some other factor peculiar to her, in which case the results obtained from her progeny cannot be viewed with much reliability. Therefore, it appears that o-methoxybenzoic acid at doses in excess of 2000 mg/kg/day caused an increase in the incidence of intrauterine death.

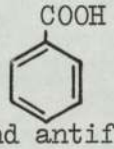
A very precise embryopathic effect of the drug was seen in the foetal weights which were significantly smaller in all rats given 1000 mg/kg/day or more ($P<0.001$ in all cases). There was no clear dose-relationship, but in all groups it was associated with a retardation in maternal weight gain and a lower degree of ossification in the foetal skeletons.

Examination of Table 11.2 will show that 20 of the 379 foetuses had subcutaneous haemorrhages. All of these occurred in test groups where

the incidence (6.7%) was appreciably higher than the spontaneous incidence in AH A rats (1.63%). Although there is no dose-related response, it is possible that the test compound may have increased this incidence. The single case of fused placentae could not have been drug-induced because it was established before treatment commenced. However, the appearance of a case of microcaudia in the 4000 mg/kg group is perhaps more pertinent. This malformation occurred in rabbits treated with different salicylates (Chapter 7) and has been induced also in rats by this range of drugs. There was no malformation in the litter of the single rat given 5000 mg/kg/day but, as was discussed above, this result may not be reliable. Consequently, when reviewing the possible teratogenicity of o-methoxybenzoic acid, this defect should not be totally disregarded.

This study involved a particularly innocuous compound. A massive dose, greatly in excess of amounts of aspirin used previously, was necessary to induce maternal toxicity; yet the only indisputable embryopathic effect was a retardation of foetal growth. However, there is evidence of a possible increase in intrauterine death and in the incidence of an indigenous anomaly. The most significant feature, though, was a lack of any birth defect of a type associated with salicylates. Such a result suggests that the acetyl group is necessary for aspirin teratogenicity.

(b) Benzoic acid



Benzoic acid has antibacterial and antifungal activity and is an efficient preservative in foods and pharmaceutical preparations. After absorption, it is conjugated with glycine in the liver to form hippuric acid.

Two experiments into the teratogenicity of benzoic acid were undertaken and the protocol for these is given in Table 11.3. The dams in Experiment 11.C were killed 21 days post coitum, as a result of which their foetuses were larger. Only the dams given 1250 mg/kg/day showed any toxic effect; they gained less weight than the controls during treatment (a mean of 7g compared with 21g) and failed to recover completely when dosing stopped.

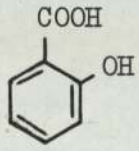
Table 11.4 shows that this was the only group to show any significant embryotoxicity. The mean number of live foetuses per litter was 78% lower than the controls and was associated with a sevenfold increase in the incidence of embryonic death ($P<0.05$ in both instances). Further, the live foetuses in this group were 20% smaller than the controls ($P<0.01$), indicating that the drug retarded foetal growth. These effects were associated with the failure of the dams to increase weight to the same extent as the controls.

The rats given 1000 mg/kg/day also showed evidence of an embryotoxic effect but, in this case, it was not significant statistically. The number of live foetuses was less than half the control value and resorption was increased by a factor of six. In view of a similar but enhanced effect seen with the higher regimen, it is probable that the increased intrauterine death in the 1000 mg/kg group was a drug effect.

One of the 315 foetuses examined was malformed. As with o-methoxybenzoic acid, it was a case of microcaudia. However, its appearance in the lowest dose group with no anomaly at much higher dose levels suggests that benzoic acid was not the causative agent.

Like o-methoxybenzoic acid, benzoic acid induced both maternal and embryonic toxicity but no teratogenic effect. These results also suggest that the acetyl group is necessary for the teratogenic effect of aspirin.

(c) Salicylic acid



In spite of the failure to produce birth defects with o-methoxybenzoic acid and benzoic acid, it was decided to investigate salicylic acid. This appears to be the most relevant compound, being the principal metabolite of aspirin after the hydrolysis of the acetyl group. It has bacteriostatic and fungicidal properties and is used therapeutically in different skin diseases.

Birth defects were produced in the first experiment, the protocol for which is presented in Table 11.5. The weight of the test dams did not differ appreciably from that of the controls throughout treatment but, during late pregnancy it fell below that of the controls. Three rats did not become pregnant.

Table 11.6 shows the results of the investigation. The control group contained an unusually small number of live foetuses in each litter and showed a relatively high incidence of embryonic death. Consequently, the reduction in foetal number in the 300 mg/kg group is not significant and cannot be regarded as a drug effect.

The mean weight of the live foetuses in the 250 and 300 mg/kg groups was significantly lower than the control value ($P < 0.05$ in both instances). The reduction was by a margin of 26% in the former group

and by 21.5% in the latter. However, the rats given the intermediate dose of 275 mg/kg/day had foetuses of normal size. Subsequent investigations with salicylic acid have confirmed that treatment with 250 mg/kg/day results in the development of significantly smaller foetuses. This observation clarifies the results obtained in the present experiment; the lack of effect in the 275 mg/kg group was considered to be atypical and it was concluded that salicylic acid at dose levels in excess of 250 mg/kg/day retards the normal growth of rat foetuses.

All three dose levels produced birth defects of known association with aspirin. Thus, such congenital malformations can be induced in the absence of the acetyl group which, therefore, is not essential for their pathogenesis.

To date, this study had yielded divergent results; the experiments with o-methoxybenzoic acid and benzoic acid suggested that the acetyl group was essential for the induction of birth defects but the teratogenic effects of salicylic acid were incompatible with this. It was clear that the acetyl group was not essential for aspirin teratogenicity but it was possible that it may enhance it. Therefore, at this juncture, it was decided to study further the possible role of the acetyl group by comparing directly the embryopathic effects of aspirin and salicylic acid. Acetic acid is also formed during aspirin hydrolysis, and the possible role of this compound in aspirin teratogenesis was also considered.

In these investigations, equimolecular levels of the drugs were used and the protocol is given in Table 11.7. The two investigations were undertaken in the same experiment (11.F) but each will be considered separately. Three rats did not become pregnant.

(d) The comparative embryopathic effects of aspirin and salicylic acid

This study involved only the aspirin and salicylic acid groups in Experiment 11.F. At the dose levels administered, both compounds were toxic to the pregnant rats, but salicylic acid was less so than doses of aspirin containing equimolecular weights of salicylic acid. These conclusions were derived from observations on maternal weight change during pregnancy. While the controls gained a mean of 24g during the treatment period, the rats given 325 mg/kg/day of aspirin gained only 0.5g and those given an equivalent amount of salicylic acid (250 mg/kg/day) gained 11g. Further, when treatment was discontinued, the test animals failed to grow to the same extent as the controls. This was evident from the maternal weight change over the full gestation period. The controls gained a mean of 113g during this time while the aspirin rats gained 68g and those given salicylic acid gained 103g.

This pattern was repeated at the higher dose levels but here the effect was more pronounced. During the treatment period, the rats given 455 mg/kg/day of aspirin lost a mean of 5g while those given the equivalent dose of salicylic acid (350 mg/kg/day) gained 7g. In addition, these aspirin rats gained only 38g over the whole gestation period while the salicylic acid ones gained 71g.

The lower maternal weights in the test groups reflect the lower number of foetuses surviving to term and the smaller size of these foetuses (discussed below). The respective weights of the dams demonstrated the toxicity of the salicylic acid, and the addition of the acetyl group to this molecule enhanced this effect.

The results of the investigation are presented in Table 11.8. The increased toxicity of aspirin over equivalent amounts of salicylic acid was also apparent in the relative embryopathic effects of these drugs.

These effects are expressed as histograms in Fig. 11.1. The high doses of both compounds caused intrauterine death, thereby reducing the number of live foetuses in each litter significantly below the control value. No foetus survived 455 mg/kg/day of aspirin while the mean number per litter after an equivalent dose of salicylic acid (350 mg/kg/day) was only one ($P < 0.001$ in both instances), the latter case being 91% lower than the controls. The lower dose of aspirin (325 mg/kg/day) also reduced the mean number of foetuses alive at term; in this case it was 65% below the control value ($P < 0.01$). These three test groups showed an associated increase in the incidence of resorption ($P < 0.001$ in all cases), there being no instance in the control animals. There was an increase in resorption in the rats given salicylic acid at 250 mg/kg/day ($P < 0.001$), but no accompanying decrease in foetal number. In spite of this, it was considered that this regimen adversely affected embryonic survival in utero.

These results demonstrate that, although all four regimens were detrimental to the survival of the embryos, the effect of aspirin was more harmful than equivalent doses of salicylic acid. At the higher dose levels, aspirin killed all the embryos while a mean of only one foetus per litter developed after salicylic acid treatment. Further, intrauterine resorption in the aspirin group was 50% greater than in the corresponding salicylic acid one. Perusal of the results obtained with the lower doses will show that, after aspirin was administered, there were 65% fewer foetuses alive at term than with salicylic acid. This difference is significant ($P < 0.01$) and was associated with over twice the number of resorption sites - a difference which was almost certainly significant biologically, but was not so statistically. The evidence presented shows that salicylic acid caused embryonic death in rats, and that its effect was enhanced by the addition of the acetyl group to the

molecule to form aspirin.

The increased embryotoxicity of aspirin over salicylic acid was also reflected in the weight of the foetuses alive at term. All groups of test foetuses were significantly smaller than the controls ($P<0.001$ in all cases) and showed less advanced ossification. Those from dams given 350 mg/kg/day of salicylic acid were 45% smaller while no foetus survived the equivalent dose of aspirin. However, the foetuses from the lower dose of aspirin (325 mg/kg/day) were 44% smaller, compared with a reduction of 34% seen with 250 mg/kg of salicylic acid.

At the lower dose levels, the foetuses in the aspirin group were 15% smaller at term than those from the equivalent salicylic acid one (a difference with no statistical significance). No direct comparison could be made at the higher level because there was no foetus in the aspirin group. The effects on foetal weight were accentuated by a high proportion of grossly malformed foetuses which typically were very small. These results show that aspirin retarded foetal growth to a greater extent than did salicylic acid.

A consideration of the percentage of surviving foetuses showing gross abnormalities confirms the enhanced embryopathic effects of aspirin over salicylic acid. In this section of the investigation, 94 foetuses were examined carefully and 18 of these showed serious malformations. Details of these are presented in Table 11.8, which also lists two other foetuses, both controls, which showed subcutaneous haemorrhages. As with the data relating to foetal weight, no direct comparison could be made at the high dose levels because no foetus survived the aspirin treatment; but, one of the four salicylic acid foetuses (25%) was malformed. However, at the lower levels, eight of the 15 foetuses in the aspirin group (53%) showed birth defects, but

this compares with only nine out of 32 (28%) in the equivalent salicylic acid group.

This study confirmed the previous findings that the acetyl group of the aspirin molecule is not essential for its embryopathic activity in rats. However, it also showed that this action is not entirely due to its salicylic acid metabolite. This suggested that the other primary hydrolytic metabolite of aspirin - i.e. acetic acid - may play some role in enhancing its effect. This hypothesis was tested in the other section of Experiment 11.F.

(e) The role of acetic acid in relation to the embryopathic effects of aspirin

(Table 11.7)

This study involved all the groups in the Experiment 11.F. Aspirin was compared with equivalent doses of salicylic acid and acetic acid and with a combination of these two metabolites. The dose levels of the mixture were estimated as a 2.30:1 by weight ratio of salicylic acid: acetic acid. If acetic acid is an important factor, the results obtained from its mixture with salicylic acid should parallel those obtained with aspirin alone. A direct comparison of aspirin and salicylic acid was made above and discussion of these data will not be repeated in this section.

All regimens except acetic acid alone retarded maternal weight gain during pregnancy. As discussed above, the effects produced by aspirin were more severe than those associated with salicylic acid. However, there was a close correlation between the effects of the salicylic acid/acetic acid mixture and those of salicylic acid alone. This was particularly so at the lower dose levels where both groups of rats gained a mean of 11g during the treatment period. Further, over the whole

gestation period, the rats given salicylic acid gained only 8g less than the mean of 111g gained by those given the mixture.

At the higher levels, the mixture appeared to be slightly more toxic than salicylic acid but considerably less so than aspirin. Thus, after 350 mg/kg/day of salicylic acid, rats gained a mean of 7g during dosing while those given the mixture at 502 mg/kg/day lost 1g. Over the whole gestation period, there was a mean 71g gain associated with salicylic acid treatment, but only a 60g gain when acetic acid was added. The lower maternal weights were associated with litters containing lower numbers of foetuses which were also smaller.

Comparison with the results obtained with aspirin shows that considerable maternal toxicity, indicated by weight change, was induced by salicylic acid. This was slightly enhanced at higher levels when acetic acid was administered with it. However, the effects of the mixture were less severe than those produced by aspirin alone. Acetic acid alone did not affect maternal weight gain.

The results of the experiment are presented in Table 11.8 and Fig. 11.1. The mean number of foetuses alive at term in each litter was reduced by aspirin and by salicylic acid, with or without acetic acid, but not by acetic acid alone. The effect of aspirin was more severe than that of salicylic acid - see above (d). However, the results from the salicylic acid/acetic acid mixture were remarkably similar to those from the salicylic acid alone. Neither preparation reduced the foetal number at the lower doses, but the high levels produced significant effects. The 502 mg/kg dose of the mixture reduced this value by 86% compared with the controls, while the equivalent salicylic acid dose (350 mg/kg) reduced it by 91% ($P<0.001$ in these two cases). Both regimens also significantly increased the incidence of intrauterine resorption above the controls,

where there was no case, as did the lower level of 250 mg/kg of salicylic acid alone ($P<0.001$ in all cases). This feature was not seen with the corresponding dose of the mixture (359 mg/kg/day).

In a comparison between the different test groups, the foetal number in the rats given aspirin at 325 mg/kg/day was reduced by 70% of the value in those given the mixture at 359 mg/kg/day ($P<0.002$). This difference was greater than that between the aspirin and salicylic acid alone where the level of significance was $P<0.01$. The difference between the controls and the salicylic acid groups, with or without acetic acid, was less marked than was the case with aspirin. The lower level of aspirin (325 mg/kg) reduced the foetal number by 65% and infinitely increased resorption ($P<0.001$ in both instances), and the higher dose of 455 mg/kg/day of aspirin killed every conceptus.

It was deduced from these results that aspirin alone was lethal to a greater percentage of conceptuses than equivalent doses of salicylic acid, irrespective of acetic acid being added. Indeed, the adverse effects of the salicylic acid/acetic acid mixture were slightly less severe than those induced by salicylic acid alone. Again, acetic acid alone produced no apparent effect.

The somewhat milder effect of the mixture compared with salicylic acid was also seen in the data from foetal weight determinations. At the lower dose of the mixture (359 mg/kg/day), the foetuses were only 23% smaller than the controls ($P<0.01$), compared with 34% with salicylic acid, 250 mg/kg/day and 44% with aspirin, 325 mg/kg/day ($P<0.001$ in the latter two cases). The difference between the weights in the aspirin and the mixture groups was also significant; the former foetuses were 28% smaller ($P<0.05$). Further, at the higher levels, the foetuses from dams given the mixture at 502 mg/kg/day were 30% smaller than the controls

($P<0.02$), whereas those from dams given salicylic acid at 350 mg/kg/day were 45% smaller ($P<0.001$); no foetus survived the equivalent dose of aspirin (455 mg/kg/day). The low foetal weights in the salicylate groups were associated with consistently smaller foetuses showing a low degree of ossification, together with a high proportion of malformed progeny which were almost invariably smaller.

As was the case with intrauterine survival of the conceptuses, foetal growth was also adversely affected by all salicylate preparations. The most profound effect was associated with aspirin which was followed, in decreasing order, by the salicylic acid alone and the mixture.

This pattern of acetic acid reducing the embryopathic effects of salicylic acid was also seen in the production of congenital abnormalities. The remaining 157 foetuses were examined in addition to the 94 mentioned above and, from these, 12 more malformed progeny were observed. Again, acetic acid appeared completely innocuous and produced no detectable effect. However, its influence on salicylic acid was apparent in that only 10 malformed foetuses were produced from the 50 (20%) from dams given the lower level of 359 mg/kg/day of the mixture. (This compared with 28% in the equivalent salicylic acid group and 53% in the aspirin one.) At the higher dose levels, relevant comparison was made extremely difficult by the very low number of foetuses alive at term. Here, only one out of four in the salicylic acid, 350 mg/kg group and two out of six in the mixture, 502 mg/kg group showed birth defects.

Again it was apparent that the embryopathic effects of salicylic acid were enhanced by the addition of the acetyl group to the molecule to form aspirin. Yet the effects were slightly reduced when equivalent amounts of acetic acid were administered together with salicylic acid.

Discussion

The initial experiments described in this chapter yielded conflicting results. Firstly, the failure of o-methoxybenzoic acid and benzoic acid to induce birth defects suggested that the acetyl group of aspirin played an integral part in its teratogenicity. That salicylic acid is teratogenic in the rats proves that this is not the case. o-Methoxybenzoic acid is a stable compound and its innocuous characteristics were probably due to its failure to convert to salicylic acid. Further, the replacement of the hydrogen ion of the hydroxyl group of salicylic acid by a methyl group to form o-methoxybenzoic acid diminishes the physiological activity (Dyson and May, 1911). It was possible to administer much higher doses of o-methoxybenzoic acid and benzoic acid than was the case with aspirin. The low degree of toxicity of benzoic acid may have been due to the lack of a hydroxyl group in the ortho position, which is its only structural difference from salicylic acid, as it is known that hydroxyl groups generally increase the physiological activity and toxicity of aromatic compounds (Dyson and May, 1911).

In the preceding chapter, it was found that phenol did not induce congenital malformations in the rats. The production of birth defects by salicylic acid proved the essential nature of the carboxyl group for such an effect because this group is the only structural difference between the two compounds. It is known that the entrance of a carboxyl group into an aromatic compound lowers its toxicity (Dyson and May, 1911). In the present case, salicylic acid was better tolerated by the rats than was phenol. It was evident from this and preceding studies that birth defects were induced only in the presence of salicylic acid.

The comparative study with equivalent doses of aspirin and salicylic acid demonstrated the enhanced toxicity of the former compound. This

may have been caused by the aspirin being considerably less bound to plasma protein than salicylate, as was shown in 1946 by Lester, Lolli and Greenberg. (The possible relationship between protein binding and teratogenicity was discussed in Chapter 7.) It also suggested that acetic acid, the other hydrolytic product of aspirin, may play some role in the teratogenesis. However, this was not the case as its addition to teratogenic doses of salicylic acid did not increase their embryopathic effects. Indeed, there was some evidence to suggest that it may have decreased these effects. The differences in the results between the salicylic acid/acetic acid mixture and salicylic acid alone were small and not significant statistically. Nevertheless, the embryopathic effects of the mixture were always less severe than those of salicylic acid.

It appears, therefore, that salicylic acid can induce all the embryopathic effects attributed to aspirin, but the activity of the discrete aspirin molecule is of a greater magnitude than that of equivalent doses of salicylic acid. This enhanced activity appears to be imparted by the acetyl group, providing that it is an integral part of the molecule. After its hydrolysis to acetic acid, which is a slow reaction, it appears to have little further effect.

Conclusions

(1) o-Methoxybenzoic acid and benzoic acid, which differ structurally from aspirin only in their lack of an acetyl group in the ortho position, did not induce birth defects in AH A rats. This suggested that the acetyl group of the aspirin molecule was necessary for its teratogenicity.

(2) However, the production of birth defects with salicylic acid in the rat refuted the initial suggestion and demonstrated that the acetyl

group was definitely not essential for the teratogenic activity of aspirin.

(3) In a comparative study, it was demonstrated that the acetyl group enhanced the embryopathic effects of aspirin over salicylic acid.

(4) Thus, the acetyl group of the aspirin molecule is effective in teratogenesis only before it becomes hydrolysed to acetic acid.

FIG. 11.1

COMPARISON OF THE INCIDENCE OF INTRAUTERINE DEATH AND BIRTH DEFECTS, AND OF THE MEAN FOETAL WEIGHT IN RATS TREATED WITH ASPIRIN, SALICYLIC ACID, ACETIC ACID AND A MIXTURE OF SALICYLIC ACID AND ACETIC ACID - WITH RANGE OF EXPERIMENTAL VARIATION (Experiment 11. F)

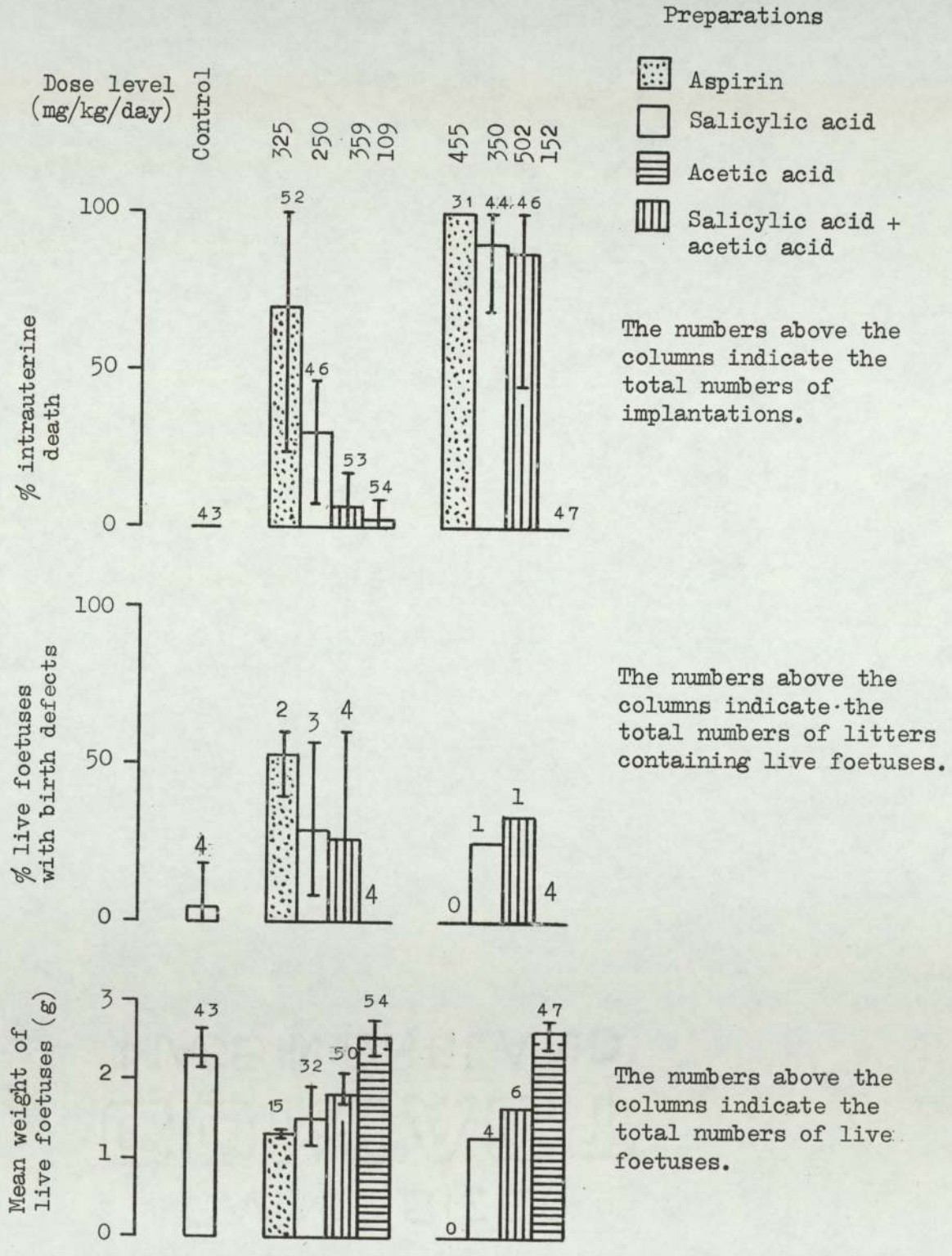


TABLE 11.1

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF O-METHOXYBENZOIC ACID

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
11.A	Control	8-12	3
	250	8-12	4
	500	8-12	4
	750	8-12	4
	1000	8-12	8
11.B	Control	8-12	3
	2000	8-12	8
	3000	8-12	4
	4000	8-12	4
	5000	8-12	4

TABLE 11.2

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF O-METHOXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
11.A	Control	3	39	0	13.0	2.58	1	0.33	0.001	0	0	0
	250	4	47	0	11.75	2.63	4	1.0	0.028	0	0	2[1]
	500	1	14	0	14.0	2.33	0	0	0	0	0	1[1]
	750	4	48	0	12.0	2.42	4	1.0	0.012	0	0	1[1]
	1000	7	67	0	9.57	2.10*	8	1.13	0.147	0	0	4[1]
11.B	Control	3	40	0	13.33	2.65	3	1.0	0.176	0	0	0
	2000	8	75	0	9.38	1.98*	23	2.88	0.167	0	0	6[1]
	3000	3	13	0	4.33*	1.76*	29	9.67	0.159	0	0	4[1]+1[2]
	4000	3	25	0	8.33	1.92*	7	2.33	0.159	0	0	1[1]+1[3]
	5000	1	11	0	11.0	1.99*	0	0	0	0	0	0

* Statistically significantly different from the controls.

1 Subcutaneous haemorrhages.

2 Fused placentae.

3 Microcaudia, subcutaneous haemorrhage.

TABLE 11.3

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF BENZOIC ACID

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
11.C	Control	8-12	3
	250	8-12	5
	500	8-12	5
	750	8-12	4
11.D	Control	8-12	4
	1000	8-12	5
	1250	8-12	4

TABLE 11.4

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF BENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
11.C	Control	3	43	0	14.33	3.80	1	0.33	0.330	0	0	0
	250	5	68	0	13.60	3.72	3	0.60	0.127	0	0	1[1]
	500	5	76	0	15.20	3.56	2	0.40	0.022	0	0	0
	750	4	48	0	12.0	3.86	4	1.0	0.228	0	0	0
11.D	Control	4	45	0	11.25	2.32	6	1.50	0.054	0	0	0
	1000	5	25	0	5.0	2.20	46	9.20	0.259	0	0	0
	1250	4	10	0	2.50*	1.84*	42	10.50*	0.194	1	0.25	0

* Statistically significantly different from the controls.

1 Microcaudia.

TABLE 11.5

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF SALICYLIC ACID

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
11.E	Control	8-12	6
	250	8-12	6
	275	8-12	6
	300	8-12	6

TABLE 11.6

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF SALICYLIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
11.E	Control	4	34	0	8.50	2.09	8	2.0	0.026	2	0.50	0
	250	6	52	2	8.67	1.55*	1	0.17	0.120	0	0	$5^{1}+5^{2}$
	275	5	53	0	10.60	2.07	16	3.20	0.038	0	0	4^{2}
	300	6	46	0	7.67	1.64*	29	4.83	0.092	0	0	$1^{2}+1^{3}$

* Statistically significantly different from the controls.

