EFFECT OF PODOPHYLLIN AND TEMPERATURE ON SKELETAL DEVELOPMENT OF THE HOLTZMAN ALBINO RAT

A Thesis

Presented to

The Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

by

Julian Jonathan Dwornik, B.A., M.Sc.

May 1969

EFFECT OF PODOPHYLLIN AND TEMPERATURE ON SKELETAL DEVELOPMENT OF THE HOLTZMAN ALBINO RAT

An Abstract of a Thesis

Presented to

The Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

by

Julian Jonathan Dwornik, B.A., M.Sc.

May 1969

Pregnant Holtzman albino rats were given podophyllin by stomach tube in doses ranging from 0.012 to 1.6 milligrams per 100 grams body weight of the animal for various periods from the eighth to twelfth days of gestation. Most fetuses, as well as ten-day-old newborn rats of podophyllin-treated mothers, were cleared in 1 per cent potassium hydroxide, the skeletons stained with alizarin red S, and the specimens examined under a dissecting microscope. Remaining fetuses were cross sectioned with a razor-blade and then studied under a dissecting microscope.

Some evidence was obtained to suggest that podophyllin is capable of inhibiting intrauterine growth. Severely runted viable fetuses were observed on the twenty-first day of pregnancy in animals that had received 0.1 milligram of the drug daily. This was noted in 4.2 per cent of these animals. This was not, however, observed in any fetuses of treated control or untreated control (normal) animals.

Although considerable variation was noted in fetal ossification in animals treated with podophyllin, there were no major skeletal malformations. It was not reasonable to conclude that the drug was responsible for these variations because time as a variable, was not incorporated into the experimental design. Changes have been proposed for similar experiments in order to make future studies more meaningful even in the absence of major skeletal defects.

Widespread variation in ossification was noted in fetuses of animals in the various treated and the one untreated control group.

Although there are a number of factors that may account for this variability, fluctuating environmental temperature is believed responsible, since it was not possible to maintain a constant temperature throughout the entire course of this two-year investigation.

No visceral variations were observed in razor-blade cross sectioned fetuses of animals that had been treated with podophyllin. In skeletons of ten-day-old newborn animals from mothers treated with podophyllin, slightly dumbbell vertebral centra were observed, but it cannot be proved that this was a podophyllin effect.

In another study, pregnant Holtzman albino rats were exposed to various fixed (gradient) temperatures ranging from 60 to 90 degrees Fahrenheit, and to a fluctuating temperature of 65 and 90 degrees Fahrenheit for twenty-one days of gestation. Using the alizarin technique, it was noted that fetuses of mothers exposed to fixed temperatures of 70, 75, and 80 degrees Fahrenheit had the fewest number of skeletal variations.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Professor Emeritus I. Maclaren Thompson, former Head of the Department of Anatomy, and to his successor, Dr. Keith L. Moore, for giving me the opportunity to study in the department. In addition I wish to express my appreciation to Dr. Moore for his kind encouragement and friendly guidance of this research project and the preparation of this thesis. I also want to thank Dr. James B. Longley, Professor and Chairman, Department of Anatomy, University of Louisville, for giving me assistance in completing the revisions of this thesis.

I am also indebted to the following persons who have made it possible for me to complete this thesis: Dr. R. G. LaFlèche, Mr. G. Geddes, Miss J. C. Hay, and Mrs. O. Neuendorff, for their laboratory assistance; and Mr. M. Stover, for photographic assistance. I wish to thank Mr. S. Vivian, for his helpful guidance in the statistical analysis. The author is also grateful to Mr. E. Clune, and the late Mr. E. Peterson, for their capable care of the animals. Finally I wish to thank my wife Diane, and my daughter Pamela, for their patience and understanding during the writing of this manuscript.

This study was supported by funds made available to Dr. K. L. Moore, from the Medical Research Council of Canada and from the University of Manitoba.

TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER

I INTRODUCTION

At present there are many laxatives (Carter's liver pills, posalfin, choleflavin), and also some cold and influenza preparations sold which contain podophyllin (Excerpta Medica Foundation, 1964). Clark and Parsonage (1957) reported a case history of a woman who, after having ingested podophyllin, developed peripheral neuropathy; this symptom was also noted in some individuals who had taken thalidomide (Fullerton and Kremer, 1961). Cullis (1962) has reported one case of congenital deformities in which the mother had taken podophyllin (among other substances) from the fifth to the ninth week of her pregnancy, this being a critical period in organogenesis. The baby had multiple anomalies. Some of the defects observed were similar to those seen in children born to mothers who had taken thalidomide. This weakly suggests that podophyllin might be a teratogen, especially because it is an antimitotic drug.

A search of the literature has not revealed any studies indicating that podophyllin has been sufficiently tested as a possible teratogen in mammals. However, because it is well established that podophyllin is a mitosis-inhibiting drug (Karnofsky, 1965), it may also be a teratogenic agent. Consequently substances containing podophyllin should not be taken by pregnant women, or by women in the child-bearing age.

The main objective of the present investigation has been to study podophyllin with special emphasis on its possible teratogenic effects in rats, in an attempt to determine if it might cause some of the human developmental anomalies now attributed to chance. Attempts have been made to improve some of the current methods used for testing potential teratogens in the rat; this has included a study designed to establish the temperature at which minimum normal skeletal variations occur during development.

II REVIEW OF THE LITERATURE

Origin of Podophyllin, Its Properties, and Constituents

Podophyllin was discovered accidentally in 1835 by Dr. John King (Zakon, 1952). Podophyllin being a resin is also referred to as podophyllum resin, resina podophylli, and podophyllinium (Hartwell and Schrecker, 1958). Podophyllum, from which the resin podophyllin is extracted (yield not less than 5 per cent), consists of the dried rhizomes and roots of *Podophyllum peltatum* Linnaeus (family: Berberidaceae); common names: May apple, mandrake, Indian apple, wild lemon, duck's foot (Kelly and Hartwell, 1954; Remington's Practice of Pharmacy, 1958). This herbaceous perennial is an indigenous, North American, plant flowering in May and bearing fruit in late summer or autumn.

Remington's Practice of Pharmacy (1958) states that the resin podophyllin occurs as an amorphous powder, varying in colour from light brown to greenish-yellow. The powder turns darker when exposed to light or temperatures exceeding 77 degrees Fahrenheit (25 degrees Centigrade); therefore it should be stored in a dark, cool place. Podophyllin has a characteristic odour and a bitter, acrid taste (The Extra Pharmacopoeia, 1952). The resin which is soluble in alcohol (completely or almost) forms a slightly opalescent, faintly acid solution (Sullivan, 1949; The Extra Pharmacopoeia, 1952). The resin is also soluble in normal solutions of potassium and sodium hydroxide. It is partially soluble in hot water (precipitates on cooling), chloroform, ether, and a dilute solution of ammonia, but insoluble in cold water. The podophyllin used in this study is a commerical extract from *Podophyllum peltatum* and will be referred to as either podophyllin or resin.

Hartwell and Schrecker (1958) have indicated that twelve well-characterized compounds have been isolated from either the roots and rhizomes of *Podophyllum peltatum* or its resin. Of these, eight appear to be present in the resin podophyllin. The following are the names of the individuals and the substances which they obtained from podophyllin. Podwissotzki (1881) was the first to isolate (in 1880) and name podophylotoxin (in 1881); a colourless, crystalline substance. In addition he noted the presence of a pigment which he called podophylloquercetin. He later recognized its close similarity to quercetin in analysis, melting point, and certain physical and chemical properties. Dunstan and Henry (1898) were able to completely purify this pigment and almost conclusively established the identity of podophylloquercetin with quercetin.

For approximately fifty years there was no interest in podophyllin. Then Kaplan (1942) reported that topical application of podophyllin in oil (to twenty patients) cured condylomata acuminata (venereal warts). In an effort to determine the substance and/or substances (in the resin) responsible for producing cell death in the condylomata, investigators analyzed the resin using modern techniques. Hartwell (1947) extracted alpha-peltatin. Hartwell and Detty (1948) obtained another form and called it beta-peltatin. In 1954, two additional compounds were isolated; Kofod and Jørgensen (1954) extracted dehydropodophyllotoxin and Kelly and Hartwell (1954), picropodophyllin glucoside. According to Hartwell and Schrecker (1958), the picropodophyllin glucoside was possibly produced by epimerization of podophyllotoxin glucoside. Kofod and Jørgensen (1955) also obtained desoxypodophyllotoxin.

The last substance to be isolated (present only in trace amounts) was 4'-demethylpodophyllotoxin (Bartek et al., 1955; cited by Hartwell and Schrecker, 1958).

The Pharmacological Actions of Podophyllin and Its Constituents in Man and Other Mammals

There are records extending back several hundred years indicating that the root of *Podophyllum peltatum* was used as a purgative and emetic (Kelly and Hartwell, 1954). Following the discovery of podophyllin by King, in 1835 (Zakon, 1952), investigators have studied this drug and its components since 1880 for pharmacological and physiological effects in man and other mammals (Kelly and Hartwell, 1954). Previous studies were restricted primarily to the ability of these compounds to exhibit catharsis or to act as choleretics.

More recently, attempts have been made to treat cancer patients with podophyllin and its compounds using parenteral or oral routes. However, a limiting factor has been the severe gastrointestinal discomfort which results from the use of high doses of either podophyllin, podophyllotoxin, alpha, or beta-peltatin (Kelly and Hartwell, 1954).

Podophyllotoxin was generally thought to be the active component of podophyllin when purgation was the criterion (Dixon, 1902; Viehoever and Mack, 1938). It has since been indicated, however, that a cathartic action is also produced by alpha-peltatin, beta-peltatin, and 4'-demethylpodophyllotoxin (Greenspan and Leiter, 1949; Kelly and Hartwell, 1954).

Podophyllin and podophyllotoxin, when used as purgatives, produce griping and unpleasant sensations sometimes accompanied by nausea and vomiting (Kelly and Hartwell, 1954). Podophyllin, although not an ideal laxative, is still used in the preparation of cathartic pills and proprietary medicines. Some of these are: choleflavin, posalfin, Carter's liver pills and also some cold and influenza tablets (Excerpta Medica Foundation, 1964).

Effects on Intestine and Gall Bladder. Studies have been done to determine the mechanism by which podophyllin and its constituents produce catharsis.

Gruber, Richardson and Bryan (1932) intravenously injected podophyllin (varying from 20 to 35 milligrams) to non-anesthetized dogs with Thiry-Vella loops. They noted a loss of tone in the intestine and disappearance of rhythmic contractions; these effects were followed by the recurrence of rhythmical contractions. They also injected podophyllin (25 milligrams dissolved in 50 per cent alcohol) directly into the intestinal loop and observed temporary relaxation of the gut which was followed by an increase in general tonus, marked peristaltic activity, and bluish-black discoloration of the exposed intestinal surface.

A similar study was conducted in dogs by Kelly, Truant and Smith (1949) using podophyllotoxin (0.5 and 0.75 milligram per kilogram suspended in saline). They noted an irregular decrease in amplitude of contractions, with a slight decrease in tone which increased at times. The rhythmicity of the contractions remained normal and typical intestinal activity returned in three hours.

The Extra Pharmacopoeia (1941) described podophyllin and podophyllotoxin as cholagogues, however, there is little agreement on this statement among investigators. Some workers have stated that podophyllin and podophyllotoxin enhance the production of bile (Bain, 1898; Bauer and Spiegel, 1919; Stewart and Ryan, 1928; Petrovsky and Pavlenko, 1939).

It has also been indicated by Baldi (1883), Pitini and Fernandez (1914 to 1918), and Steinmetzer (1926), that podophyllin does not enhance the production of bile (cited by Kelly and Hartwell, 1954).

More recent studies have conclusively shown that podophyllin, podophyllotoxin, and Carter's Little Liver Pills containing podophyllin, (administered intravenously, orally, and duodenally to dogs and humans) do not produce any detectable effect on the formation of bile, evacuation of the gall bladder, or on the passage of bile into the duodenum (Ivy, Annegers and Atkinson, 1942; Ivy, Roback and Stein, 1942; Ivy, DeHoog and Gutmann, 1945; Ivy, Roth and Gutmann, 1945; Case and Powers, 1946).

Effects on the Cardiovascular and Respiratory Systems. The cardiovascular effects of nonlethal doses of podophyllin and podophyllotoxin are relatively mild, transitory and irregular in occurrence (Neuberger, 1891; Boyd, 1928; Gruber, Richardson and Bryan, 1932; Hazelton, 1942; Philips, Chenoweth and Hunt, 1948; Kelly, 1951). Podophyllin and podophyllotoxin administered at lethal doses, however, result in failing respiration and muscular dystrophy accompanied by a fall in blood pressure to shock levels (Boyd, 1928; Gruber, Richardson and Bryan, 1932; Philips, Chenoweth and Hunt, 1948; Kelly, 1951; Sullivan, Follis and Hilgartner, 1951).

Philips, Chenoweth and Hunt (1948) administered lethal doses (amounts not stated) of podophyllotoxin to dogs. Initially they observed respiratory stimulation. Terminally respiration was laboured and slow, and death followed respiratory failure. Fatal doses given to rats and cats, but not dogs, often caused severe pulmonary damage. Greenspan and Leiter (1949), using various routes, administered alpha-peltatin, beta-peltatin, and podophyllotoxin (at sublethal or lethal doses) to mice, rats, rabbits, and dogs and noted a respiratory depression.

Effects on the Hematopoietic System. The early literature does not contain any references on the action of podophyllin or its compounds to the hematopoietic system (Kelly and Hartwell, 1954). However, in recent years evidence of damage to this system has been acquired with the result that attempts are now being made to treat individuals having lymphomas and leukemias.

Sánchez Caballero and Ergueta Collao (1949) intraperitoneally administered podophyllin (first 0.25 milligram, and later 0.50 milligram each two or three days) to white female rats weighing 125 to 245 grams. In one group, each animal received an accumulated dose of 10.5 milligrams of podophyllin distributed over a period of sixty to seventy-five days; this produced a marked decrease in the number of white blood cells. In another group each animal received 10.5 milligrams of podophyllin given over a period of forty days; a 50 per cent leukopenia was observed in each of these animals. In the second groups studied the animals died.

Greenspan and Leiter (1949) gave podophyllotoxin, alpha-peltatin, and beta-peltatin parenterally, in the maximum tolerated single dose to mice, rats, rabbits, and dogs. Following injection of sublethal or lethal doses, they observed a definite pattern of response in formed elements of peripheral blood and bone marrow. One half to two hours after injection, a leukopenia developed which was followed by leukocytosis (with degenerating neutrophiles and lymphocytes). Normoblastosis and a terminal leukopenia were observed twenty-four to forty-eight hours after injection. At lethal doses they noted degeneration and aplasia of bone.

Kelly et al. (1949) studied the effects of podophyllotoxin (by intramuscular and intravenous routes) in normal white and CAF mice with sarcoma 37. Within one hour after intraperitoneal injection of podophyllotoxin (1 milligram per kilogram) they observed a 50 per cent decrease in the leukocyte count. The counts stayed at this level for several days. They also noted similar results in animals given three doses at two day intervals. In these animals the counts were low for ten days following the last treatment, however, after fourteen days, the counts were back to normal levels.

Waterman (1950) studied the effects of podophyllotoxin (0.2 to 0.4 milligram) on an inbred strain of mice which developed leukosis. He observed cellular damage in the spleen and lymph nodes.

Kelly et al. (1951b) studied blood changes in adrenalectomized rats treated with a single intraperitoneal injection of podophyllotoxin at toxic doses. Within one hour they observed a significant leukopenia (mononuclear, polymorphonuclear, eosinophilic). By twenty-four hours, the

normal leukocyte count was re-established and no other changes were detected. They also noted that following treatment with podophyllotoxin, the ratio of spleen weight to total body weight was increased in adrenalectomized rats and decreased in normal animals. These investigators suggested that the spleen was involved in producing leukopenia which was present in the adrenalectomized rats.

When Kelly et al. (1952) administered a single toxic dose of podophyllotoxin intraperitoneally (10 milligrams per kilogram) to intact and adrenalectomized rats, they concluded that it exerted a direct cytotoxic action of the hematopoietic and thymicolymphatic systems. A relatively nontoxic injection of podophyllotoxin (1 milligram per kilogram) appeared to be a combination of, or a balance between, a specific cytotoxic action of the drug and a nonspecific adrenotropic effect. These authors also observed evidence of cytological damage in the bone marrow, spleen, thymus, lymph node, and adrenal gland.

Eyestone (1953) subcutaneously injected podophyllotoxin, alpha-peltatin, and beta-peltatin, to normal mice, rats, rabbits, and dogs. He stated that fatal doses of these drugs caused severe depletion of the hematopoietic cells in the bone marrow; the cells most strikingly affected being the lymphocytes, thymocytes, and myeloid cells. There were no residual effects detected in animals recovering from single or multiple injections of the different drugs.

Effects on the Nervous System. Podwissotski (1880 - 1882) subcutaneously injected dogs with a lethal dose of podophyllotoxin (0.005

gram per animal). Approximately two hours after injection, the animals walked awkwardly on the hind legs and fell down easily (cited by Viehoever and Mack, 1938).

Dudley (1890) observed depression, and other disorders, in two individuals (wife and husband) who had each taken about 5 grains (0.32 gram) of podophyllin. The wife died thirty-one hours after taking the drug but the husband remained in a depressed state for a period of two or three weeks and finally recovered.

Disque (1913) subcutaneously injected cats with a solution of podophyllin (0.01 gram in 1 per cent alcohol per animal) and observed paralysis. He also noted the same disorder in these animals when the drug (0.01 gram) was given in pill form.

Philips, Chenoweth and Hunt (1948) administered fatal doses (amount not stated) of podophyllotoxin to dogs. They observed muscular weakness, hind-leg ataxia, and eventual prostration.

MacCardle and Perrault (1949) subcutaneously administered podophyllin (30 micrograms per gram of body weight) to chickens with Rous sarcomas. Nineteen hours after injection, these birds were unable to stand due to loss of muscular coordination. Histological studies, using silver stain, of the central nervous system revealed the following: many cerebellar Purkinje cells were completely degenerated or damaged; there was fragmentation or loss of dendrites; degeneration of neurofibrils, and chromatolysis. Cell bodies in the red nucleus showed vacuolization and loss of pigment. In the spinal cord the lower motor neurons appeared normal. These workers also noted that whereas motor end organs of the femoral and pectoral muscles normally have bulbous endings, the

end organs in the treated chickens consisted of dense plaques of fine granules. Similar studies were done using mice with intramuscularly implanted sarcoma 37. These animals were also subcutaneously injected with podophyllin (30 micrograms per gram of body weight). Twenty-four hours after injection functional and structural cerebellar damage were not observed in these animals.