1 Subcutaneous haemorrhages.

2 Scoliosis, hemivertebrae, fused ribs, sternal anomalies.

3 Craniorrhachischisis, scoliosis, kyphosis, hemivertebrae, fused ribs, sternal anomalies, exophthalmia.

TABLE 11.7

PROTOCOL FOR THE INVESTIGATIONS INTO THE COMPARATIVE TERATOGENICITY OF ASPIRIN AND SALICYLIC ACID, AND INTO THE POSSIBLE EFFECT OF ACETIC ACID IN ASPIRIN TERATOGENICITY

Experiment code	Compound	Dose level (mg/kg/day)	Days of administration	No. of rats mated
11.F		Control	8-12	4
	Aspirin	325	8-12	4
		455	8-12	4
	Salicylic acid + acetic acid	359	8-12	4
		502	8-12	4
	Salicylic acid	250	8-12	4
		350	8-12	4
	Acetic acid	109	8-12	4
		152	8-12	4

TABLE 11.8

RESULTS OF THE INVESTIGATIONS INTO THE COMPARATIVE TERATOGENICITY OF ASPIRIN AND SALICYLIC ACID, AND INTO THE POSSIBLE EFFECT OF ACETIC ACID ON ASPIRIN TERATOGENESIS

Experiment code	Compound	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
				Alive	Dead								
11.F		Control	4	43	0	10.75	2.36	0	0	0	0	0	[1]
	Aspirin	325	4	15	0	3.75*	1.31*	37	9.25*	0.157	0	0	[2]+[3]+[4]+[5]+[6]+[7]+[8]+[9]
		455	2	0	0	0*	0*	31	15.50*	0.136	0	0	0
	Salicylic acid	250	3	32	1	10.67	1.55*	13	4.33*	0.158	0	0	[3]+[4]+[6]+[10]+[11]+[12]+[13]+[14]+[15]
		350	4	4	0	1.0*	1.29*	40	10.0*	0.198	0	0	[11]
	Salicylic acid + acetic acid	359	4	50	0	12.50	1.81*	3	0.75	0.213	0	0	[1]+[10]+[11]+[16]+[17]+[18]+[19]+[20]+[21]+[22]+[23]
		502	4	6	0	1.50*	1.66*	40	10.0*	0.154	0	0	[10]+[24]
	Acetic acid	109	4	54	0	13.50	2.57	1	0.25	0.020	0	0	0
		152	4	47	0	11.75	2.61	0	0	0	0	0	0

* Statistically significantly different from the controls.

[1] Subcutaneous haemorrhages.

[2] Cheiloschisis, subcutaneous haemorrhages.

[3] Palatoschisis, cheiloschisis.

[4] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, arthrogryposis, curly tail, exophthalmia, fused ribs, sternal anomalies, hemivertebrae.

[5] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, arthrogryposis, exophthalmia, fused ribs, sternal anomalies, hemivertebrae.

[6] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, arthrogryposis, fused ribs, sternal anomalies, hemivertebrae.

[7] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, exophthalmia, fused ribs, sternal anomalies, hemivertebrae.

[8] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, ablepharia, fused ribs, sternal anomalies, hemivertebrae.

[9] Craniorrhachischisis, scoliosis, kyphosis, arthrogryposis, exophthalmia, fused ribs, sternal anomalies, hemivertebrae.

[10] Craniorrhachischisis, scoliosis, kyphosis, fused ribs, sternal anomalies, hemivertebrae.

[11] Exencephalia, macroglossia.

[12] Dead foetus with craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, arthrogryposis, exophthalmia, ablepharia, fused ribs, sternal anomalies, hemivertebrae.

[13] Craniorrhachischisis, scoliosis, kyphosis, omphalocele, fused ribs, sternal anomalies, hemivertebrae.

[14] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, arthrogryposis, curly tail, fused ribs, sternal anomalies, hemivertebrae.

[15] Craniorrhachischisis, scoliosis, kyphosis, arthrogryposis, omphalocele, curly tail, fused ribs, sternal anomalies, hemivertebrae.

[16] Exencephalia.

[17] Arthrogryposis.

[18] Craniorrhachischisis, scoliosis, kyphosis, exophthalmia, ablepharia, fused ribs, sternal anomalies, hemivertebrae.

[19] Craniorrhachischisis, scoliosis, kyphosis, exophthalmia, ablepharia, curly tail, macroglossia, fused ribs, sternal anomalies, hemivertebrae.

[20] Craniorrhachischisis, scoliosis, kyphosis, macroglossia, fused ribs, sternal anomalies, hemivertebrae.

[21] Craniorrhachischisis, scoliosis, kyphosis, eventration of the abdominal viscera, fused ribs, sternal anomalies, hemivertebrae.

[22] Eventration of the abdominal viscera.

[23] Generalised subcutaneous oedema.

[24] Myeloschisis.

CHAPTER 12

THE POSITIONS OF THE HYDROXYL GROUPS IN RELATION TO SALICYLATE TERATOGENICITY

Introduction

Previous investigations showed that salicylic acid is teratogenic in the rat but benzoic acid is not. Since the only structural difference between these two compounds is the presence of a hydroxyl group in the ortho position in salicylic acid, it was apparent that this group is essential for the production of birth defects. Because of this, experiments were undertaken to investigate the importance of hydroxyl groups in relation to salicylate teratogenesis.

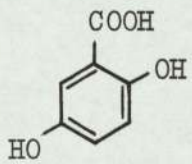
The monohydroxy (or monophenolic) salicylic acid has two therapeutically inert isomers which have their hydroxyl group in either the meta or para position. Unlike its isomers, salicylic acid forms a chelate ring with its hydroxyl group sharing its hydrogen ion with the adjacent carbonyl of the carboxyl group. This formation of a chelate ring is associated with the anti-rheumatic property of salicylic acid. That its isomers do not form such a ring explains their inactivity as anti-inflammatory agents (Clarke, Clarke and Mosher, 1958). Larsson and Boström (1965) found that intramuscular injection of approximately 500 mg/kg of parahydroxybenzoic acid did not induce birth defects in mice. Further, my own investigations, not presented in this thesis, have shown that oral administration of up to 5000 mg/kg/day of para- or meta- hydroxybenzoic acid appeared totally innocuous in AH A rats. Consequently, neither compound formed a part of this investigation. However, the presence of hydroxyl groups in the 2 or 6 position

permits the formation of a chelate ring. Because of this, a series of commercially available dihydroxybenzoic acids was studied for embryopathic activity in rats. The trihydroxybenzoic acids were not included because the most active of these (2,3,6-trihydroxybenzoic acid) is not available commercially.

Experimental Work

The investigations were carried out with 2,5-dihydroxybenzoic acid and its isomers 2,3-,2,4-,2,6- and 3,4-dihydroxybenzoic acid, all of which will be considered individually. The standard techniques described in Chapter 5 were employed throughout.

(a) 2,5-Dihydroxybenzoic acid



Gentisic acid or 2,5-dihydroxybenzoic acid is a metabolite of salicylic acid formed by its oxidation in the body. Approximately 1% can be recovered from the urine of man after aspirin ingestion (Milne, 1963). Its chemical structure is similar to that of salicylic acid but it has a second hydroxyl group in the 5 position. It is reported to have anti-rheumatic activity comparable with that of salicylic acid and is less toxic (Clarke et al, 1958). It was originally thought to be the active metabolite of salicylic acid but it is ineffective in doses equivalent to the amount of metabolite produced.

The investigations in pregnant rats involved two experiments and the protocol for these is given in Table 12.1. This compound was

remarkably non-toxic in the rat, and levels of up to 3000 mg/kg/day were administered without any noticeable effect. All test dams gained weight to the same extent as the controls, both during and after the treatment period. Four rats did not become pregnant.

The results of the investigations are presented in Table 12.2. The rats given 1000 mg/kg/day had 28% fewer live foetuses in each litter than the controls. This difference was found to be significant with the 'Student's' t test ($P<0.05$), but was not so with the Mann-Whitney U test. It was considered that this was not a drug-effect since the relevant group showed no associated increase in the incidence of intrauterine death, and rats treated with doses two or three times greater had normal litter numbers.

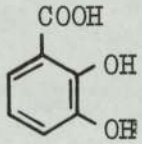
There was no effect on foetal weight or on the production of anomalies. Subcutaneous haemorrhages occurred in all test groups and in one of the controls. These lesions are known to occur spontaneously in AH A rats and there was no increase in the incidence in the treated animals. The case of microcaudia in a control foetus was obviously not related to the drug.

The one structural difference between salicylic acid and gentisic acid is extremely important. The additional hydroxyl group in the 5 position negates the toxic and embryopathic effects of salicylic acid in rats. This failure to produce any toxicity may indicate that the drug is not readily absorbed orally in these rats.

The experiments with gentisic acid were not persued beyond this point. As doses up to 3000 mg/kg had produced no detectable effect, it was considered impractical to increase the level. Attention was

then directed towards the isomers of gentisic acid.

(b) 2,3-Dihydroxybenzoic acid



The first isomer of gentisic acid to be studied was also a metabolite of salicylate, namely 2,3-dihydroxybenzoic acid (pyrocatechuic acid). It is very effective in the treatment of rheumatic fever and its therapeutic dose is only one half of that used for salicylic acid. The enhanced anti-rheumatic properties have been associated with the presence of a second hydroxyl group in the 3 position.

The study using 2,3-dihydroxybenzoic acid involved two experiments, and the protocol for these is given in Table 12.3. In view of the reported low toxicity in man (Clarke et al, 1958), high doses were used initially. However, all levels showed some adverse effects on the rats and doses of 1500 mg/kg and above were too toxic for the treatment schedule to be completed. The affected animals became lethargic, anorexic and had staring coats and an excessive oronasal discharge. As the level immediately below this (1250 mg/kg/day) was tolerated by the dams and produced no embryopathic effect, a second experiment involving graded doses between these limits was undertaken.

This compound killed 14 rats but no obvious pathological change was found. Four of these received only 2 doses of 2000 mg/kg before they died, and the two lower levels of 1750 and 1500 mg/kg each killed two of the four dams after three doses, whereafter treatment was discontinued. Although all doses of the drug in Experiment 12.D each killed two of the four dams, four of these rats died after the 12th

day of gestation, so the treatment schedule was completed. Three does were not mated successfully.

All dose levels of 2,3-dihydroxybenzoic acid adversely affected maternal weight change during treatment. In Experiment 12.C, the controls gained a mean of 14g while the rats in the 1000 mg/kg group gained 4g and there was no change in the 1250 mg/kg animals. Those given 1500 mg/kg and 1750 mg/kg lost 2g and 5g respectively during the three days of dosing while the controls gained 7g during the same period. In the follow-up study (Experiment 12.D), the controls gained a mean of 19g while the survivors at 1300 mg/kg gained 9g, the one at 1400 mg/kg gained 8g, and there was no weight change in those given 1450 mg/kg. After the treatment was withdrawn, all groups of rats recovered and, by term, had grown to the same extent as the controls.

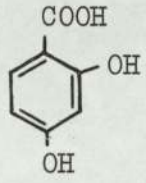
In summarising the effects of pyrocatechuic acid in pregnant rats, the toxicity of these high doses was apparent in that all levels of 1300 mg/kg and above killed some of the animals and retarded the weight gain of the survivors during treatment. The lower doses also affected maternal weight gain but were not lethal. All surviving rats recovered when the treatment was discontinued and proceeded to gain weight normally.

The results of the investigation are presented in Table 12.4. There was no effect upon the survival of the conceptus or on its growth rate. Further, the abnormalities observed were considered to be not drug-induced. Subcutaneous haemorrhages are indigenous lesions in AH A rats and their incidence was not enhanced by the drug. Further, it was apparent that the one case of microcaudia was not associated with the treatment as there were 121 normal foetuses from dams given

a similar or higher dose level.

This investigation involved a compound which, at high dose levels, was toxic to a proportion of the pregnant rats but left the progeny of the surviving dams unaffected. Hence, an additional hydroxyl group in the 3 position of the salicylic acid molecule prevented its teratogenic effect in rats.

(c) 2,4-Dihydroxybenzoic acid



2,4-Dihydroxybenzoic acid or β-resorcylic acid is a gentisic acid isomer which has low toxicity. Two experiments on its possible embryopathic effects were undertaken and the protocol for these is given in Table 12.5. The highest dose level of 2000 mg/kg/day caused lethargy and resulted in the deaths of two of the four rats two days after the dosing schedule was completed. These were the only two intercurrent deaths and, again, there was no obvious pathological change. Although the surviving does had normal progeny, it was decided to carry out a second experiment using doses between this toxic level and 1500 mg/kg/day where all the dams had survived. Three rats did not become pregnant.

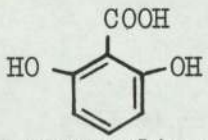
Only the rats given the two highest dose levels showed any effect on maternal weight during the treatment period. The dams given 2000 mg/kg/day lost a mean of 3g compared with an 18g gain by their controls, and those given 1900 mg/kg/day gained 1g while their controls gained 22g. All other groups gained weight to the same extent as their

respective controls. When the two toxic regimens were withdrawn, the dams recovered and, by term, had grown to a size comparable with the controls.

The results of these experiments are presented in Table 12.6. Again, there was no effect upon the number of foetuses in each litter or their weight. Further, although subcutaneous haemorrhages and one case of microcaudia were observed, they were not related to the test compound.

High doses of 2,4-dihydroxybenzoic acid were toxic to the pregnant rats but had no adverse effect on the conceptuses. Hence, a second hydroxyl group in the 4 position of the salicylic acid molecule prevents the teratogenic effect in rats.

(d) 2,6-Dihydroxybenzoic acid



The potential of ϒ-resorcylic acid (2,6-dihydroxybenzoic acid) to form a chelate ring from either hydroxyl group enhances its anti-rheumatic activity (Clarke et al, 1958). This gentisic acid isomer is reported to be about 10 times more potent in the treatment of acute rheumatic fever than either salicylic acid or gentisic acid but is not used therapeutically because it is correspondingly more toxic (Sarett, 1965).

Two experiments were undertaken and the protocol for these is given in Table 12.7. The rats could not tolerate the high dose of 1000 mg/kg; they became lethargic, anorexic and had staring coats and a nasal discharge. Because of this, treatment was discontinued after

two doses, by which time the dams had lost a mean of 33g while the controls had gained 6g. As the rats could tolerate 750 mg/kg/day, the second experiment (12.H) was designed to examine any effect produced between this dose and 1000 mg/kg/day.

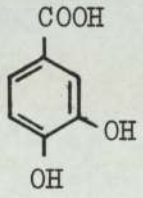
All treatments affected maternal weight gain during dosing. In Experiment 12.G, while the controls gained a mean of 15g, the dams given 500 mg/kg/day lost 2g, and those given 750 mg/kg/day lost 11g. In the second experiment (12.H), the controls gained 22g per rat while those given 800 mg/kg/day gained 2g, and those given 900 mg/kg/day showed no weight change. The discontinuation of treatment was followed by a rapid improvement in the dams' condition and, by term, their weight gain was comparable with that of the controls. Although there was no clear dose-related response, all levels of γ-resorcylic acid prevented the pregnant rats from gaining weight normally, but no intercurrent death occurred. Four rats were not mated successfully.

The results of the investigations are presented in Table 12.8, which shows that the test compound induced no embryopathic effect; there was no effect on the number of live foetuses in each litter or their weights. The mean foetal weight in the 900 mg/kg group was 6% lower than the control value but the difference is not significant. There was a high incidence of subcutaneous haemorrhages in the test and control groups of Experiment 12.G; hence there was no specific drug-effect. The anomalies in the second experiment (12.H) were restricted to the control group.

2,6-Dihydroxybenzoic acid induced considerable maternal toxicity during the treatment period but evoked no apparent effect upon the

conceptus. Hence, the addition of a second hydroxyl group in the 6 position of the salicylic acid molecule prevents the teratogenicity in rats.

(e) 3,4-Dihydroxybenzoic acid



The absence of a hydroxyl group in the 2 position means that 3,4-dihydroxybenzoic acid (protocatechuic acid) is chemically less similar to salicylic acid than are the other isomers of gentisic acid. Furthermore, no hydroxyl group is present in the 6 position so that a chelate ring cannot be formed. However, if its hydroxyl groups are in the ortho position, it can become oxidised into a quinone. Consequently, its efficacy in treating rheumatic fever is limited and it is reported to have appreciable toxicity in man (Clarke et al, 1958). It has been included in these investigations for completeness and because it is another isomer of gentisic acid.

Three experiments were undertaken in this study and the protocol for these is given in Table 12.9. Because of the alleged toxicity, comparatively low doses were administered initially but these were superseded by higher levels as its low toxicity in rats became apparent. One control rat did not become pregnant.

There was a possible effect on maternal weight gain during dosing in Experiment 12.I. The controls gained a mean of 18g while the 250 mg/kg group gained 17g. There was obviously no effect here but the

weight gain in the 500 mg/kg group was only 14g per rat and this may represent a mild effect of the regimen. This was suspected because, in the second experiment (12.J), the compound retarded maternal weight gain at both levels; the dams given 1000 mg/kg/day gained a mean of 18g, and those given 2000 mg/kg/day gained 11g compared with a 26g gain by the controls. This effect was more evident in the final experiment (12.K) where the controls gained 22g per rat while the 2500 mg/kg group lost 1g, and the 3000 mg/kg group showed no weight change. Further, the test dams in this experiment showed obvious toxic symptoms during dosing; they became lethargic, anorexic and had staring coats and an oronasal discharge. Although no dam died during the course of the experiment, it was considered unreasonable to increase the dose further. The discontinuation of the treatment was accompanied by a remarkable recovery in the condition of the rats. They increased weight quickly and, by term, had grown to the same extent as the controls.

In summary, it is difficult to determine the lowest dose which affected maternal weight gain. There was definite evidence at doses of 1000 mg/kg and above and there may have been a mild effect at the 500 mg/kg level.

The results of this study are presented in Table 12.10. There was no effect upon the mean number of live foetuses per litter. In the 1000 mg/kg group this value was 33% lower than the controls but was not accompanied by an increase in resorption or a lower maternal weight at term. It was significant ($P<0.05$) according to the 'Student's' *t* test, but was not so according to the Mann-Whitney *U* test. The conclusions from the latter statistical test seem the more reliable

because up to three times this dose level had no effect upon litter number.

The same experiment (12.J) also yielded test foetuses which were significantly smaller than the controls; those in the 1000 mg/kg group were 12% smaller, and those in the 2000 mg/kg group were 13% smaller ($P<0.002$ in both cases). It is unlikely that the small size of these foetuses was drug-induced because the much higher doses given in Experiment 12.K produced no such effect. It appears, therefore, that protocatechuic acid did not affect foetal growth.

The most interesting feature of this investigation was the case of exencephalia observed in one of the 48 foetuses from dams given 2000 mg/kg/day. This is a malformation described consistently in association with salicylate teratogenesis and its appearance in the highest dose group of that experiment suggested that it may have been a drug-effect. In that event, the dose would have been in the region of the lowest teratogenic level. Unfortunately, the much higher doses produced no malformation and it seems likely, therefore, that the exencephalia was not induced by the drug. Of the other anomalies recorded, fused placentae are indigenous in the rats, as are the subcutaneous haemorrhages which appeared in test and control groups. Further, the oedematous foetus occurred in a control litter. Therefore, there is no indication that any abnormality described here was drug-induced.

Large doses of up to 3000 mg/kg/day of 3,4-dihydroxybenzoic acid had induced considerable maternal toxicity but evoked no embryopathic effect.

Discussion

In view of the report published by Clarke et al (1958) concerning the enhanced therapeutic activity of 2,3- and 2,5-dihydroxybenzoic acid over salicylic acid, it appears surprising that these compounds are not used more frequently in the treatment of certain rheumatic diseases. An extensive search of the literature and a number of consultations has yielded no categorical explanation for this. However, the views expressed were typified by Deakin (1971) who considered that, subsequently, these drugs may have proven less effective than initially seemed to be the case. Furthermore, the introduction of cortisone at about this time may have detracted from the interest shown in them.

The investigations described in this chapter were concerned with the possible embryopathic effects of gentisic acid and its isomers, and were undertaken to assess the importance of the hydroxyl group in respect of number and position. With the exception of 3,4-dihydroxybenzoic acid, all compounds were similar to salicylic acid in having a hydroxyl group in the 2 position, but each had a second hydroxyl group at another point on the benzene ring. Hence, these could form a chelate ring, a feature associated with their anti-rheumatic activity (Clarke et al, 1958). Protocatechuic acid is refractory in not having a hydroxyl group in a position in which it might form a chelate ring.

Doses of gentisic acid up to 3000 mg/kg/day were given without any toxic symptom being observed. Consequently, investigations with this compound were not persued. The isomers studied induced toxic changes in the female rats and the maximum tolerated doses were administered. These doses were extremely large but none of them

affected the conceptus in any way. The dams could tolerate less 2,6-dihydroxybenzoic acid than any of the other compounds; this was followed in descending order of toxicity by 2,3-, 2,4-, 3,4- and 2,5-dihydroxybenzoic acid. It is not possible to correlate this with the levels of salicylic acid inducing comparable maternal toxicity because doses in excess of those inducing congenital malformations were not used.

Neither gentisic acid nor any one of its isomers had any embryopathic effect in a total of 1261 test foetuses examined in detail. Consequently, the addition of a second hydroxyl group to the 3,4,5 or 6 position of the salicylic acid molecule prevented its teratogenic activity in rats, as did the replacement of the ortho hydroxyl group with ones in the meta and para positions. These findings demonstrated that the single hydroxyl group in the 2 position was essential for the teratogenesis of salicylic acid. There was no teratogenicity in the absence of this group (benzoic acid) or with the addition of a second hydroxyl group.

There appears to be no relation between the ability to form a chelate ring system and teratogenesis. This was proven because all the compounds with a hydroxyl group in the 2 or 6 position could form a chelate ring but, of these, only salicylic acid induced birth defects.

Conclusions

(1) Gentisic acid in doses up to 3000 mg/kg/day induced no toxic effect in the rats.

(2) Isomers of gentisic acid induced maternal toxicity but showed no embryopathic activity at maximum tolerated levels.

(3) The discrete salicylic acid molecule with a single phenolic hydroxyl group is the structure teratogenic in the rat.

(4) The addition of a second hydroxyl group on to any point of the benzene ring of the salicylic acid molecule negated the teratogenesis.

(5) There was no relation between teratogenesis and the formation of a chelate ring.

TABLE 12.1

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,5-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
12.A	Control	8-12	4
	250	8-12	4
	500	8-12	4
	750	8-12	4
	1000	8-12	4
12.B	Control	8-12	4
	2000	8-12	4
	3000	8-12	4

TABLE 12.2

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,5-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
12.A	Control	3	39	0	13.0	2.44	7	2.33	0.249	0	0	0
	250	4	61	0	15.25	2.35	3	0.75	0.277	0	0	3[1]
	500	3	43	0	14.33	2.39	1	0.33	0.390	0	0	3[1]
	750	4	52	0	13.0	2.42	1	0.25	0.018	0	0	1[1]
	1000	3	28	0	9.33*	2.25	1	0.33	0.019	0	0	2[1]
12.B	Control	4	52	0	13.0	2.33	0	0	0	0	0	1[1]+1[2]
	2000	4	44	0	11.0	2.47	1	0.25	0.042	0	0	1[1]
	3000	3	41	0	13.67	2.55	1	0.33	0.009	0	0	1[1]

* Statistically significantly different from the controls.

[1] Subcutaneous haemorrhages.

[2] Microcaudia.

TABLE 12.3

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,3-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
12.C	Control	8-12	4
	1000	8-12	4
	1250	8-12	4
	1500	8-10*	4
	1750	8-10*	4
	2000	8-9*	4
12.D	Control	8-12	3
	1300	8-12	4
	1400	8-12	4
	1450	8-12	4

* It was intended to treat these rats from days 8-12 but the dose levels were too toxic for the regimen to be completed.

TABLE 12.4

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,3-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
12.C	Control	4	49	0	12.25	2.29	6	1.50	0.063	0	0	0
	1000	3	35	0	11.67	2.23	6	2.0	0.125	0	0	0
	1250	3	50	0	16.67	2.37	9	0	0	0	0	1[1]+1[2]
	1500	2	20	0	10.0	2.25	7	3.50	0.265	0	0	0
	1750	2	21	0	10.50	2.30	0	0	0	0	0	1[1]
	2000	0										
12.D	Control	3	40	0	13.33	2.33	0	0	0	0	0	0
	1300	2	30	1	15.0	2.22	0	0	0	0	0	0
	1400	1	13	0	13.0	2.26	0	0	0	0	0	0
	1450	2	29	0	14.50	2.11	0	0	0	0	0	0

1 Subcutaneous haemorrhages.

2 Microcaudia.

TABLE 12.5

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,4-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
12.E	Control	8-12	4
	1000	8-12	4
	1500	8-12	4
	2000	8-12	4
12.F	Control	8-12	4
	1600	8-12	4
	1700	8-12	4
	1800	8-12	4
	1900	8-12	4

TABLE 12.6

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,4-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
12.E	Control	3	42	0	14.0	2.33	2	0.67	0.123	0	0	0
	1000	4	61	0	15.25	2.40	1	0.25	0.033	1	0.25	0
	1500	2	26	0	13.0	2.24	1	0.50	0.480	0	0	3[1]
	2000	2	31	0	15.50	2.37	3	1.50	0.029	0	0	0
12.F	Control	4	52	0	13.0	2.33	0	0	0	0	0	1[1]+1[2]
	1600	4	39	0	9.75	2.52	0	0	0	0	0	0
	1700	4	47	0	11.75	2.40	2	0.50	0.020	0	0	0
	1800	4	49	0	12.25	2.36	3	0.75	0.179	0	0	0
	1900	4	46	0	11.50	2.40	5	1.25	0.170	0	0	1[1]

[1] Subcutaneous haemorrhages.

[2] Microcaudia.

TABLE 12.7

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,6-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
12.G	Control	8-12	4
	500	8-12	4
	750	8-12	4
	1000	8-9*	4
12.H	Control	8-12	4
	800	8-12	4
	900	8-12	4

* It was intended to treat these rats from days 8-12 but the dose level was too toxic for the regimen to be completed.

TABLE 12.8

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF 2,6-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
12.G	Control	4	40	0	10.0	2.15	8	2.0	0.254	0	0	5[1]
	500	3	38	0	12.67	2.41	1	0.33	0.20	0	0	2[1]
	750	4	48	1	12.0	2.01	5	1.25	0.096	0	0	4[1]
	1000	3	38	0	12.67	2.28	4	1.33	0.027	0	0	1[1]
12.H	Control	4	52	0	13.0	2.33	0	0	0	0	0	1[1]+1[2]
	800	4	46	0	11.50	2.33	2	0.50	0.086	0	0	0
	900	2	25	0	12.50	2.19	0	0	0	0	0	0

[1] Subcutaneous haemorrhages.

[2] Microcaudia.

TABLE 12.9

PROTOCOL FOR THE INVESTIGATIONS INTO THE TERATOGENICITY OF 3,4-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	Days of administration	No. of rats mated
12.I	Control	8-12	4
	250	8-12	4
	500	8-12	4
12.J	Control	8-12	4
	1000	8-12	4
	2000	8-12	4
12.K	Control	8-12	2
	2500	8-12	4
	3000	8-12	4

TABLE 12.10

RESULTS OF THE INVESTIGATIONS INTO THE TERATOGENICITY OF 3,4-DIHYDROXYBENZOIC ACID IN RATS

Experiment code	Dose level (mg/kg/day)	No. of rats	No. of foetuses		Mean No. of live foetuses per litter	Mean weight of live foetuses (g)	No. of resorption sites	Mean No. of resorption sites per litter	Mean weight of resorption sites (g)	No. of resorption scars	Mean No. of resorption scars per litter	No. of abnormalities
			Alive	Dead								
12.I	Control	3	42	0	14.0	2.33	2	0.67	0.123	0	0	0
	250	4	47	0	11.75	2.53	4	1.0	0.012	0	0	0
	500	4	59	0	14.75	2.55	3	0.75	0.236	0	0	0
12.J	Control	4	61	0	15.25	2.46	5	1.25	0.043	0	0	1[1]
	1000	4	41	0	10.25*	2.16*	10	2.50	0.124	0	0	0
	2000	4	48	0	12.0	2.14*	5	1.25	0.058	0	0	1[2]+1[3]
12.K	Control	2	26	0	13.0	2.28	4	2.0	0.156	0	0	1[4]
	2500	4	57	1	14.25	2.11	4	1.0	0.079	0	0	3[4]
	3000	4	48	0	12.0	2.23	6	1.50	0.162	0	0	3[4]

* Statistically significantly different from the controls.

1 Generalised subcutaneous oedema.

2 Exencephalia.

3 Fused placentae.

4 Subcutaneous haemorrhages.

CHAPTER 13

PATHOLOGY OF SOME CONGENITAL MALFORMATIONS IN RATS

Introduction

In attempting to understand the aetiology of certain congenital defects, consideration must be given to their pathology. This chapter is concerned with both macroscopic and microscopic details of the malformations observed during the conduct of this thesis. The histological and staining techniques used are described in Chapter 5. All affected foetuses studied had the same gestational age - i.e. 20 days post coitum.

This thesis has involved the detailed examination of 1534 foetuses whose dams were treated with potentially teratogenic regimens of salicylates, and 176 of these (11.5%) showed anomalies. It is a feature of many teratogens that only a percentage of the embryos exposed become malformed; possible reasons for this are considered in the General Discussion (Chapter 14). In all, 40 different defects were observed in the present study. Many of the affected foetuses had more than one defect, and the frequency with which each anomaly occurred is presented in Table 13.1.

Histopathological Report and Discussion

All macroscopic photographs may be compared with the control presented in Fig. 13.1 but photomicrographs of controls are included with each series of sections. Most of the abnormalities will be considered individually but, where a particular primary defect has resulted in more than one malformation, these malformations will be discussed together.

(a) Neural tube defects

Failure of the neural tube to close gives rise to three basic congenital malformations, all of which were induced by salicylates. If the defect is restricted to the spinal region, spina bifida results; but if the brain is the only region affected, exencephalia is the malformation. A combination of these two conditions is craniorrhachischisis which involves the whole of the neural tube (Fig.13.2).

The normal central nervous system develops from the neural plate which is originally flat and single-layered. It thickens and becomes depressed axially to form a neural groove. This groove deepens as the folds of neural plate become elevated and meet and fuse above it to give rise to a neural tube. The region in which fusion occurs initially is the thorax, from which it progresses both rostrally and caudally. Hence, defects arising from a non-closure of the neural tube may reflect the stage of development at which the teratogen was effective. A prolonged effect starting at the time of normal closure may cause complete craniorrhachischisis, but one starting later may cause exencephalia and/or spina bifida. There are many possible interstages which are determined by the precise time in development at which the teratogen becomes effective and the duration of its action.

The experimental production of congenital malformations which arise from a defect in neural tube closure was reviewed by Kalter (1968). A wide array of teratogens inducing such damage includes vitamin deficiency or excess, alkylating agents, alkaloids, antibiotics, antimetabolites, hypoglycaemics, steroids, anticholesterolaemics, antihistamines, thalidomide, trypan blue, alloxan, urethan, hydroxyurea, industrial solvents, detergents, chemosterilants, x-rays, viruses, and deprivation of food, oxygen or heat. These defects also occur spontaneously in many

species, notably large animals.

The pathogenesis of this group of abnormalities was originally thought to be a secondary opening of the closed neural tube. This theory was proposed by von Recklinghausen (1886) who attributed the opening to stresses involved in flexure. Until quite recently, some authors supported this explanation (e.g. Gruenwald, 1947a, 1974b). However, as long ago as 1929, Sternberg had suggested that these malformations could arise from a primary failure of the neural tube to close. This is now generally accepted as being the aetiology of these abnormalities and they are initiated at the blastula or gastrula stage of development. The changes commence in the mesodermal cells in the cephalic region in which a shrinkage of the cytoplasm and an accumulation of fluid causes an enlargement of the intercellular spaces. This is accompanied by a reduction of the mitotic rate, and leads to cellular necrosis. The necrotic area later spreads caudally and adversely affects the notochord (Marin-Padilla, 1965a, 1965b, 1966). This would interfere with the inductive properties of the mesoderm upon the ectoderm, and the subsequent induction of skeletal development by the ectoderm.

Spina bifida - the most severe form of which is myeloschisis (Fig. 13.2A) - was first produced with salicylates by Goldman and Yakovac (1964a). The failure of the neural tube to close and separate from the ectoderm prevents the mesoderm, which should form the neural arch, from meeting and closing over the developing cord. The fissure, which is typically median and posterior, is generally restricted to the lumbar region and involves a variable number of vertebrae. The classification upon which the present cases were based was that presented by Cameron (1956). This involves four classes of spina bifida which, in ascending order of severity, are termed spina bifida occulta, meningocele,

meningomyelocele and myelocele (or, more correctly, myeloschisis). Spina bifida occulta involves a failure in the closure in the neural arch elements but the spinal cord remains in its canal; in meningocele the meninges protrude through the gap in the vertebral arches but the cord still remains in the canal; in meningomyelocele both the meninges and the cord protrude through the gap; and in myeloschisis the neural plate remains unclosed. This system was largely adopted by Warkany, Wilson and Geiger (1958) who listed the additional class of myelocystocele - which involves the cystic enlargement of the central canal.

The cases of myeloschisis described by Warkany et al (1958) differed from those induced by salicylates in AH A rats in only one characteristic. In the former, the spinal ganglia were fused in the midline, whereas this feature was not observed in the present study. However, all other aspects appeared similar and it was considered justifiable to classify the defect described in this thesis as myeloschisis. The lesions also showed a marked similarity to rachischisis as described by Hamilton, Boyd and Mossman (1945).

The present examples were in the lower lumbar and sacral regions (Fig. 13.3B) and were remarkably similar to the vertebral involvement in craniorrhachischisis (Fig. 13.3C). The neural plate typically had remained widely open along its length, a feature seen in some foetuses with craniorrhachischisis (Fig. 13.4B) but restricted to the thoracic and anterior lumbar regions of others. However, in the foetuses with myeloschisis and in the corresponding (lumbar) region of some of those with craniorrhachischisis, a condition similar to a false spinal canal was observed (Fig. 13.4C). These canals were lined with ependymal cells but were deemed 'false' because they showed little association with the possible partial closure of the neural tube. They were observed in

more than one region of the neural tissue and were rarely more than 100μ long. Their position typically arose dorsally and passed caudally to the ventral surface as a narrow canal. In other foetuses, the canals may have developed from the neural plate starting to roll over and fusing at a later time. Alternatively, they may have arisen because of the subsequent cellular proliferation and secondary fusion of neural tissue resulting from the very close proximity of these cells.

It is noteworthy that the longitudinal sections (Fig. 13.3) showed an enlarged subarachnoid space between the neural tissue and the centra. This region was occupied by a loose network of meningeal tissue which was probably pia arachnoid. This feature was not apparent in the transverse sections (Fig. 13.4). Warkany et al (1958) also mentioned its clarity particularly in sagittal sections, but Warkany and Takacs (1959) described it also from transverse sections. It appears, therefore, that an enlarged subarachnoid space is a feature associated with some, but not all, cases of myeloschisis and craniorrhachischisis.

The abnormal neural tissue leads to malformation of the associated skeletal structures. The chondrification centres of each half of the neural arch were present but their growth had been inhibited and the elements had become splayed out (Fig. 13.4).

One spinal characteristic in the present study was restricted to craniorrhachischisis. It involved the posterior sacral and caudal regions where the spinal cord was not exposed. The neural plate had rolled over to form a cord but had not fused completely. It had become overlayed with connective tissue and skin, but had not become enclosed by the neural arch (Fig. 13.4D). Warkany and Takacs (1959)

described a basically similar foetus but, in their example, the neural tube in this region was complete and the neural arch and associated soft tissues had developed normally. This difference appears to reflect the duration of action of the teratogen in relation to the stage of embryonic development.

The vertebral involvement in craniorrhachischisis and myeloschisis indicates their similar pathogenesis. This is also the case with the cranial features of craniorrhachischisis and exencephalia (Fig. 13.5), both defects having been observed during the original work of Warkany and Takacs (1959). Like spina bifida, the primary anomaly is in the formation of the neural tube and its separation from the associated ectoderm. Patten (1952, 1953) showed that the hyperplastic brain was an early overgrowth of tissue which occurred after the neural tube had failed to close. The overgrowth is not checked because the primary neural defect has failed to induce the development of the cranial bones. The proliferation of cells, which is typically in the cerebral and cerebellar regions, may not be symmetrical (Fig. 13.5C) and tends to increase in the ventricular areas where partial occlusion may occur (Fig. 13.14B). The abnormal and unrestricted brain growth leads to folding and the origin of channels connecting with the ventricles. This is followed by cellular degeneration and, in some cases studied here, extensive bleeding into the cavities (Fig. 13.5B). Overgrowth also

occurs to some extent in the spinal region of foetuses with craniorrhachischisis and myeloschisis, where the tissue assumes a deeply convex shape (Fig. 13.4C).

In all of the cases examined, the overgrowing brain tissue extended further anteriorly in exencephalia than in craniorrhachischisis; this is illustrated by the series of transverse sections cut through the eyes (Fig. 13.6). This may have been related to the severe kyphosis in the cervical region of foetuses with craniorrhachischisis where the spinal curvature had resulted in the head occupying an abnormally far back position, as can be seen in Fig. 13.13B.

(b) Facial and palatine clefts

The non-closure of the neural tube constitutes the primary cause of a series of discrete malformations. Similarly, the pathogenesis of a wide range of facial and palatine clefts is related to a common primary defect. Normally, an ectodermal invagination separates the lips from the bony portion of the upper and lower jaws. This is followed by the fusion of various parts of the maxillary processes to the frontonasal process. The defects arise from a failure in this fusion. All of the clefts which are theoretically possible between these parts can occur, and they may involve the palate, the lips, the face and/or the maxillae. Warkany and Takacs (1959) first showed that salicylate treatment can

induce palatoschisis (cleft palate) and cheiloschisis (hare lip).

Palatoschisis (Fig. 13.7A) arises from the defective fusion of the palatine processes of the maxillae with each other or with the posterior margins of the primitive palate. The sections in the area of the cleft show agenesis of the middle region of the palatine processes of the maxillae and the associated soft tissue (Fig. 13.7C). The shelves formed by these outgrowths are foreshortened, but the tip of each has developed its own group of raphe cells which, in the normal palate, form a median ridge (Fig. 13.7B). The epithelium on the vestigial shelf is normal except in the region of the actual cleft; here the typical stratified squamous, keratinising buccal epithelium tapers to a single layer of squamous epithelial cells. This layer extends around the whole surface of the cleft and is continuous with the olfactory epithelium overlaying the palatine shelf (Fig. 13.7E).

Overlapping of the palatine processes of the maxillae was described in foetal mice by Larsson, Ericson and Boström (1963) after they had treated the dams with salicylate. However, this defect in palatine development was not observed in the present study.

Cheiloschisis (Fig. 13.8A) is an anomaly which involves the maxillary processes and the frontonasal process. The cases examined histologically were all associated with cleft palates; they showed folded nasal bones and poorly developed maxillary turbinal bones, producing a short nose (Fig. 13.8C). This condition may be similar to the 'short snout'

observed by Trasler (1965) in relation to aspirin-induced facial clefts in mice. The lower jaw may also be correspondingly short. The transverse section illustrates the very reduced height of the nasal cavity associated with the defective development of the premaxillae, maxillae, nasals and nasal septum (Fig. 13.8E).

The spontaneous development of facial and palatine clefts has been recorded in a variety of species including mice, rats, rabbits, guinea pigs, cats, cattle, horses and man - one in every 1000 humans (Carter, 1964), particularly in Japanese (Neel, 1958). The experimental production of cleft palate in laboratory animals has been associated with a wide range of teratogens. These include vitamin A deficiency (Hale, 1935) or excess (Cohlan, 1953); deficiency of riboflavin (Warkany and Schraffenberger, 1944) or folic acid (Evans, Nelson and Asling, 1951); deprivation of oxygen (Ingalls et al, 1950) or food (Kalter, 1954); puncturing the amnion (Trasler, Walker and Fraser, 1956); stress (Rosenzweig, Blanstein and Anderson, 1970); thyroidectomy (Langman and van Faassen, 1955); irradiation (Warkany and Schraffenberger, 1947); lathyrogens (Steffek, 1969); nucleic acid antagonists (Murphy, Dagg and Karnofsky, 1957); neuroleptic drugs (Roux, 1959); antihistamines (King, 1963); adrenocorticotrophic hormone (Heiberg, Kalter and Fraser, 1959); oestrogens (Nishihara, 1958); progestogens (Takano, Yamamura, Suzuki and Nishimura, 1966); and hydrocortisone (Kalter and Fraser, 1952). The glucocorticoids appear to be the most efficient experimental teratogen for palatoschisis,

with cortisone inducing this defect in 100% of A/Jax mouse foetuses (Fraser, 1961). However, mice show a tremendous strain variation in their susceptibility to cortisone-induced cleft palate, which may be related to the precise time of palatine closure in the different strains, and its pathogenesis appears to be more prevalent in the winter months (Kalter, 1959).

The mechanisms of cleft palate development were reviewed by Fraser (1961) and Larsson (1962). It may be caused by a recessive gene (Fitch, 1957) or a mechanical obstruction by the tongue - as is effected when the amnion is punctured and the reduced pressure results in a raising of the tongue - (Trasler et al, 1956), or by the head being too wide in cases of oxycephalia (Stark and Ehrmann, 1958). However, cortisone-induced cleft palate arises from a delay in shelf growth (Walker and Fraser, 1957) and was thought to be mediated through an effect of cortisone on the placenta (Kozaki, Takakusu and Ban, 1964).

Quite recently, a novel hypothesis was proposed by Humphrey (1969). Having indicated the importance of the opening and closing of the foetal mouth for normal palatine shelf elevation, she suggested that narcotic or tranquillizer drugs may reduce foetal reflexes, thereby suppressing the opening of the mouth. This would delay shelf elevation, prolong palatopharyngeal and palatoglossal epithelial contact and result in adhesions and cleft palate. In the absence of any pertinent information on the effects of salicylates on foetal reflexes, it is not possible to

evaluate this hypothesis in terms of cleft palate evoked by salicylates.

It appears that, by a process of elimination, the pathogenesis of the cases reported in this thesis is similar to that associated with cortisone. Palatoschisis has never been observed spontaneously in over 140,000 AH A rats born in our breeding unit; therefore it is unlikely that it involved a recessive gene. There was no evidence of the tongue obstructing shelf growth, neither was the defect associated with macroglossia or oxycephalia. The only other possible causes are the early development of oral cysts (Scott, 1955), or the failure of the epithelium on the shelf edge to break down and allow fusion (Barry, 1961). Larsson (1962) did not accept either explanation for cleft palate formation, and no support for them was afforded by the present cases. Therefore, it seems reasonable to conclude that salicylate-induced cleft palate may, like that produced by cortisone, results from a delay in shelf growth.

(c) Otocephalia

The otocephalic syndrome involves an intergradation of defects in which the ears may develop more ventrally and towards the midline, the jaws and mouth may be reduced or absent, the nose may be proboscoid or absent, and the eyes may be closer together, united or absent. Consequently, in complete otocephalia, the only remnant of the head is the ears which have become fused and appear at the apex of the neck.

A definite shortening of the lower jaw was observed in foetuses with micrognathia (Fig. 13.9A), and those with agnathia contained no bony structure whatsoever in what was a very rudimentary lower jaw (Fig.13.10A). Both abnormalities arise from a failure in the development of the mandibular part of the first branchial arch. Both degrees of the

malformation were associated with microstomia and aglossia. Synotia, which frequently occurs with these defects, was not observed.

The lower jaw of the foetus with micrognathia had shortened bony elements and some musculature which may have been the remnants of that normally associated with the tongue (Fig. 13.9B). Similar muscles appeared in the case of agnathia but, in this foetus, there was total agenesis of the mandible and the upper incisors were reduced. This latter feature was not found with micrognathia but here the lower incisors had not formed, and the upper jaw elements (premaxillae, maxillae and nasals) were reduced in length (Fig. 13.9C). Both micrognathia and agnathia were associated with accentuated ridges on the buccal roof. In agnathia, the epithelium lining the buccal floor showed less stratification and the region of keratinisation ended much nearer to the mouth. This foetus also showed buccal atresia; the nasal cavity opened into the pharynx but the buccal cavity was separated from it by a septum. Further, it had no submaxillary salivary glands and the convolutions of the maxillary turbinal bones were much reduced (Fig. 13.10B).

The jaw defects, which are novel in association with aspirin teratogenesis, appear to be a low grade of otocephalia (Geoffroy-Saint-Hilaire,1837). However, the present cases do not fall precisely into his classification system, nor within those of Taruffi (1884), Blanc (1895) or Wright and Wagner (1934), although showing many of the features described by these workers. This syndrome occurs spontaneously in many species including mice, guinea pigs, cats, dogs, sheep, cattle, swine and humans, and agnathia has been induced experimentally in foetal rats by treating the dams with rabbit anti-rat kidney antiserum.

The cases of micrognathia described in association with salicylates appeared to have no otic defect. The upper jaw hypoplasia seems similar to the condition referred to as 'brachygnathia superior' by Jubb and Kennedy (1963). However, while their definition involves only the premaxillae, in the cases reported here the nasals and maxillae were also affected. The single case of agnathia appeared to have normally positioned pinnae, but the very nature of the defect resulted in the malposition of the otic capsules, a feature which cannot be seen from the figure (Fig. 13.10).

Low grade otocephalia may be associated with hydrocephalia, and this latter malformation was observed in the foetus with micrognathia, (Fig. 13.9). Further, in the most elaborate classification of these defects, Blanc (1895) described a brain with a single anterior cavity full of fluid - a condition typically associated with cyclopia. This indicates an intergradation between otocephalia and cyclocephalia, which are the two divisions into which Geoffroy-Saint-Hilaire (1837) classified facial defects. In cyclocephalia, the eyes approach the midline and the ultimate defect is cyclopia.

(d) Hydrocephalia

The large number of teratogens implicated in the production of hydrocephalia in different species was reviewed by Kalter (1968). These include vitamin deficiency, alkylating agents, alkaloids, antimetabolites, antibiotics, anticholesterolaemics, hypoglycaemics, thalidomide, trypan blue, alloxan, viruses, x-rays, oxygen and heat deprivation, large amounts of naturally-occurring body compounds and sugar. Like other central nervous system malformations, hydrocephalia occurs spontaneously in many species, particularly large animals. This defect results from

an accumulation of cerebrospinal fluid in the cranial vault. If the fluid is within the ventricles, the condition is known as internal hydrocephalia, or if between the brain and the dura mater, external hydrocephalia.

The brain of the early foetus has thin cerebral walls and a large choroid plexus which secretes copious amounts of cerebrospinal fluid. In the normal brain, the fluid flows from the lateral ventricles through the foramen of Munro to the third ventricle. It then passes through the aqueduct of Sylvius to the fourth ventricle; from there, via the foramina of Luschka and Magendie, to the cysterna magna, and then to the subarachnoid space. There is a carefully controlled balance between the production of fluid and its absorption into the circulation. About 80% of the absorption takes place through the villi of the cerebral meninges and the remainder is through the spinal meninges.

Interference with the balanced flow of the fluid typically results in hydrocephalia. In a very small proportion of cases it is caused by excessive cerebrospinal fluid production or by a failure in the absorption. Russell (1949) reported that a least 99% of the cases in children involve the obstruction of the passage of the fluid into the subarachnoid space. The vast majority of these obstructions are malformations of the central nervous tissue in the region of the foramina or aqueduct. These malformations involve the complete or partial blockage of the ducts, usually by forking or atresia and less frequently by stenosis or septum formation.

The demonstration of the actual causative malformation is extremely difficult and, although Warkany and Takacs (1959) found a blocked aqueduct, none could be found in the foetus examined in the present study. However, it does show the effects of the malformation in the gross dilatation of the lateral ventricle (Fig. 13.9B). Other sections in the series showed that the fourth ventricle was also affected and

that there was excess fluid between the brain and the dura mater. This indicates that both internal and external hydrocephalia were present.

(e) Macroglossia

Salicylates can prevent the development of the tongue, as was seen in association with the lower jaw malformations. These compounds can also instigate a definite hyperplasia of the muscle mass of the tongue, a condition termed macroglossia or megaloglossia (Figs. 13.2B and 13.11). In this condition, the longer thicker tongue protrudes through the mouth and is almost certainly similar to the 'protruding tongue' to which Warkany and Takacs (1959) referred. Histologically there was little difference between the malformed and the normal tongues except that the former showed a slight reduction in the number of blood vessels near the tip.

(f) Eye defects

The protrusion of the eyes in the exophthalmic foetuses (Fig. 13.2B) was due to a grossly enlarged lens - macrophakia - (Fig. 13.12B). In this respect, the condition resembled that described by Gillman et al (1948) with trypan blue teratogenicity in rats. In both cases the lens filled the anterior chamber and was in very close proximity to the cornea. However, unlike the eyes described by Gillman et al (1948), and also those reported by Larsson, Ericson and Boström (1963) in association with salicylate teratogenesis in mice, there was no histological feature in the lenses in this study which could not be found also in control sections. Other regions of the globe were not affected, with the possible exception of the retina whose blood vessels tended to be hyperaemic. Unfortunately, Goldman and Yakovac (1964a) gave no details of the salicylate-induced exophthalmia in rats, neither did Miki (1967) after

producing the condition with large doses of vitamin A. Therefore, no comparison can be made with the defects produced here.

Exophthalmia was frequently associated with ablepharia which constitutes a persistence of the embryonic state whereby the eyelids fail to form (Figs. 13.2B and 13.12B). Normally, the lids grow towards each other and become joined by a region of highly vacuolated, specialised epithelial cells (Fig. 13.12C) which will ultimately rupture to allow the lids to open. The affected foetuses showed total agenesis of the lids, and the head epithelium was in direct continuity with the cornea. However, in the region whence the lids should have originated, the epithelium had formed a wedge of the highly vacuolated cells described above (Fig. 13.12D) and keratinisation of the head epithelium had stopped at this point. Warkany and Takacs (1959) first observed salicylate-induced failure of the eyelids to develop but made no mention of the presence of specialised epithelial cells.

There is an interesting similarity between this effect and that seen in palatoschisis (Fig. 13.7). The pathogenesis of both malformations was followed by a reversion to the normal pattern of development - a feature which has been associated with other malformations. The classic example in man (and various experimental animals, too) must be the phocomelia induced by thalidomide, where hands and feet may develop after agenesis of the long bones. Another example was seen in the caudal region of foetuses with craniorrhachischisis where the neural and skeletal tissues had assumed normal conformation.

(g) Miscellaneous axial skeletal defects

In addition to the axial skeleton defects associated with malformations of the central nervous system, described above, kyphosis (Fig. 13.13B) and scoliosis (Figs. 13.2C and 13.14B) were induced. These malformations will be considered together because they always occurred together. Consequently, the observations made for each were complicated by the other defect. The centra were markedly less developed than normal; the most advanced stage seen featured hypertrophied chondrocytes, compared with the normal where the calcified cartilage was breaking down and osteoblasts were secreting the subperiosteal bone. This contrasts sharply with the 'precocious' development described by Lansdown (1970). In the present study, the hemivertebrae and irregular fusion of adjacent vertebrae associated with scoliosis had altered the shape and position of the intercentral tissue. However, this showed no histological difference from the controls.

It was difficult to obtain comprehensive sections of these malformations because of the involvement of the associated anomalies. This was particularly so with scoliosis (Fig. 13.14B), but the unilateral agenesis of some of the vertebral elements was apparent, as was its effect on the ribs on the concave side. They were no longer parallel to each other and now showed irregular fusions.

Rib abnormalities usually arise secondarily to vertebral ones, a point typified by fused ribs which unite posteriorly when two or more ribs arise from one vertebral process (Fig. 13.15A). Ribs are generally absent in association with the agenesis of part or all of the vertebra but, when two adjacent vertebrae are fused together, the two ribs arise as one and divide a short distance from the spine. The cellular

structure of the malformed ribs appeared normal (Fig. 13.15C). The defects were invariably associated with sternal anomalies which typically involved fusion or agenesis of the sternebrae.

The scoliosis observed was classified as 'congenital structural scoliosis' (Browne, 1967) because it involved malformations of the vertebrae and ribs. The general pattern of vertebral and rib anomalies appeared similar to those induced with salicylates by Warkany and Takacs (1959). Similar defects have been associated with x-rays (Kriegel, Langendorff and Shibata, 1962), trypan blue (Gillman et al, 1948), thalidomide (King and Kendrick, 1962), tolbutamide (Smithberg, 1961), alloxan (Klosovskii, 1963), streptomycin (Warkany and Takacs, 1965) and various anticancer drugs (Murphy and Chaube, 1962).

Variable degrees of agenesis of the caudal vertebrae and the associated soft tissues (microcaudia) were observed in both test and control foetuses (Fig. 13.16A). Macroscopically, the tail varied from a very short stump to a longer, hair-like appendage. The level at which the foetal rat vertebral column ended also varied. In one case the whole lumbar region was absent but, more frequently, the last complete vertebra appeared normal. This was followed by a histologically normal remnant of the centrum of the posteriorly adjacent vertebra (Fig.13.16C), whereafter no skeletal element was present.

The cases of curly tail studied (Fig. 13.17) represented an extension of scoliosis, being caused by hemivertebrae in the caudal region.

Tail defects occur spontaneously in a number of species, notably mice, rats, dogs, cats, pigs and cattle. The termination of the nerve cord seen in tailless kittens by Kerruish (1964) was typical of some of the defects induced by salicylates in the present study. Salicylates were first linked with 'undersized tails' by Chebotar (1967) but no detail of the pathology was presented.

The tail defects reported in this thesis had no associated urinogenital or intestinal anomalies, as may occur in Manx cats and AH beagle dogs. Spina bifida may also be linked with tail defects, as was found by Gillman et al (1948) with trypan blue and by Chebotar (1967). However, no such case was observed in our rats.

Other teratogens known to induce tail defects include x-rays (Russell, Russell and Major, 1951), cyclizine (Tuchmann-Duplessis and Mercier-Parot, 1963), imipramine (Larsen, 1963), and the alkaloids colchicine (Wiesner, Wolfe and Yudkin, 1958) and deserpidine (Tuchmann-Duplessis and Mercier-Parot, 1961).

Although serious defects are frequently associated with malformations of the tail, they do not occur invariably. Indeed, Gillman et al (1948) reported spina bifida in only some of their foetuses with abnormal tails. Further, when tail defects were observed during the experiments reported in this thesis, there was usually no associated abnormality. Consequently, when tail defects occur on their own, they may be considered not very important if extrapolated to man. However, the serious nature of the malformations frequently associated with them should not be overlooked.