Sullivan, Follis and Hilgartner (1951) used mice and rats to study the toxicology of podophyllin and podophyllotoxin. Each animal received, subcutaneously or orally, a single LD_{50} dose of either podophyllin or podophyllotoxin (dosage 8 to 90 milligrams per kilogram). Eight hours after podophyllin administration, the animals were observed to have a dragging gait and hyper-extended posterior extremities. At nine hours there was a period of excitation, during which the animals ran wildly, followed by spastic convulsions. Similar observations were seen in animals given podophyllotoxin.

Ward et al. (1954) reported a case of an eighteen-year-old pregnant girl who had a large condyloma acuminata of the vulva. Twenty-five per cent podophyllin ointment was applied to the lesion. Approximately twenty-five hours after application the patient was in a stupor. At thirty hours all deep tendon reflexes were absent. The autopsy findings were as follows: the leptomeninges of the brain were congested and showed a few mononuclear and red cells; the cortex exhibited rarefaction, and the white matter (appearing fragmented and swollen) contained many capillaries with wide perivascular spaces. Occasionally neuronophagia and increased glial elements were observed.

Clark and Parsonage (1957) reported on a twenty-five-year-old woman who had orally taken podophyllin (2.8 grams in 1 ounce of 75 per cent ethanol). Their clinical findings were as follows:

> "There was, however, a fairly severe degree of general weakness of the limbs, which were hypotonic and exhibited gross incoordination with associated pseudo-athetotic movements of the outstretched hand and fingers. The plantar responses were flexor in type, but the tendon-jerks were either extremely sluggish or absent, and there was impairment distally in the limbs of all modalities of sensation, postural sense being lost in the digits."
>
> "A substantial amount of recovery of function had occurred at the end of 6 months after ingestion of the podophyllin, but a residual neurological deficit was still evident at the end of 16 months."

Effects on Other Tissues and Organs. There are reports of individuals who have suffered local irritating reactions following contact with podophyllin. Rosner (1946) observed one patient with an "all red and puffed up" eye. This was followed by an ulceration of the surface epithelium and considerable loss of sight. The eye regained its normal appearance and vision one month after the onset of the condition. Keim (1947) and Lane (1947) each noted a similar incident.

Keim (1947) also observed extensive and severe dermatitis in the cubital fossa. Also noted were superficial vesiculation with denudation and considerable edema in other areas. Lane (1947) observed pronounced erythema and edema of the penis, scrotum, perineum, and perianal region.

Additional toxic symptoms have been observed in various species of animals and man given either the resin or one of its compounds. These

have been: damage to the liver, prostration, and death (Viehoever and Mack, 1938; Sullivan, Follis and Hilgartner, 1951; Ward et al., 1954).

Metabolism. Kelly et al. (1951 a), and Kelly and Hartwell (1954) investigated the metabolism of podophyllotoxin (one of the constituents of podophyllin). They developed a bioassay method, based on the lethality of podophyllotoxin to the chick embryo, and applied it to the study of the disposition of podophyllotoxin by the intact animal, and of the action of animal tissues on the drug *in vitro*. Acetone extraction of material from the carcasses of mice and rats killed at intervals following injection with podophyllotoxin was inoculated into the yolk sac of chick embryos. They noted that the drug had been metabolized by the intact animal within one hour to a product which was nonlethal to the chick embryo and insoluble in acetone. Furthermore there was no evidence to indicate that the unaltered drug was accumulated or retained in the blood, liver, spleen, kidney, or tumour tissue.

These investigators also showed that incubation of podophyllotoxin with homogenates (of carcass, blood serum, red blood cells, liver, spleen, kidney, intestine, and tumour tissue) of untreated rats and mice did not appear to alter or destroy the drug in four hours. Using this method, they did not obtain any evidence of unaltered podophyllotoxin excretion in rats and dogs.

Kocsis, Walaszek and Geiling (1957) studied the disposition of biosynthetically labelled C^{14} podophyllotoxin in normal and tumour-bearing mice and hamsters. Normal mice were each injected (subcutaneously) with 1 milligram of C^{14} podophyllotoxin; each of the tumour-

bearing mice were injected with half of this dose (0.5 milligram). Kocsis and his colleagues noted that the mice with sarcoma 180 excreted approximately two times more radioactivity in the urine (in the four hours following drug injection) than the non-tumour-bearing animals. Urinary excretion of radioactive-labelled podophyllotoxin was retarded in mice bearing Ehrlich ascites tumours.

Hamsters with fibrosarcomas were each injected (subcutaneously) with 0.5 milligram of C^{14} podophyllotoxin (per 100 grams body weight). These workers observed that the normal hamsters excreted (in the urine) about two times more radioactivity than the hamsters with fibrosarcomas; this was at all time intervals investigated up to 72 hours.

Kocsis and his associates did not detect any unchanged podophyllotoxin in the urine of mice treated four hours before with C^{14} podophyllotoxin. According to them, results on two mice given 1 milligram of C^{14} podophyllotoxin indicated that between 5 and 6 per cent of the injected dose can be recovered from the carcass and from 0.3 to 1.2 per cent in the intestines of such animals. Mice given C^{14} podophyllotoxin (250 micrograms; the abbreviation mcgs. appeared in the original article and was interpreted to mean micrograms) were able to convert (in twenty-four hours) between 5 and 7 per cent of the injected dose to $C^{14}O_2$. In hamsters, however, it was noted that they converted (in six hours) a much smaller percentage of the labelled drug to $C^{14}O_2$ than did mice in this same period of time (Kocsis, Walaszek and Geiling, 1957).

Freedberg (1965) studied *in vivo* and *in vitro* the effects of podophyllin upon macromolecular metabolism. In an *in vivo*, *in vitro* study, the dorsal surfaces (on one side of each animal) of epilated

guinea pigs were painted with a 25 per cent podophyllin solution. Punch biopsies were taken at various time intervals (following podophyllin application) and incubated in the presence of C^{14} leucine. The epidermis (separated from the dermis) was then studied to determine the amount of labelled amino acid in the epidermal proteins. Freedberg observed (following a single application of podophyllin) a marked stimulation of amino acid incorporation into the epidermal proteins. He also noted that within two hours, protein synthesis in the epidermis increased (six times) and then decreased to control levels. Similar results (as in the previous experiment) were observed using L-methionine-methyl C^{14} and of C^{14} leucine.

Freedberg, in an *in vitro* study, placed untreated slices of guinea pig skin into tubes containing various concentrations of podophyllin. Podophyllin caused inhibition rather than stimulation of amino acid incorporation into protein. Furthermore, the inhibitory effect was dose related with the concentrations of substance approximating those in the *in vivo* studies.

Podophyllin, tested on ribonucleic acid synthesis (using modifications of the slice technique) showed inhibition of total C^{14} uridine incorporation and a decrease in the specific activity of epidermal ribonucleic acid. This inhibition was observed as early as fifteen minutes after the application of podophyllin. Stimulation of ribonucleic acid synthesis was never observed.

Freedberg injected podophyllin (3, 15, and 30 milligrams per 100 grams body weight) into mice (each dose to separate groups). Twenty hours later the mice were killed and liver slices from these animals

were incubated with C^{14} leucine. Liver slices from animals treated with a 3 milligram dose of podophyllin did not stimulate the liver proteins to incorporate C^{14} leucine. However, liver slices, taken from mice given 15 and 30 milligrams of podophyllin (doses per 100 grams body weight), stimulated incorporation of C^{14} leucine into liver proteins. Freedberg, also observed that stimulation occurred within four hours (after podophyllin injection of 15 milligrams per 100 grams body weight) and progressed for the next fifteen to twenty hours. Similar studies, on incorporation of C^{14} orotic acid into liver ribonucleic acid, showed no effect at the above mentioned doses of podophyllin.

Freedberg, further studied the effect of podophyllin and podophyllotoxin on cell-free amino acid incorporation. A cell-free amino acid-incorporating system was prepared from the livers of mice which earlier (fifteen hours) had intraperitoneally been given podophyllin (3 and 15 milligrams per 100 grams body weight) and podophyllotoxin (5 milligrams per 100 grams body weight). The preparations made from animals receiving podophyllin (3 and 15 milligrams per 100 grams body weight) stimulated amino acid incorporation whereas those preparations made from the podophyllotoxin treated mice caused inhibition. When the dose of podophyllotoxin (given to mice) was lowered to 1 milligram per 100 grams body weight, stimulation was also observed.

Freedberg states, "The fact that podophyllin caused increased amino acid incorporation associated with either inhibition of RNA synthesis (skin system) or no change in RNA synthesis (liver system) pointed to a direct effect upon the protein synthetic pathway. This

possibility has been confirmed by the cell-free experiments in which we have shown that podophyllin resulted in more efficient ribosomal protein synthesis."

Effects of Podophyllin and Its Constituents on the Enzyme Systems

A single subcutaneous injection of podophyllotoxin, alpha, or beta-peltatin to mice results in a marked drop in the cytochrome oxidase activity of homogenates from implants of sarcoma 37 (Waravdekar and Leiter, 1949; Waravdekar, Domingue and Leiter, 1952; Leiter, Paradis and Waravdekar, 1953). In addition these investigators observed the same effects in other excised transplantable tumours of the same animal. In the tumour tissue, the cytochrome oxidase activity dropped most rapidly during the first eight hours following drug injection, however, in homogenates of spleen, liver, kidney, and testes, the drop in activity was not as marked; even at above lethal doses.

Miller, Davison and Smith (1949) noted *in vitro*, the effect of podophyllotoxin in a variety of enzyme systems. They observed respiratory inhibition in the following tissues: kidney, thymus, tumour, spleen, lymph nodes, testis, and brain of rats, also sarcoma 37 implants in mice, and chick embryos. Inhibition became more pronounced with time. Several enzymes tested by these investigators were not inhibited by podophyllotoxin.

Clinical Uses of Podophyllin and Its Constituents

It has been mentioned previously that Kaplan (1942) was probably

the first to report on the use of podophyllin for the treatment of condylomata acuminata. Since that time numerous investigators have used this drug and its constituents to treat various types of disease, including tumours.

Kelly and Hartwell (1954) reviewed the biological effects of this resin and also its chemical composition. They found that since 1942, workers have been using podophyllin and its compounds extensively for the treatment of various disorders. Some of these are: condyloma acuminata, granuloma inguinale, verrucae, molluscum contagiosum, tinea capitis, nonspecific dermatoses, metabolic diseases, benign new growths, and malignant new growths.

Currently many investigators are doing experimental studies on tumours using podophyllin and its constituents. Some of these are: Chatard, 1957; Jakobi, 1956; Liska, 1956; Kozakiewicz, 1958; Greiner, Klotz and Lang, 1959; Richardson, 1959; Firstater, 1961; Tomin, 1967.

Effects of Podophyllin and Its Constituents on the Cell

Kaplan (1942) appears to be the first to record the use of podophyllin for the treatment of condylomata acuminata; more commonly called venereal warts (Sullivan and King, 1947; Kelly and Hartwell, 1954). Prior to this time the cytological effects of this drug were either unknown or were not recorded. Since then King and Sullivan (1946) (1947), while treating condylomata acuminata, noted a similarity of effects in epithelial cells exposed to either podophyllin or colchicine. Shortly thereafter, it was established that podophyllin, and later that

some of its compounds, were mitotic inhibitors. Many studies on the cytological effects of podophyllin and/or its compounds have since been done in a variety of plants and animals (Kelly and Hartwell, 1954).

Effects on Mammalian Cells. Padawer and Gordon (1956) studied the effects of colchicine and podophyllotoxin on rat and hamster mast cells. They injected podophyllotoxin or colchicine intraperitoneally to rats (1 milligram per 100 grams body weight). In the rats, one to three hours after podophyllotoxin injection, they obtained mast cells from the peritoneal fluid which were damaged similar to cells of rats which had been given colchicine. These results, reproduced *in vitro* by the addition of colchicine or podophyllotoxin to rat peritoneal fluid, also suggested to them that these drugs may act directly on the cells.

Hamsters, known to be more resistant to colchicine than rats, also required more colchicine (10 milligrams per 100 grams body weight) to induce damage to their mast cells.

Podophyllotoxin produced changes in the peritoneal fluid mast cells (in both rats and hamsters) identical to those observed following colchicine treatment (Padawer and Gordon, 1956). One dose of podophyllotoxin (1 milligram per 100 grams body weight) was sufficient to produce effects in mast cells of both species, but two different doses of colchicine were required (1 milligram and 10 milligrams per 100 grams body weight to rats and hamsters respectively).

Spendlove et al. (1964) studied *in vitro* the effect of podophyllin (and seven other antimitotic agents) on a variety of mammalian cells infected with intracellular reovirus antigen (three types; 1, 2 and 3).

These workers state that intracellular reovirus antigen localizes in the area occupied by the achromatic figure of mitotic cells. In untreated interphase cells the reovirus forms a filamentous, intracytoplasmic reticulum, but in the presence of certain spindle poisons it consolidates into clumps.

Spendlove and his associates observed that podophyllin (concentrations of 40 to 50 micrograms per liter) clumps (in human amnion cells) the antigen of the three types of intracellular reovirus. They also noted that the antigen of type 1 reovirus was consolidated into clumps in various cells, however, different concentrations of podophyllin were required to produce clumps in each of the infected cell types.

These results indicate that spindle poisons affect intracellular reovirus antigen indirectly by their action on the spindle or on a closely related organelle of similar physicochemical structure (Spendlove et al., 1964).

Effects on Plant Cells. Sullivan and Wechsler (1947) put onion root tips (*Allium cepa*) into a saturated aqueous solution of podophyllin. Later they noted mitotic inhibition of the meristematic tissue in late prophase, that spindle formation was impaired in metaphase, and that there was subsequent absence of the anaphase and telephase figures. Sullivan (1947) observed similar results when he soaked petunia leaves in either podophyllin or colchicine solutions (cited by Kelly and Hartwell, 1954).

Miller, Davison and Smith (1949) in a germination study exposed radish and cucumber seeds, and corn kernels to 0.001, 0.01, and 0.2 per cent solutions of podophyllotoxin. They did not observe any effect of the drug on germination.

Effects of Podophyllin and Its Constituents on Marine Eggs, Planarians, and Newts

Cornman (1947) studied the effect of podophyllin on *Arbacia* (sea urchin) egg cleavage. He noted that the effect of this resin appeared to represent the combined effects of podophyllotoxin and quercetin; the latter two compounds present in podophyllin. Cleavage was slowed slightly over a wide range of concentrations (0.4 to 2.0 milligrams per liter). At threshold concentrations (4.0 to 6.0 milligrams per liter) cleavage of some eggs was completely inhibited but in the remainder there was only moderate delay. Continued study showed that podophyllotoxin and quercetin were more active than colchicine. *Arbacia* was not as sensitive to the effects of podophyllotoxin as were two other echinoderms; the *Asterias* (starfish) eggs and *Echinarachnius* (sand dollar). Cornman (1949) also noted that *Asterias forbesii* eggs, subjected to a podophyllin concentration of 1 milligram per liter, resulted in the mitotic or meiotic spindle being quickly inactivated at prophase, metaphase, or anaphase.

Additional studies (using podophyllin, some of its compounds, and colchicine) were done using the previously mentioned marine eggs and those of the sea urchins *Tripneustes esculentus* and *Lytechinus variegatus*, and the sea slug *Chromodoris* (Cornman and Cornman, 1951). Results of these experiments showed that the cytological effects (destruction or crippling of the achromatic meiotic or mitotic figure) produced by these drugs were essentially alike. Furthermore, podophyllotoxin was shown to be the most active. Quercetin was capable of severely retarding division but did not impede any stage of development.

Goldberg and Black (1948) studied the effects of podophyllin in regenerating *Planaria dorotocephala*. Planarias were decapitated directly anterior to the pharynx. The posterior sections were treated with podophyllin and the type and rate of regeneration were observed. To the cut surfaces they applied podophyllin paste and allowed it to remain there for a duration of one to five minutes. The planarias were then washed off thoroughly with water. In these worms the regeneration time for the heads was from ten to thirty-three days as compared to five days for the untreated controls. In subsequent experiments adverse effects were also observed. In many of the podophyllin treated planarias the regenerated heads were atypical, many of them presenting either single median or lateral eyes.

Sentein (1951 a and b) investigated the effect of podophyllin and colchicine using newt eggs, *Triturus helveticus*. He noted many similar effects using the two drugs. Polyploidy resulted if podophyllin and colchicine were used before fission commenced. If however, segmentation had started then the new fissures were inhibited and the formation of blastomeres with polynuclei were observed. In addition both drugs were capable of producing spindle depolarization.

Effects of Podophyllin and Its Constituents on Other Organisms

Grainger (1947) studied the effect of podophyllin on S and R strain *Eberthella typhosa*. One gram of podophyllin was added to each of two flasks containing 100 milliliters of nutrient broth; the S strain was placed into one flask and the R strain was placed into the other one.

Daily subcultures were made from these flasks on nutrient agar plates. Podophyllin did not have any effect on the colonial character of these two strains of bacteria.

Reiss and Doherty (1949) (1951) used podophyllin (1 per cent in aquaphor and 0.2 per cent in carbowax) for the treatment of tinea capitis (ring worm of the scalp) which was primarily caused by *Microsporum audouini* and *Microsporum lanosum*. Although no final conclusions were made, their results suggested to them that podophyllin could be considered as a therapeutic agent for treatment of tinea capitis.

Young (1951) tested the effect of podophyllotoxin *in vitro* and *in vivo* on *Microsporum audouini*. In the *in vitro* study, infected hairs were placed in 1.0 and 3.3 per cent podophyllotoxin solutions in 90 per cent alcohol. The hairs were left in the solutions for periods ranging from one minute to forty-eight hours and then cultures on Sabouraud's dextrose medium at room temperature. The hairs in the 1 per cent solution of podophyllotoxin showed retarded growth after five minutes, advanced inhibition occurred in thirty mintues, and complete cessation of growth was observed at sixty minutes. The *in vivo* study consisted of fourteen Negro patients with *Microsporum audouini* infections of their scalps. To these, daily (for thirty days excluding Saturdays and Sundays) topical applications of 3.3 per cent podophyllotoxin in 90 per cent ethanol resulted in both a clinical and mycological cure of nine individuals (64 per cent).

Contrary to the *in vitro* results observed by Young (1951), Monash (1952) repeated the study using the same concentration of podophyllotoxin and showed that this drug is not an effective fungicide for hairs infected with *Microsporum audouini*.

Robbins, Bourke and Smith (1950) studied, using fertile New Hampshire Red eggs, the effect of podophyllotoxin (plus three hundred and fourteen other chemicals) on *Rickettsia typhi* infection (commonly referred to as *Rickettsia moorseri*). On the seventh day after incubation of the eggs, they injected 0.0008 milligram of podophyllotoxin per egg along with 0.2 milliliter of rickettsial suspension. They were able to ascertain that podophyllotoxin was ineffective against *Rickettsia typhi* infection.

Schubert (1948 a) tested over four hundred compounds, including podophyllin, on mice infected with *Shistosoma mansoni*. Intraperitoneal injections of podophyllin (0.08 milligram in 0.25 milliliter of water per day) to mice (infected eight weeks prior to the test) for twelve days were not effective in treating this condition. Schubert (1948 b) did another study in which he tried to determine the effectiveness of eighty drugs (including podophyllin) in stopping the development of the adult shistosoma. Treatment was for five days, being initiated immediately after exposure of the mice to infection. Podophyllin was not effective in the treatment of this parasite.

Cornman and Cornman (1951) noted that protozoa and diatoms were viable in podophyllin concentrations (100 milligrams per liter) which had previously poisoned some marine eggs.

Taylor (cited by Kelly and Hartwell, 1954) noted that amoeba cultures were slightly inhibited by podophyllin.

Effects of Podophyllin and Its Constituents on Mammalian Development

Man. There has been only one case recorded, in which it was known with certainty that a woman during pregnancy had taken podophyllin and other substances contained in herbal "slimming tablets" (Cullis, 1962). The report was as follows:

> "A woman, aged 24, with mild pre-eclamptic toxaemia was induced surgically at term. The baby, a female weighing 4 lb. 14 oz., had multiple deformities. The right thumb and radius were absent. There was an extra thumb on the left side. There is probably a septal defect in the heart. The right external ear was malformed, and there were skin tags on the right cheek and in the region of the right ear.
>
> "The mother had taken herbal "slimming tablets" for 3-1/2 weeks from the 5th to the 9th week of her pregnancy--a critical period of foetal development. Two tablets had been taken three times a day. They consist of: Ext. sacred bark gr. 1/6 (10 mg.), P. podophyllin gr. 1/2 (30 mg.), Ext. fuci gr. 1/3 (20 mg.), and Ext. boldea fragrans gr. 1-1/2 (100 mg.)."

In addition, Cullis mentioned that podophyllin, like thalidomide, could cause polyneuritis and that it was also a cell-poison which inhibits mitosis. He advocated a ban on all substances with possible teratogenic effects--such as podophyllin. This case, although indicating a possible teratogenic action of podophyllin, does not prove it. The association may be coincidental.

Mouse. Didcock, Picard and Robson (1952) intravenously (0.05 milligram to 2.0 milligrams) and subcutaneously (0.1 to 2.5 milligrams) administered podophyllotoxin in 10 per cent alcohol to pregnant mice (days not stated). An effect was usually obvious twenty-four hours after

treatment when a profuse blood-stained vaginal discharge was seen, commonly followed by reabsorption, but occasionally by abortion. Also large and apparently normal placentas were observed *in utero* of fetuses which were dead and undergoing reabsorption.

Wiesner and Yudkin (1955) administered podophyllotoxin either as a solution in aqueous alcohol or as a suspension of microcrystals in water. Swiss mice each received a single subcutaneous dose of 0.25 milligram either on the same day that mating was confirmed (by vaginal plug) or three, six, twelve, or fourteen days thereafter. Occasionally some of the animals showed interruptions in their pregnancies if given the drug within the first twenty-four hours after copulation. These animals were mated once more, given podophyllotoxin, and again pregnancy did not continue. This had been repeated for three successive pregnancies and each time similar results were noted. The subsequent fourth pregnancies were not treated and they went to term quite normally. Pregnancies did not continue in animals treated three or more days after copulation.

Rabbit. Didcock, Picard and Robson (1952) also included rabbits in their study. A solution of podophyllotoxin in absolute alcohol was given to pregnant rabbits (days and routes not stated). A dose of 18 milligrams per kilogram terminated pregnancy in two out of five mothers treated. When 35 milligrams per kilogram were given, three out of four mothers terminated their pregnancies. There were no deaths in either of the two groups of mothers.

Didcock, Jackson and Robson (1956) further investigated the effect of podophyllotoxin on pregnancy in rabbits. They injected the resin (0.2 to 100 micrograms) into either the amniotic fluid or placenta around the seventeenth day of gestation. Laparotomies were done five to eight days after injection. They noted that the toxic effects of podophyllotoxin were apparently exerted directly on the fetus and not on the placenta. Furthermore this drug, a spindle poison, was capable on consistently interrupting pregnancy in doses which were not toxic to the mother.

Rat. Didcock, Picard and Robson (1952) administered podophyllin (0.25 milligram per 100 grams intravenously or 0.6 milligram per 100 grams subcutaneously) to pregnant rats. The resin was suspended in 10 per cent alcohol. The days of drug administration were not stated. The effects were similar to that observed in mice.

Thiersch (1963) intraperitoneally administered podophyllotoxin and podophyllin separately to groups of pregnant rats (strain, Long-Evans). Both compounds were suspended in distilled water. All animals were killed on the day before term and the surviving fetuses were fixed, cleared, and stained with alizarin.

The podophyllotoxin (0.5 to 50 milligrams per kilogram) was given daily on various days of gestation between and including the sixth to the sixteenth days of gestation. The podophyllin (0.5 to 20 milligrams per kilogram) was given daily from and between the sixth to the twenty-first days of gestation.

Thiersch, observed that the single Ld_{50} for the adult rat was approximately 15 milligrams per kilogram. Both compounds were more toxic to the fetuses than to the mothers. The eleventh and twelfth days seemed to be the critical days for drug administration because when the podophyllotoxin (5.0 milligrams per kilogram) and podophyllin (5.0 milligrams per kilogram) were each administered to rats on the eleventh and twelfth days of pregnancy, there were resorption rates of 95 and 90 per cent respectively. Both drugs (0.5 or 1.0 milligram per kilogram) when given daily over longer periods of pregnancy resulted in a loss of 18 to 75 per cent of the fetuses. It was observed that generally both drugs stunted fetal growth. Using alizarin-stained fetuses, macroscopic anomalies were not detected.

Effects of Temperature on Pregnant Mammals

Sundstroem (1927) placed mated rats in a hot room environment (temperature not given) and in two instances only, observed pregnancies which proceeded to full term with viable offspring. In the other mated animals the majority of the fetuses were dead and the occurrence of typical resorptions were observed. In addition Sundstroem, reviewed the literature on the physiological effects of tropical climate.

Ogle (1934) subjected mice to a warm, humid environment. The temperatures used were 88 to 92 degrees Fahrenheit (31.1 to 33.3 degrees Centigrade) and the relative humidity was approximately 75 per cent. Of the total matings observed, only a low percentage resulted in pregnancy.

Also the litter size was reduced and only a few of these were viable. Furthermore animals reacted most unfavourably to reproduction when exposed to fluctuating environmental conditions (temperature and relative humidity).

Ingalls, Curley and Prindle (1952), observed in pregnant mice an average of more than eight offspring per litter, however, during the hottest part of the summer they noted that reproduction almost ceased.

MacFarlane, Pennycuik and Thrift (1957) observed a resorption rate of 7 per cent when pregnant Wistar rats were subjected to temperatures of 71.6 to 82.4 degrees Fahrenheit (22 to 28 degrees Centigrade) and relative humidity 60 to 80 per cent. However, when the temperature was 95 degrees Fahrenheit (35 degrees Centigrade) the resorption rate increased to 58 per cent. A noticeable decrease in resorption rates, at 95 degrees Fahrenheit, occurred following the administration of either vitamins and protein, progesterone or thyroxine. Also there was a highly significant reduction in resorptions if the animals were first acclimatized (from two to ten weeks) at 95 degrees Fahrenheit.

Intrauterine Development of the Rat

Long and Burlingame (1938 - 1944) studied the development of various external features in prenatal rat development. They related these observations to the days on which particular structures appeared and the equivalent number of somites. At eight and one-half days the blastocyst was formed and by eight and three-quarter days three germ layers were visible. On the ninth day the mesoderm had reached its

greatest extent and by nine and one-half days they observed blood filled spaces. Although there were no somites present on the tenth day, there were some on day ten and one-quarter (four to five). The earliest evidence of the optic fovea was noted on days ten and one-half (seven somites). The mandibular arches were widely separated ventrally by the eleventh day (sixteen somites). The anterior limb bud formed on days eleven and one-quarter (twenty-one somites), appearing as lateral swellings between the sixth and tenth somites. Also the hyoid arch was present. The posterior lumb bud also appeared as a lateral swelling on day twelve and one-half at the thirty-four somite stage. Growth of the anterior limb continued to be more advanced than in the posterior. Each of the limbs became longer and the distal ends flattened, became five-angles, and then lobed. Eventually the lobes became transformed into digits with claws. By days nineteen and one-half, the digit on the inner margin of the anterior foot was reduced in size to a mere knob and never acquired a claw. In addition the fifth toe was also smaller than the remaining three toes. The middle digits on the hind foot were of equal length with the inner and outer toes somewhat shorter.

Long and Burlingame, have shown that within a relatively short time period (days eight and one-half to twelve and one-half) a considerable number of organs in the embryo of the rat undergo rapid differentiation and growth.

Skeletal ossification during pre and postnatal rat development was observed by Walker and Wirtschafter (1957). Their studies were done using alizarin-stained specimens and roentgenography.

Wright et al. (1958) used the Long-Evans rat to study embryonic skeletal development. They studied intramembranous and endochondral bone formation using both toluidine blue and alizarin red S which were modified after the original techniques of Miller (1921) and Dawson (1926) respectively (cited by Wright et al., 1958). Using toluidine blue, the earliest appearance of skeletal cartilage was in the third to ninth ribs (day fifteen). Furthermore the earliest appearance of bone (in the entire skeleton) was observed in the body of the mandible on the fifteenth day. From day fifteen and one-half to sixteen, the majority of bones (whether initially membranous or endochondral) made their first appearance.

Ossification of the vertebral arches started at the first cervical and proceeded caudally. Some ossification of the cervical and thoracic arches was observed by the seventeenth day although the rostral ones were better developed. From the thoracic region, ossification proceeded to the lumbar, sacral, and caudal regions. In the caudal region, the first and second arches were ossified by the twentieth day, and the third on the twenty-first day. Ossification of the more distal caudal arches occurred after birth.

Contrary to the sequence noted in the arches, ossification of the vertebral bodies started in the midthoracic region and progressed caudad with greater rapidity than rostrad. The thoracic, four to thirteen, appeared on the eighteenth day; the caudal vertebral bodies, one to three, on day twenty; and the cervical, three to seven, on the twenty-first day.

Ossification of the ribs (three to nine) started on day fifteen and one-half and continued to day seventeen and one-half; at this time all thirteen pairs of ribs were complete. Here also the spread was in both directions and similar to that observed in the vertebral bodies; slightly more rapidly caudally than cranially.

Ossification of the first two sternebrae was noted to commence on day nineteen. The third, fourth, and xyphoid process, appeared on day nineteen and one-half. The fifth sternebra did not ossify until the twentieth day and at this time the sternebral sequence was complete.

Wright et al., in addition observed the remainder of the skeleton to in general contain definitive bone. There were some exceptions, these being in some of the smaller bones found within the foot, skull, and vertebral column. In most of these instances, however, they usually observed either an ossification centre or expanding bone, this depending upon the bone being studied.

In contrast to the previous investigators who have indicated that the development time of some structures occurs at a specific time, Farris and Griffith (1962) have stated, "accurate designations of time must be avoided." These authors therefore use the number of somites present for determining the time-period when various structures undergo development. Farris and Griffith refer to the observations of Long and Evans (1922) who showed that approximately one third of the ova at ovulation did not develop to term. Also it was probable that many of the uncleaved eggs, probably immature, did not complete development.

Long and Evans also observed in one instance that development of the eggs had not started until forty-eight hours after ovulation. Farris and Griffith in addition, refer to the experiments of Pincus (1936) who had shown that the immature egg is potent enough to develop after fertilization. Pincus also indicated that at the time of ovulation, the eggs in the rat are in various stages of maturation.

III MATERIALS AND METHODS

Part I: Podophyllin Study

Strain of Animal Used. Holtzman albino rats (descended from the Sprague-Dawley strain) were used in this study. They were purchased from the Holtzman Company, Madison, Wisconsin.

Randomization of Animals. Virgin female rats (200 to 270 grams) were formed into thirty-three groups (Table I, next page) of ten animals each for podophyllin treatment, alcohol or water control, and untreated control (normal), by the following randomizing procedure. Example, twenty females were placed in separate cages numbered from one to twenty. Papers numbered from one to twenty were folded in half, placed in a box, mixed, and drawn. The first ten numbers drawn were assigned animals to be treated with the drug and the last ten numbers drawn were assigned animals in the ethanol or water control group; a similar method of randomizing was used for all thirty-three groups. The rats were ear-marked and six females were placed in each breeding cage.

Breeding of Animals. Two males were placed in each of the breeding cages between five and eight o'clock in the evening and removed between nine and ten o'clock the following morning. Vaginal smears were then taken.

Vaginal Smears. Slides were numbered to correspond with those of the ear-marked females. The smears were taken using commercially prepared swabs (Q-Tips). Prior to taking the smear, the swab was dipped in distilled water and rolled between the thumb and index finger to make it smooth. This prevented unnecessary trauma to the vagina.

Table I

Animal Groups in the Podophyllin Study

Group Number	Treatment Day(s) of Gestation	Day/Dose Mg./100 Gm.	Alcohol	Water	Stomach Tube
I	8-12	0.012	+	-	+
II	8-12	0.025	+	-	+
III	8-12	0.05	+	-	+
IV	8-12	0.1	+	-	+
V	8-12	0.2	+	-	+
VI	8-12	0.4	+	-	+
VII	10-12	0.012	+	-	+
VIII	10-12	0.025	+	-	+
IX	10-12	0.05	+	-	+
X	10-12	0.1	+	-	+
XI	10-12	0.2	+	-	+
XII	10-12	0.4	+	-	+
XIII	10	0.1	+	-	+
XIV	10	0.4	+	-	+
XV	10	0.8	+	-	+
XVI	10	1.6	+	-	+
XVII	8-12	0.1	-	+	+
XVIII	10-12	0.1	-	+	+
XIX	10	0.1	-	+	+
XX	10-12	0.1	-	+	+
XXI	10-12	0.1	-	+	+
XXII	8-12	-	+	-	+
XXIII	10-12	-	+	-	+
XXIV	10	-	+	-	+
XXV	8-12	-	-	+	+
XXVI	10-12	-	-	+	+
XXVII	10	-	-	+	+
XXVIII	8-12	-	-	-	+
XXIX	10-12	-	-	-	+
XXX	10	-	-	-	+
XXXI	-	-	-	-	-
XXXII	-	-	-	-	-
XXXIII	-	-	-	-	-

Explanation of Table I.

In groups I to XVI, the podophyllin-alcohol suspension was administered by stomach tube.

In groups XVII to XXI, the podophyllin-water suspension was administered by stomach tube.

In groups XXII to XXIV, no podophyllin was given; only alcohol by stomach tube.

In groups XXV to XXVII, no podophyllin or alcohol was given; only water by stomach tube.

In groups XXVIII to XXX, no podophyllin, alcohol, or water was given. The stomach tube was inserted into the animal's esophagus and then withdrawn.

Groups XXXI to XXXIII, the normals for the study, were not subjected to any treatment.

To take the smear, the animal was firmly grasped as follows: the palm of the left hand covered its back and the thumb and fingers gripped it under the fore-legs. This prevented the rat from biting the operator and when correctly done did not cause excessive pressure to either the esophagus or the trachea. Using the right hand, the swab was inserted into the vagina and gently rolled. It was then withdrawn and rolled onto the corresponding slide thereby making a smear of epithelial cells and spermatozoa (if present). The slides were stained in methylene blue for two minutes, dipped in cold water once to wash off excess stain, and allowed to air dry.

The day on which sperms were observed (Figure 1) was considered day zero of gestation.

Environment and Care of Animals. Mated females were placed in separate cages. All animals in groups I to XXXIII were kept in the regular animal quarters.

The lights, controlled by an automatic timer, were turned on at eight o'clock in the morning and off at eight o'clock in the evening.

The temperature, although usually constant at 78 degrees Fahrenheit, at times varied plus or minus 7 degrees. On one occasion it varied (within an eighteen hour period) from 66 to 88 degrees Fahrenheit. Some of the animals in groups III, IV, XXII, XXIII, XXVIII, and XXXI were subjected to this extreme variation.

The relative humidity was also another factor which could not be controlled. It varied from 19 to 65 per cent.

All animals were maintained on a diet of Victor Fox Cubes (protein, 25 per cent; fat, not less than 5 per cent; fibre, not more than 4.5 per cent). Fresh water was provided daily.

Preparation of Podophyllin. The podophyllin-alcohol solution was prepared by first partially dissolving and suspending it in 10 cubic centimeters of absolute ethanol. To this mixture 90 cubic centimeters of distilled water were added. The podophyllin-water solution was prepared by adding 100 cubic centimeters of distilled water to the drug. The mixture was then homogenized. Each concentration of podophyllin was used for one month, discarded, and then a new solution was prepared. This was done to prevent any possible chemical changes which might occur.

Groups I to XXI received the drug either in 10 per cent alcohol or in distilled water on the basis of 0.1 cubic centimeter of solution

per 100 grams body weight.

Alcohol control groups (XXII, XXIII, XXIV) and water control groups (XXV, XXVI, XXVII) received either 0.1 cubic centimeter of 10 per cent ethanol or water respectively per 100 grams body weight.

Apparatus For Tubal Administration. The apparatus (Figure 2) used for tubal administration of the drug was as follows: a blunted 18G Luer-Lok needle was attached to a 1 cubic centimeter tuberculin syringe; a two and one-half inch piece of polyethylene tubing (inside diameter 0.045 inch, outside diameter 0.062 inch) was fitted over the needle. This apparatus was used for administration of all test solutions (drug, ethanol, and water), and without any solution, administered in the stomach tube control groups.

Caesarean Section. With the exception of the females in groups XXI and XXXIII, all animals were killed on the twenty-first day of gestation with an overdose of chloroform to prevent cannibalism of the young. A midline abdominal incision was made, and the uterine horns were excised and carefully examined for the presence of resorption sites or macerated fetuses. Resorbed implantation sites and resorbed placentas were also recorded as resorption sites. The fetuses were removed from the uterine horns, weighed, and crown-rump length measured. The fetuses (excluding those in groups XX and XXXIII) were then eviscerated and fixed in individual jars containing 95 per cent ethanol for at least one week. This prevents maceration of the fetuses when they are

placed in potassium hydroxide to be cleared. Placentas were also measured and the average diameter was recorded for each group.

Fetuses in groups XX and XXXII were fixed in modified Davidson's solution, but were not cleared or stained as were the specimens in other groups. Instead free-hand transverse razor blade cuts were made in the head, neck, thoracic, abdominal, and pelvic regions. The sections were examined for possible defects with the aid of a dissecting microscope (7.5X).

Following examination, the placentas, macerated fetuses, and resorption sites were each placed in separate jars containing modified Davidson's solution. These along with the cut sections in groups XX and XXXII were stored for possible future reference.