(h) Ventral body wall defects

The vertebral column defects, particularly kyphosis, were often associated with soft tissue anomalies. Retroflexion in the neck region, for example, resulted in the lateral bowing of the oesophagus (Fig.13.13B). However, the defects more typically linked with kyphosis are omphalocele (Fig. 13.18) and eventration of the abdominal viscera (Figs. 13.13B and 13.19).

Omphalocele, which was first related to salicylate teratogenicity by Goldman and Yakovac (1964a), is the herniation of varying amounts of the abdominal viscera into the umbilical cord. Its pathogenesis involves an early interference with the development of the body stalk, which

separates the embryo from the wall of the blastocyst and converts into the umbilical cord. The size of the caecum in relation to that of the umbilical orifice is considered to be important for the normal withdrawal of the midgut loops into the perivisceral cavity. The failure to return is associated with a developmental defect in the normal closure of the abdominal ring, and the persistence of this embryonic condition results in an omphalocele. The example sectioned was typical of all those observed in that the only viscera within the umbilical cord were intestines (Fig. 13.18C).

The eventration of various abdominal viscera occurs through a congenital fissure in the ventral body wall (Fig. 13.13B), and was first produced with salicylates by Warkany and Takacs (1959). The margins of the aperture are continuous with the amnion and no proper umbilical cord develops. The foreshortened umbilical vessels pass from the placenta to the foetus in the amnion wall, and the placenta may adhere to the viscera. The defect arises in a manner similar to omphalocele, but the gap in the abdominal wall is much more extensive and may even extend rostrally into the thorax. There is no barrier to withold the viscera which become exteriorised.

The terminology of these ventral body wall defects has lost its precise meaning. Even Potter (1952) suggests that omphalocele is synonymous with eventration of abdominal viscera or abdominal hernia, but Morrison (1963) does restrict the use of omphalocele (or exomphalos or amniocele) to the umbilical hernia. However, the most constructive discussion comes from Willis (1958), who clearly distinguishes between umbilical hernia (omphalocele) and eventration of abdominal viscera. His adoption of this latter term in preference to gastroschisis, which erroneously implies a split in the stomach, is much more acceptable.

In six cases sectioned serially (e.g. Fig. 13.13B), the exteriorised liver, intestines and pancreas were histologically normal, as was the lung tissue which was displaced so far posteriorly that some lobes had become eventrated and others were situated next to the bladder. This differs from the findings of Warkany and Takacs (1959), who described foetuses with visceral eventration in which the livers were enlarged and showed additional subdivision of the lobes. In the present study, the differences between the lungs of the normal and the malformed were related to the former having been ventilated. The lung displacement was associated with no remnant of diaphragm development. The vascular connection between the bladder and the normal umbilicus still remained but was with the posterior border of the fissure.

(i) Cardiovascular defects

The most significant histological change in the viscera was the cardiac defect (Fig. 13.20B), and an extensive search of the literature indicates that it has not been described previously. This hypoplastic heart was of normal shape externally but was found to have extremely thin ventricular walls. Furthermore, the interauricular tissue was represented by a complex of many distorted or deformed blood vessels which was indicative of an angioma, appearing similar to those described by Willis (1948, 1958). However, the cardiac muscle cells appeared normal. The heart contained very little blood, but that present appeared normal. There was no discrete pericardium but a thin sheet of connective tissue present in some regions was fused with surrounding connective tissue.

Congenital cardiovascular malformations have been produced in experimental animals by inducing deficiency of a number of vitamins. These include vitamin A (Warkany and Roth, 1948), folic acid (Baird, Nelson, Monie and Evans, 1954), riboflavin (Nelson, Baird, Wright, and

Evans, 1956) and pantothenic acid (Nelson, Wright, Baird and Evans, 1957). They may also be produced with large doses of vitamin A (Kalter and Warkany, 1961), trypan blue (Wilson, 1955), thalidomide (Roux, Cahen and Dupuis, 1965), glucocorticoids (Clavert, Buck, Rumpler and Ruch, 1965) and streptonigrin (Warkany and Takacs, 1965). Further, x-rays (Wilson et al, 1953), hypoxia (Ingalls et al, 1952) and hypothermia (Sobin, 1955) have also been associated with cardiovascular abnormalities.

The first report linking salicylates with such defects was made by Monie (1964), but detailed pathology was not presented until the paper by Takacs and Warkany (1968). The malformation described in this thesis appears different from these. However, the production of such defects in rats with salicylates made the paper by McColl (1966) particularly interesting. In the only report of salicylate teratogenicity in rabbits, he referred exclusively to aortic arch anomalies.

(j) Limb defects

As well as defects in the axial skeleton, malformations were also observed in the appendicular skeleton. Arthrogryposis multiplex congenita, which results in limb joints becoming rigid in flexion or extension, has not been associated previously with salicylate teratogenesis and, in the present study, involved extended hindlimbs in the affected foetuses (Fig. 13.21A). The term itself is a misnomer, strictly meaning crooked joints, but the condition to which it refers is one of stiff joints. There was typically no bony defect but the soft tissue changes usually found were not apparent (Fig. 13.21C). Arthrogryposis occurs spontaneously most frequently in large animals, and is a primary defect of the muscles which become hypoplastic and may show both atrophic and hypertrophic fibres. The sarcolemma nuclei become prominent and the muscle fibres

are infiltrated and replaced with fatty and fibrous tissue. It is frequently associated with a reduction in the number of anterior horn cells in the spinal cord. It was not possible to examine anterior horn cells in the present cases as all except one, which was subjected to skeletal straining, were associated with craniorrhachischisis.

The abnormality produced was classed as arthrogryposis only because of the rigid extension of the affected limbs. It was not possible to articulate the joints and the fixed position is evident from the figure (Fig. 13.21).

Polydactylia (Fig. 13.22) was the other limb malformation associated with salicylates. It is the commonest individual malformation of the extremities and is caused by an excessive division of the limb bud at the level of the metacarpals (or metatarsals) or the phalanges. It occurs in guinea pigs, where it may be associated with a semidominant gene (Wright, 1934), in mice where it may be caused by an autosomal dominant (Lyon, Phillips and Searle, 1964) or recessive (Danforth, 1930)gene, and in rats, also associated with an autosomal recessive gene (Kalter, 1968). Further, it has been induced experimentally by radiation (Green, 1964), thiamylal (Tanimura, 1963) and myleran (Kameyama, 1967).

The cases reported in this thesis involved one extra digit which appeared next to the 1st proximal phalanx. Previously, Trasler (1965) had induced polydactylia in mice treated with aspirin but did not mention the level at which it arose.

(k) Oedema and haemorrhage

Subcutaneous oedema was found as a generalised condition extending over most of the foetus (Fig. 13.23A), or restricted to the region below the lower jaw - bottle-jaw oedema - (Fig. 13.23B). A description of

bottle-jaw oedema in foetal rats was given by Warkany and Takacs (1959) after they had treated the dams with salicylate. However, the generalised condition was not reported until 1966 (Baba and Nagahama), although Lapointe and Harvey (1964) had produced it in hamsters with salicylamide. In man, it is not uncommon in the neonate but soon regresses. It is most severe after intrauterine anoxia when fluid leaks from the capillaries exposed to low oxygen levels. The fluid from the foetal blood is replaced from the dam's blood.

In the present cases, the region most severely affected was the subcutaneous connective tissue, but the dermis and underlying muscles were slightly involved in some foetuses. The cells were extremely large and the contents had displaced the nucleus to a peripheral position (Fig. 13.23D). Many cell walls were ruptured.

Oedema tends to be self-limiting because the swollen tissue is more resistant to subsequent stretching. The hydrostatic pressure of the fluid becomes nearly the same as that in the capillaries, and tissue fluid production almost stops. The walls of the lymphatic vessels are pulled open to facilitate drainage; this is brought about because the increased hydrostatic pressure stretches the fibres attached to the vessel walls (Ham and Leeson, 1950).

The effects of anoxia, which can cause oedema, may be instrumental in producing the subcutaneous haemorrhages seen extensively in AH A rats (Fig. 13.10A). Certainly, in the case of premature human infants, low negative pressure on the skin induces haemorrhage by rupturing the thin walls of the vessels in which there is circulatory stasis. Anoxia produces congestive circulatory failure with overfilling of the heart chambers and pooling of blood in the viscera. The thin-walled foetal vessels become engorged, and circulatory stasis and tissue anoxia lead to haemorrhage. Visceral haemorrhage was described by Eriksson (1969) in

relation to salicylate teratogenesis but none was found in the present study.

Fig. 13.24 shows a haemorrhage typical of those seen in AH A rats. The blood was escaping from the ruptured vessel and dispersing through the subcutaneous tissue and the dermis. This lesion appears different from the 'thin-walled capsules' containing blood which were described by Larsson, Boström and Ericson (1963) in association with salicylate teratogenicity in mice. In all of the cases examined in the present study, the blood cells did not occupy any discrete region but had spread through the tissue. Hence, there may be a similarity between this lesion and the salicylate-induced diffuse haemorrhages observed in mice by Larsson, Ericson and Boström (1963).

Oedema and haemorrhage, both of which may arise as sequels to moderate hypoxia, constitute part of the oedema syndrome described in chicks by Grabowski (1964), but applicable also to mammals. Varying degrees of this syndrome have been recorded in association with many teratogens, such as typan blue (Gillman et al, 1948), antihistamines (King, Weaver and Narrod, 1965), hydrocortisone (Hoar, 1962), dicoumarol (Kraus, Perlow and Singer, 1949), large amounts of pituitary extract, adrenocorticotrophic hormone, vasopressin or adrenaline (Jost, 1950, 1951, 1953), and a deficiency of vitamin E (Mason, 1943), vitamin K (Browne, Fudge and Richardson, 1947), linoleic acid (Martinet, 1952) and pantothenic acid (Giroud, Lefebvres, Prost and Dupuis, 1955). In his paper discussing embryonic oxygen deficiency, Grabowski (1970) cited a number of experiments in which it was suggested that oedema or haemorrhage may have been an early stage in the pathogenesis of different malformations. Moreover, he suggested that many of the heart and blood vessels anomalies attributed to trypan blue may have arisen because

of oedema and disturbed haemodynamics in the formative stages. A similar implication was made in relation to certain aspects of salicylate teratogenesis by Larsson, Boström and Ericson (1963). Such a consideration may be extremely important, particularly in the light of work undertaken with children suffering with cerebral palsy by Ito (1968). He thought that the brain lesion in the majority of these children may have been caused by intracranial haemorrhage which resulted from anoxia.

Grabowski's (1970) evidence is interesting, but valid comment of it in relation to salicylate teratogenesis cannot be made without detailed studies on the pathogenesis of the defect to determine whether it arose at the stage when the teratogen was administered. The present studies on oedema and subcutaneous haemorrhages associated with salicylate treatment of the dams involved only the full term foetus. However, cases have been seen in which oedema and haemorrhage have not been associated with either a teratogen or another defect. Others have been cases of bottle-jaw oedema in foetuses with multiple defects including severe kyphosis. This suggests that the oedema may have been secondary to an interference with normal drainage caused by malformation.

(1) Fused placentae

The final anomaly to be considered is fused placentae (Fig. 13.25) which develop spontaneously in AH A rats. The condition occurs when ordinary dichorionic, dizygotic twins are too closely implanted and involves two implantation sites (McLaren and Michie, 1959a), and a fusion line is always apparent. It contrasts with the common placenta of true twins which is single from the start of development. The present cases bear no relation to the treatment; this is because

implantation and the subsequent development of the lesion had occurred before dosing was commenced.

The fused placentae (Fig. 13.25C) have two separate labyrinths and the basal zone of each, with its layer of giant cells, extends between them. The decidua basalis, which is fibrotic and atrophic at this stage, is common to both placentae and does not infiltrate between them. The terminology of the different placental zones tends to vary with different authors, but that used here was taken from the comprehensive account by Davies and Glasser (1968).

Conclusions

The foregoing descriptions show that a wide range of congenital defects may be produced in AH A rats by salicylates. Whilst the overall percentage of foetuses showing malformations was 11.5%, the range in individual groups of rats given a potentially teratogenic regimen was 0 - 100%. This compares with the incidence reported by Warkany and Takacs (1959) who found defects in 47% of the foetuses in the methyl salicylate groups and 32% in the sodium salicylate ones. The incidence of each anomaly in the present study is illustrated in Fig. 13.26. The malformation observed most frequently was scoliosis, which was always associated with fused ribs, sternal anomalies and hemivertebrae, and occurred in 42% of abnormal pups. Further, all foetuses with kyphosis also had these malformations and all cases of craniorrhachischisis were associated with these five defects. Another correlation was apparent in the case of ablepharia which was invariably found in exophthalmic pups. There was no such association in rabbits (Chapter 7).

Certain abnormalities were observed for the first time with salicylate teratogenesis; these included agnathia, micrognathia, microstomia, aglossia, buccal atresia, hypoplasia of the premaxillae, maxillae and nasal bones, hypoplasia and agenesis of the incisors, agenesis of the submaxillary glands, the pericardium and the diaphragm, arthrogryposis and curly tail. Further, polydactylia has been recorded only in mice (Trasler, 1965). The areas of subcutaneous haemorrhage occur spontaneously in AH A rats and their incidence was not enhanced by the salicylate. They may be similar pathologically to the salicylate-induced diffuse haemorrhages in mice described by Larsson, Ericson and Boström (1963).

Table 13.1 shows that 17 defects were observed on only one or two occasions. Of these, only hydrocephalia and folded nasals have been induced by other workers with salicylates. However, cardiac hypoplasia, angioma, agenesis of the pericardium and the diaphragm, and hypoplasia of the nasals, maxillae and premaxillae all appeared in foetuses which also showed other defects previously associated with salicylates. Moreover, they came from dams treated with potentially teratogenic doses of salicylates and had siblings with characteristic defects. Consequently, the pathogenesis of these malformations was attributed to the salicylate. The remaining novel defects were all degrees of otocephalia which was observed in three foetuses. This syndrome was believed to have been induced by the salicylate because the drug was administered at the period during which these parts normally develop.

In retrospect, salicylates can produce a broad spectrum of malformations, and all of these novel ones occurred after a potentially teratogenic regimen had been given to a susceptible species. Therefore, it was considered that these defects, although present in low numbers, were caused by the salicylate.

It was demonstrated in Chapter 8 that the teratogenic effect of salicylate extends throughout the period of organogenesis which, in AH A rats, is generally 8-12 days post coitum. However, specific defects reflect the precise time of the administration of the teratogen, as can be seen from Fig. 13.26. Naturally, this relates to the system developing during treatment and it is worth re-iterating the association of this feature with the axial grade of embryonic development. Generally, defects of the anterior region were induced early in organogenesis and those of the extremities arose towards the end. It appears that the embryopathic activity of salicylate is not restricted to any particular organ system or to any specific time period. The non-specific nature of the defects observed indicates that the embryopathic action of salicylate does not result from a direct insult on the conceptus. Instead, it suggests a generalised effect, possibly on the embryo-placenta unit.

FIG. 13.1

MACROSCOPIC APPEARANCE OF NORMAL RAT FOETUSES

Fig. 13.1A
Lateral view.

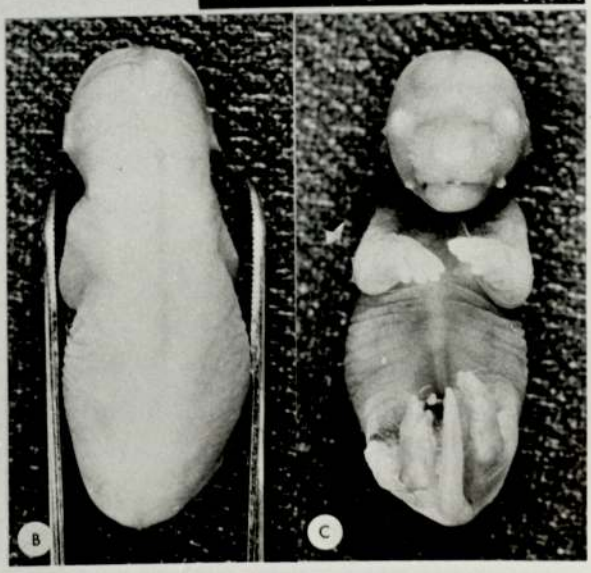


Fig. 13.1B
Dorsal view.

Fig. 13.1C
Ventral view.

FIG. 13.2

MACROSCOPIC APPEARANCE OF RAT FOETUSES SHOWING MALFORMATIONS CAUSED BY A PRIMARY NON-CLOSURE OF THE NEURAL TUBE

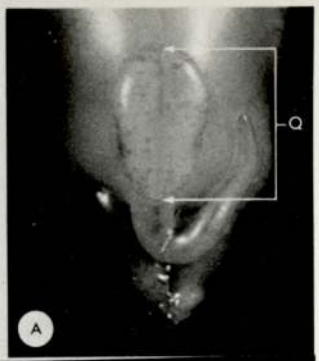


Fig. 13.2A

Dorsal view of the posterior region of a foetus showing myeloschisis (Q).

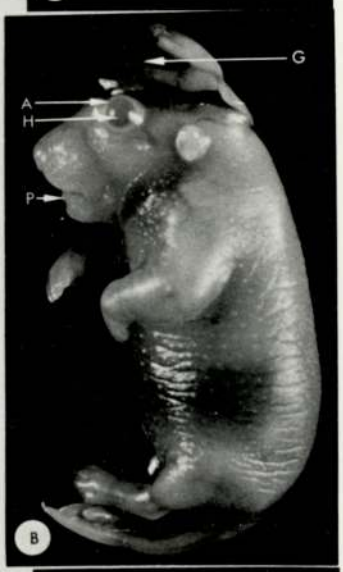


Fig. 13.2B

Laternal view of a foetus showing exencephalia (G). This pup also shows ablepharia (A), exophthalmia (H) and macroglossis (P).

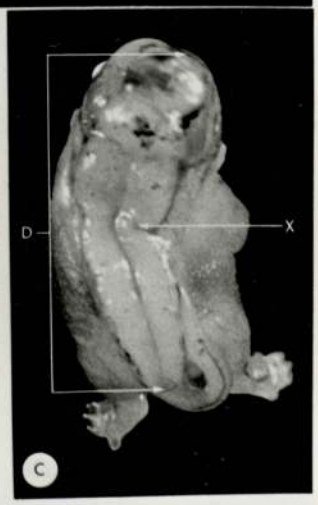


Fig. 13.2C

Dorsal view of a foetus showing craniorrhachischisis (D). This pup also shows scoliosis (X).

FIG. 13.3

LONGITUDINAL SECTIONS THROUGH THE POSTERIOR REGION OF RAT FOETUSES SHOWING THE CENTRAL NERVOUS TISSUE AND ITS ASSOCIATED STRUCTURES

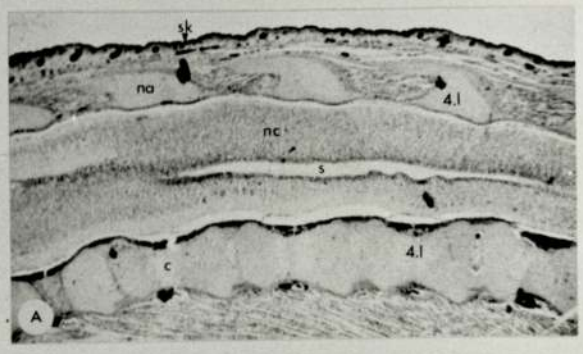


Fig. 13.3A

Lumbar region of a normal foetus showing the nerve cord (nc), with its spinal canal (s), enclosed within the centra (c) and neural arches (na) of the vertebrae. These are surrounded by muscle and connective tissue and overlaid with skin (sk). The 4th lumbar vertebra (4.1) is located.

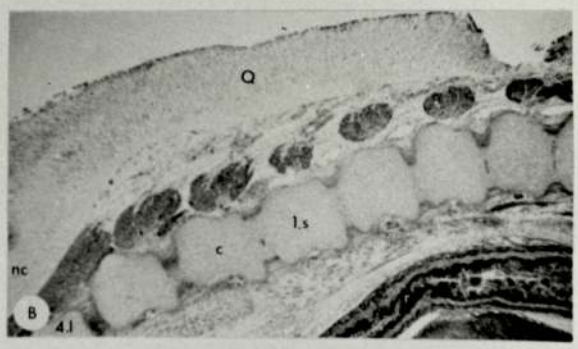


Fig. 13.3B

The corresponding region of a foetus with myeloschisis (Q). The nerve cord (nc) appears normal down to the level of the 4th lumbar vertebra (4.1), whereafter it has failed to close and has remained exteriorised. The centra (c) appear normal but the neural arches have not enclosed the cord which has no overlaying tissue. The number of vertebrae appear normal, as is shown by the position of the 1st sacral (1.s.) one in relation to the rectum (r).

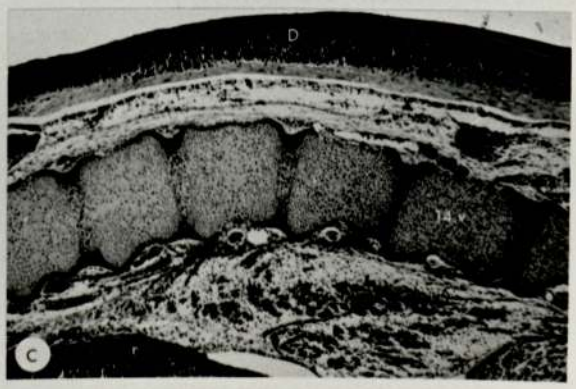


Fig. 13.3C

The corresponding region of a foetus with craniorrhachischisis (D). The appearance of the unclosed neural tissue and its associated structures is identical to that seen in myeloschisis (Fig. 13.3B). The multiple defects of this foetus made it impossible to differentiate clearly the region of the vertebral column. This section shows the 14th vertebra (14.v) but is at the level of the rectum (r).

FIG. 13.4

TRANSVERSE SECTIONS THROUGH THE DORSAL REGION OF RAT FOETUSES SHOWING THE CENTRAL NERVOUS TISSUE AND ITS ASSOCIATED STRUCTURES

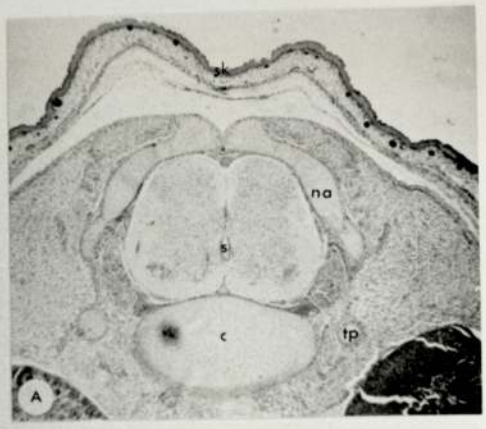


Fig. 13.4A

Section through the anterior lumbar region of a normal foetus showing the nerve cord (nc) with its spinal canal (s). The associated vertebra shows the normal configuration of the centrum (c), transverse processes (tp) and neural arch (na). This is overlaid by muscle, connective tissue and skin (sk).

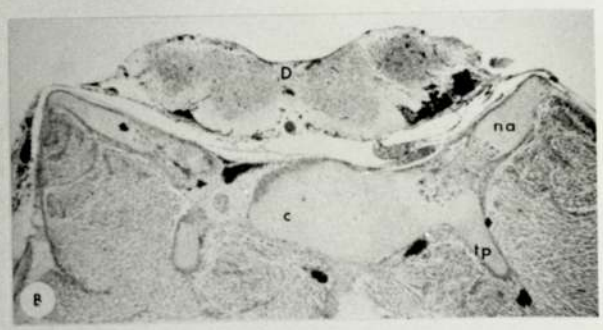


Fig. 13.4B

The corresponding region of a foetus with craniorrhachischisis. The neural tissue (D) has remained open and the neural arch elements (na) are widely splayed. There is no overlaying epidermal tissue, but the centrum (c) and transverse processes (tp) have remained normal.

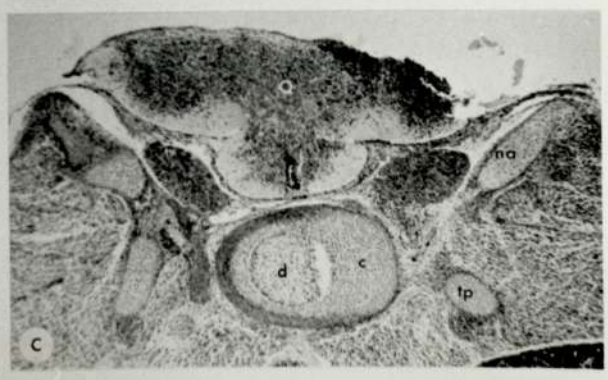


Fig. 13.4C

The posterior lumbar region of a foetus with myeloschisis. The neural tissue (Q) shows a massive overgrowth and the development of what may be a false spinal canal (f). The vertebral elements are similar to those described in Fig. 13.4B, but this section includes an intervertebral disc (d).

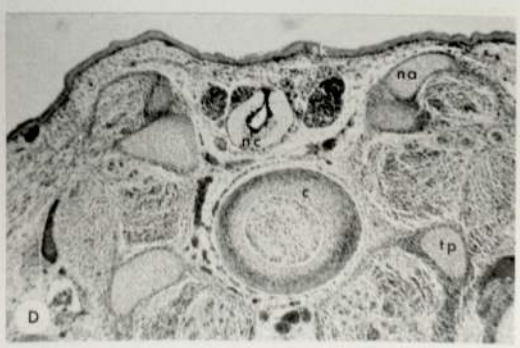


Fig. 13.4D

The posterior 'lower sacral' region of a foetus with craniorrhachischisis showing the almost complete closure of the nerve cord (nc) in this region. The neural arch elements (na) retain the typical splayed position but muscle, connective tissue and skin (sk) overlay the neural tissue.

FIG. 13.5

TRANSVERSE SECTIONS THROUGH RAT FOETAL BRAINS AND THEIR ASSOCIATED STRUCTURES

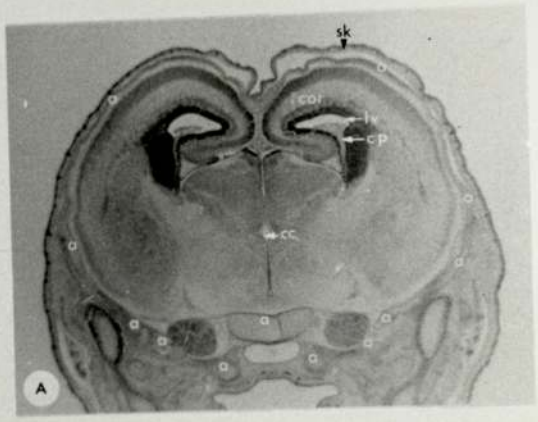


Fig. 13.5A

Section through the brain of a normal pup showing the central canal (cc), lateral ventricles (lv), choroid plexus (cp) and cortex (cor). The whole is protected by the cranial bones (a) and enclosed with skin (sk).

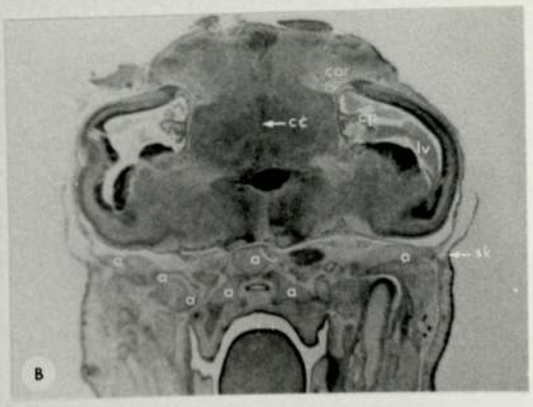


Fig. 13.5B

The corresponding region of a foetus with craniorrhachischisis. The cranial bones (a) have developed only below the brain and, like the skin (sk), fail to enclose it. The brain tissue is hyperplastic particularly in the cortical region (cor).