Application of Quinine to Newborn Rats. Fetuses in groups XXI and XXXIII were permitted to go to term. At birth the number of liveborn and stillborn animals in each litter was recorded. The dorsal and ventral surfaces of all live newborn rats in these litters were then painted with a supersaturated aqueous solution of quinine to prevent cannibalism. It has been observed that if the mother is treated during pregnancy there is an increased tendency for cannibalism to occur. Quinine painting protected most of the young from being eaten by their mothers. On the tenth day after birth, the surviving newborn rats were killed. The lengths and weights of these animals were recorded, as were also the lengths and weights of those animals that died before the tenth day. All newborn animals (this included newborn dying before the tenth day and those killed on the tenth day after birth) were then eviscerated,

placed in separate jars, and fixed in 95 per cent alcohol. The alizarin technique was used to study the skeletons of all newborn animals, but primary interest was in skeletons of the ten-day-old rats.

Preparation of Twenty-One-Day-Old Fetuses For Study. The fetuses in groups I to XIX and XXII to XXXI were cleared and stained using Dawson's method (Gurr, 1962). Removal of the skin was not necessary. The following is an outline of the procedure.

1. After fixation, place fetuses in acetone for one week to remove body fat. Change acetone on the third day.
2. Wash in 95 per cent alcohol for two days.
3. Place in one per cent potassium hydroxide for three or four days to clear the soft tissue. Change the solution on the second day; at this time remove any remaining blood clots or viscera.
4. Place in alizarin red S for two days to stain the bones.
5. Destain the soft tissues in Mall's solution (glycerine, potassium hydroxide, water) for two days.
6. Dehydrate in glycerine for two days in each concentration of 50, 70, and 90 per cent solutions.
7. Store in 100 per cent glycerine for examination.

The fetal skeletons were examined under a dissecting microscope (7.5X); those in group XXXI for normal variations, and all remaining groups (excluding those in groups XX, XXI, XXXII, and XXXIII) for possible anomalies.

Preparation of Ten-Day-Old Newborn Rats For Study. In groups XXI and XXXIII the offspring (including some stillborn and liveborn animals that did not live for ten days after parturition) were also cleared and stained using Dawson's method. Most of the specimens were in the solutions for times which were different from those in the former outline, as follows:

1. Following fixation, place specimens in acetone for two weeks to remove body fat. Change acetone every third day.
2. Wash in 95 per cent alcohol for four days. Change alcohol after the second day.
3. Place in one per cent potassium hydroxide for nine days to clear the soft tissues; change the solution every second day. On the eighth day skin the specimens and then return to the potassium hydroxide solution for one more day.
4. Place in alizarin red S for two days to stain the skeletons.
5. Destain the soft tissues in Mall's solution for two days. Change the solution after the first day.
6. Same as in the previous outline.
7. Same as in the previous outline.

The skeletons were examined with the aid of a dissecting microscope (7.5X); those in group XXXIII for normal variations, and those in group XXI for possible anomalies.

Examination. Forty-five responses (observations) were recorded. The majority of these responses were analyzed in most mothers or their offspring. Table II shows the study method and the observations recorded for each of the groups.

Before disclosing the individual responses, their organization will be described. For example, responses ten, eleven, and twelve were referred to as a *block* because responses ten and eleven separately measured a variation in the same structure; responses ten and eleven were then added together to give a total for response twelve. The remainder of the responses were blocked in a similar manner.

The reason for considering the grouped responses is that sometimes a block (for example, responses ten, eleven, and twelve) contains responses (responses ten or eleven) which when individually analyzed are not statistically significant. However, if the values for these responses are combined, the total (response twelve) when analyzed will then become significant.

It will be noted that in these responses, the word *number* is used to designate one response within a block. The word *total* is used to show the sum of the values in the responses (contained within a block) beginning with the word *number*; the exception was in response seventeen, which contained the totals of responses thirteen, fourteen, and fifteen (response sixteen was omitted).

The word *young* (in responses five to forty-five) was used to designate both the twenty-one-day-old fetuses and the ten-day-old newborn rats.

Table II

Responses Studied in Each Group

Groups	Age of Young Examined	Method of Study	Responses Recorded and/or Analyzed
I - XIX	21 day fetuses	alizarin	1 - 45
XXII - XXXI	21 day fetuses	alizarin	1 - 45
XX + XXXII	21 day fetuses	cross-section	1 - 6
XXI + XXXIII	10 day newborn survivors only	alizarin	3, + 5 - 45

Explanation of Table II.

In groups I to XIX, XXII to XXXI, XX, and XXXII, the number of living fetuses was recorded in each group. The number of living fetuses in groups I to XIX and XXII to XXXI were used to express ratios in the statistical analysis of most of the forty-five responses (refer to statistical method, page 50).

In groups XXI and XXXIII, the values in the above responses were recorded from those animals which survived for ten days after birth. The observations were not statistically analyzed.

The following is a description of the responses which were recorded and statistically analyzed in most of the groups; any other unusual observations, apart from these, are separately mentioned in the results.

1. Total number of implantation sites per mother.
2. Total number of resorptions per mother.
3. Total number of stillborn animals per mother.
4. Average placental diameter (in millimeters) per mother.

5. Average length (in millimeters) of young per mother.
6. Average weight (in grams) of young per mother.
7. Total number of young per mother with wide cranial sutures or abnormal ossification in cranial bones that participate in forming the major sutures.
8. Total number of young per mother with uneven length of incisors in the maxilla or mandible.
9. Total number of young per mother with retarded ossification in the hyoid bone.
10. Number of young per mother with uneven ossification in the anterior arch of the atlas.
11. Number of young per mother with absence of ossification in the anterior arch of the atlas.
12. Total number of young per mother with uneven or absence of ossification in the anterior arch of the atlas.
13. Number of young per mother with one ossification centre in the odontoid process.
14. Number of young per mother with two separated ossification centres in the odontoid process.
15. Number of young per mother with two joined ossification centres in the odontoid process.
16. Number of young per mother with absence of an ossification centre in the odontoid process.
17. Total number of young per mother with one centre or two separated or joined centres in the odontoid process.

18. Number of young per mother with absence of either one or two cervical centra in consecutive order starting with the second cervical.
19. Number of young per mother with absence of three or more cervical centra in consecutive order starting with the second cervical.
20. Number of young per mother with absence of one or more cervical centra in nonconsecutive order starting with the second cervical.
21. Total number of young per mother with absence of cervical centra irrespective of the number that were absent.
22. Total number of young per mother with absent thoracic or lumbar or sacral vertebral centra.
23. Number of young per mother with slightly dumbbell vertebral centra in the cervical, thoracic, lumbar, or sacral regions.
24. Number of young per mother with dumbbell vertebral centra in the cervical, thoracic, lumbar, or sacral regions.
25. Number of young per mother with duplicated vertebral centra in the cervical, thoracic, lumbar, or sacral regions.
26. Total number of young per mother with various forms of dumbbell and duplicated centra in the cervical, thoracic, lumbar, or sacral regions.
27. Total number of tail centra per mother's litter.
28. Number of young per mother with fusion of vertebral arches.

29. Number of young per mother with absent vertebral arches.
30. Total number of young per mother with abnormal vertebral arches; fusion, abnormal ossification, or absence.
31. Total number of young per mother with anomalous or absent ribs (excluding forms of supernumerary ribs).
32. Number of young per mother with supernumerary ribs; unilateral in the cervical or lumbar regions.
33. Number of young per mother with supernumerary ribs; bilateral in the cervical or lumbar regions.
34. Total number of young per mother with supernumerary ribs; unilateral or bilateral in the cervical or lumbar regions.
35. Number of young per mother with slightly retarded ossification in the fifth sternebra.
36. Number of young per mother with retarded ossification in the fifth sternebra.
37. Number of young per mother with almost no ossification in the fifth sternebra.
38. Number of young per mother with no ossification in the fifth sternebra.
39. Total number of young per mother with abnormally retarded or absent fifth sternebra.
40. Total number of young per mother with other sternebrae affected (excluding the fifth); retarded or absence of ossification.

41. Total number of young per mother with abnormal or absent ossification in sternebrae regardless of the sternal centre affected.
42. Total number of young per mother with abnormal or absent ossification in bones of the pelvic girdle.
43. Total number of young per mother with an ossification centre in the coracoid process.
44. Total number of young per mother with presence of an ossification centre in the calcaneus or other tarsal bones; one or both feet.
45. Total number of young per mother with absent metatarsal or metacarpal bone(s); one or both feet.

Statistical Method. The statistical analysis was done on a computer (/360 Model 65) at the University of Manitoba. The Fortran program was written by Mr. S. Vivian, Department of Pharmacology and Therapeutics, University of Manitoba.

The statistical method used for analyzing groups I to XIX and XXII to XXXI was the analysis of variance with an orthoganal set of single degree of freedom comparisons (Steel and Torrie, 1960 a). In addition, orthoganal polynomials were used to establish the dose response curve for the equally spaced dose levels in groups I to VI, VII to XII, and XIV to XVI (Steel and Torrie, 1960 a).

Before the actual statistical analysis was done, the raw values in most instances had to be expressed differently than when

they were initially recorded; this was done with the aid of a computer. Values for response one were neither expressed as ratios nor transformed. Original values for responses two and three were each expressed as a ratio per total number of implantation sites per mother and angular transformed (Steel and Torrie, 1960 b). Values for responses four, five, and six were not expressed as ratios, but were square root transformed (Steel and Torrie, 1960 b). The values for responses seven to forty-five were each expressed as a ratio of the total number of events over litter size; in all instances, except response twenty-seven, these ratios were angular transformed prior to the statistical analysis (Steel and Torrie, 1960 b). Because of these transformations, most of the mean values in the Results are not true arithmetic means, but they are an index (closely resembling percentages) from which the significance of differences were made.

Six major contrasts were used to analyze each of the forty-five responses. Contrasts one, two, and three were designed to assist in establishing an overall effect and to also determine which treatment days were most effective; either all or most of the groups were analyzed. Contrasts four (a) to (e) were used to analyze those groups of animals which received treatments from days eight to twelve of gestation. Contrasts five (a) to (e) were used to analyze those groups of animals which received treatments from days ten to twelve of pregnancy. Contrasts six (a) to (f) were used to analyze those groups of animals which received treatments on the tenth day only of gestation.

In some of these contrasts, the word *treatment(s)* was used to refer to either, a) podophyllin in alcohol, b) podophyllin in water, c) alcohol only, d) water only, e) stomach tube only or any combination of these.

The following is a list of the contrasts, and also the comparisons which were made in each of the contrasts.

1. At least one of the treatments is significantly effective.

   N* ←------→ A, B, C

2. Either days of treatment eight to twelve and ten to twelve or day ten only is significantly more effective in producing a response.

   A, B ←------→ C

3. Either days of treatment eight to twelve or ten to twelve is significantly more effective in producing a response.

   A ←------→ B

4(a). At least one of the treatments is significantly effective.

   S ←------→ W, Al, DW, DAl

(b). In the absence of any significant podophyllin effect, the alcohol is significantly effective.

   W, DW ←------→ Al, DAl

(c). The podophyllin administered in water is significantly effective.

   W ←------→ DW

(d). The podophyllin administered in alcohol is significantly effective.

Al ◄------► DAl

(e). The podophyllin in alcohol dose response curve has a significant, i) linear component, ii) quadratic component, iii) cubic component, iv) quartic component, and v) fifth or higher order component.

DAl - 1, 2, 3, 4, 5, 6

5(a). At least one of the treatments is significantly effective.

S ◄------► W, Al, DW, DAl

(b). In the absence of any significant podophyllin effect, the alcohol is significantly effective.

W, DW ◄------► Al, DAl

(c) The podophyllin administered in water is significantly effective.

W ◄------► DW

(d). The podophyllin administered in alcohol is significantly effective.

Al ◄------► DAl

(e). The podophyllin in alcohol dose response curve has a significant, i) linear component, ii) quadratic component, iii) cubic component, iv) quartic component, and v) fifth or higher order component.

DAl - 1, 2, 3, 4, 5, 6

6(a). At least one of the treatments is significantly effective.

S ◄------► W, A1, DW, DA1

(b). In the absence of any significant podophyllin effect, the alcohol is significantly effective.

W, DW ◄------► A1, DA1

(c). The podophyllin administered in water is significantly effective.

W ◄------► DW

(d). The podophyllin administered in alcohol is significantly effective.

A1 ◄------► DA1

(e). The 0.1 milligram podophyllin dose in alcohol is significantly different than the mean of the three higher doses of podophyllin in alcohol (0.4, 0.8, and 1.6 milligrams).

DA1 - 4 ◄------► DA1 - 6, 7, 8

(f). The podophyllin in alcohol dose response curve has a significant, i) linear component, and ii) quadratic or higher order component.

DA1 - 6, 7, 8

* Key to Symbols.

N = Normals

A = All treatments administered on days 8-12.

B = All treatments administered on days 10-12.

C = All treatments administered on day 10 only.

S = Stomach tube only.

W = Water only.

Al = Alcohol only.

DW = Drug (podophyllin) in water.

DAl = Drug (podophyllin) in alcohol.

DAl - 1 = 0.012 milligram podophyllin dose in alcohol.

DAl - 2 = 0.025 milligram podophyllin dose in alcohol.

DAl - 3 = 0.05 milligram podophyllin dose in alcohol.

DAl - 4 = 0.1 milligram podophyllin dose in alcohol.

DAl - 5 = 0.2 milligram podophyllin dose in alcohol.

DAl - 6 = 0.4 milligram podophyllin dose in alcohol.

DAl - 7 = 0.8 milligram podophyllin dose in alcohol.

DAl - 8 = 1.6 milligrams podophyllin dose in alcohol.

The various components listed in contrasts four (e), five (e), and six (f) were used to determine the shapes of dose response curves. A linear component is one in which the slope either rises or declines with dose levels. A quadratic component is one which has the shape of a parabola; it can be either U-shaped or shaped as an inverted U. A cubic component is one which is shaped similar to a parabola (has somewhat the shape of an S on its side). A quartic component is one which has either an M-shape or an inverted M-shape. A fifth or higher order component is one which is quite complicated; it is similar to an M but instead of two peaks it will have three or more peaks to it (it can also be inverted).

Part II: Temperature Study

Strain of Animal and Randomization. Virgin female Holtzman rats (200 to 270 grams) were formed into eight groups (Table III) of randomly selected animals each containing ten rats.

Because the presence of spermatozoa in a vaginal smear is only evidence that copulation has occurred but is in no way an indication that fertilization will follow, and because previous studies have shown that 10 to 15 per cent of the animals do not become pregnant, fourteen animals were selected to ensure that there would be ten pregnant animals in each group. However, only the first ten animals pregnant (when the Caesarean sections were done) were used; the remainder were discarded.

Breeding of Animals and Vaginal Smears. Method and technique were the same as in the podophyllin experiments; refer to page 37.

Environment Chamber and Care of Animals. Following mating in the regular animal room, animals in each group were transferred into the environment chamber (Coldstream Products of Canada Limited) and placed in separate cages.

The lighting was controlled in the same manner as it was in the regular animal quarters; refer to page 40.

The temperature, once set, remained constant (plus or minus 1 degree Fahrenheit) for each of the animals within a group from days zero to twenty-one of gestation; the exception was in group HTE where there was a variation of plus or minus 2 degrees Fahrenheit. The

Table III

Animal Groups in the Temperature Study

Group	Days of Gestation	Temperature(s) in Degrees Fahrenheit	Per cent Relative Humidity
ATE	0-21	90 ± 1	50 ± 2
BTE	0-21	85 ± 1	50 ± 2
CTE	0-21	80 ± 1	50 ± 2
DTE	0-21	75 ± 1	50 ± 2
ETE	0-21	70 ± 1	50 ± 2
FTE	0-21	65 ± 1	50 ± 2
GTE	0-21	60 ± 1	50 + 25 - 2
HTE	0-21	65 ± 2 and 90 ± 2	50 + 25 - 2

Explanation of Table III.

Groups ATE to GTE were exposed to the temperatures indicated in the table; days zero to twenty-one of gestation. The relative humidity was constant at 50 per cent (exception was group GTE where it varied from 50 to 75 per cent).

Group HTE was exposed to an alternating temperature of 65 degrees Fahrenheit for eight hours and then 90 degrees for sixteen hours; days zero to twenty-one of gestation. The relative humidity fluctuated from 50 to 75 per cent.

temperature was reset each time for new groups. The animals in group HTE were exposed (from days zero to twenty-one of pregnancy) to an alternating temperature; at nine o'clock in the morning the control was

set at 65 degrees Fahrenheit and at five o'clock in the evening, 90 degrees.

The humidistat, for control of relative humidity, was set at 50 per cent (plus or minus 2 per cent) for all temperature settings, however, it did not always maintain this setting; in groups GTE and HTE the relative humidity varied from 48 to 75 per cent.

The animals were fed and watered in the same way as in the podophyllin study; refer to page 40.

Caesarean Section. The procedure was the same as in the podophyllin study; refer to page 41. The first ten pregnant animals in each of the groups (ATE to HTE) were killed on the twenty-first day of gestation.

Preparation of Twenty-One-Day-Old Fetuses for Study. The fetuses were cleared and stained in the same manner as in the podophyllin study; refer to page 43.

Fetal skeletons were examined under a dissecting microscope (7.5X) to determine if temperature has an effect on ossification and also to try and establish a temperature at which normal ossification occurs, but with a minimum amount of variation in the fetuses of a group.

Examination. The same responses (numbers one to forty-five) as those recorded in the podophyllin study (refer to page 46) were also recorded for the temperature study.

Statistical Method. The statistical method used was the same as in the podophyllin study; refer to page 50.

Each of the values for the responses (numbers one to forty-five) were expressed in the same manner as in the podophyllin study; refer to page 51.

The contrasts used in analyzing each of the forty-five responses were as follows:

T - 1. The varying temperature group (group HTE; temperature 65 and 90 degrees Fahrenheit) is significantly different than the mean of the fixed (gradient) temperature groups (groups ATE, BTE, CTE, DTE, ETE, FTE, GTE; 90, 85, 80, 75, 70, 65, and 60 degrees Fahrenheit respectively).

T - 2. The temperature (90, 85, 80, 75, 70, 65, and 60 degrees Fahrenheit) response curve has a significant, i) linear component, ii) quadratic component, iii) cubic component, iv) quartic component, and v) fifth or higher order component.

IV RESULTS

Part I: Podophyllin Study

The statistical analysis was done on groups I to XIX and XXII to XXXI.[1] The major podophyllin effects are summarized by Table IV. The effects that appeared to be due to ethanol are shown in Table V. Strangely a number of water treatment effects usually occurred when given on day ten only of pregnancy. In the Discussion an attempt will be made to explain the ethanol and water effects. The stomach tube, when administered on days eight to twelve, ten to twelve, and day ten only of gestation, appears to be responsible for producing the following effects: retarded ossification in the hyoid bone, duplicated vertebral centra, fused vertebral arches, absence of ossification in the fifth sternebra, and retarded or absence of ossification in sternebrae other than the fifth sternal centre. The frequency (in per cent) of the major responses, as related to the treatments administered to the various groups, is summarized in Table VI. The possible stomach tube effects are considered in more detail in the Discussion.