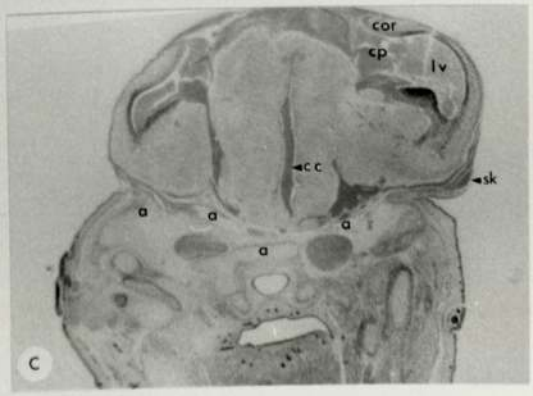


Fig. 13.5C

The corresponding region of a foetus with exencephalia showing a condition very similar to craniorrhachischisis. This section shows the asymmetrical cortical overgrowth (cor), and the ventricles (lv) and their inter-communicating cavities filled with blood.

FIG. 13.6

TRANSVERSE SECTIONS THROUGH THE HEADS OF RAT FOETUSES AT THE LEVEL OF THE EYES, SHOWING THE ANTERIOR DEVELOPMENT OF THE BRAIN

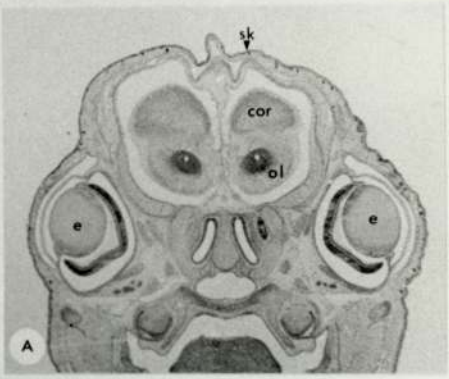


Fig. 13.6A

Normal foetus showing the central nervous tissue in the region of the eyes (e). In this plane the olfactory lobes (ol) are overlaid with the most anterior part of the cerebral cortex (cor). The skin (sk) covers the head.

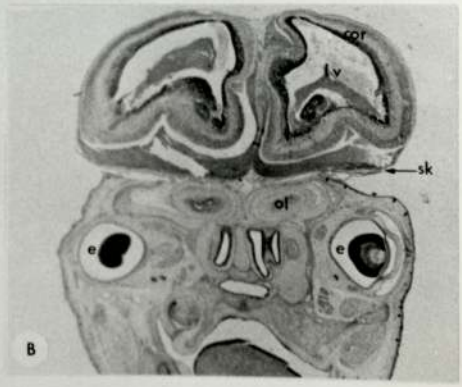


Fig. 13.6B

The corresponding region of an exencephalic foetus. The olfactory lobes (ol) are present but the hyperplastic cortex (cor) has grown anteriorly and the lateral ventricles (lv) appear in this plane in an extremely dilated form. The development of the skin (sk) is arrested at the point indicated by the arrow.

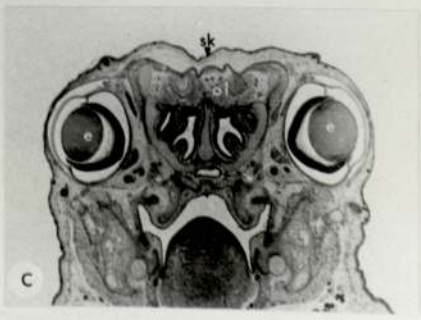


Fig. 13.6C

The corresponding region of a foetus with craniorrhachischisis. Again, there is olfactory lobe development (ol) but no indication of any cortex. The covering of skin (sk) appears normal.

FIG. 13.7

PALATOSCHISIS IN THE RAT FOETUS

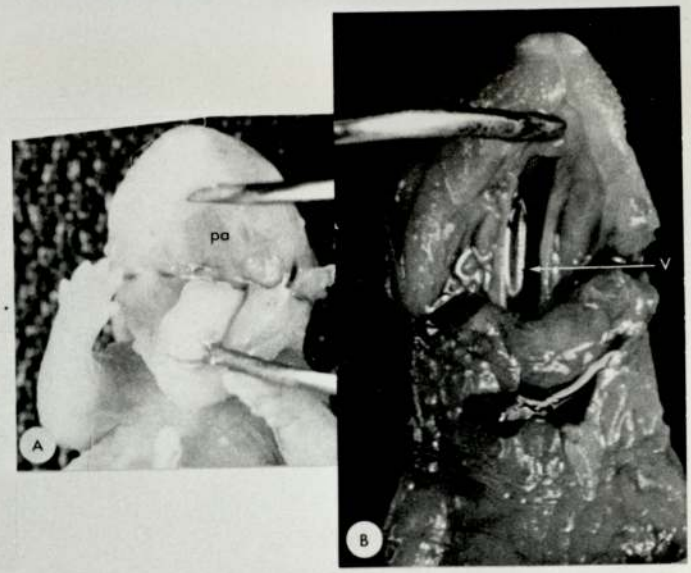


Fig. 13.7A

Macroscopic appearance of a normal palate (pa).

Fig. 13.7B

Macroscopic appearance of a palate showing a median cleft (V).

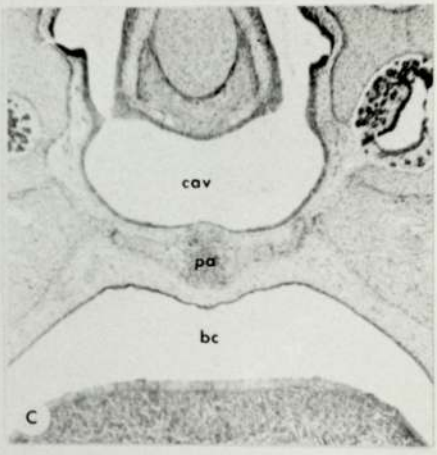


Fig. 13.7C

Transverse section through a normal palate (pa), separating the buccal cavity (bc) from the nasal cavity (cav).

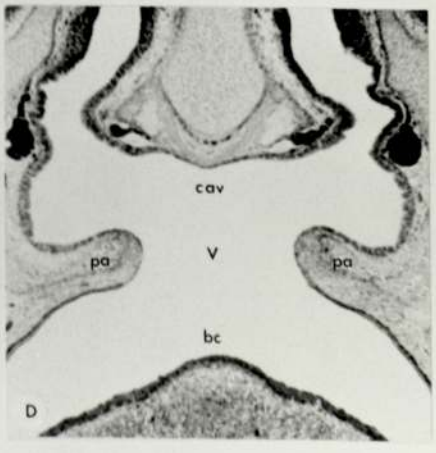


Fig. 13.7D

Transverse section through the region of the cleft (V) in the palate of a foetus with palatoschisis. The palatine shelves (pa) are foreshortened and have failed to meet in the midline. Consequently, the buccal cavity (bc) opens directly into the nasal cavity (cav).

FIG. 13.7 Contd/

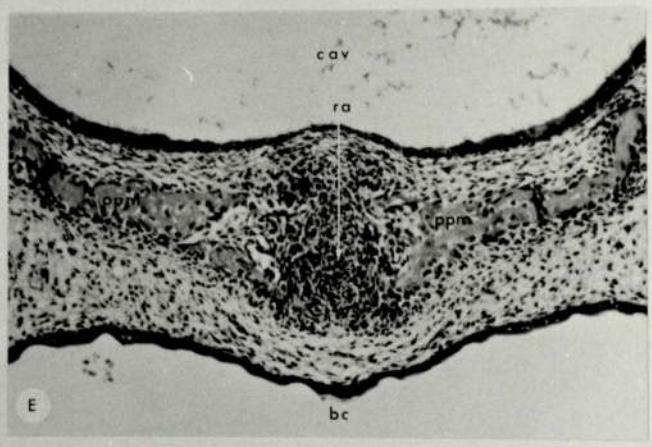


Fig. 13.7E

High power detail of a normal palate. The palatine processes of the maxillae (ppm) grow towards the midline where they fuse and form a median ridge - the raphe (ra).

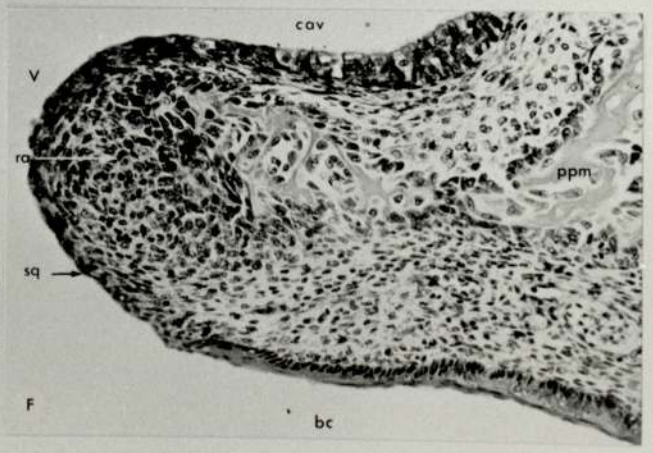


Fig. 13.7F

High power detail of one palatine shelf of a foetus with palatoschisis. The palatine process of the maxilla (ppm) and its associated soft tissue is foreshortened but the raphe cells (ra) have developed normally. The stratified squamous epithelium of the buccal roof has become a monolayer (sq) which extends around the cleft (V), whereafter normal olfactory epithelium is present.

FIG. 13.8

CHEILOSCHISIS IN THE RAT FOETUS

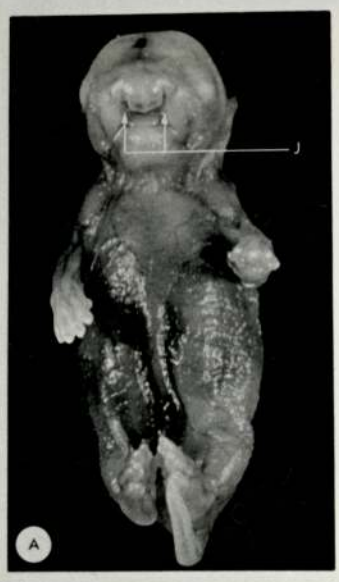


Fig. 13.8A

Macroscopic appearance of bilateral cheiloschisis (J).

Fig. 13.8B

Longitudinal section through the head of a normal rat foetus. This shows the normal conformation of the nasal bones (n), the maxillary turbinal bones (mt), the palate (pa) separating the nasal cavity (cav) from the buccal cavity (bc), the tongue (tn), the lower jaw (man) with its incisors (ic), the oesophagus (oes), the trachea (tr), submaxillary salivary glands (ssg), lateral ventricle (lv) and subarachnoid space (as). This section may be used to control all longitudinal sections through heads with defects.

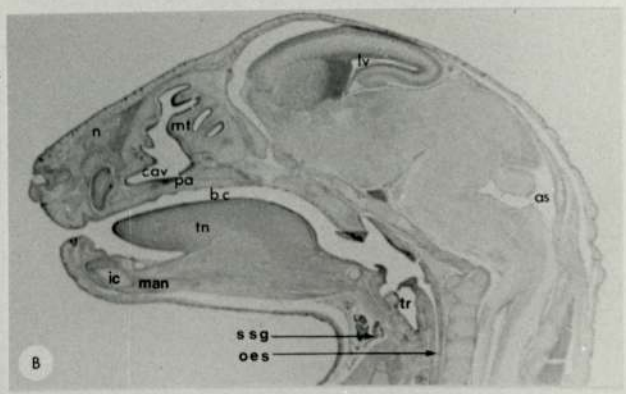


Fig. 13.8C

Longitudinal section through the head of a foetus with cheiloschisis (J). This malformation is associated with palatoschisis (V) and folded nasal bones (Hf). The maxillary turbinal bones (mt) are poorly developed giving a more capacious nasal cavity (cav).

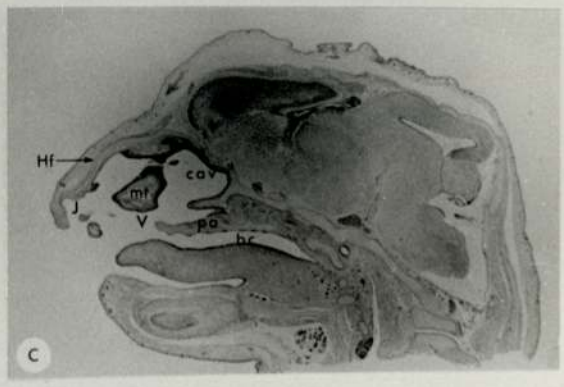


FIG. 13.8 Contd/

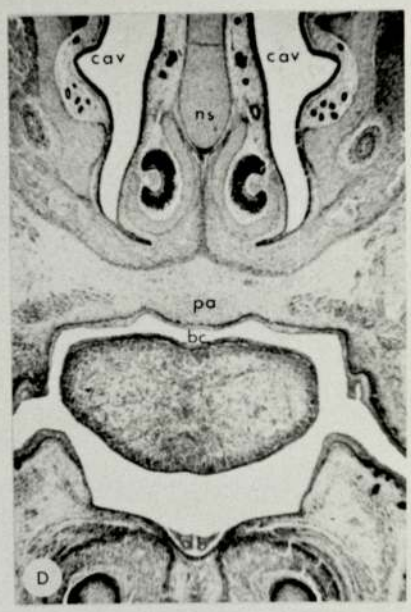


Fig. 13.8D

Transverse section through the anterior nasal zone of a normal foetus showing the nasal cavity (cav) separated from the buccal cavity (bc) by the anterior region of the palate (pa). This plane also shows the median nasal septum (ns).

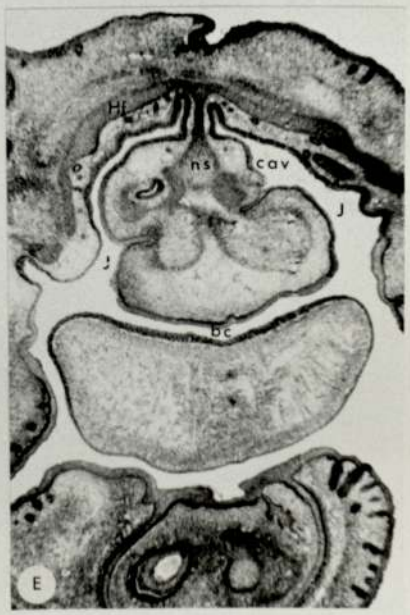


Fig. 13.8E

The corresponding section in a foetus with bilateral cheiloschisis (J). This leaves the nasal cavity (cav) opening into the buccal cavity (bc) on both sides of the midline. The nasal septum (ns) is small and squat so that the height of the nasal region is much reduced. Further, the nasal bones show irregular folding (Hf).

FIG. 13.9

MICROGNATHIA, MICROSTOMIA, AGLOSSIA AND HYDROCEPHALIA IN THE RAT FOETUS

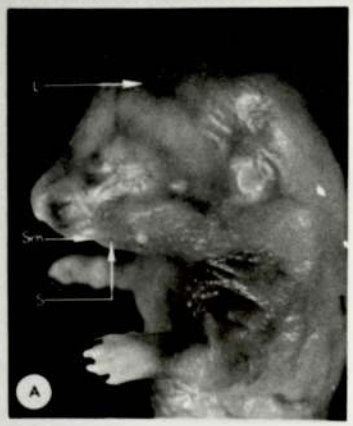


Fig. 13.9A

Macroscopic appearance of a foetus showing a domed head typical of hydrocephalia (L), and micrognathia (S) and microstomia (Sm).

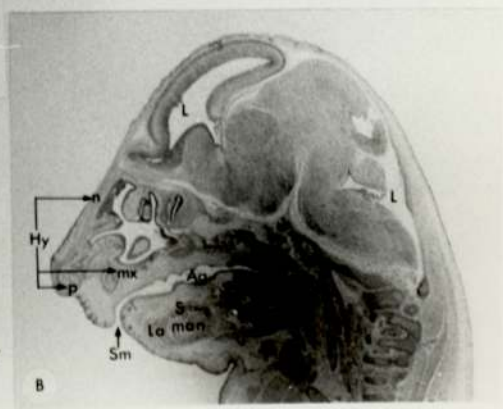


Fig. 13.9B

Longitudinal section through the head of this foetus. Cerebrospinal fluid has accumulated in the lateral ventricle and the subarachnoid space to give both internal and external hydrocephalia (L). The small lower jaw (s) contains a hypoplastic mandible (man) and has no incisors (La). No tongue has developed (Ag) and the mouth is very small (Sm). The nasals (n), maxillae (mx) and premaxillae (pm) are foreshortened (Hg). For a control section see Fig. 13.8B.

FIG. 13.10

AGNATHIA, MICROSTOMIA, AGLOSSIA AND BUCCAL ATRESIA IN THE RAT FOETUS

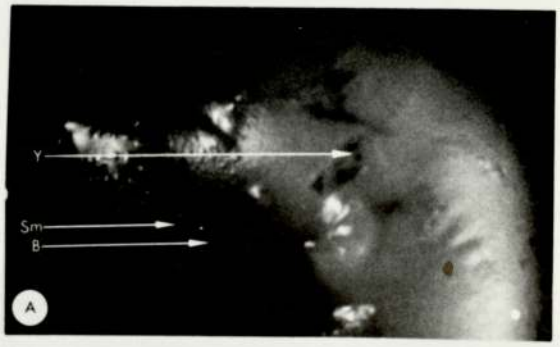


Fig. 13.10A

Macroscopic appearance of the head of a foetus showing agnathia (B), microstomia (Sm) and subcutaneous haemorrhages (Y).

Fig. 13.10B

Longitudinal section through the head of this foetus. The very small mouth (Sm) opens into the buccal cavity (bc), the roof of which has accentuated ridges. There is no tongue (Ag) and the buccal cavity is separated from the pharynx by a septum (Ca) which is continuous with the soft palate (pa). The nasal cavity (cav) opens into the pharynx and there is a normal oesophagus (oes) and trachea (not shown in this section). The lower jaw remnant has no bony element (B) and there is complete agenesis of the submaxillary glands (Ya). Further, the upper incisors are hypoplastic (Lh).

For a control section see Fig. 13.8B.

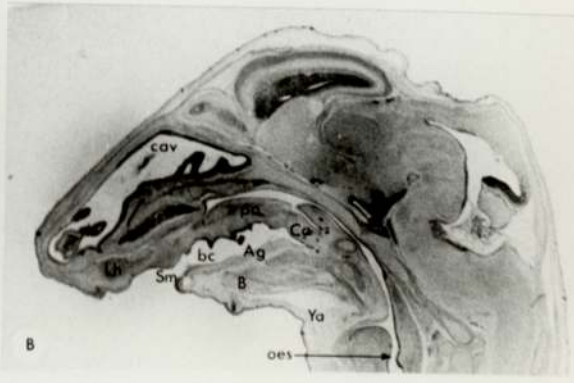
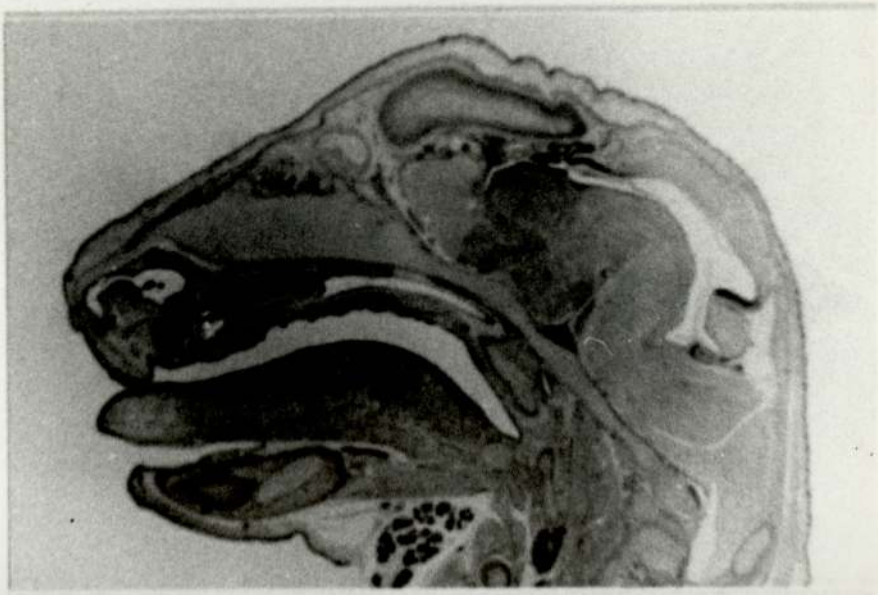


FIG. 13.11

LONGITUDINAL SECTION THROUGH THE HEAD OF A RAT FOETUS SHOWING MACROGLOSSIA (P)

The macroscopic appearance is illustrated in Fig. 13.2B.



For a control section see Fig. 13.8B

FIG. 13.12

SECTIONS THROUGH THE EYES OF RAT FOETUSES SHOWING EXOPHTHALMIA AND ABLEPHARIA

The macroscopic appearance is illustrated in Fig. 13.2B.

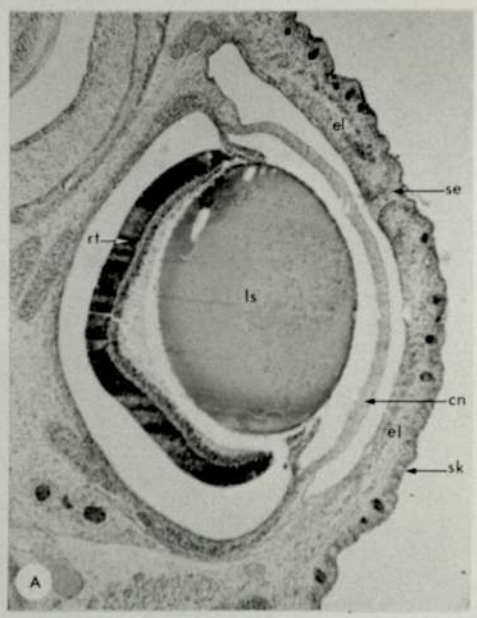


Fig. 13.12A

Longitudinal section through a normal rat foetal eye and adnexa showing the retina (rt), the lens (ls) and the cornea (cn). The eye lids (el) are covered with skin (sk) and are separated by a group of highly specialised epithelial cells (se) which ultimately rupture to allow the lids to open.

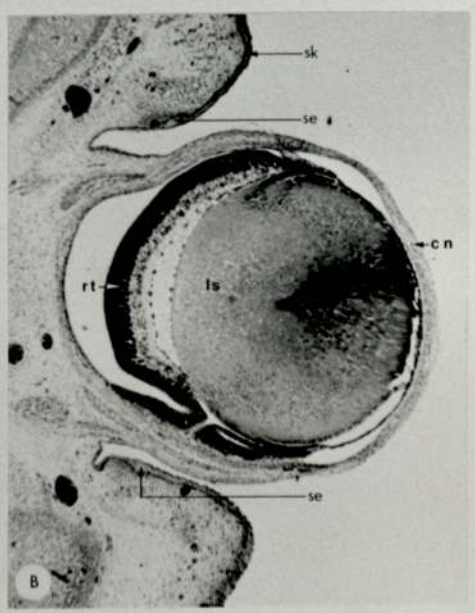


Fig. 13.12B

The corresponding section in a foetus with exophthalmia and ablepharia. The reflexed folding of the retina (rt) is an artefact. The exophthalmia is caused by the grossly enlarged lens (ls) which fills the anterior chamber to become closely associated with the cornea (cn). There has been no development of the eye lids, but the facial skin (sk) continues as far as the focus of specialised epithelial cells (se). These are the cells which normally develop between the eyelids but, in the ablepharon, they appear in the region from which the lids should have grown.

FIG. 13.12 Contd/

Fig. 13.12C

High power detail of the specialised epithelial cells (se) between normally developed eyelids (el). These cells are covered externally with a layer of keratin (kr).

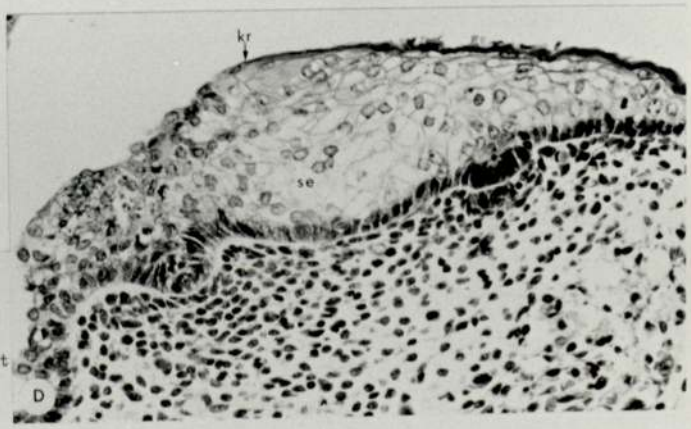


Fig. 13.12D

High power detail of the specialised epithelial cells (se) of the ablepharon. These cells are homologous with those shown in Fig. 13.12C, and the layer of keratin (kr) partly covers them but is not present in regions closer to the eye.

FIG. 13.13

LONGITUDINAL SECTIONS THROUGH WHOLE RAT FOETUSES, SHOWING MULTIPLE BIRTH DEFECTS.

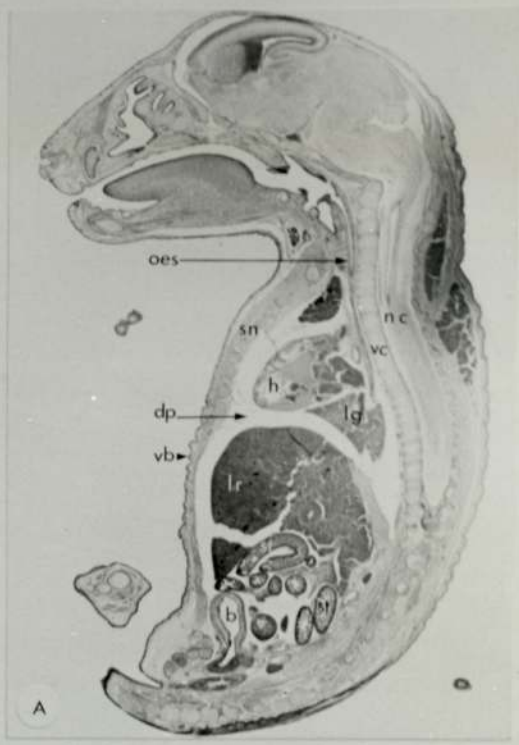


Fig. 13.13A
Normal foetus showing the position and conformation of the vertebral column (vc) and nerve cord (nc), the oesophagus (oes), the sternum (sn), the heart (h), the lungs (lg), the diaphragm (dp), the ventral body wall (vb), the liver (lr), the intestine (st) and the bladder (b).

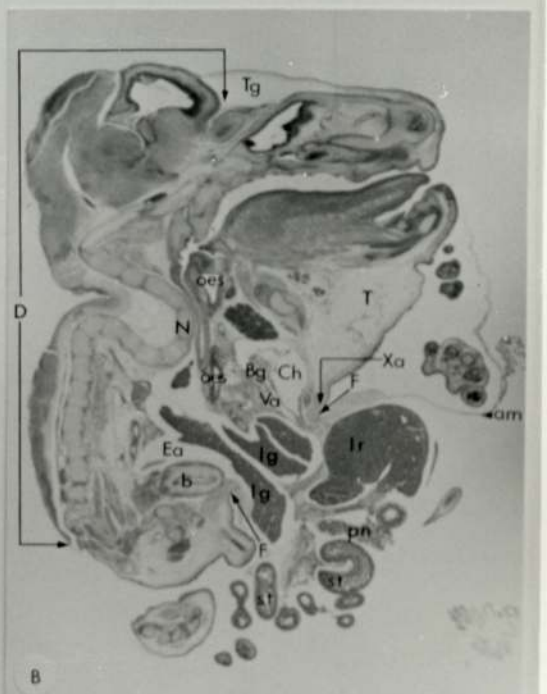


Fig. 13.13B
Grossly malformed foetus with craniorrhachischisis (D), and kyphosis (N) in which the extreme retroflexion has deflected the oesophagus (oes) laterally along its mid-region. The defective development of the ventral body wall (F), which is continuous with the amnion (am) has resulted in the eventration of the liver (lr), pancreas (pn), intestines (st) and lung (lg). Part of the lung retained lies adjacent to the bladder (b). The sternum is represented by a single, small sternebra (Xa) and there is no diaphragm (Ea). The hypoplastic heart (Ch) has no pericardium (Va) and the interauricular region contains an angioma (Bg). Subcutaneous oedema is generalised (Tg) but primarily associated with the lower jaw (T).

FIG. 13.14

LONGITUDINAL SECTIONS THROUGH WHOLE RAT FOETUSES SHOWING SCOLIOSIS

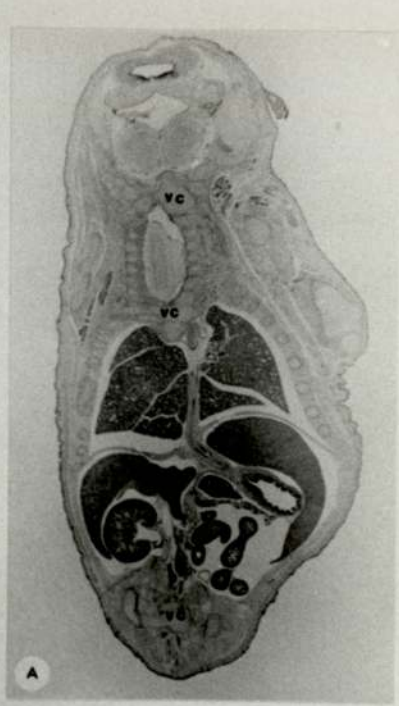


Fig. 13.14A

Normal foetus showing the straight vertebral column (vc).

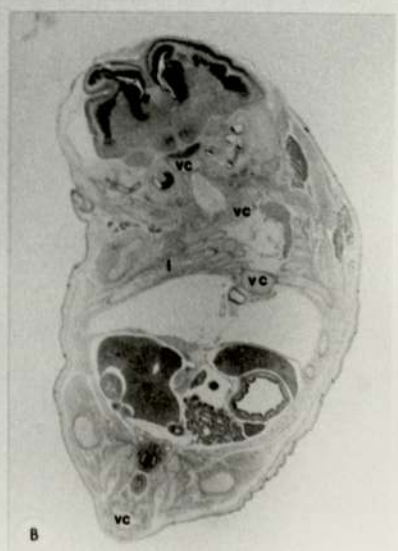


Fig. 13.14B

Foetus with scoliosis. The vertebrae (vc) no longer occupy a straight line and the ribs on the concave side are fused (I). This pup also showed kyphosis which prevented a series of adjacent vertebrae from occupying one plane. The lateral ventricles (lv) show asymmetrical, partial occlusion which has resulted from unrestricted growth of the brain tissue - a sequel to the craniorrnachischisis also affecting this foetus. The lack of lung tissue in this section is an artefact.

FIG. 13.15

FUSED RIBS IN THE RAT FOETUS

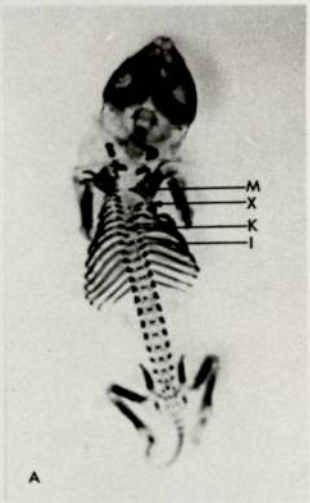


Fig. 13.15A

Stained preparation of a foetal skeleton showing scoliosis (X) which is associated with hemivertebrae (K) and fused ribs (I). This pup also shows inhibited ossification of the cervical centra (M).

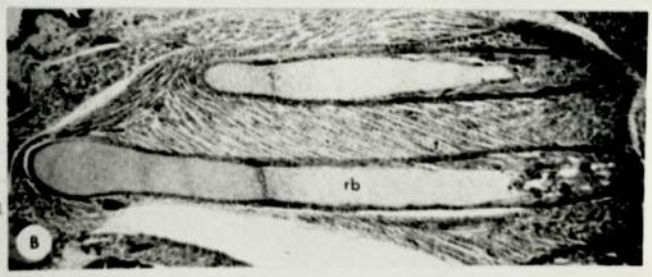


Fig. 13.15B

Longitudinal section through part of a normal foetal rib (rb).

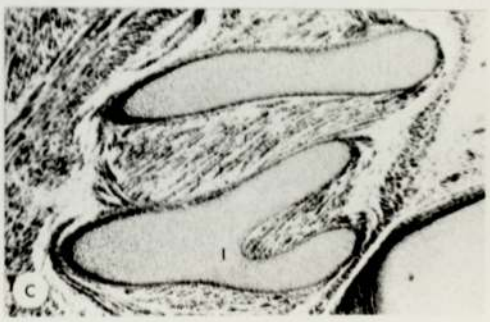


Fig. 13.15C

Corresponding section through part of the affected ribs in the region of the fusion (I). These ribs appeared normal histologically.

FIG. 13.16

MICROCAUDIA IN THE RAT FOETUS

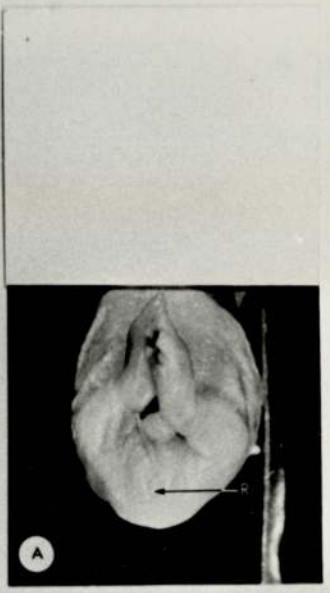


Fig. 13.16A

Macroscopic appearance of the lower ventral region of a foetus with microcaudia (R).

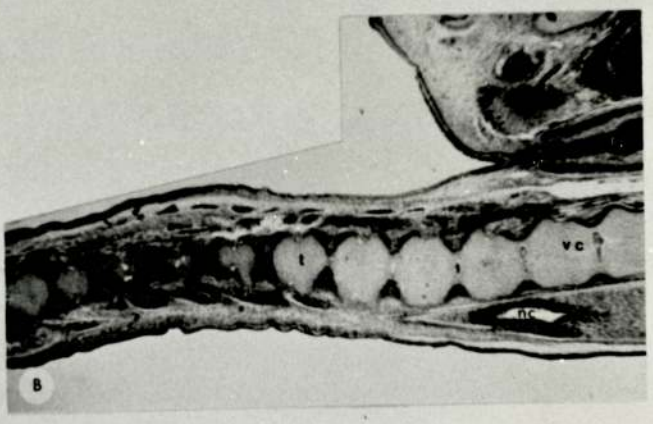


Fig. 13.16B

Longitudinal section through the proximal caudal region of a pup with a normal tail. The nerve cord (nc) extends just beyond the rectum (r) and the vertebrae (vc) continue into the tail (t).

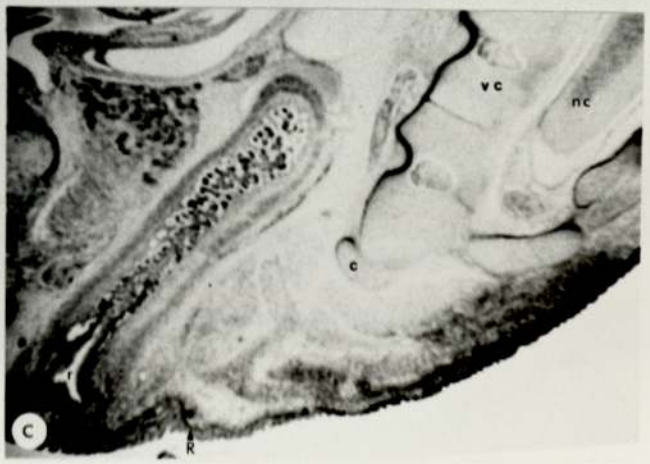


Fig. 13.16C

Corresponding region of a pup with microcaudia. The nerve cord (nc) and the vertebral column (vc) have not developed beyond the rectum (r) and there is no post anal tail. The vertebral column terminates with a normal vertebra followed by a remnant of the centrum (c) of the posteriorly adjacent one. The only evidence of a tail is a rudimentary stump (R).

FIG. 13.17

MACROSCOPIC APPEARANCE OF CURLY TAIL

TAIL (E) IN THE RAT FOETUS

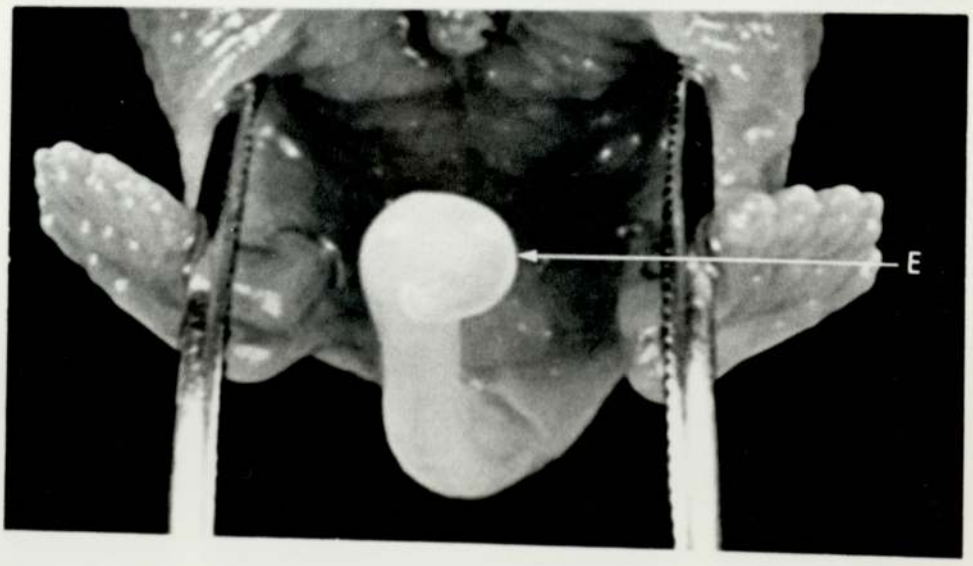


FIG. 13.18

OMPHALOCELE IN THE RAT FOETUS

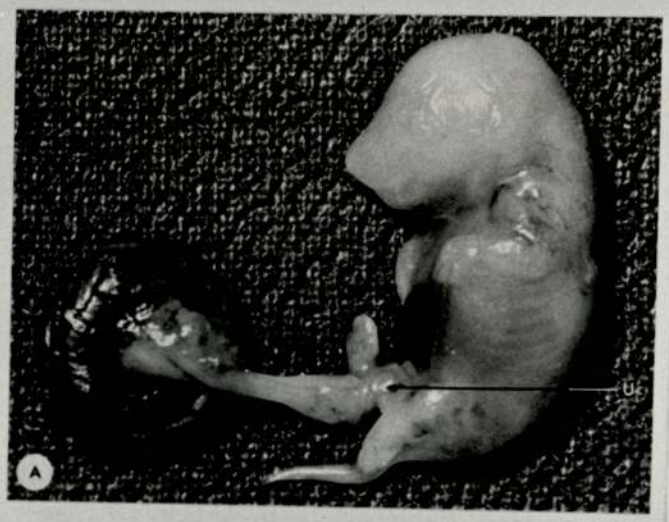


Fig. 13.18A

Macroscopic appearance of a foetus with omphalocele (Uc), in which the intestine remains within the umbilical cord.

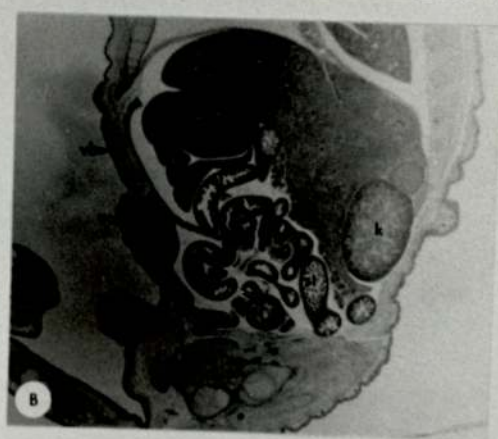


Fig. 13.18B

Longitudinal section through the abdomen of a normal foetus showing the ventral body wall (vb) with the umbilicus (u). Enclosed within the cavity are the liver (lr), the kidneys (k), the bladder (b) and the intestines (st).

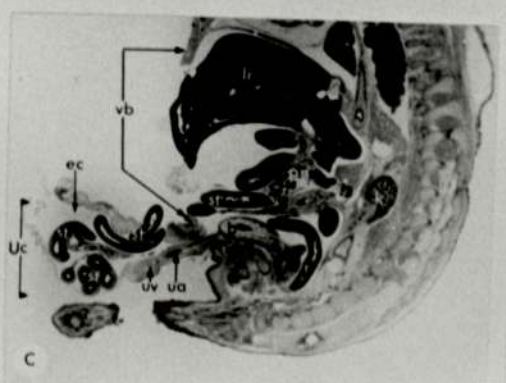
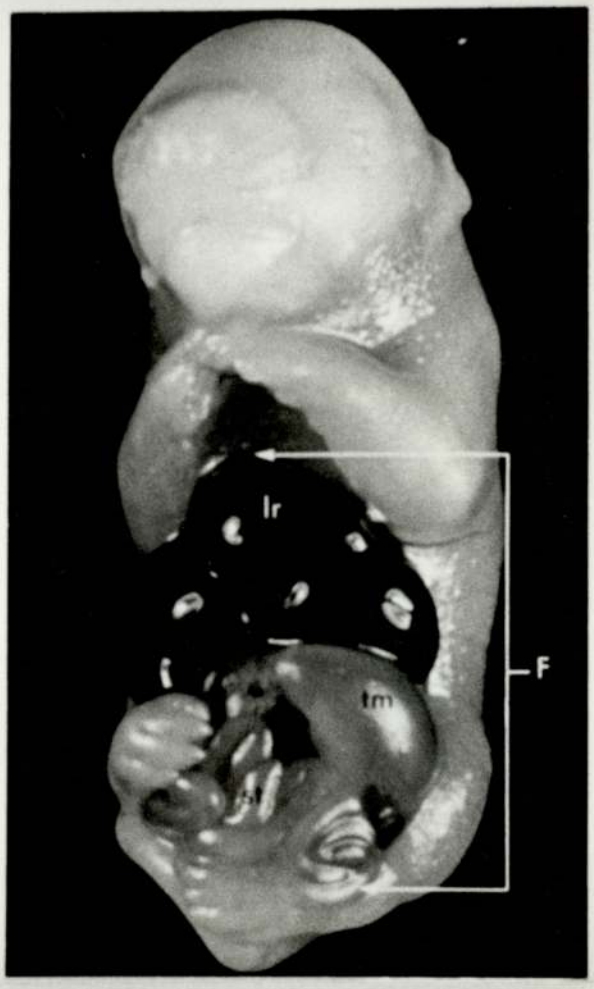


Fig. 13.18C

Corresponding section through a foetus with omphalocele (Uc). The missing section of ventral body wall (vb) between the arrows is an artefact. Some regions of the intestines (st) have remained within the extraembryonic coelom (ec). Other viscera, including the liver (lr), the kidneys (k), the pancreas (pn) and the bladder (b), are **not** affected. The section indicates that the proximal region of the rectum (r) is also within the exocoel. The umbilical vein (uv) and artery (ua) are also located.

FIG. 13.19

MACROSCOPIC APPEARANCE OF THE VENTRAL ASPECT OF A RAT FOETUS SHOWING EVENTRATION OF THE ABDOMINAL VISCERA (F).



The exteriorised organs include the liver (lr), the stomach (tm) and the intestines (st).

A longitudinal section of a foetus with this defect is illustrated in Fig. 13.13B.

FIG. 13.20

LONGITUDINAL SECTIONS THROUGH RAT FOETAL HEARTS SHOWING HYPOPLASIA, ANGIOMA AND PERICARDIAL AGENESIS.

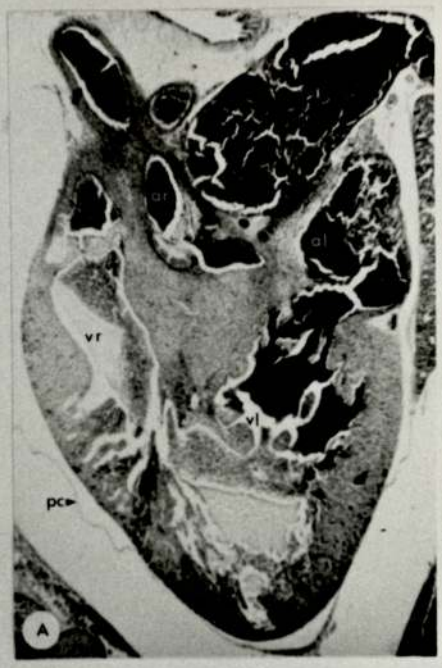


Fig. 13.20A

Normal heart showing the left (al) and right (ar) auricles and left (vl) and right (vr) ventricles and the pericardium (pc).

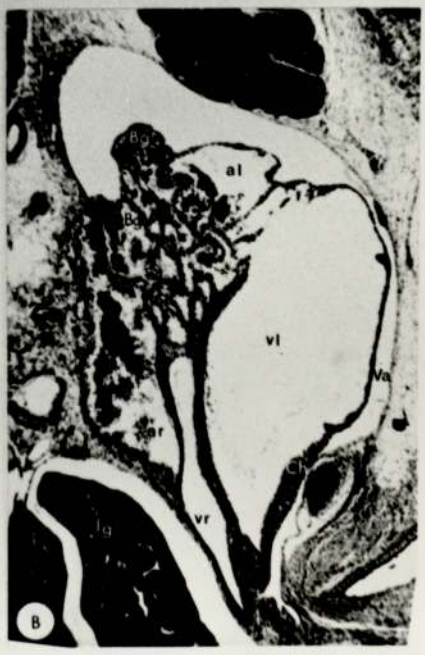


Fig. 13.20B

Corresponding section of the affected heart showing extreme hypoplasia of all walls. The left ventricle (vl) is enlarged but the size of the right one (vr) and the auricles (al and ar) is not appreciably affected. The region between the auricles is occupied by an angioma (Bg). No pericardium is apparent (Va). The lung (lg) is also located.

FIG. 13.21

ARTHROGRYPOSIS IN THE RAT FOETUS

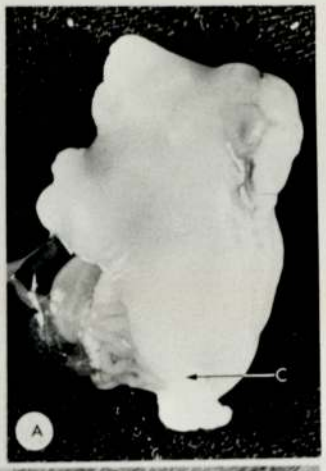


Fig. 13.21A

Macroscopic appearance of arthrogryposis (C) involving the distal region of the hindlimbs fixed in extension. This foetus showed multiple defects.

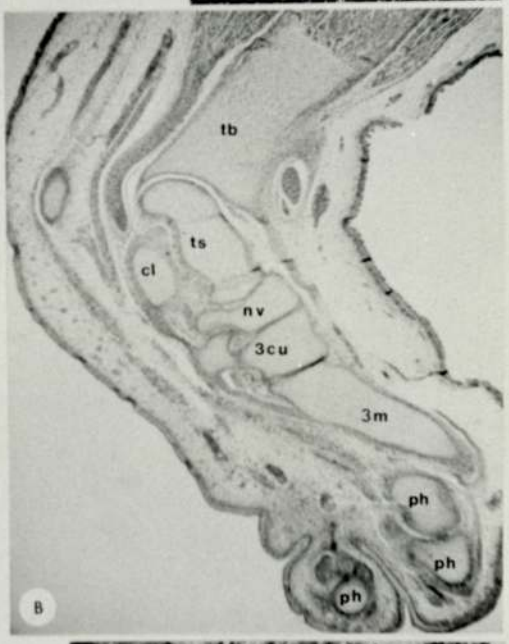


Fig. 13.21B

Longitudinal section through the distal region of a hindlimb of a normal foetus. The bones located include the tibia (tb), the talus (ts), the calcaneus (cl), the navicular (nv), the 3rd cuneiform (3.cu), the 3rd metatarsal (3.m) and phalanges (ph).

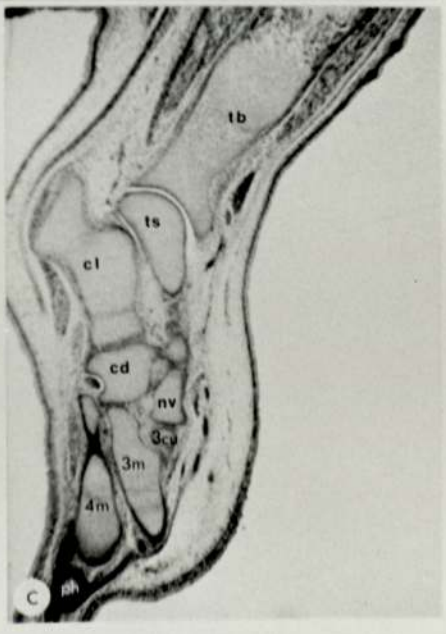
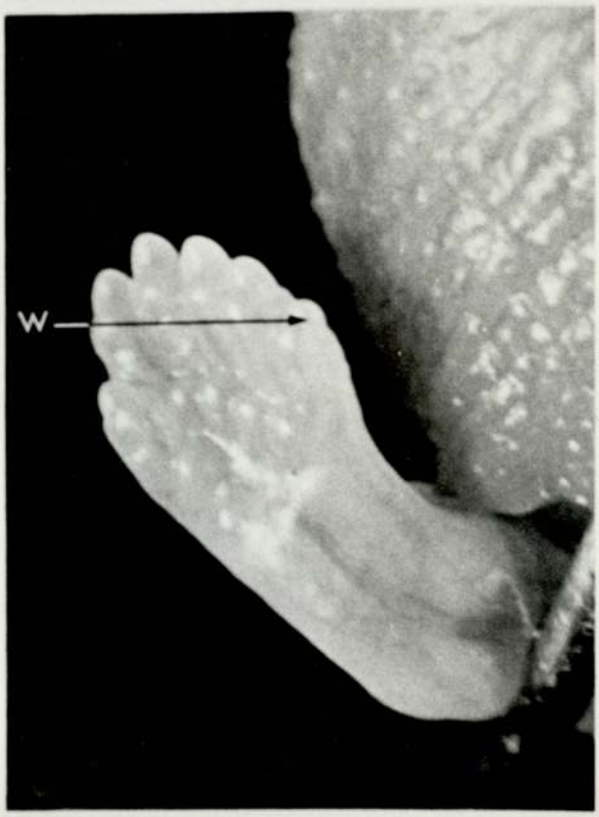


Fig. 13.21C

Corresponding region of an affected foetus showing the normal conformation of the tibia (tb), the talus (ts), the calcaneus (cl), the cuboid (cd), the navicular (nv), the 3rd cuneiform (3.cu), the 3rd (3.m) and 4th (4.m) metatarsals and phalanges (ph).

FIG. 13.22

MACROSCOPIC APPEARANCE OF THE RIGHT PES OF A RAT FOETUS WITH POLYDACTYLIA



The additional digit (W) is adjacent to the 1st digit.

FIG. 13.23

SUBCUTANEOUS OEDEMA IN THE RAT FOETUS

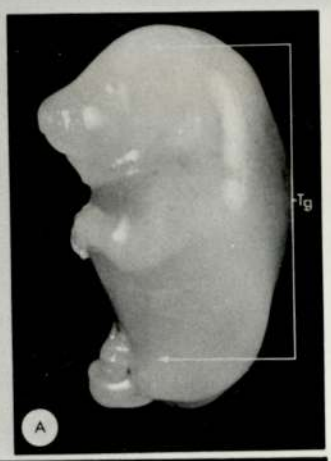


Fig. 13.23A

Macroscopic appearance of a foetus with generalised subcutaneous oedema (Tg).

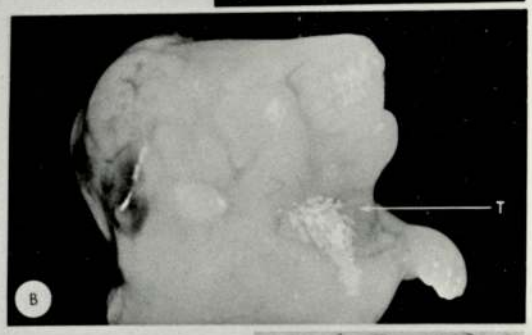
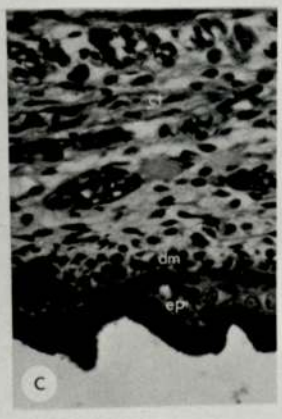


Fig. 13.23B

Macroscopic appearance of the head of a foetus with bottle-jaw oedema (T). Here the only tissue affected is associated with the lower jaw.