The results observed in each response have been grouped together where possible and presented as effects on various structures. A response had to contain at least one significant effect before a table (with the means) was included in the text. The means in the tables and the text are in most instances not arithmetic, but are transformed means closely resembling percentages that were derived by the statistical procedure outlined; refer to pages 50 and 51.

---

[1]A complete and detailed summary of the entire statistical analysis is shown in Table I of the Appendix. Table II in the Appendix contains the total number of liveborn twenty-one-day-old fetuses examined in each of the above groups.

Table IV

Major Podophyllin Effects on Various Structures

Day(s) of Treatment During Pregnancy	Anterior Arch of Atlas		Absence of Ossification in Cervical Centra*	Dumbbell Centra	Super-numerary Ribs	Slightly Retarded Ossification in Fifth Sternebra	Absence of Ossification in Calcaneus
	Unevenly Ossified	Absence of Ossification					
8-12	+	+	+	+	+	+	+
10-12	+	-	-	-	-	+	+
10	-	-	+	+	-	-	+

Explanation of Table IV.

The podophyllin, suspended in 10 per cent ethanol, and water, had a significant or highly significant effect (designated by a "+") by retarding, completely deleting or stimulating ossification of the structure.

A negative symbol (-) indicates that the podophyllin did not have any significant effect on the structure.

*Two responses are included, responses eighteen and twenty; refer to page 48.

Table V

Ethanol Effects on Various Structures

Day(s) of Treatment During Gestation	Absence of Ossification in Anterior Arch of Atlas	Absence of Ossification in Cervical Centra*	Retarded Ossification in Fifth Sternebra
8-12	-	-	+
10-12	+	+	-
10	-	-	-

Explanation of Table V.

A negative symbol (-) shows that the 10 per cent ethanol did not have any significant effect on the structure.

A positive sign (+) indicates that the ethanol had a significant or highly significant effect on the structure.

*This was response eighteen; refer to page 48.

Table VI

Frequency of Major Responses as Related to the Various Treatments Administered

Response	Percentage of Fetuses with Response/Group(s) Studied					
	Untreated Control (Normal)	Stomach Tube Controls	Water Controls	Alcohol Controls	Podophyllin in Water	Podophyllin in Alcohol
Uneven Incisors (8)*	11.8	17.0	17.4	18.9	17.2	15.9
Incompletely Ossified Hyoid (9)*	0	0	0.6	0	2.5	1.6
Anterior Arch of Atlas (12)*	15.7	38.8	74.5	31.4	76.9	43.5
Dumbbell Vertebral Centra (26)*	2.0	2.5	2.8	3.2	3.7	4.2
Fused Vertebral Arches (30)*	0	0.3	0	0	0.9	1.2
Incompletely Ossified Sternebrae (41)*	15.7	36.3	31.5	28.2	36.0	38.0
Presence of Ossification in Calcaneus (44)*	60.8	64.4	65.7	81.4	61.2	62.9

Explanation of Table VI.

The percentages indicated were derived by taking total number of events in a response over the total number of fetuses that were obtained in various groups treated on days eight to twelve, ten to twelve, and day ten only of gestation. There were 102 fetuses in group XXXI, untreated control (Normal). Groups XXVIIIto XXX were stomach tube controls; there were 317 fetuses. Groups XXV to XXVII were water controls; there were 321 fetuses. Groups XXII to XXIV were alcohol controls; there were 312 fetuses. Groups XVII to XIX were treated with podophyllin in water; there were 325 fetuses. Groups I to XVI were treated with podophyllin in alcohol; there were 1654 fetuses.

*This indicates the response number in the list of responses on pages 46 to 50.

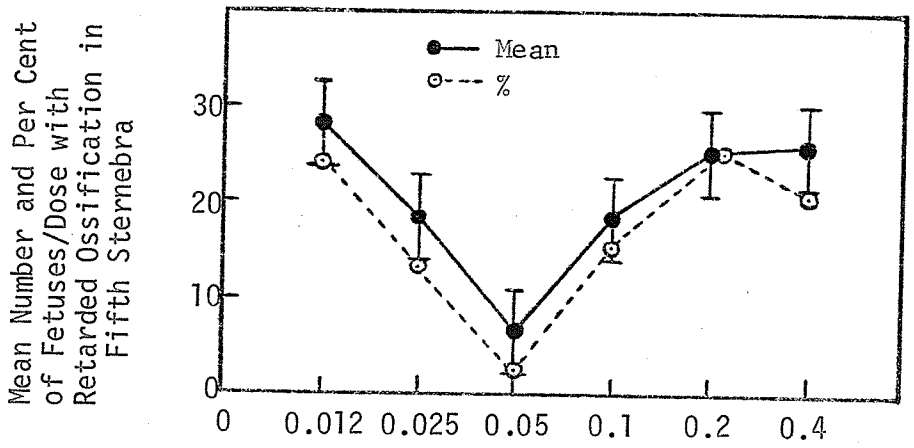
In these results a highly significant observation was one in which alpha was less than 0.01. A significant effect was one in which alpha was less than 0.05, but greater than 0.01. When alpha was greater than 0.05, but less than 0.1, this suggested that the effect might have been significant (borderline); however, when alpha was 0.1 or greater, this is taken to mean that the observation was definitely not significant.

The most frequently observed podophyllin dose response curves were quadratic and cubic shapes. To assist in visualizing their shape, these two curves have been plotted in Graphs 1 and 2 using data in responses thirty-six and eight respectively.

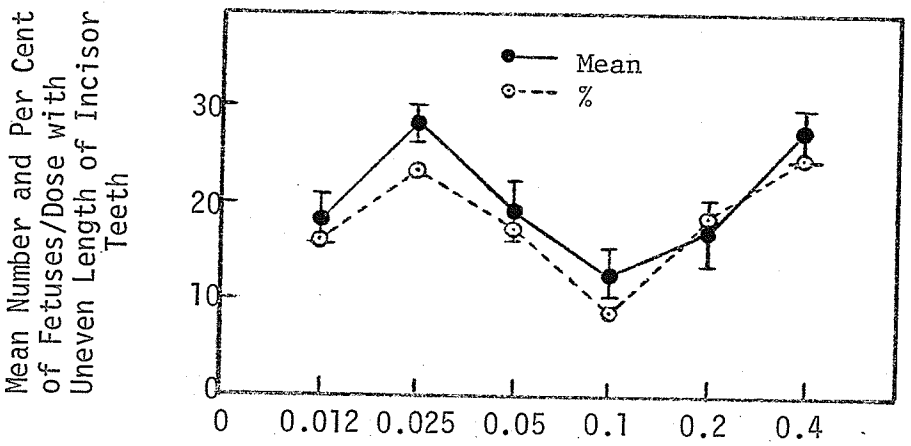
Effects on Implantation, Resorption, and Stillbirth Rate. Responses one, two, and three were analyzed. One was total number of implantation sites per mother, two was total number of resorptions per mother, and three was total number of stillborn animals per mother. Although there were no significant results in any of these responses, there was an interesting observation in response two; complete litter resorption was noted in one animal (third in group XVI) on the twenty-first day of gestation. The animal had received 1.6 milligrams of podophyllin in alcohol on day ten only of pregnancy. From the sizes of these resorptions it appeared that embryonic death had occurred between the tenth to twelfth days of gestation. Entire litter resorption was not observed in any of the pregnant control rats.

1. This podophyllin dosage response curve is a quadratic component. Because the means are not arithmetic, per cents have also been calculated so comparisons can be made. Note that the means are largest at the low (0.012 milligram) and high (0.4 milligram) podophyllin doses administered in 10 per cent ethanol by stomach tube; this suggests that these dose levels are either inhibiting or delaying ossification. Be comparison because the mean is smallest at the intermediate dose (0.05 milligram), this suggests that this dose, in this instance, is either least effective or it is stimulating ossification. Group I received 0.012 milligram of drug, Group II received 0.025 milligram, Group III received 0.05 milligram, Group IV received 0.1 milligram, Group V received 0.2 milligram, and Group VI received 0.4 milligram of podophyllin.

2. This podophyllin dosage response curve is a cubic component. Note that the largest means are at the 0.025 and 0.4 milligram drug doses administered in 10 per cent alcohol by stomach tube. This suggests that these doses may be either delaying or retarding even tooth eruption. Because the means are smallest at the 0.012 and 0.1 milligram podophyllin doses, this suggests that these doses may be either least effective or they may be stimulating even tooth eruption. The groups receiving the drug doses are the same as those mentioned in the explanation of Graph 1.



Graph 1



Graph 2

Effects on Placental Development. Response four, average placental diameter (in millimeters) per mother, was analyzed. The dose response curve for groups I to VI, treated with podophyllin in alcohol on days eight to twelve of gestation, had a highly significant quadratic (U-shaped curve) and cubic component (S-shaped curve); examples in Graphs 1 and 2. The means of the placental diameter for groups I to VI were 16.3, 16.3, 16.0, 14.8, 15.8, and 16.6 respectively (R-4, Table VII). These means suggested that the low and high podophyllin doses (0.012, 0.025, 0.05, 0.2, and 0.4 milligram) was either stimulating the placentas to become larger or that these doses were the least effective. By contrast, the mean for the 0.1 milligram drug dose suggested that podophyllin was inhibiting placental development because the mean was smallest; 14.8.

A significant podophyllin (in water) effect was observed when the drug was administered to animals in group XVIII on days ten to twelve of pregnancy. The mean placental diameter for animals in group XVIII was 16.5 millimeters, but the mean for the water control group (XXVI) was 15.1 millimeters (R-4, Table VII). This suggested that podophyllin (0.1 milligram) might stimulate an increase in placental diameter.

The dose response curve for groups VII to XII, treated with podophyllin in alcohol on days ten to twelve of gestation, had a highly significant quadratic component. The means for groups VII to XII were 16.7, 15.9, 14.9, 14.9, 15.5, and 16.5 millimeters respectively (R-4, Table VII). Once again, the dose response curve suggested that

Table VII

Mean Placental Diameter (R*-4) Per Group in Podophyllin Study

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension** 10% OH	H_2O	Stomach Tube	Mean/Group R-4
I	8-12	0.012	+	-	+	16.3
II	8-12	0.025	+	-	+	16.3
III	8-12	0.05	+	-	+	16.0
IV	8-12	0.1	+	-	+	14.8
V	8-12	0.2	+	-	+	15.8
VI	8-12	0.4	+	-	+	16.6
XVII	8-12	0.1	-	+	+	15.6
XXII	8-12	-	+	-	+	15.6
XXV	8-12	-	-	+	+	15.5
XXVII	8-12	-	-	-	+	16.2
VII	10-12	0.012	+	-	+	16.7
VIII	10-12	0.025	+	-	+	15.9
IX	10-12	0.05	+	-	+	14.9
X	10-12	0.1	+	-	+	14.9
XI	10-12	0.2	+	-	+	15.5
XII	10-12	0.4	+	-	+	16.5
XVIII	10-12	0.1	-	+	+	16.5
XXIII	10-12	-	+	-	+	15.3
XXVI	10-12	-	-	+	+	15.1
XXIX	10-12	-	-	-	+	16.0
XIII	10	0.1	+	-	+	15.5
XIV	10	0.4	+	-	+	15.7
XV	10	0.8	+	-	+	15.8
XVI	10	1.6	+	-	+	15.7
XIX	10	0.1	-	+	+	15.6
XXIV	10	-	+	-	+	16.0
XXVII	10	-	-	+	+	15.7
XXX	10	-	-	-	+	15.7
XXXI	-	-	-	-	-	16.2

Explanation of Table VII.

A plus (+) indicates that either ethanol or water was used as a suspension medium. This symbol also shows that the stomach tube was used.

A minus (-) indicates that a suspension medium was not used,

shows that the stomach tube was not used, and also indicates that podophyllin was not administered.

The dosage of 10 per cent ethanol or water given to control animals was 0.1 cubic centimeter per 100 grams body weight of the rat.

*R means response; this symbol is used in the remaining tables.

**Suspension medium, either 10% ethanol or water.

---

the low and high drug doses either stimulated the placentas to become larger or they were least effective; the intermediate doses (0.05 and 0.1 milligram) apparently inhibited placental development because the means were lowest (14.9 for each group).

Effects on Fetal Length and Weight. This was a combination of the results in responses five and six; response five was average fetal length (in millimeters), and six was average fetal weight (in grams) of young per mother. There was a highly significant difference in the lengths and weights of fetuses between groups that received treatments on days eight to twelve and ten to twelve, and those groups that received treatments on day ten only of pregnancy. The means (R-5 and R-6, Table VIII) indicated that treatments on days eight to twelve and ten to twelve were more effective since the mean lengths and weights of fetuses from animals treated on these days were generally lower than in fetuses of animals treated on day ten only.

Highly significant differences, attributed to alcohol, were noted between fetal length in groups I to VI and XXII, which received podophyllin in alcohol and alcohol only (days eight to twelve), and

Table VIII

Mean Fetal Length (R*-5) and Weight (R-6) Per Group in Podophyllin Study

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-5	Mean/Group R-6
I	8-12	0.012	+	-	+	43.5	5.5
II	8-12	0.025	+	-	+	43.1	5.4
III	8-12	0.05	+	-	+	43.0	5.4
IV	8-12	0.1	+	-	+	41.9	5.3
V	8-12	0.2	+	-	+	42.2	5.1
VI	8-12	0.4	+	-	+	43.8	5.5
XVII	8-12	0.1	-	+	+	44.8	5.6
XXII	8-12	-	+	-	+	42.9	5.5
XXV	8-12	-	-	+	+	43.7	5.4
XXVIII	8-12	-	-	-	+	42.0	5.3
VII	10-12	0.012	+	-	+	43.5	5.5
VIII	10-12	0.025	+	-	+	43.4	5.4
IX	10-12	0.05	+	-	+	42.5	5.1
X	10-12	0.1	+	-	+	40.9	4.9
XI	10-12	0.2	+	-	+	42.6	5.3
XII	10-12	0.4	+	-	+	43.9	5.5
XVIII	10-12	0.1	-	+	+	42.3	5.1
XXIII	10-12	-	+	-	+	41.8	5.3
XXVI	10-12	-	-	+	+	42.9	5.2
XXIX	10-12	-	-	-	+	42.1	5.4
XIII	10	0.1	+	-	+	43.6	5.6
XIV	10	0.4	+	-	+	43.5	5.6
XV	10	0.8	+	-	+	43.4	5.6
XVI	10	1.6	+	-	+	43.4	5.7
XIX	10	0.1	-	+	+	43.5	5.2
XXIV	10	-	+	-	+	44.3	5.7
XXVII	10	-	-	+	+	44.3	5.5
XXX	10	-	-	-	+	42.9	5.3
XXXI	-	-	-	-	-	41.5	5.3

Explanation of Table VIII and Key to Symbols.

Refer to pages 69 and 70.

those groups (XVII and XXV) which received podophyllin in water and water only (days eight to twelve). The means (R-5 , Table VIII) of the fetal lengths for groups I to VI and XXII ranged from 41.9 to 43.8 millimeters, but the means for groups XVII and XXV were 44.8 and 43.7 millimeters.

Although an alcohol effect was observed above, the dose response curve for groups I to VI, treated with podophyllin in alcohol on days eight to twelve, had a significant quadratic component. The mean fetal length for groups I to VI was 43.5, 43.1, 43.0, 41.9, 42.2, and 43.8 millimeters respectively (R-5, Table VIII). These means suggested that the low and high podophyllin doses either stimulated an increase in fetal length or they were least effective whereas the 0.1 milligram dose probably inhibited growth because the mean was lowest (41.9).

In contrast to the previous observations, it was noted that podophyllin in alcohol, given on days ten to twelve, was significantly effective. The means (R-5, Table VIII) of fetal length for the podophyllin treated groups (VII to XII) were 43.5, 43.4, 42.5, 40.9, 42.6, and 43.9 millimeters; the mean for the alcohol control group (XXIII) was 41.8 millimeters. This indicated that, under these specific conditions, podophyllin generally seemed to cause an increase in fetal length. The exception was in group X, where each animal received 0.1 milligram of podophyllin; mean was 40.9. This mean was low because the third animal in the group contained thirteen extremely small viable fetuses; their mean length was only 20.2 millimeters (Figure 3).

There were also some mothers in other podophyllin treated groups which had small viable twenty-one-day-old fetuses. Group XIII received

0.1 milligram of podophyllin in alcohol on day ten only of pregnancy. The fifth mother had fourteen offspring; one fetus was 22.0 millimeters long. Group XVIII received 0.1 milligram of podophyllin in water on days ten to twelve of pregnancy. The third animal had thirteen fetuses whose mean length was 37.6 millimeters. Also in this group, the fifth mother had eight fetuses. Of these, three were runted (Figure 4), each measuring 22.0 millimeters, but the other five were normal size (approximately 42.0 millimeters; Figure 4). Twenty-one-day-old fetuses of this size have not been previously observed in any control mothers.

Interestingly, when fetal lengths were analyzed in groups VII to XII and XXIII, it is recalled that podophyllin generally caused an increase in fetal length, however, there was no significant dose response curve for groups VII to XII. By contrast, in the analysis of fetal weights for groups VII to XII and XXIII, there was no significant podophyllin effect, but the dose response curve for groups VII to XII had a significant quadratic component. The means of the weights for groups VII to XII were 5.5, 5.4, 5.1, 4.9, 5.3, and 5.5 grams (R-6, Table VIII). These means showed that low and high podophyllin doses either caused an increase in fetal weights or these doses were least effective. The intermediate dose (0.1 milligram) caused a decrease in weight. The reason for the low mean of 4.9, observed in group X, was that the weights of the previously mentioned thirteen viable runts (used in calculating fetal length) were also included in the computing of these means.

Effects on Cranial Sutures. Response seven was total number of young per mother with wide cranial sutures or abnormal ossification of cranial bones which participate in forming the major sutures. The only significant observation was that treatments administered on days ten to twelve of pregnancy were more effective than when the same treatments were administered on days eight to twelve; the means are shown in Table IX (R-7). The groups treated on days ten to twelve had more young with wide cranial sutures than the groups treated on days eight to twelve. There were no other significant observations.

Effects on Teeth. Response eight was analyzed (total number of young per mother with uneven length of incisor teeth in the maxilla or the mandible; Figures 5 and 6). A highly significant drug effect was observed when group XVII, given podophyllin (0.1 milligram) in water on days eight to twelve, was compared with group XXV, given only water on these same days of gestation. The mean (R-8, Table IX) number of fetuses with uneven incisors in the podophyllin group (XVII) was 14.0, but in the water control group (XXV) it was 28.6. From these means it appeared that podophyllin suppressed the development of uneven incisors, or, conversely, it stimulated even tooth eruption.