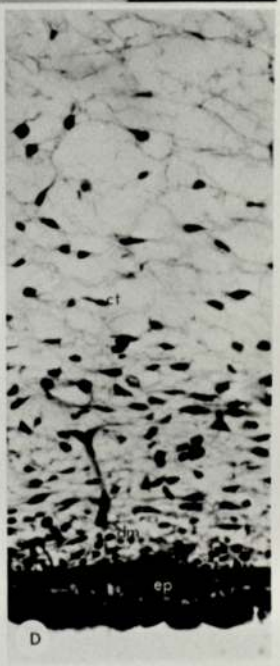


Fig. 13.23C

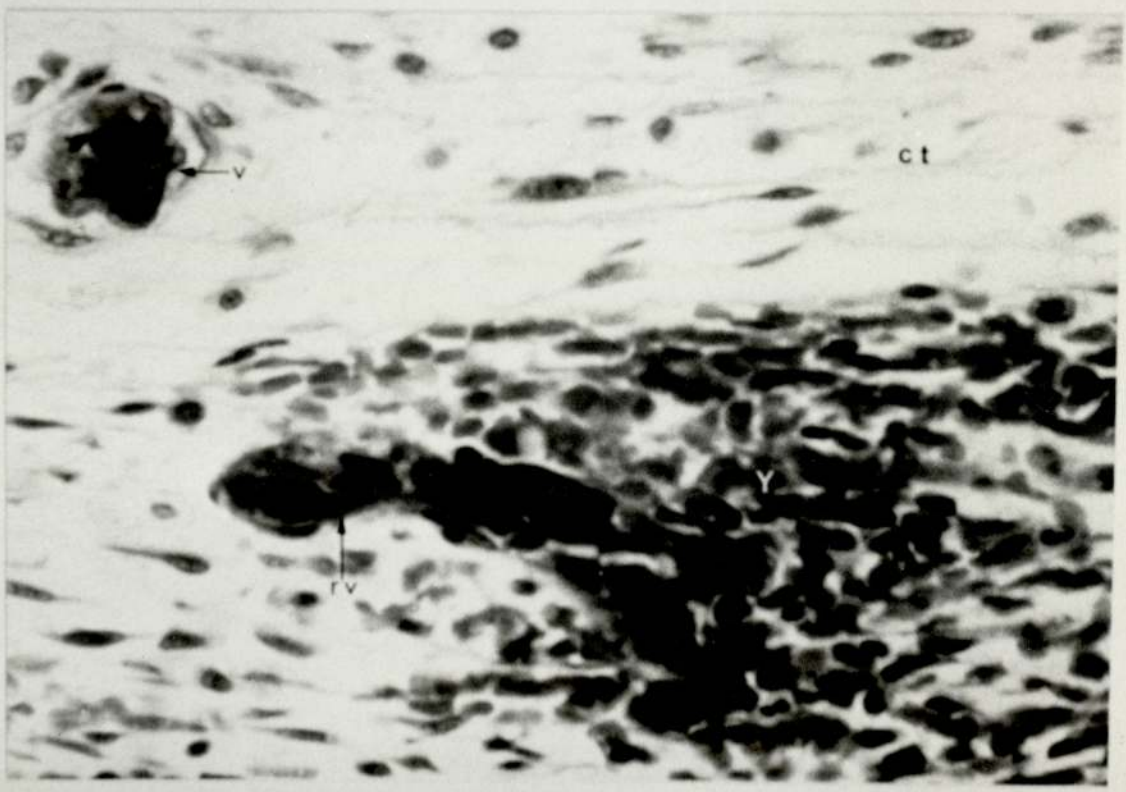
Section through the epidermis (ep), the dermis (dm) and the subcutaneous connective tissue (ct) associated with the lower jaw of a normal foetus.

Fig. 13.23D

Corresponding region of an oedematous foetus. The epidermis (ep) has remained unchanged, but the cells of the dermis (dm) and the connective tissue (ct) are distended and the fluid has displaced their nuclei to a peripheral position.

FIG. 13.24

SECTION THROUGH THE SUBCUTANEOUS TISSUE OF A RAT FOETUS SHOWING A HAEMORRHAGE (Y)



The photograph shows a normal, intact vein (v) and one that has ruptured (rv) and allowed the blood to disperse through the connective tissue (ct).

FIG. 13.25

FUSED PLACENTAE IN RATS

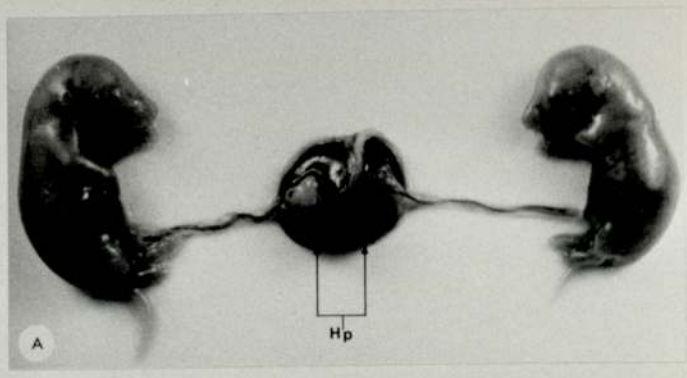


Fig. 13.25A

Macroscopic appearance of two rat foetuses whose placentae are fused (Hp).

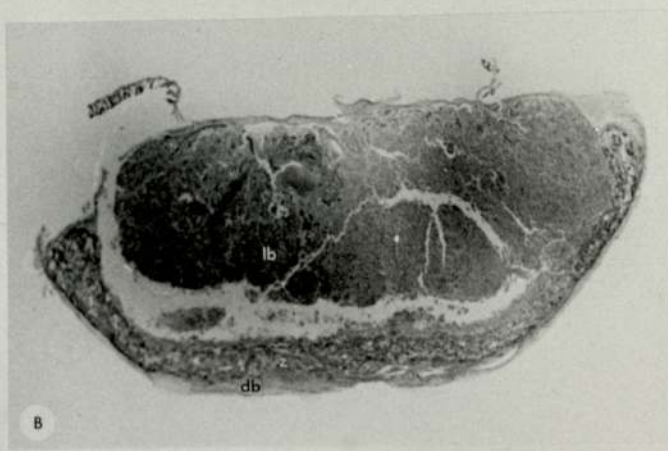


Fig. 13.25B

Tangential section through a normal rat placenta showing the labyrinth (lb), the basal zone (z) and the decidua basalis (db).

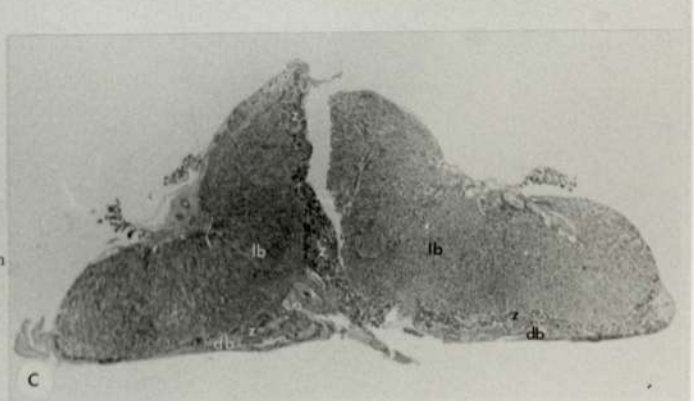


Fig. 13.25C

Corresponding section through two fused placentae. The labyrinths (lb) are discrete but, in the region of fusion, there is a common basal zone (z), having a single layer of giant cells. The decidua basalis (db) is common to both placentae but does not infiltrate into the fusion zone.

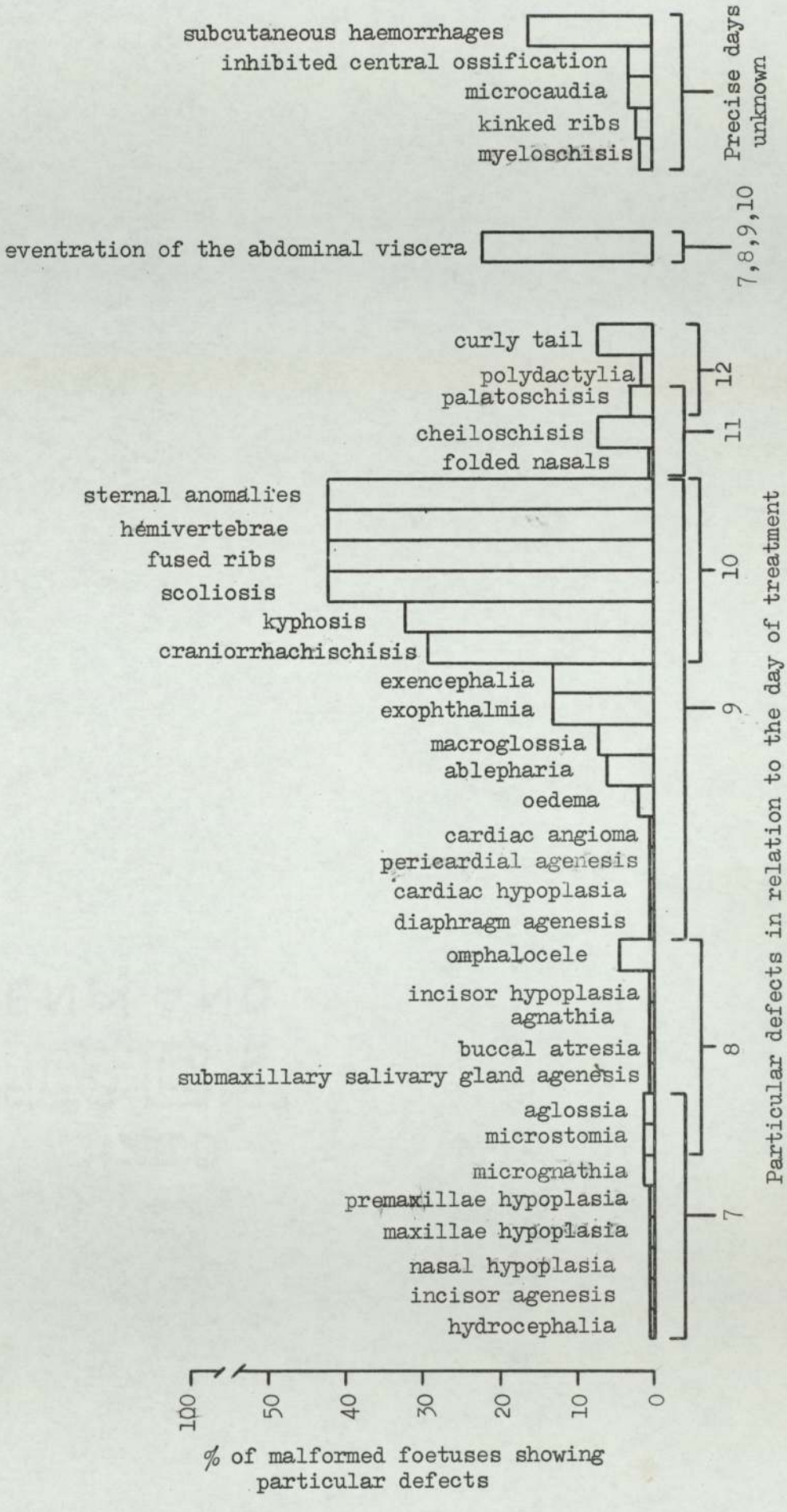


FIG. 13.26

THE INCIDENCE OF INDIVIDUAL BIRTH DEFECTS IN AH A RATS MALFORMED BY SALICYLATES

TABLE 13.1

INCIDENCE OF INDIVIDUAL BIRTH DEFECTS PRODUCED BY SALICYLATES IN AH A RATS

1534 foetuses were exposed to potentially teratogenic regimens.

Birth defects observed in 176 foetuses	No. of foetuses affected	% of foetuses affected
Scoliosis	74	42
Fused ribs	74	42
Hemivertebrae	74	42
Sternal anomalies	74	42
Kyphosis	56	32
Craniorrhachischisis	52	29
Eventration of the abdominal viscera	40	22
Subcutaneous haemorrhage	28	16
* Arthrogryposis	25	14
Exencephalia	23	13
Exophthalmia	23	13
Cheiloschisis	12	7
Macroglossia	12	7
* Curly tail	12	7
Ablepharia	11	6
Omphalocele	8	4.5
Palatoschisis	5	3
Microcaudia	5	3
Inhibited central ossification	5	3
Kinked ribs	4	2

TABLE 13.1 CONTINUED

Birth defects observed in 176 foetuses	No. of foetuses affected	% of foetuses affected
Oedema	4	2
Myeloschisis	3	1.5
Polydactylia	3	1.5
*Microstomia	2	1
*Micrognathia	2	1
*Aglossia	2	1
*Agnathia	1	0.6
Hydrocephalia	1	0.6
*Buccal atresia	1	0.6
*Cardiac hypoplasia	1	0.6
*Angioma	1	0.6
Folded nasals	1	0.6
*Agenesis of the incisors	1	0.6
*Hypoplasia of the incisors	1	0.6
*Agenesis of the pericardium	1	0.6
*Agenesis of the diaphragm	1	0.6
*Agenesis of the submaxillary glands	1	0.6
Hypoplasia of the nasals	1	0.6
Hypoplasia of the maxillae	1	0.6
Hypoplasia of the premaxillae	1	0.6

* Not previously associated with salicylate teratogenicity.

SECTION E

GENERAL DISCUSSION

CHAPTER 14

GENERAL DISCUSSION

Introduction

In the last quarter of the 19th century, it was discovered that salicylates can cross the placenta (Zweifel, 1887) and may be harmful to the conceptus (Furbringer, 1875; Binz, 1893). However, their potential embryopathic effects were not realised fully until 1959 when Warkany and Takacs produced birth defects in developing rats by treating the dams during pregnancy. In the subsequent avalanche of publications in the 1960's, salicylates were found to be teratogenic in mice (Larsson, Boström and Ericson, 1963), hamsters (Lapointe and Harvey, 1964) and rabbits (McColl, 1966) but not so in monkeys (e.g. Wilson, 1968). Among the many authors, only Larsson and his co-workers (1963, 1963, 1965, 1968, 1969) and Goldman and Yakovac (1963, 1964a, 1964b, 1965) attempted to elucidate the mechanism of the embryopathic action. The results of the former team suggested that salicylate-induced inhibition of mucopolysaccharide synthesis may be instrumental but, as was discussed in Chapter 3, other anti-inflammatory agents have this facility but are not teratogenic. The most important conclusion reached by Goldman and Yakovac was that the uncoupling of oxidative phosphorylation played no major role in the teratogenic action.

This thesis was undertaken to investigate a possible relationship between the embryopathic effects and the chemical structure of salicylates and related compounds. This aspect was accompanied by

a detailed histopathological study of many of the birth defects induced, as this was considered essential in attempting to understand their aetiology.

Principal Conclusions

The studies undertaken with Stride Dutch rabbits yielded no drug-induced congenital malformation. Aspirin, sodium salicylate and salicylic acid were all given at maximum tolerated doses and induced both maternal and embryonic toxicity. Although McColl (1966) induced cardiovascular defects in rabbit offspring by treating the pregnant does with 200 mg/kg of aspirin for an unspecified period, no comparable effect was observed with similar or larger doses by any other investigator (Earley and Hayden, 1964; Ikeda et al, 1965; Loosli et al, 1964; Schardein et al, 1969). Even McColl et al (1967) did not repeat this result and failed to mention this earlier observation in the later publication. The experiments presented in this thesis involved aspirin administered in doses twice as high as had been given previously - Loosli et al (1964) gave 300 mg/kg - and three times that reported to be teratogenic by McColl (1966).

There is no report in the literature of sodium salicylate, salicylamide or salicylic acid having been tested for teratogenesis in the rabbit. Therefore, direct comparisons cannot be made. It is noteworthy, however, that the dose of salicylamide used in the present study was nearly three times that found to produce birth defects in hamsters by Lapointe and Harvey (1964).

Trasler's.(1965) observations of a strain difference in mice in their teratogenic response to aspirin may help to explain the findings

of McColl (1966). It is possible that he used a different, more susceptible strain of rabbits than did other investigators, but he gives no indication of this in either the 1966 or the 1967 papers. In the absence of any detailed information concerning the experiments McColl undertook in 1966, valid discussion of his findings cannot be presented.

That all other reports indicate that salicylates are not teratogenic in rabbits suggests that this species is not generally susceptible. This may be related to the fate of the salicylate in the rabbit - a possibility which was discussed in Chapter 7. Certainly, the appreciable toxicity produced in both the dams and the progeny indicates that substantial amounts of the drugs had been absorbed. Further consideration was not given to this topic because it was the aim of the thesis to study birth defects. As none was produced in the rabbit, attention was directed towards the rat - the species in which Warkany and Takacs (1959) first demonstrated salicylate teratogenicity.

The sensitivity of AH A rats to the embryopathic effects of aspirin was established. A broad spectrum of defects was produced by treating the dams on any day between the 8th and the 12th post coitum. Most defects were induced by treatment at one specific time during organogenesis and reflected the particular system developing at that time. A relationship between the time at which an insult is applied and the resulting developmental defect was first suggested by Stockard (1921) who was studying the effects of hypoxia and hypothermia on different stages of sea minnow embryogenesis. In the application of this theory to mammalian teratology, allowance has to be made for the pre-implantation stages when the mammalian embryo appears to be generally insensitive to teratogenic insult. Wilson (1961) suggested

that this may be related to the embryonic cells retaining their totipotency before their differentiation to form specific parts of the whole organism. Subsequent implantation is followed by organogenesis which is characterised by the rapid division, migration and differentiation of the cells. At this stage, which occurs between 7 and 8 days post coitum in AH A rats, the circulatory systems of the embryo and the dam become intimately associated. This leaves the embryo exposed to most substances present in the maternal blood, and it becomes sensitive to teratogens. This extremely vulnerable period of organogenesis ceases after the 12th day post coitum and is followed by foetal growth. This latter period is the longest stage of development, during which only relatively few teratogens are effective.

Although birth defects generally reflect the organ primordia developing at the time of the insult, as was the case in the present study, Wilson et al (1953) produced congenital renal anomalies by irradiating rats on the 9th day of pregnancy. Attention is drawn to this because the metanephros does not appear until the 12th day. Another example of such a lack of synchronisation between the time of treatment and the resulting defect was reported by Murphy (1959); he induced skeletal abnormalities by administering alkylating agents to rats at a developmental stage when the notochord was the only apparent skeletal structure.

Treatment on individual days during organogenesis showed that the 9th day post coitum yielded the highest percentage of both malformed foetuses and intrauterine deaths. This indicated that day 9 is the most sensitive to the embryopathic effects of salicylates - a feature consistent with the observations of Warkany and Takacs (1959).

The relationship between the incidence of malformation and intrauterine death is generally a parallel one and, as well as the present study, many experiments illustrate this (e.g. Landauer, 1958; Millen and Woollam, 1957; Nelson, Asling and Evans, 1952; Waddington and Carter, 1952; Wilson, 1955).

In some cases, it appears that malformation and resorption are both sequels of the same insult, with embryonic death resulting from severe deformity. This was shown by Wilson and Warkany (1949) who found hypovitaminosis A-induced malformations, which were rarely apparent at term, in the early embryonic stages. However, Wilson (1961) cited unpublished investigations by Smithberg (1960) which showed that both phenomena can occur independently.

That drugs such as thalidomide and some antibiotics produce specific deformities is a good indication that they act directly on the embryo (Sullivan, 1970). Salicylates differ from this in that they can produce a broad spectrum of malformations involving most of the organ systems; this suggests a non-specific effect. Furthermore, salicylates given to mice during late pregnancy induced premature parturition, killed some of the foetuses and caused haemorrhaging in others (Eriksson, 1970). This result was consistent with my own observations in AH A rats given aspirin during late pregnancy; the progeny from these showed the additional characteristic of being significantly smaller than the controls. All of this evidence suggests that the salicylate embryopathic effect is not a precise one on the embryo, but rather is on some aspect of the conceptus-placenta unit.

In addition to the multiple defects already associated with salicylates, seventeen novel anomalies were described in this thesis.

Prior to the present study, the only noteworthy histological descriptions had been made by Warkany and Takacs (1959), who examined foetuses with multiple gross malformations. Consideration was given to such foetuses in the present study and, in addition, the many other defects observed were examined. Detailed discussion of this aspect of the work and the implications made from it were presented in Chapter 13 and will not be re-iterated here. Suffice it to say that aspirin appears to have an overall effect upon the differentiating embryonic systems, as do other teratogens which produce a broad spectrum of defects, such as trypan blue, x-rays, vitamin deficiency and many of the antimetabolites. The scant knowledge of the mechanism of action of most teratogens means that comparisons have to be made from the resulting defects. Careful histological examination of the salicylate-induced malformations suggests that they arose by a mechanism different from defects described for trypan blue (Gillman et al, 1948) or deficiencies of different vitamins (Nelson et al, 1957; Warkany and Schraffenberger, 1944) or x-rays (Wilson, 1954) or antimetabolites (Murphy et al, 1957).

This does not preclude chemically different teratogens from acting by a common mechanism. Runner (1959) observed remarkably similar skeletal defects in foetal mice after treating their dams with insulin, iodoacetate, a folic acid antagonist or by food deprivation. Yet chemically similar teratogens do not necessarily produce identical malformations, as Murphy (1959) showed with closely related alkylating agents.

It was not surprising that identical defects were produced by the different aspirin preparations investigated in the present study. The

differences in response to the various regimens appeared to be related to efficiency of absorption, with the more soluble forms allowing a higher blood level to be attained in a shorter time.

Discussion of the effects produced by removal or substitution of the groups in the aspirin molecule, and by altering the number and/or position of the hydroxyl groups in the salicylic acid molecule was presented in the experimental section (Chapters 10, 11 and 12). The relationship between chemical structure and teratogenic activity in AH A rats is illustrated in Table 14.1. Congenital malformations were produced in rats only when salicylic acid was present; the lack of a comparable effect by the other compounds investigated appears to be related to their failure to convert to salicylic acid. It is noteworthy that phenol, which differs chemically from salicylic acid only by possessing no carboxyl group was not teratogenic in the rat. The reverse is the case in Stride Dutch rabbits, where phenol is teratogenic but salicylic acid is not.

It was rather surprising that the addition of a second hydroxyl group to the salicylic acid molecule negated the embryopathic activity. This was contrary to what may have been expected from the general concept that the addition of these groups to aromatic compounds increases their physiological activity and toxicity (Dyson and May, 1911).

Variability of Teratogenic Response

One of the most interesting features apparent in studies on birth defects is the lack of uniformity of response to teratogens observed in a given group of animals and within individual litters. In the present study, for example, 366 rats given potentially teratogenic

regimens contained foetuses alive at term. Of these dams, only 92 (25%) had malformed pups in their litters. Further, the 366 rats produced a total of 1534 foetuses of which 176 (from the 92 litters) were malformed. It was found that the proportion of malformed foetuses in each litter was not related to the dose level of aspirin (Table 14.2). Further, the mean percentage of malformed pups decreased as the litter size increased (Fig. 14.1). This was not surprising as litters with fewer foetuses tend to be associated with more sensitive dams which, therefore, have a higher incidence of intrauterine mortality. The smaller total number of foetuses in these litters means that the proportion of malformed pups is correspondingly higher.

This confirms that the effects of aspirin on the individual pups within a litter are not independent of the litter from which they came. The probability of getting a given number of malformed pups in litters of different sizes was calculated from the data obtained from the different experiments with aspirin, and is presented in Table 14.3. The most marked low probabilities occurred where large litters contained a high proportion of malformed foetuses. The probability of getting 11 malformed pups out of a litter of 13 is $<10^{-8}$ - that is, it is virtually impossible to obtain this result if the effects on all 13 pups are independent. Similarly, the probability of getting nine malformed pups out of 14 is 3×10^{-6} (again, virtually impossible). To get even four malformed out of a litter of 12, the probability is 9×10^{-4} (that is one in 1000 cases).

These calculations serve to show that the effects of aspirin on individual embryos are not independent of the rest of the litter. Yet, some influence determines whether a given pup - but not necessarily

its siblings - is sensitive to the teratogenic action. The most striking difference between the siblings of a litter is their genetic constitution. Consequently, the possibility of an interaction between the drug and the genotype of the embryo was considered. That such an interaction can occur is demonstrated by extrinsic factors increasing the incidence of so-called 'spontaneous' defects (e.g. Runner, 1959). In the present study, the appearance of the more common spontaneous defects in AH A rats was not enhanced by salicylate. Further, attention is drawn to the experiments in which thalidomide was given to armadillos (Marin-Padilla and Benirschke, 1963); those animals show polyembryony so that all siblings in a given litter are identical genetically. However, thalidomide malformed some pups but left other litter-mates unaffected. From a clinical view-point, a number of cases of mono-zygotic twins of which one was normal while the other was deformed have appeared in the literature (e.g. Gedda and Del Porto, 1969; Pearn, 1969; Potter, 1952). Therefore, it appears unlikely that salicylates interact with specific embryonic genotypes to malform some pups while leaving siblings unscathed.

Interest was taken in the physical aspects of the foetuses, namely their position in the uterus, their size and the size of their placentae. That the uterine site may be important in relation to differential embryonic susceptibility to teratogenesis is suggested by the haemodynamic theory of foetal growth. This states that the nutrient available for foetal growth depends partly on the pressure of maternal blood, which is greater at the ovarian and cervical ends of the uterine horns than between them. Indeed, foetal guinea pigs (Ibsen, 1928),

swine (Waldorf, Foote, Self, Chapman and Casida, 1957) and mice (McLaren and Michie, 1959b) which were implanted at the ends of the uterine horns were larger than their siblings which occupied intermediate positions. It would be reasonable to surmise that embryos obtaining more nutrient would also be exposed to a greater teratogenic insult. In this event, foetuses positioned at the ends of the uterus should be more disposed to deformity.

This was not the case, although conflicting results have been obtained previously in relation to this phenomenon. Woollam and Millen (1961, 1962) found that mouse conceptuses at the ovarian end were less sensitive to the teratogenic effects of hypervitaminosis A or hypoxia than their siblings at the cervical end. This was confirmed in one study by Kalter (1963) but, in another, the reverse was the case. The discrepancy in these findings suggests that the uterine site is not an important general consideration in differential susceptibility. This was found to be so in rats treated with trypan blue (Beaudoin and Kahkonen, 1963) and is also true of the present study. The appearance of the conceptus at term in relation to uterine site in AH A rats treated with potentially teratogenic salicylate regimens is presented in Table 14.4. There is nothing to suggest that teratogenic response varied with the site of implantation.

The possibility that the weight of the foetus, particularly in relation to that of its placenta, may be related to teratogenic susceptibility was considered. The results of this survey are presented in Table 14.5. That teratogens reduce the weight of apparently normal foetuses has been reported on many occasions (e.g. Kalter and Warkany,

1957; Beaudoin and Kahkonen, 1963; Endo, 1966) and was confirmed in the present study. Further, the malformed foetuses were smaller than their normal siblings - a feature also observed by Kalter (1957) and Endo (1966), and other workers - and, as Trasler and Fraser (1958) also found, showed retarded development. This led Kalter (1968) to suggest that embryos more disposed to a delay in development may be more sensitive to some teratogens. However, in the absence of information on embryonic growth around the time of the teratogenic insult, a valid appraisal of this idea cannot be made at the present time.

In studies referring to foetal weight, consideration is rarely, if ever, given to the relative size of the placenta. A large placenta would afford a greater surface area for the passage of, among other things, teratogens which cross the placenta. This may be instrumental in disposing particular embryos to malformation. The present study (Table 14.5) indicated that the size of the placenta varied in relation to that of the foetus irrespective of malformation. However, there is no evidence of whether this was the case at the time of administration of the teratogen or occurred subsequently, and this clearly is an area which warrants further study.

Having been unsuccessful in attempting to equate foetal factors with teratogenic susceptibility, a study was made of the dams involved. The incidence of cleft palate induced by cortisone (Kalter, 1956), 6-amino-nicotinamide (Pinsky and Fraser, 1959) and 5-fluorouracil (Dagg, Schalager and Doerr, 1966) was inversely related to maternal weight. However, with the latter teratogen (5-fluorouracil), there was a direct relation between digital defects and maternal weight. Hence, there is no indication of a general influence of maternal weight. This was found

in the present study when the weight of the dams at the time of treatment with the salicylate was considered. The 92 does which had malformed pups had a mean weight of 268g, the remaining 274 which had no malformed pups had a mean weight of 249g, and a further 77 rats which showed only intrauterine resorption had a mean weight of 281g.