It was also noted that when podophyllin in alcohol was administered to groups I to VI on days eight to twelve of pregnancy, the dose response curve for these groups had a highly significant cubic component (Graph 2, page 67). The means for groups I to VI were 18.5, 28.4, 19.2, 12.9, 17.0 and 27.4 respectively (R-8, Table IX). These means suggested that the low and

Table IX

Mean Number of Fetuses Per Group in Podophyllin Study with Wide Cranial Sutures (R*-7), Uneven Length of Incisor Teeth (R-8), and Retarded Ossification in the Hyoid Bone (R-9)

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-7	Mean/Group R-8	Mean/Group R-9
I	8-12	0.012	+	-	+	0.9	18.5	0.9
II	8-12	0.025	+	-	+	0.8	28.4	0.8
III	8-12	0.05	+	-	+	1.0	19.2	1.0
IV	8-12	0.1	+	-	+	2.7	12.8	0.9
V	8-12	0.2	+	-	+	1.1	17.0	1.1
VI	8-12	0.4	+	-	+	0.9	27.4	0.9
XVII	8-12	0.1	-	+	+	0.9	14.0	0.9
XXII	8-12	-	+	-	+	1.0	20.0	1.0
XXV	8-12	-	-	+	+	1.0	28.6	3.4
XXVIII	8-12	-	-	-	+	0.9	23.9	0.9
VII	10-12	0.012	+	-	+	0.9	29.3	0.9
VIII	10-12	0.025	+	-	+	2.5	14.4	0.9
IX	10-12	0.05	+	-	+	3.8	18.8	4.6
X	10-12	0.1	+	-	+	13.0	13.1	9.7
XI	10-12	0.2	+	-	+	2.6	6.6	0.9
XII	10-12	0.4	+	-	+	0.9	27.1	4.0
XVIII	10-12	0.1	-	+	+	6.4	20.0	9.2
XXIII	10-12	-	+	-	+	0.9	19.2	0.9
XXVI	10-12	-	-	+	+	0.8	19.2	0.8
XXIX	10-12	-	-	-	+	2.8	10.5	1.0
XIII	10	0.1	+	-	+	2.4	22.2	2.4
XIV	10	0.4	+	-	+	1.1	16.7	1.1
XV	10	0.8	+	-	+	6.0	18.7	6.0
XVI	10	1.6	+	-	+	1.7	8.3	3.2
XIX	10	0.1	-	+	+	2.4	27.7	2.4
XXIV	10	-	+	-	+	0.9	29.5	0.9
XXVII	10	-	-	+	+	0.9	20.7	0.9
XXX	10	-	-	-	+	2.5	29.0	0.9
XXXI	-	-	-	-	-	6.4	13.9	0.9

Explanation of Table IX and Key to Symbols.

Refer to pages 69 and 70.

high doese (0.025 and 0.4 milligram) generally stimulated uneven incisoral eruption, but the 0.012 and 0.1 milligram doses either suppressed this effect or they were less effective than some of the other doses.

Strangely, the statistical analysis showed that water administered on days ten to twelve of pregnancy was producing a significant effect. The mean for the stomach tube control, group XXIX, was 10.5, but the means for groups VII to XII, XVIII, XXIII, and XXVI ranged from 6.6 to 29.3 (R-8, Table IX). Because this does not appear possible, it is not accepted. An attempt will be made to discuss this possibility, since it occurred in a number of other instances.

It was noted, however, that the dose response curve for groups VII to XII, given podophyllin in alcohol on days ten to twelve of gestation, had a highly significant quadratic component. The means (R-8, Table IX) for groups VII to XII were 29.3, 14.4, 18.8, 13.1, 6.6, and 27.1 respectively. These means once again suggested that the low and high doses of podophyllin stimulated uneven tooth eruption, but the 0.2 milligram dose either prevented uneven incisoral growth or it was least effective because the mean for the latter dose was only 6.6.

The analysis again implied that water treatements administered on day ten only were causing a highly significant effect. The mean for the stomach control group (XXX) was 29.0, but the means for groups XIII to XVI, XIX, XXIV, and XXVII varied from 8.3 to 29.5 (R-8, Table IX).

Effects on Hyoid Bone. Response nine was total number of young per mother with retarded ossification of the hyoid bone (Figure 7).

There was a significant difference between the mean for the normal group (XXXI) and the means for all the other groups receiving treatments (R-9, Table IX). Because the mean for the normal group was 0.9, but for the other groups the means ranged from 0.8 to 9.7, this indicated that the stomach tube was responsible for this effect since there were no other significant observations in this response.

In spite of this, it was still interesting to note that ten podophyllin treated mothers had thirty-five fetuses with this defect. By comparison, only one alcohol control animal had two fetuses with retarded ossification in the hyoid bone.

Effects on Vertebrae. Following the examination of the alizarin stained specimens, it was observed that there was a considerable degree of variability in the ossification of some individual vertebra or areas of the vertebral column. Because these variations existed, the observations were placed into twenty-one separate categories and then analyzed (responses ten to thirty inclusive). Responses ten to twelve concerned those observations recorded in the anterior arch of the atlas, responses thirteen to seventeen involved those variations noted in the ossification of the odontoid process (dens), responses eighteen to twenty-one involved variations noted in the number (present or absent) and sequence of ossification centres in the cervical centra, and response twenty-two concerned those fetuses which had absence of ossification centres in the thoracic, lumbar, and sacral regions. Responses twenty-three to twenty-six were related to the number of young which had various dumbbell-formed and duplicated centra in all regions of the vertebral

column, and response twenty-seven was associated with the number of ossified tail centra per mother's litter. Responses twenty-eight to thirty concerned the number of fetuses which had abnormally ossified (retarded), fused or absent vertebral arches in all regions of the column.

The above responses were analyzed, grouped together, and presented as effects on the following structures:

A. Anterior arch of atlas.

B. Odontoid process.

C. Cervical centra (number present, absent, and sequence).

D. Thoracic, lumbar, and sacral centra (number absent).

E. Incidence of dumbbell and duplicated centra (all regions).

F. Tail centra.

G. Vertebral arches.

A. Anterior Arch of Atlas. As stated, the analysis was done on responses ten to twelve; response ten was number of young per mother with uneven ossification in the anterior arch of the atlas (Figure 8), eleven was number with absent anterior arch (no ossification; Figure 9), and response twelve was total number of young with uneven or absence of ossification in the arch. The latter was a summary of effects in responses ten and eleven.

In responses ten to twelve, a highly significant difference was noted in the mean numbers of fetuses with these effects in groups receiving treatments on days eight to twelve and ten to twelve when compared with the means for those groups receiving treatments on day ten only of gestation.

From the means (R-10, R-11, and R-12, Table X) it appeared that treatments administered on day ten only were most effective since the means were generally higher.

In response ten (uneven ossification), the administration of podophyllin in alcohol on days eight to twelve of pregnancy produced a significant effect. The means for the podophyllin groups (I to VI) ranged from 25.3 to 45.0, but the mean for the alcohol control group (XXII) was 23.1 (R-10, Table X). These means suggested that podophyllin was preventing even ossification in the anterior arch.

A significant drug effect was also observed when the podophyllin (in alcohol) was given to groups VII to XII on days ten to twelve of gestation. The means for the drug treated groups (VII to XII) varied from 25.3 to 42.5, but the mean for the alcohol control group (XXIII) was 19.5 (R-10, Table X). In addition, the dose response curve, for groups VII to XII, had a significant cubic component; the means for groups XII to XII were 34.9, 35.6, 42.5, 25.3, 26.5, and 37.4 respectively (R-10, Table X). These means indicated that the lower and higher podophyllin doses were generally inhibiting even ossification; the intermediate dose (0.05 milligram) either stimulated even ossification or it was least effective since the mean number of observed effects was lowest (25.3).

The results of the analysis (in response ten) for groups receiving treatments on day ten only of gestation were not consistent with those groups which received treatments on days eight to twelve and ten to twelve of gestation. A significant alcohol effect was detected when the

Table X

Mean Number of Fetuses Per Group in Podophyllin Study with Uneven (R*-10), Absence (R-11), and Uneven or Absence of Ossification (R-12) in the Anterior Arch of the Atlas

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-10	Mean/Group R-11	Mean/Group R-12
I	8-12	0.012	+	-	+	34.6	7.4	36.8
II	8-12	0.025	+	-	+	45.0	2.5	45.6
III	8-12	0.05	+	-	+	25.3	1.0	25.3
IV	8-12	0.1	+	-	+	33.4	0.9	33.4
V	8-12	0.2	+	-	+	36.2	2.8	36.8
VI	8-12	0.4	+	-	+	40.3	4.1	41.4
XVII	8-12	0.1	-	+	+	60.1	12.7	69.8
XXII	8-12	-	+	-	+	23.1	1.0	23.1
XXV	8-12	-	-	+	+	52.6	9.2	58.3
XXVIII	8-12	-	-	-	+	21.4	0.9	21.4
VII	10-12	0.012	+	-	+	34.9	0.9	34.9
VIII	10-12	0.025	+	-	+	35.6	0.9	35.6
IX	10-12	0.05	+	-	+	42.5	2.5	44.1
X	10-12	0.1	+	-	+	25.3	9.7	34.1
XI	10-12	0.2	+	-	+	26.5	0.9	26.5
XII	10-12	0.4	+	-	+	37.4	2.6	38.0
XVIII	10-12	0.1	-	+	+	48.2	19.7	64.4
XXIII	10-12	-	+	-	+	19.5	0.9	19.5
XXVI	10-12	-	-	+	+	60.6	13.1	67.2
XXIX	10-12	-	-	-	+	23.0	1.0	23.0
XIII	10	0.1	+	-	+	44.2	10.0	47.6
XXIV	10	0.4	+	-	+	55.3	14.4	64.9
XV	10	0.8	+	-	+	52.3	4.5	53.8
XVI	10	1.6	+	-	+	39.4	9.0	42.5
XIX	10	0.1	-	+	+	56.7	12.7	64.9
XXIV	10	-	+	-	+	49.9	7.4	54.9
XXVII	10	-	-	+	+	57.1	7.9	61.9
XXX	10	-	-	-	+	57.1	10.8	62.4
XXXI	-	-	-	-	-	17.9	5.1	19.7

Explanation of Table X and Key to Symbols.

Refer to pages 69 and 70.

means for the podophyllin in water group (XIX) and water control group (XXVII) were compared with the means for the podophyllin in alcohol groups (XIII to XVI) and the alcohol control group (XXIV). The means, indicative of the number of fetuses which had unevenly ossified anterior arches in groups XIX and XXVII were 56.7 and 57.1, but the means for groups XIII to XVI and XXIV ranged from 39.4 to 55.3 (R-10, Table X). In this particular instance, the means suggested that alcohol may have enhanced ossification of this centre since the means were lower in the groups given podophyllin in alcohol and alcohol only, than in the groups given podophyllin in water and water only.

Interestingly, in response ten the dose response curve for podophyllin in alcohol (administered day ten only; groups XIII to XVI) had a significant linear component. The means (R-10, Table X) for groups XIII to XVI were 44.2, 55.3, 52.3, and 39.4; these means showed that as dosage increased, the number of fetuses with unevenly ossified arches decreased. This suggested that the high podophyllin dose (1.6 milligrams) was probably least effective since the mean was lowest.

A highly significant podophyllin effect was observed in response eleven (no ossification in the anterior arch). The animals in groups I to VI had been given podophyllin in ethanol on days eight to twelve of pregnancy. The means for the podophyllin treated groups (I to VI) varied from 0.9 to 7.4, but the mean for the alcohol control group (XXII) was 1.0 (R-11, Table X). In addition, the podophyllin dose response curve for groups I to VI had a significant quadratic component. The means for groups I to VI were 7.4, 2.5, 1.0, 0.9, 2.8, and 4.1 respectively (R-11,

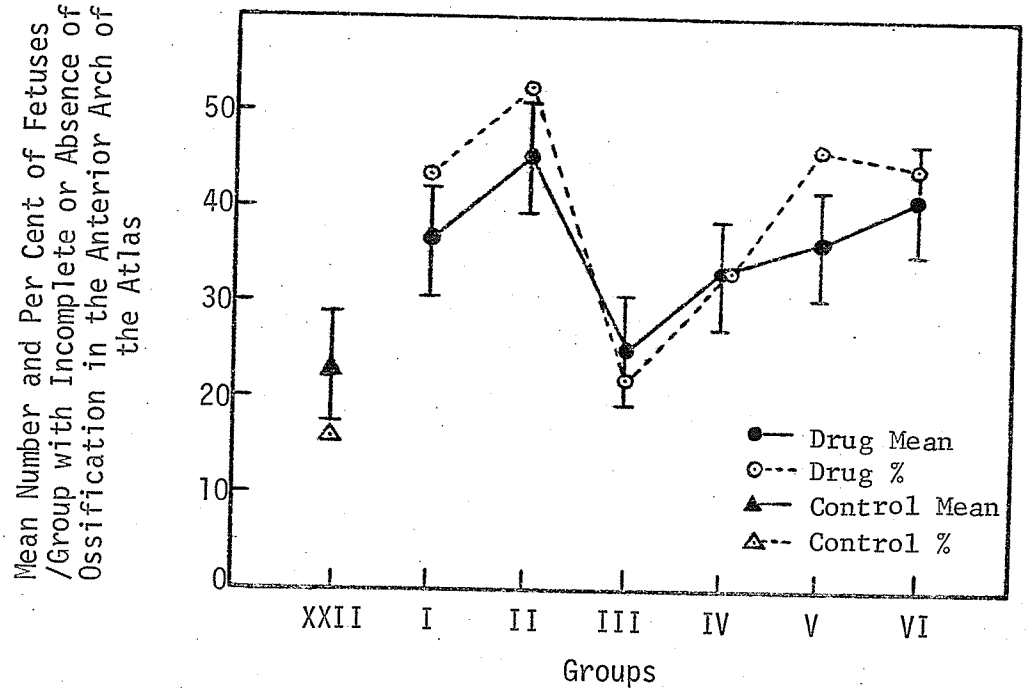
Table X). These values suggested that the low and high doses were inhibiting ossification in the arch, but the intermediate doses were either stimulating ossification or they were least effective, since the means were lowest (1.0 and 0.9).

A highly significant alcohol effect, similar to that noted in response ten for groups treated on day ten only, was also observed in response eleven for groups which received treatements on days ten to twelve of pregnancy. The means for the podophyllin in water group (XVIII) and water control group (XXVI) were 19.7 and 13.1 respectively, but in the podophyllin in alcohol groups (VII to XII) and the alcohol control group (XXIII) the means ranged from 0.9 to 9.7 (R-11, Table X). These means suggested that alcohol was enhancing ossification because they were low in groups VII to XII and XXIII.

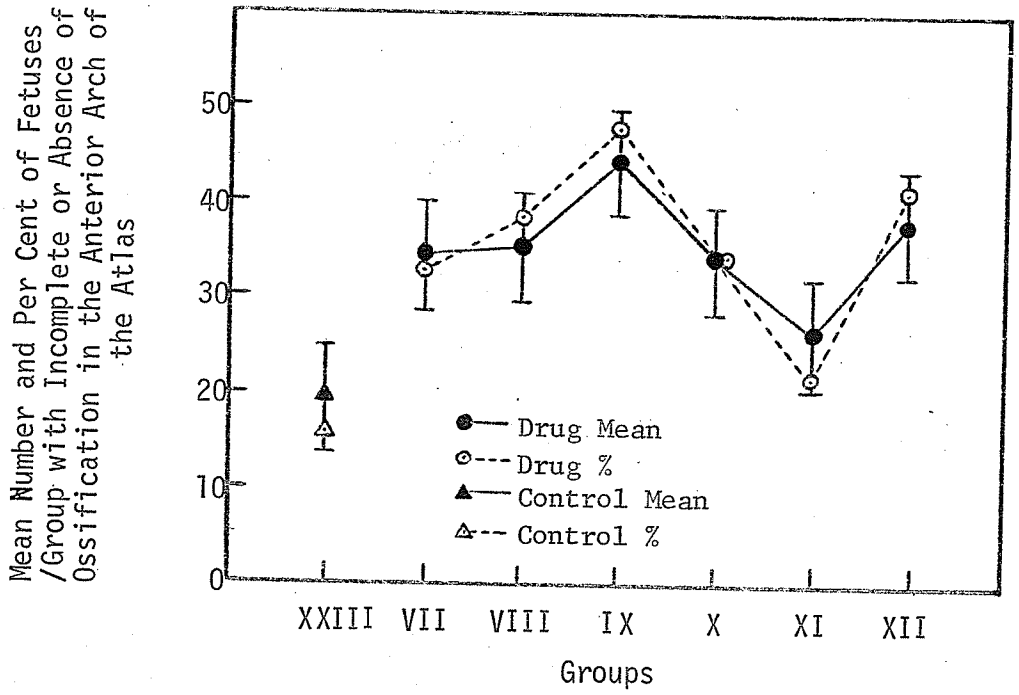
The raw data in responses ten and eleven were added together for each of the groups and once again analyzed in response twelve; uneven ossification and absence of the anterior arch. The overall effects and means (compare R-10 with R-12 in Table X) were generally similar to those observed in response ten (uneven ossification of the anterior arch). The results in response twelve are summarized in Graphs 3 and 4.

B. Odontoid Process. The responses analyzed were as follows: thirteen was number of young per mother with one ossification centre, fourteen was number with two separated ossification centres, fifteen was number with two joined ossification centres, sixteen was number with absence of an ossification centre, and seventeen was number of young with one ossification centre or two separated ossification centres or two joined ossification

Graphs 3 and 4



Graph 3



Graph 4

centres in the odontoid process. The latter was an addition of the values in responses thirteen to fifteen.

Response thirteen (one centre); in determining which treatment days were most effective, groups given treatments on days eight to twelve and ten to twelve were compared with groups given treatments on day ten only of gestation. The groups receiving treatments on days eight to twelve and ten to twelve had a highly significant increase in the number of fetuses with one ossification centre (R-13, Table XI). These results consequently produced a significant increase in the mean number of fetuses which had two centres joined in the odontoid process (response fifteen; R-15, Table XI). The observations, apart from indicating that treatments on days eight to twelve and ten to twelve were more effective, also implied that one of the treatments may have produced an effect.

There were no significant podophyllin, alcohol, or stomach tube effects in responses thirteen to seventeen. However, when the number of fetuses in the stomach tube control group was compared against the number of fetuses in the water control group, alcohol control group, the drug in water group, and the podophyllin in ethanol groups, highly significant and significant water treatment effects were observed in responses thirteen, fifteen, and seventeen (Table I, Appendix). In responses thirteen (one centre) and fifteen (two joined centres) the effect was noted in groups which received treatments on days eight to twelve of gestation (R-13 and R-15, Table XI), but in response seventeen (one centre, two separated centres, two joined centres) the effect occurred in groups treated on day ten only of pregnancy (R-17, Table XI). Although the analysis showed that water treatment was producing an effect, it does not seem possible.