The possibility that the teratogen may have affected food and/or water intake of the 'sensitive' dams was also considered. It was interesting that dosing increased the water intake of both test and control animals (but not of the sham-dosed ones), but those given the salicylate drank more (Fig. 14.2). Conversely, salicylate depressed food consumption, a feature not seen in the control groups. Although there was some difference in the response of test and control rats, there was remarkable uniformity between the rats in each group, irrespective of the outcome of pregnancy.

Retrospective examination of data obtained during the conduct of this thesis has shed little new light on the problem of inter- and intralitter variability of response to teratogens. It is possible that the answer to this problem will come from multifactorial analyses rather than consideration of any one feature.

Clinical Implications from Salicylate Teratogenicity

The problems involved in extrapolating results from animal experiments to what may happen in humans are enormous and, at present, insoluble. Variation of teratogenic response occurs not only between different animal classes, different mammalian orders, different genera, different species, different strains and different litters, but also between different siblings in polyembryonous animals. Therefore, in

considering clinical implications in teratogenesis, precise information can be derived only from human studies; but this does not preclude animal test systems from providing useful indications of what may happen in man. The situation with salicylates is complicated still further because it is not always possible to extrapolate results from aspirin to other salicylates (Palazzo and Strani, 1965). The present study on salicylate teratogenesis is consistent with this finding.

Probably one of the most useful correlations between animal and clinical studies is the histopathological examination of malformed progeny. The trauma associated with the birth of humans may totally disrupt defects such as craniorrhachischisis in which vulnerable tissues are exposed. Studies on intact malformed experimental animals may provide information towards understanding the aetiology of the defect and its subsequent prevention in man.

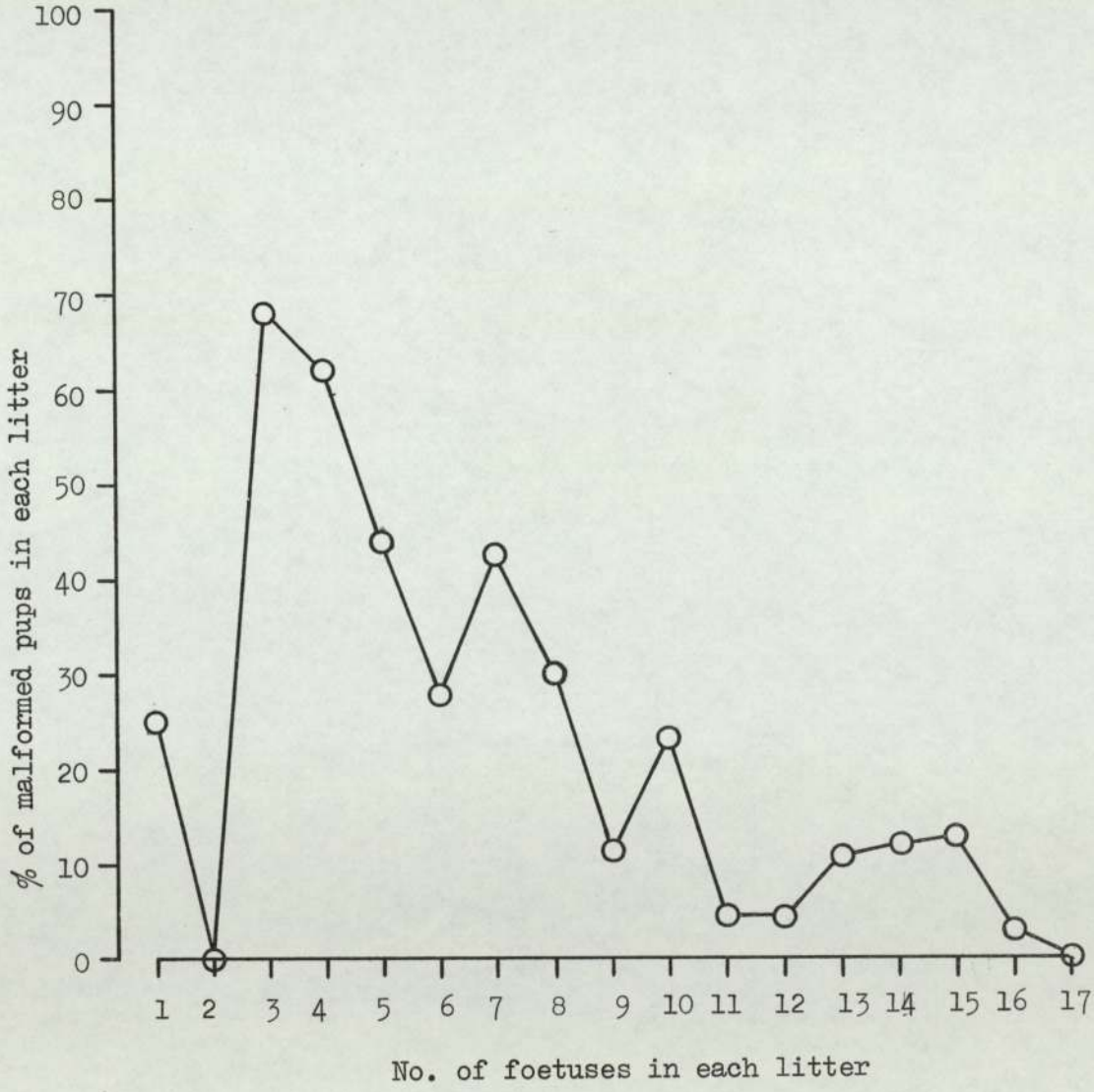
The true value of experimental animals in understanding human birth defects may not yet be realised. A significant consideration must be the embryo at the stage when the defect arises. The timing of the induction of defects can be controlled in animals, and there is obvious scope for investigation into the anatomical and biochemical changes that occur. The prevention of birth defects can be a practical possibility only when their pathogenesis is more fully understood.

However, the question of whether salicylates are teratogenic in humans still remains. The literature contains three retrospective surveys and a few isolated cases where the pregnant woman has taken salicylate during pregnancy and the child has birth defects. The discussion of these reports was presented in Chapter 3 but, in

conclusion, it is worth stressing the unsatisfactory nature of retrospective studies involving freely available drugs. The answer to the problem will come only when a controlled prospective survey is undertaken and attempts made to correlate any malformation with the stage of embryonic development at which the drug was taken - as well as attempting to relate specific defects to specific drugs.

FIG. 14.1

RELATIONSHIP BETWEEN LITTER SIZE AND THE PERCENTAGE OF FOETUSES MALFORMED



N.B. The range of experimental variation was not illustrated because it was between 0 and 100% in most cases.

FIG. 14.2

MEAN DAILY FOOD AND WATER CONSUMPTION OF RATS DOSED FROM THE 8TH TO THE 12TH DAYS OF PREGNANCY

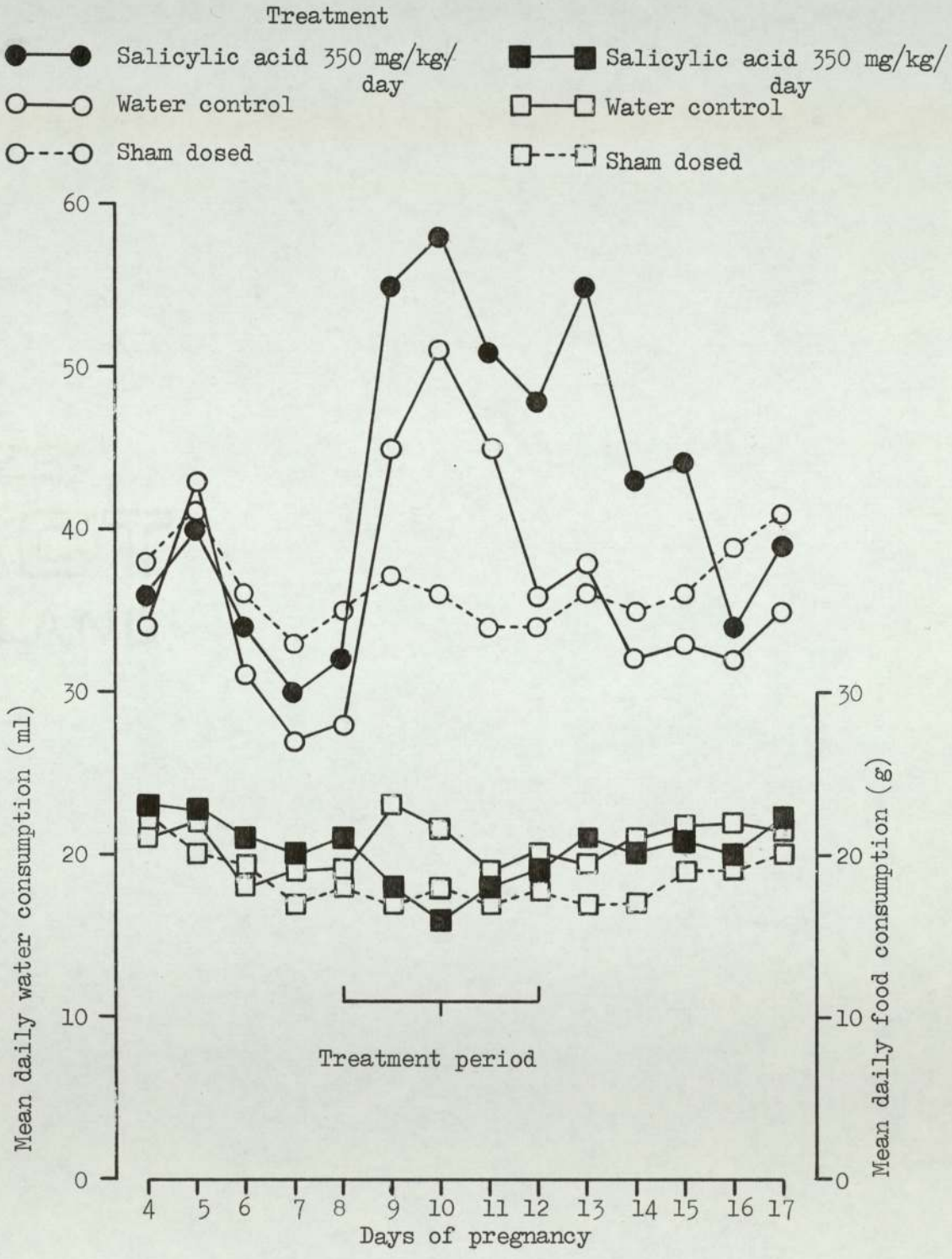


TABLE 14.1

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND EMBRYOPATHIC ACTIVITY IN AH A RATS

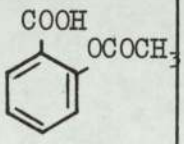
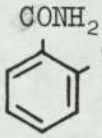
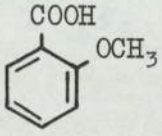
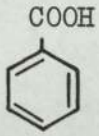
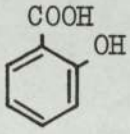
Compound		Maximum tolerated dose (mg/kg/day, days 8-12)		Teratogenicity
Name	Structure	Dam	Embryo	
Acetylsalicylic acid 200*		>500	250	+
Soluble		>455	325	+
Micronised		>350	>350	+
Micronised+ Mannoxol OT/P		>350	>350	+
Salicylamide		1500	1500	-
Phenol		350	350	-
o-Methoxy-benzoic acid		5000	5000	-
Benzoic acid		>1250	>1250	-
Salicylic acid		>350	350	+

TABLE 14.1 contd/

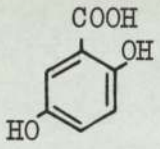
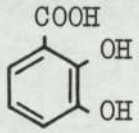
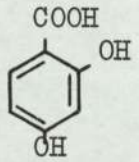
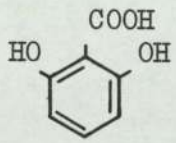
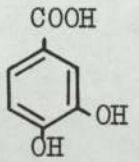
Compound		Maximum tolerated dose (mg/kg/day, days 8-12)		Terato-genicity
Name	Structure	Dam	Embryo	
2,5-Dihydroxy-benzoic acid		>3000	>3000	-
2,3-Dihydroxy-benzoic acid		1450	1450	-
2,4-Dihydroxy-benzoic acid		2000	2000	-
2,6-Dihydroxy-benzoic acid		900	900	-
3,4-Dihydroxy-benzoic acid		>3000	>3000	-

TABLE 14.2

THE RELATIONSHIP BETWEEN THE DOSE LEVEL OF SOLUBLE ASPIRIN AND THE PROPORTION OF MALFORMED PUPS IN EACH LITTER

Dose level (mg/kg/day, days 8-12)	200	225	250	275	300	350
Mean proportion of malformed pups per litter	0.257	0.506	0.085	0.225	0.068	0.464

TABLE 14.3

THE PROBABILITY OF GETTING A GIVEN NUMBER OF MALFORMED FOETUSES IN A LITTER, ASSUMING THAT THE EFFECTS ON ALL PUPS IN THE LITTER ARE INDEPENDENT

Total No. of live foetuses in the litter	No. of malformed pups in the litter. 0	1	2	3	4	5	6	7	8	9	10	11
1	3	1										
2	2	0	0									
3	1	0	0	2								
4	1 .02	0	1	0	2 .15							
5	2	0	1	0	1	1 .016						
6	2	0	0	0	0	1 .007	0					
7	0	0	1	1	1 .22	0	0	0				
8	1	0	1	0	0	1 .042	0	0	0			
9	4	0	2	1 .057	0	0	0	0	0	0		
10	1	1	0	0	0	0	1 .011	0	0	0	0	
11	8	0	1	1 .01	0	0	0	0	0	0	0	0
12	10	0	1	0	1 *	0	0	0	0	0	0	0
13	7	3	1	0	0	0	0	0	0	0	0	1 ⊠
14	5	0	1	0	0	0	0	0	0	1 ∅	0	0
15	1	0	1	0	1 .089	0	0	0	0	0	0	0
16	1	1	0	0	0	0	0	0	0	0	0	0
17	1	0	0	0	0	0	0	0	0	0	0	0

* 9×10^{-4} ⊠ $< 10^{-8}$ ∅ 3×10^{-6}

TABLE 14.4

THE PERCENTAGE OF RESORPTION SITES AND NORMAL AND MALFORMED FOETUSES AT TERM IN RELATION TO UTERINE SITE

Uterine site 1= cervical end	No. of implantation sites	% of implantation sites terminating as		
		Normal foetuses	Malformed foetuses	Resorption sites
1	138	45	16	39
2	134	44	15	41
3	133	46	15	39
4	131	45	23	32
5	122	48	18	34
6	105	44	22	34
7	82	49	18	33
8	50	58	20	22
9	24	29	21	50
10	12	39	25	36
11	7	14	0	86
12	2	50	0	50
13	1	0	0	100
14	1	0	0	100

TABLE 14.5

THE RELATIONSHIP BETWEEN AH A RAT FOETAL AND PLACENTAL WEIGHTS AND THE SUSCEPTIBILITY TO SALICYLATE TERATOGENESIS

Category	No. of foetuses	Mean weight of foetuses (g)	Mean weight of placentae (g)	Foetal weight: placental weight ratio
Normal control foetuses	1016	2.34	0.96	0.410
Normal foetuses from rats exposed to potentially teratogenic regimens of salicylate	1716	1.98	0.81	0.409
Malformed foetuses	176	1.60	0.68	0.425

S E C T I O N F

APPENDICES

APPENDIX 1

DIETARY CONSTITUENTS

The constituents of the Oxoid pasteurized breeding diet fed to the rats in the present study are listed in Appendix 1, Table 1, and are those published in 1969 by Oxoid Ltd. The only relevant information which B.O.C.M. would divulge about their coney rabbit pellets was that this diet contained more grass meal than most other diets, and it had the normal blend of added vitamins and minerals. However, they did provide the percentages of oil, protein and fibre, and these also appear in Appendix 1, Table 1.

APPENDIX 1 TABLE 1

CONSTITUENTS OF THE DIETS FED TO THE RATS AND THE RABBITS IN THE PRESENT STUDY

Constituent	Oxoid pasteurized breeding diet	B.O.C.M. coney rabbit pellets
Crude oil	3.8%	2.5%
Crude protein	20.5%	18 %
Crude fibre	2.6%	14 %
Digestible crude oil	3.1%	
Digestible crude protein	17.9%	
Digestible crude fibre	1.2%	
Digestible carbohydrate	49 %	
Essential fatty acids	1.7%	
Non-essential fatty acids	2.0%	
Oleic acid	0.7%	
Linoleic acid	1.1%	
Lysine	1.24%	
Methionine	0.48%	
Cystine	0.34%	
Tryptophan	0.26%	
Valine	0.98%	
Leucine	1.52%	
Isoleucine	0.95%	

APPENDIX 1 TABLE 1 contd/

Constituent	Oxoid pasteurized breeding diet	B.O.C.M. coney rabbit pellets
Threonine	0.69%	
Phenylalanine	0.95%	
Histidine	0.45%	
Arginine	1.17%	
Tyrosine	0.67%	
Glycine	1.70%	
Non-essential amino acids	7.51%	
Calcium : Phosphorus ratio	1.0 : 1.04	
Calcium	0.77%	
Phosphorus	0.80%	
Sodium	0.50%	
Chlorine	0.77%	
Magnesium	0.20%	
Potassium	0.87%	
Sulphur	0.27%	
Iron	210 mg/kg	
Copper	30 mg/kg	
Manganese	85 mg/kg	
Cobalt	1 mg/kg	
Zinc	28 mg/kg	
Iodine	2 mg/kg	
Vitamin A (includes vitamin A content of carotene)	5390 iu/kg	
Vitamin D_3	1480 iu/kg	
Vitamin E	97 mg/kg	
Vitamin B_1	5.5 mg/kg	
Vitamin B_2	9.5 mg/kg	
Niacin	54.2 mg/kg	
Pantothenic acid	18.6 mg/kg	
Choline	2000 mg/kg	
Biotin	0.13 mg/kg	
Folic acid	0.40 mg/kg	
Vitamin B_6	11.5 mg/kg	
Inositol	2800 mg/kg	
Vitamin B_{12}	37.9 µg/kg	
Vitamin K	1.5 mg/kg	
Carotene	0.5 mg/kg	

APPENDIX 2

DETERMINATION OF THE PARTICLE SIZE OF MICRONISED ASPIRIN

The particle size of the micronised aspirin was determined microscopically according to the technique described in the British Standards Institution publication B.S. 3406: Part 4: 1963. A brief synopsis of this method, which utilises a light microscope, is presented here. The procedure involves the determination of the number and volume or weight of the sizes of particles in a powder. This is effected with the use of circles having the same projected area as the particles, and can be employed for particles whose size is between 0.8 and 150 μ.

A representative sample of the powder is dispersed in light liquid paraffin with Mannoxol OT/P on a glass slide and examined microscopically. The particles can be compared with standardised reference circles which are simultaneously visible on a graticule. The number of particles in each size class can then be determined and used to calculate the relative volumes within the different size classes. This is based on the assumption that particles of all sizes have the same shape. Then the relative volumes are an indication of size distribution by volume. If all sizes of particles have the same density, size distribution by volume is the same as size distribution by weight.

The large number of particles on the slide makes it impractical to count every one. Consequently, counts are restricted to sample areas and the accuracy of the technique largely depends upon the uniform dispersion of the particles and the number counted.

The particle size of the micronised aspirin was determined by this method and the results are presented in Appendix 2, Table 1.

APPENDIX 2 TABLE 1

PARTICLE SIZE OF MICRONISED ASPIRIN:

CALCULATED ON A WEIGHT BASIS

Lower limit of particle size in class (μ)	Percentage in class (on weight basis)	Standard error
9.37		
6.63	10.49	1.20
4.68	9.32	0.68
3.31	6.53	0.35
2.34	24.93	2.55
1.66	31.72	2.07
1.17	6.26	0.85
0.83	8.48	0.81
0.64	2.28	0.28

APPENDIX 3

SKELETAL STAINING TECHNIQUE

(1) Skin and eviscerate the foetus and fix in 70% alcohol.

(2) De-fat in acetone.

(3) Wash in 70% alcohol.

(4) Stain in toluidine blue for 60 hours.

(5) Wash and differentiate in 96% alcohol.

(6) Macerate in 1% aqueous potassium hydroxide.

(7) Stain in 0.001% alizarin red S in 1% aqueous potassium hydroxide for 3 days.

(8) Clear through graded glycerin (25%, 50%, 75% aqueous).

(9) Store in pure glycerin.

Toluidine blue stain

Toluidine blue	6 g
0.5% Hydrochloric acid	20 ml
70% Alcohol - to	1000 ml

APPENDIX 4

RADIOGRAPHIC TECHNIQUE

The rabbit foetuses were x-rayed with a Newton-Victor K.5 portable x-ray machine, with the film 60 cm vertically below the anode, for ¾s. The current was 15 mA and the kV was approximately 62. Ilford Ilfex envelope-packed x-ray films 20.3 x 25.4 cm were used. The foetuses were positioned on the plate by means of a simple jig designed specifically for that purpose. The exposed films were developed in Ilford Phen-X x-ray developer for 3½ minutes at 22°C and fixed in Ilford Ilfofix for 10 minutes at 22°C. The dry radiographs were examined with the use of a Kodak Coldlight Illuminator, Series 2.

APPENDIX 5

MAYER'S HAEMATOXYLIN AND EOSIN TECHNIQUE

(1) De-wax in xylol.

(2) Wash in absolute alcohol.

(3) Wash in water.

(4) Stain in Mayer's haematoxylin for 1½ minutes.

(5) Blue in tap water.

(6) Stain in 1% aqueous eosin for 1 minute.

(7) Differentiate in tap water.

(8) Dehydrate in absolute alcohol.

(9) Clear in xylol.

(10) Mount in D.P.X.

Mayer's haematoxylin

Haematoxylin	1 g
Distilled water	1000 ml
Potassium alum	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate	50 g

APPENDIX 6

DETERMINATION OF SERUM-SALICYLATE LEVELS

A synopsis of the method described by Keller (1947) is presented below.

Reagents

(A) 1% hydrated ferric nitrate in 0.07N nitric acid.

(B) Diluted ferric nitrate. Dilute 5 parts of Reagent A with 4 parts of water.

(C) 0.038N nitric acid. 0.07N nitric acid can be obtained by making 4.69 ml of concentrated nitric acid (sp gr 1.42, 70.5%) up to 1000 ml with water. 0.038N nitric acid can be obtained by diluting 5 parts of this reagent with 4 parts of water.

(D) Stock salicylate solution. Dissolve 1 mg of salicylic acid in 1 ml water and dilute 1 to 10 with water (0.1 mg salicylic acid per ml).

Preparation of a standard curve

Sera were pooled from five rats which had not received salicylate. The procedure outlined below (1-4) was undertaken using these sera. The optical density represents the colour intensity obtained from the reaction of acidified ferric nitrate with normal serum. This value is substracted from the total optical density obtained.

Take seven tubes and to six of them add 0.5, 1, 2, 3, 4 and 5 ml of Reagent D. Make all seven tubes up to 5 ml with water. These represent blood salicylate levels of 0-50 mg. To each tube add 5 ml of 1% ferric nitrate solution. Read these on the colorimeter, after

first reading the blank tube to adjust the transmission to 100%. Then add the value for the normal serum to each reading. The standard curve can be prepared by plotting these values against the corresponding blood levels on a rectangular graph.

Procedure

(1) Add 9 ml of Reagent B to 1 ml of serum in a colorimetric tube.

(2) Add 9 ml of Reagent C to 1 ml of serum in another tube.

(3) Set the wavelength at 530 mm μ and adjust the colorimeter to 100% transmission with the acidified serum. Read the colour intensity of the reacted serum.

(4) Read the serum-salicylate level directly from the standard curve.

APPENDIX 7

INCIDENCE OF SPONTANEOUS CONGENITAL MALFORMATIONS IN AH A RATS

Malformation syndrome	No. of cases observed	Incidence (%)
Subcutaneous haemorrhages	313	1.631
Fused placentae	14	0.073
Kinked ribs	9	0.050
Microcaudia	6	0.031
Curly tail	3	0.016
Omphalocele	2	0.010
Generalised subcutaneous oedema	2	0.010
Giant foetus, limb flexures	1	0.005
Giant foetus	1	0.005
Omphalocele, limb flexures, subcutaneous haemorrhages	1	0.005
Eventration of the abdominal viscera, scoliosis, hemivertebrae, fused ribs	1	0.005
Exencephalia	1	0.005
Diaphragmatic hernia	1	0.005
Bilateral auricular hypertrophy	1	0.005
Unilateral micromelia, subcutaneous haemorrhages	1	0.005
Polydactylia, phalangeal agenesis	1	0.005
Limb flexure	1	0.005

APPENDIX 8

INCIDENCE OF SPONTANEOUS CONGENITAL MALFORMATIONS IN STRIDE DUTCH RABBITS

Malformation syndrome	No. of cases observed	Incidence (%)
Limb flexures	70	0.907
Splayed limbs	12	0.155
Ablepharia	8	0.104
Microcaudia	4	0.052
Ablepharia, exencephalia	3	0.039
Partial splenic agenesis	3	0.039
Splenic hypertrophy	2	0.026
Renal hypertrophy	2	0.026
Acephalia, scoliosis, hemivertebrae, fused ribs, kyphosis, eventration of the abdominal viscera, limb flexures, phalangeal agenesis	1	0.013
Cranioschisis, palatoschisis, macroglossia, partial pinnal agenesis	1	0.013
Eventration of the abdominal viscera, limb flexures, microcaudia, imperforate anus, urinogenital fistula	1	0.013
Hydrocephalia	1	0.013
Lipoid necrosis of the ocular lens	1	0.013
Talipes equinovarus, phalangeal agenesis	1	0.013
Eventration of the abdominal viscera	1	0.013
Meningocele	1	0.013
Scoliosis, hemivertebrae, fused ribs	1	0.013
Kinked ribs	1	0.013
Ventricular hypertrophy	1	0.013
Cranioschisis, hydrocephalia, micrognathia	1	0.013
Arthrogryposis	1	0.013
Microcaudia, limb flexures	1	0.013
Phalangeal agenesis	1	0.013

APPENDIX 9

INCIDENCE OF SPONTANEOUS CONGENITAL MALFORMATIONS IN AH BEAGLE DOGS

Malformation syndrome	No. of cases observed	Incidence (%)
Palatoschisis	8	0.257
Unilateral radial agenesis	2	0.064
Anencephalia, cranial teratoma, ablepharia, macrophthalmia, palatoschisis	1	0.032
Palatoschisis, microcaudia, urorectal fistula, agenesis of the external genitalia	1	0.032
Anencephalia, palatoschisis, unilateral radialagenesis	1	0.032
Palatoschisis, bilateral hare lip, pulmonary agenesis, liver stasis	1	0.032
Microcaudia, unilateral renal agenesis, unilateral renal hypertrophy, urorectal fistula, agenesis of the external genitalia	1	0.032
Stillborn, macroglossia, partial pinnal agenesis, oedema in the hind limbs	1	0.032
Palatoschisis, hare lip	1	0.032
Macroglossia	1	0.032
Hydrocephalia	1	0.032
Agenesis of the ventral abdominal wall, the posterior region of the ventral thoracic wall, the xiphisternum, the liver, the stomach, the small and large intestines and the urinogenital system, imperforate anus	1	0.032
Urorectal fistula	1	0.032

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