Table XI

Mean Number of Fetuses Per Group in Podophyllin Study with One Ossification Centre (R*-13), Two Joined Ossification Centres (R-15), and One Centre or Two Joined or Two Separated Ossification Centres (R-17) in the Odontoid Process

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-13	Mean/Group R-15	Mean/Group R-17
I	8-12	0.012	+	-	+	16.0	33.1	48.6
II	8-12	0.025	+	-	+	18.2	29.7	44.7
III	8-12	0.05	+	-	+	26.6	18.0	52.0
IV	8-12	0.1	+	-	+	17.1	36.3	49.5
V	8-12	0.2	+	-	+	15.9	26.0	39.7
VI	8-12	0.4	+	-	+	24.1	22.1	45.1
XVII	8-12	0.1	-	+	+	17.7	24.9	43.2
XXII	8-12	-	+	-	+	25.2	28.4	48.5
XXV	8-12	-	-	+	+	26.5	28.4	49.1
XXVIII	8-12	-	-	-	+	31.8	15.2	44.1
VII	10-12	0.012	+	-	+	17.8	33.9	53.0
VIII	10-12	0.025	+	-	+	27.8	33.0	56.4
IX	10-12	0.05	+	-	+	21.3	23.8	41.7
X	10-12	0.1	+	-	+	27.5	23.5	45.5
XI	10-12	0.2	+	-	+	16.9	32.5	42.2
XII	10-12	0.4	+	-	+	24.1	24.2	47.2
XVIII	10-12	0.1	-	+	+	21.8	29.2	51.9
XXIII	10-12	-	+	-	+	22.7	37.7	54.2
XXVI	10-12	-	-	+	+	22.8	24.9	44.2
XXIX	10-12	-	-	-	+	27.4	25.9	49.6
XIII	10	0.1	+	-	+	26.6	35.6	54.6
XIV	10	0.4	+	-	+	12.5	33.3	44.9
XV	10	0.8	+	-	+	19.7	35.0	48.1
XVI	10	1.6	+	-	+	27.1	22.1	44.4
XIX	10	0.1	-	+	+	18.3	38.4	51.1
XXIV	10	-	+	-	+	12.5	35.0	47.2
XXVII	10	-	-	+	+	17.0	44.1	60.8
XXX	10	-	-	-	+	19.1	27.4	41.0
XXXI	-	-	-	-	-	28.4	23.5	46.2

Explanation of Table XI and Key to Symbols.

Refer to pages 69 and 70.

C. Cervical Centra (Number Present, Absent, and Sequence).

Responses eighteen to twenty-one were analyzed. Eighteen was number of young per mother with absence of ossification in either one or two cervical centra in consecutive order; starting the count with the second cervical (Figure 10). In this response it was noted that there was a highly significant increase in the number of fetuses with absent centra in animals which received treatments on days eight to twelve and ten to twelve of pregnancy (R-18, Table XII). This implied that treatments administered on these days were more effective in producing this effect than treatments which were given on day ten only of gestation.

In this same response, a significant podophyllin effect was noted when the drug was administered in alcohol on days eight to twelve. The means for groups (I to VI) receiving podophyllin varied from 46.1 to 58.7; the mean for the appropriate alcohol control group (XXII) was 60.2 (R-18, Table XII). In this instance the drug may have either stimulated ossification of these centres since there were fewer fetuses with absent centra in podophyllin treated animals than in the control group or it may be that there was considerable normal variation in ossification of these centres.

By contrast, a highly significant alcohol effect was observed in animals treated on days ten to twelve of pregnancy. The means for the podophyllin in water and water control groups (XVIII and XXVI) were 41.4 and 36.5 respectively, but the means for the podophyllin in alcohol groups (VII to XII) and the alcohol control group (XXIII) ranged from 46.0 to 54.9 (R-18, Table XII). This suggested that alcohol may inhibit

Table XII

Mean Number of Fetuses Per Group in Podophyllin Study with Absence of Ossification in One or Two Cervical Centra in Consecutive Order (R*-19), and One or More in Nonconsecutive Order (R-20) Starting the Count with the Second Cervical

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-18	Mean/Group R-19	Mean/Group R-20
I	8-12	0.012	+	-	+	47.4	9.6	6.1
II	8-12	0.025	+	-	+	51.5	10.0	14.6
III	8-12	0.05	+	-	+	58.7	16.3	10.3
IV	8-12	0.1	+	-	+	47.0	20.7	8.8
V	8-12	0.2	+	-	+	46.2	26.7	12.8
VI	8-12	0.4	+	-	+	46.1	19.5	9.8
XVII	8-12	0.1	-	+	+	46.2	15.3	13.7
XXII	8-12	-	+	-	+	60.2	11.1	11.8
XXV	8-12	-	-	+	+	40.1	21.1	17.5
XXVIII	8-12	-	-	-	+	57.4	14.7	10.5
VII	10-12	0.012	+	-	+	46.0	9.8	16.6
VIII	10-12	0.025	+	-	+	52.2	11.3	7.1
IX	10-12	0.05	+	-	+	53.4	26.9	12.2
X	10-12	0.1	+	-	+	51.9	25.3	9.7
XI	10-12	0.2	+	-	+	53.2	19.0	8.7
XII	10-12	0.4	+	-	+	50.2	9.6	10.3
XVIII	10-12	0.1	-	+	+	41.4	24.4	13.5
XXIII	10-12	-	+	-	+	54.9	16.1	10.0
XXVI	10-12	-	-	+	+	36.5	21.4	8.2
XXIX	10-12	-	-	-	+	53.3	17.4	13.3
XIII	10	0.1	+	-	+	45.7	12.3	3.9
XIV	10	0.4	+	-	+	38.2	24.4	9.0
XV	10	0.8	+	-	+	43.2	14.6	12.4
XVI	10	1.6	+	-	+	36.9	15.5	18.1
XIX	10	0.1	-	+	+	41.7	16.5	18.1
XXIV	10	-	+	-	+	38.5	10.8	10.5
XXVII	10	-	-	+	+	37.2	13.8	5.9
XXX	10	-	-	-	+	44.1	13.1	8.1
XXXI	-	-	-	-	-	56.3	16.9	7.6

Explanation of Table XII and Key to Symbols.

Refer to pages 69 and 70.

development of these centres; the means were higher in animals treated with podophyllin in alcohol, or alcohol alone, than in animals treated with podophyllin in water, or water only.

Response nineteen was number of young per mother with absence of three or more cervical centra in consecutive order, starting with the second cervical. The dose response curve for groups VII to XII, treated with podophyllin in alcohol on days ten to twelve of pregnancy, had a significant quadratic component. The means (R-19, Table XII) showing the number of fetuses affected in groups VII to XII were 9.8, 11.3, 26.9, 25.3, 19.0, and 9.6 respectively. These means suggested that the low and high podophyllin doses either stimulated ossification of these centres or they were least effective because the means were low. By contrast, the drug at the intermediate dosage levels (0.05, 0.1 and 0.2 milligram) appeared to inhibit or delay ossification of these centres.

Response twenty was number of young per mother with absence of one or more cervical centra in nonconsecutive order, starting with the second cervical (Figure 11). There were no significant observations in fetuses of animals which received treatments on days eight to twelve and ten to twelve of pregnancy. However, a significant drug effect was noted when podophyllin was administered in water to animals on the tenth day only of gestation. The mean for the podophyllin treated group (XIX) was 18.1, but for the water control group (XXVII) it was 5.9 (R-20, Table XII). This showed that podophyllin inhibited ossification of these centres since there were more fetuses with absent centra in podophyllin treated animals than in fetuses of water control mothers.

Response twenty-one was a summary of the results in responses eighteen to twenty; it was total number of young per mother with absence of cervical centra irrespective of the number absent. It was observed at a high level of significance that there were more fetuses with absent centra in animals which received treatments on days eight to twelve and ten to twelve than in fetuses of mothers which received treatments on day ten only of pregnancy.

A significant quadratic and cubic component was observed in the dose response curve for the groups (I to VI) treated with podophyllin in alcohol on days eight to twelve of gestation. The means (R-21, Table XIII) indicating the number of young affected in each of the groups were 55.7, 62.8, 78.4, 68.4, 78.3, and 68.0 respectively. The quadratic component suggested that the low and high drug doses (0.012, 0.025, and 0.4 milligram) were either stimulating ossification of the cervical centres or they were least effective, but the intermediate doses (0.05, 0.1, and 0.2 milligram) were inhibiting or delaying development of these centres. The cubic component, apart from once again showing the previous effect, also showed that the 0.05 and 0.2 milligram doses were the most effective; the means for the groups (III and V) which received the 0.05 and 0.02 milligram doses were 78.4 and 78.3 respectively.

A similar effect was also observed in animals (groups VII to XII) which received podophyllin in alcohol on days ten to twelve of pregnancy; the dose response curve had a highly significant quadratic component. The means for groups VII to XII were 61.2, 63.5, 80.9, 77.1, 71.6, and 59.6 respectively (R-21, Table XIII). These means further indicated that

Table XIII

Mean Number of Fetuses Per Group in Podophyllin Study with Absence of Ossification in Cervical Centra (R*-21) Irrespective of the Number, and Slightly Dumbbell Centra (R-23) and Dumbbell Centra (R-24) in all Vertebral Regions

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-21	Mean/Group R-23	Mean/Group R-24
I	8-12	0.012	+	-	+	55.7	2.3	5.5
II	8-12	0.025	+	-	+	62.8	7.7	2.3
III	8-12	0.05	+	-	+	78.4	11.7	1.0
IV	8-12	0.1	+	-	+	68.4	5.7	3.6
V	8-12	0.2	+	-	+	78.3	2.9	1.1
VI	8-12	0.4	+	-	+	68.0	4.1	2.9
XVII	8-12	0.1	-	+	+	64.3	2.8	0.9
XXII	8-12	-	+	-	+	74.8	5.5	1.0
XXV	8-12	-	-	+	+	63.2	2.8	1.0
XXVIII	8-12	-	-	-	+	73.1	6.7	0.9
VII	10-12	0.012	+	-	+	61.2	0.9	2.7
VIII	10-12	0.025	+	-	+	63.5	5.5	2.5
IX	10-12	0.05	+	-	+	80.9	4.4	5.1
X	10-12	0.1	+	-	+	77.1	5.1	7.0
XI	10-12	0.2	+	-	+	71.6	10.3	4.0
XII	10-12	0.4	+	-	+	59.6	4.1	4.0
XVIII	10-12	0.1	-	+	+	66.9	9.4	4.7
XXIII	10-12	-	+	-	+	71.7	2.8	2.6
XXVI	10-12	-	-	+	+	62.4	5.1	0.8
XXIX	10-12	-	-	-	+	71.0	3.6	2.8
XIII	10	0.1	+	-	+	51.4	2.5	2.5
XIV	10	0.4	+	-	+	58.3	1.1	1.1
XV	10	0.8	+	-	+	54.3	2.6	2.4
XVI	10	1.6	+	-	+	55.8	6.5	6.5
XIX	10	0.1	-	+	+	61.4	5.5	0.9
XXIV	10	-	+	-	+	46.4	5.7	0.9
XXVII	10	-	-	+	+	47.4	2.6	5.8
XXX	10	-	-	-	+	51.4	4.0	2.4
XXXI	-	-	-	-	-	73.6	4.2	0.9

Explanation of Table XIII and Key to Symbols.

Refer to pages 69 and 70.

the low and high doses were either stimulating ossification or they were least effective whereas the intermediate doses (0.05 and 0.1 milligram) were either inhibiting or delaying ossification in these centres.

D. Thoracic, Lumbar, and Sacral Centra (Number Absent).

Response twenty-two was analyzed; total number of young per mother with absent thoracic, lumbar, or sacral vertebral centra. There were no significant observations in this response.

E. Incidence of Dumbbell and Duplicated Centra (All Regions).

The analysis was done on responses twenty-three to twenty-six. Twenty-three was number of young per mother with slightly dumbbell vertebral centra (Figure 12), twenty-four was number with dumbbell centra (Figure 13), twenty-five was number of young per mother with duplicated vertebral centra (Figure 14), and response twenty-six was total number of young per mother with various forms of dumbbell and duplicated centra. The latter was an addition of the values in responses twenty-three to twenty-five.

In response twenty-three (slightly dumbbell), the dose response curve for the podophyllin in alcohol groups (I to VI) treated on days eight to twelve of gestation had a significant quadratic component. The means (R-23, Table XIII), indicative of the number of fetuses in groups I to VI with slightly dumbbell centra, were 2.3, 7.7, 11.7, 5.7, 2.9, and 4.1 respectively. These means suggested that the low and high podophyllin doses were either inhibiting this effect or that they were least effective. The intermediate podophyllin dose of 0.05 milligram appeared to be a stimulator of this effect because the mean was highest; 11.7. Apart from this observation, there were no other significant results in this response.

The podophyllin had a significant effect in response twenty-four (dumbbell centra) following the administration of the drug in alcohol to animals in groups I to VI on days eight to twelve of pregnancy (Graph 5). The means, showing (not arithmetically) the number of fetuses with dumbbell centra in each of the podophyllin treated groups (I to VI), ranged from 1.0 to 5.5, but the mean for the alcohol control group (XXII) was 1.0 (R-24, Table XIII). A highly significant drug effect was also noted when the podophyllin ethanol mixture was given to groups XIII to XVI on day ten only of pregnancy (Graph 6). The means for groups XIII to XVI were 2.5, 1.1, 2.4, and 6.5 respectively, but the mean for the alcohol control group (XXIV) was 0.9 (R-24, Table XIII).

Interestingly, although podophyllin produced significant effects in response twenty-four by causing an increase in the number of fetuses which had dumbbell centra, significant podophyllin effects were not observed in response twenty-five (duplicated vertebral centra).[1] There were no other significant effects in this response.

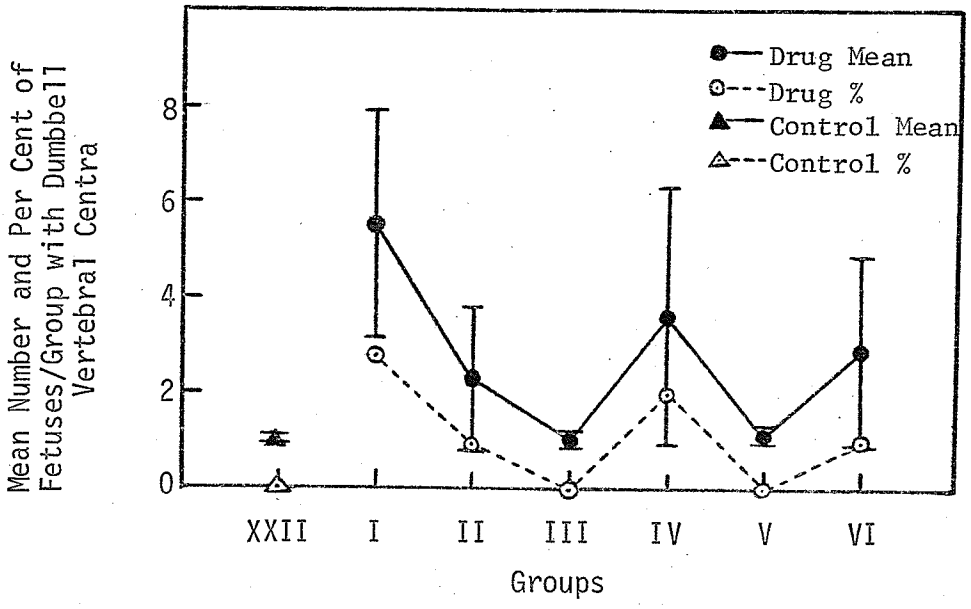
As previously stated, response twenty-six was designed to summarize the effects observed in responses twenty-three to twenty-five. Unfortunately there were no significant differences between the means in any of the groups analyzed.

F. Tail Centra. Response twenty-seven related to the total number of tail centra per mother's litter. The dose response curve for

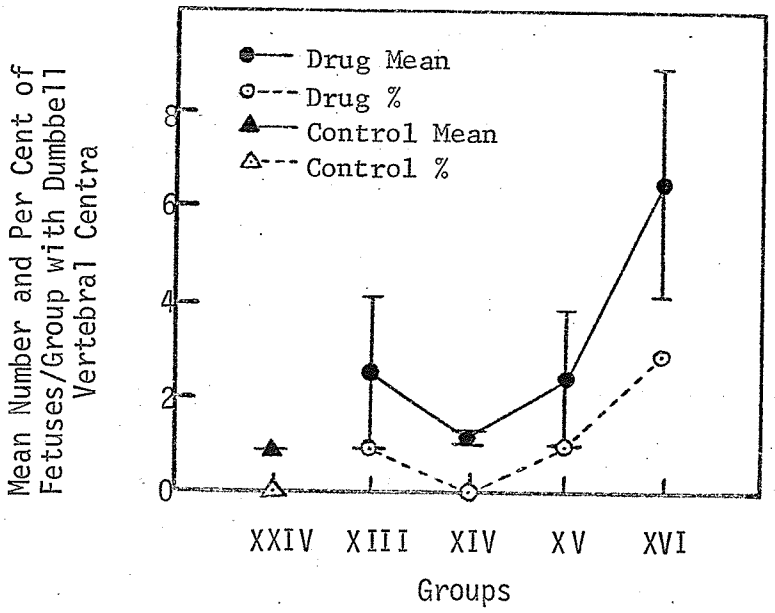
---

[1]This result occurred when the values for the groups receiving some form of treatment were compared against the value for the normal group (R-25, Table XIV).

Graphs 5 and 6



Graph 5



Graph 6

Table XIV

Mean Number of Fetuses Per Group in Podophyllin Study with Duplicated Vertebral Centra (R*-25), Mean Number of Tail Centra Per Fetus (R-27) in Each Group, and Mean Number of Fetuses Per Group with Fused Vertebral Arches (R-28)

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-25	Mean/Group R-27	Mean/Group R-28
I	8-12	0.012	+	-	+	0.9	7.0	0.9
II	8-12	0.025	+	-	+	2.2	6.7	0.8
III	8-12	0.05	+	-	+	1.0	6.8	1.0
IV	8-12	0.1	+	-	+	5.4	6.6	3.6
V	8-12	0.2	+	-	+	1.1	6.4	1.1
VI	8-12	0.4	+	-	+	0.9	6.8	0.9
XVII	8-12	0.1	-	+	+	0.9	6.9	0.9
XXII	8-12	-	+	-	+	1.0	8.0	1.0
XXV	8-12	-	-	+	+	1.0	7.1	1.0
XXVIII	8-12	-	-	-	+	0.9	6.5	0.9
VII	10-12	0.012	+	-	+	0.9	7.1	0.9
VIII	10-12	0.025	+	-	+	2.5	6.7	0.9
IX	10-12	0.05	+	-	+	3.2	6.2	3.2
X	10-12	0.1	+	-	+	2.7	5.9	0.9
XI	10-12	0.2	+	-	+	0.9	6.8	0.9
XII	10-12	0.4	+	-	+	0.9	7.1	0.9
XVIII	10-12	0.1	-	+	+	2.5	6.6	1.1
XXIII	10-11	-	+	-	+	0.9	6.8	0.9
XXVI	10-12	-	-	+	+	0.8	6.2	0.8
XXIX	10-12	-	-	-	+	2.8	6.6	1.0
XIII	10	0.1	+	-	+	0.9	7.2	0.9
XIV	10	0.4	+	-	+	1.1	7.4	1.1
XV	10	0.8	+	-	+	0.9	7.0	0.9
XVI	10	1.6	+	-	+	1.7	6.9	1.7
XIX	10	0.1	-	+	+	0.9	6.6	0.9
XXIV	10	-	+	-	+	2.5	7.3	0.9
XXVII	10	-	-	+	+	0.9	7.2	0.9
XXX	10	-	-	-	+	0.9	6.7	2.5
XXXI	-	-	-	-	-	0.9	6.4	0.9

Explanation of Table XIV and Key to Symbols.

Refer to pages 69 and 70.

the podophyllin ethanol mixture administered to groups I to VI on days eight to twelve of gestation had a highly significant quadratic component. The means for groups I to VI were 7.1, 6.7, 6.2, 5.9, and 7.1 respectively (R-27, Table XIV). These means, though not widely divergent, illustrated that the low and high drug doses either stimulated an increase in the number of tail centra per fetus or they were least effective because the means were high; the 0.05 and 0.1 milligram podophyllin doses appeared to inhibit ossification of these centres.

Once again, a significant water treatment effect was noted when administered on day ten only of gestation. Group XXX, which received the stomach tube treatment on the tenth day only of gestation, was compared with the groups which received the water (XXVII), alcohol (XXIV), drug in water (XIX), and the podophyllin in ethanol (XIII to XVI). The means are shown in Table XIV (R-27).

G. Vertebral Arches. Responses twenty-eight, twenty-nine, and thirty were analyzed. Twenty-eight was number of young per mother with fusion of vertebral arches (Figure 15), twenty-nine was number of young per mother with absent vertebral arches, and response thirty was an addition and then analysis of the raw data in responses twenty-eight and twenty-nine.

In response twenty-eight (fused arches) a significant stomach tube effect was observed; the normal group was compared against all the groups which received some form of treatment on days eight to twelve, ten to twelve, and day ten only of pregnancy. The means are in Table XIV (R-28). There were no other significant observations in this response; this also applies to responses twenty-nine and thirty.

Effects on Ribs. The analysis was done on responses thirty-one to thirty-four. Response thirty-one was total number of young per mother with anomalous or absent ribs (excluding forms of supernumerary ribs). Although there were no significant observations, there were two podophyllin treated mothers (groups XV and XVI) which had fetuses with malformed ribs (Figures 16 and 17). Ribs in fetuses of all control mothers were normal.

Response thirty-two was number of young per mother with unilateral supernumerary ribs in the cervical or lumbar regions (Figure 18), thirty-three was the number of young with bilateral supernumerary ribs (Figure 19), and response thirty-four was an addition of the values in responses thirty-two and thirty-three; it was a summary.

In response thirty-two (unilateral, mostly lumbar) a significant podophyllin in alcohol effect was noted when the drug was administered on days eight to twelve of gestation. The means for the podophyllin treated groups (I to VI) varied from 10.5 to 22.9, but the mean for the alcohol group (XXII) was 27.3 (R-32, Table XV). From these means it appeared that the podophyllin may have inhibited the occurrence of unilateral supernumerary ribs. There were no other significant observations in this response.

The only significant observation in response thirty-three (bilateral, mostly lumbar) was the cubic-shaped dose response curve for the animals in groups VII to XII which were given the podophyllin alcohol mixture on days ten to twelve of their pregnancy. The means, indicating the number of fetuses with bilateral supernumerary ribs in groups VII to XII were 14.2, 6.0, 2.7, 8.4, 14.5, and 9.0 respectively (R-33, Table XV). These

Table XV

Mean Number of Fetuses Per Group in Podophyllin Study with Unilateral Supernumerary Ribs (R*-32), Bilateral Supernumerary Ribs (R-33), and Slightly Retarded Ossification in the Fifth Sternebra (R-35)

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-32	Mean/Group R-33	Mean/Group R-35
I	8-12	0.012	+	-	+	22.9	13.2	18.9
II	8-12	0.025	+	-	+	10.5	7.7	25.2
III	8-12	0.05	+	-	+	13.6	13.6	19.8
IV	8-12	0.1	+	-	+	12.3	4.2	25.9
V	8-12	0.2	+	-	+	17.9	13.2	25.7
VI	8-12	0.4	+	-	+	16.9	14.3	22.4
XVII	8-12	0.1	-	+	+	10.3	4.4	18.4
XXII	8-12	-	+	-	+	27.3	8.2	16.4
XXV	8-12	-	-	+	+	18.2	10.6	15.5
XXVIII	8-12	-	-	-	+	19.9	7.2	17.4
VII	10-12	0.012	+	-	+	17.9	14.2	19.3
VIII	10-12	0.025	+	-	+	8.4	6.0	27.8
IX	10-12	0.05	+	-	+	13.0	2.7	22.8
X	10-12	0.1	+	-	+	20.0	8.4	22.6
XI	10-12	0.2	+	-	+	15.3	14.5	28.7
XII	10-12	0.4	+	-	+	23.7	9.0	23.3
XVII	10-12	0.1	-	+	+	15.2	12.6	14.3
XXII	10-12	-	+	-	+	24.4	13.5	18.9
XXVI	10-12	-	-	+	+	16.4	11.1	26.2
XXIX	10-12	-	-	-	+	12.7	5.2	18.1
XIII	10	0.1	+	-	+	10.7	5.3	21.8
XIV	10	0.4	+	-	+	15.8	14.7	17.0
XV	10	0.8	+	-	+	10.6	12.1	24.7
XVI	10	1.6	+	-	+	14.6	11.7	15.2
XIX	10	0.1	-	+	+	20.9	20.2	22.9
XXIV	10	-	+	-	+	14.4	7.1	24.3
XXVII	10	-	-	+	+	13.3	12.1	22.5
XXX	10	-	-	-	+	8.3	11.1	29.6
XXXI	-	-	-	-	-	26.9	4.6	18.1

Explanation of Table XV and Key to Symbols.

Refer to pages 69 and 70.

means showed that the low and high podophyllin doses were not as effective as the intermediate dose (0.05 milligram). The 0.05 milligram podophyllin dose may have inhibited this effect because the mean was lowest at this dosage.

Response thirty-four, which was an addition of the values in responses thirty-two and thirty-three, did not contain any significant results.

Effects on Sternebrae. Because there was a considerable degree of variability in sternal ossification (especially the fifth) seven different types of observations were recorded and later analyzed (responses thirty-five to forty-one). Responses thirty-five to thirty-nine were associated with degrees of ossification noted in the fifth sternal centre only; it appeared to be the most vulnerable. Response forty was concerned with degrees of ossification in sternal centres one to four and six. Response forty-one was associated with the degrees of ossification in all six sternal centres at one time; it was a summary of effects on the sternum.

The above responses, when analyzed, were grouped together and presented as effects on the following centres.

A. Fifth sternal centre.

B. First, second, third, fourth, and sixth sternal centres.

C. Sternal centres one to six inclusive.

A. Fifth Sternal Centre. Response thirty-five was number of young per mother with slightly retarded ossification in the fifth sternebra (Figure 20), thirty-six was number of fetuses per mother with retarded

ossification of this centre (Figure 21), thirty-seven was number of fetuses with almost no ossification in the fifth sternebra (Figure 22), number thirty-eight was number of young per mother with no ossification in the fifth sternal centre (Figure 23), and response thirty-nine was total number of young per mother with abnormally ossified or absent fifth sternebra. The latter was an addition of the values in responses thirty-five to thirty-eight.

In response thirty-five (slightly retarded), the podophyllin water mixture was significantly effective when administered to group XVIII on days ten to twelve of pregnancy. The mean for the podophyllin water group (XVIII) was 14.3 whereas the mean for the water control group (XXVI) was 26.2 (R-35, Table XV). From these means it appeared that the podophyllin may have either stimulated ossification of this centre, there being fewer fetuses with slightly retarded fifth sternal centres in the drug treated group than in the water control group, or it may be that the differences, though significant, nevertheless represented an sample from a group varying only within normal limits.

Also in response thirty-five, it was observed that the water was producing a significant effect when given on day ten only of gestation. The group which received the stomach tube treatment (group XXX) was compared with the remaining groups which received the other forms of treatments (groups XXVII, XXIV, XIX, and XIII to XVI). The mean for the stomach tube group (XXX) was 29.6, but the means for the water control group (XXVII), alcohol control group (XXIV), podophyllin in water group (XIX), and the drug in alcohol groups (XIII to XVI) ranged from 15.2 to 24.7 (R-35, Table XV).

There was a highly significant increase in the overall mean numbers of fetuses with retarded fifth sternal centres (response thirty-six) in the groups which were given treatments on day ten only of gestation when compared with the mean numbers of fetuses in those groups which received treatments on day eight to twelve and ten to twelve of pregnancy (R-36, Table XVI). However, comparison of the groups treated on days eight to twelve and ten to twelve revealed significantly more fetuses with this effect in the groups treated on days eight to twelve than in the groups treated on days ten to twelve of gestation (R-36, Table XVI).

The podophyllin in alcohol significantly retarded ossification in the fifth sternebra of fetuses when the animals in groups I to VI were treated on days eight to twelve of gestation. The means for the podophyllin treated groups ranged from 6.7 to 28.2, but the mean for the alcohol control group (XXII) was 10.5 (R-36, Table XVI). The dose response curve, in addition for groups I to VI, had a highly significant quadratic component. The means for groups I to VI were 28.2, 18.6, 6.7, 18.5, 25.2, and 26.0 respectively (R-36, Table XVI). These means showed that the low and high podophyllin doses were either retarding or delaying ossification, but the intermediate dose (0.05 milligram) either stimulated ossification or it was least effective since the mean was lowest.

A significant podophyllin effect was also noted in response thirty-six (retarded) when the drug in alcohol mixture was administered to groups VII to XII on days ten to twelve of gestation. The means for the podophyllin treated groups (VII to XII) varied from 6.9 to 19.0, but the mean for the alcohol control group (XXIII) was 3.9 (R-36, Table XVI).

Table XVI

Mean Number of Fetuses Per Group in Podophyllin Study with Retarded Ossification (R*-36), Almost No Ossification (R-37), and Absence of Ossification (R-38) in the Fifth Sternebra

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-36	Mean/Group R-37	Mean/Group R-38
I	8-12	0.012	+	-	+	28.2	0.9	0.9
II	8-12	0.025	+	-	+	18.6	2.2	0.8
III	8-12	0.05	+	-	+	6.7	1.0	1.0
IV	8-12	0.1	+	-	+	18.5	5.5	0.9
V	8-12	0.2	+	-	+	25.2	3.5	1.1
VI	8-12	0.4	+	-	+	26.0	0.9	0.9
XVII	8-12	0.1	-	+	+	19.0	0.9	0.9
XXII	8-12	-	+	-	+	10.5	1.1	1.0
XXV	8-12	-	-	+	+	10.8	1.0	1.0
XXVIII	8-12	-	-	-	+	11.2	0.9	0.9
VII	10-12	0.012	+	-	+	19.0	0.9	2.7
VIII	10-12	0.025	+	-	+	15.7	0.9	0.9
IX	10-12	0.05	+	-	+	17.6	4.8	0.9
X	10-12	0.1	+	-	+	6.9	2.7	9.7
XI	10-12	0.2	+	-	+	10.4	2.4	2.8
XII	10-12	0.4	+	-	+	16.0	0.9	0.9
XVIII	10-12	0.1	-	+	+	17.1	1.1	4.7
XXIII	10-12	-	+	-	+	3.9	2.6	0.9
XXVI	10-12	-	-	+	+	11.9	0.8	0.8
XXIX	10-12	-	-	-	+	8.2	1.0	2.8
XIII	10	0.1	+	-	+	31.0	0.9	2.4
XIV	10	0.4	+	-	+	23.1	1.1	6.4
XV	10	0.8	+	-	+	21.4	0.9	0.9
XVI	10	1.6	+	-	+	18.0	1.7	1.7
XIX	10	0.1	-	+	+	16.1	0.9	0.9
XXIV	10	-	+	-	+	19.0	0.9	0.9
XXVII	10	-	-	+	+	17.0	0.9	0.9
XXX	10	-	-	-	+	33.0	0.9	2.5
XXXI	-	-	-	-	-	9.1	0.9	0.9

Explanation of Table XVI and Key to Symbols.

Refer to pages 69 and 70.

This once again showed that the drug retarded ossification in the fifth sternebra.

Although a significant podophyllin effect (retarded ossification) was noted in groups (I to VI and VII to XII) treated with the drug on days eight to twelve and ten to twelve of pregnancy, there was no significant podophyllin effect when the drug in alcohol mixture was administered to the animals on day ten only of gestation. Instead a highly significant water treatment effect was observed; the group (XXX) that received the stomach tube treatment on day ten was compared with the groups (XXVII, XXIV, XIX, and XIII to XVI) which received the other forms of treatment on the same day. The mean for the stomach tube group (XXX) was 33.0, but the means for the water control group (XXVII), alcohol control group (XXIV), drug in water group (XIX), and the podophyllin in alcohol groups (XIII to XVI) ranged from 16.1 to 31.0 (R-36, Table XVI).

In response thirty-seven, number of fetuses per mother with almost no ossification in the fifth sternebra, it was noted (from the means) that treatments administered on days eight to twelve and ten to twelve of gestation were significantly more effective than when given on day ten only of pregnancy (R-37, Table XVI). A significant alcohol effect, rather than podophyllin effect, was observed in the groups that received treatments on days eight to twelve of pregnancy. The means for the water control group (XXV) and the podophyllin in water group (XVIII) were 0.9 and 1.0, but the means for the alcohol control group (XXII) and the podophyllin in alcohol groups (I to VI) ranged from 0.9 to 5.5 (R-37, Table XVI). Apart from this, there were no other significant effects observed in this response.

The only significant observation in the analysis of response thirty-eight, no ossification in the fifth sternebra, was a stomach tube effect. In thirteen of the twenty-nine groups that received some form of treatment, the means (R-38, Table XVI) were larger than the mean for the normal group (XXXI).

Response thirty-nine was an addition of the values in responses thirty-five to thirty-eight. The effects observed in this response were almost identical with those noted in response thirty-six (retarded ossification). First, there was a highly significant increase in the overall mean numbers of fetuses with abnormally retarded or absent fifth sternebra in groups that were given treatments on day ten only of gestation when compared with the mean number of young in those groups that received treatments on days eight to twelve and ten to twelve of pregnancy (R-39, Table XVII).

Second, the podophyllin alcohol mixture significantly retarded or completely deleted ossification in the fifth sternebra of the fetuses in groups I to VI; animals in these groups were given the drug at various dose levels on days eight to twelve of pregnancy. The means for the podophyllin treated groups (I to VI) varied from 25.1 to 41.4 whereas the mean for the alcohol control group (XXII) was 22.5 (R-39, Table XVII).

Third, administration of the podophyllin alcohol suspension to animals in groups VII to XII on days ten to twelve of gestation also resulted in a significant drug effect. The mean number of fetuses with abnormally ossified or absent fifth sternal centres in the podophyllin treated groups (VII to XII) ranged from 31.6 to 35.8, but the mean for

Table XVII

Mean Number of Fetuses Per Group in Podophyllin Study with Abnormal or Absence of Ossification in the Fifth Sternebra (R*-39), Abnormal or Absence of Ossification in Sternal Centres Excluding the Fifth (R-40), and Abnormal or Absence of Ossification in Any or All of the Six Sternal Centres (R-41)

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-39	Mean/Group R-40	Mean/Group R-41
I	8-12	0.012	+	-	+	36.8	13.5	38.0
II	8-12	0.025	+	-	+	35.0	12.8	36.5
III	8-12	0.05	+	-	+	25.1	11.3	25.9
IV	8-12	0.1	+	-	+	37.9	14.9	39.4
V	8-12	0.2	+	-	+	41.4	19.1	49.8
VI	8-12	0.4	+	-	+	39.6	11.9	40.1
XVII	8-12	0.1	-	+	+	28.5	20.6	35.0
XXII	8-12	-	+	-	+	22.5	16.8	27.7
XXV	8-12	-	-	+	+	21.1	9.7	25.2
XXVIII	8-12	-	-	-	+	23.7	8.0	26.0
VII	10-12	0.012	+	-	+	32.3	10.4	33.0
VIII	10-12	0.025	+	-	+	35.5	14.0	36.7
IX	10-12	0.05	+	-	+	33.4	14.7	36.7
X	10-12	0.1	+	-	+	35.8	19.8	39.3
XI	10-12	0.2	+	-	+	33.7	18.9	35.7
XII	10-12	0.4	+	-	+	31.6	10.5	32.5
XVIII	10-12	0.1	-	+	+	28.0	15.8	29.9
XXIII	10-12	-	+	-	+	21.8	14.9	23.1
XXVI	10-12	-	-	+	+	31.5	15.4	36.6
XXIX	10-12	-	-	-	+	22.5	11.6	24.2
XIII	10	0.1	+	-	+	43.8	19.3	46.1
XIV	10	0.4	+	-	+	37.0	17.4	37.0
XV	10	0.8	+	-	+	37.9	9.4	38.4
XVI	10	1.6	+	-	+	25.9	17.6	30.8
XIX	10	0.1	-	+	+	30.9	17.8	36.2
XXIV	10	-	+	-	+	36.4	13.1	40.5
XXVII	10	-	-	+	+	30.6	11.0	32.4
XXX	10	-	-	-	+	49.0	22.4	54.9
XXXI	-	-	-	-	-	22.8	2.6	22.8

Explanation of Table XVII and Key to Symbols.

Refer to pages 69 and 70.

the alcohol control group (XXIII) was 21.8 (R-39, Table XVII). This further indicated that podophyllin was retarding or completely deleting ossification in the fifth sternebra.

Fourth, there was no significant podophyllin (in alcohol) effect when administered on day ten only of gestation; this was also noted in response thirty-six (retarded ossification). Instead a highly significant water treatment effect was observed once more when the group (XXX) that received the stomach tube treatment on day ten was compared with the groups (XXVII, XXIV, XIX, and XIII to XVI) that received the other forms of treatment on day ten only of pregnancy. The mean for the stomach tube group (XXX) was 49.0, but the means for the water control group (XXVII), alcohol control group (XXIV), drug in water group (XIX), and the podophyllin in alcohol groups (XIII to XVI) varied from 25.9 to 43.8 (R-39, Table XVII).

B. First, Second, Third, Fourth, and Sixth Sternal Centres. Response forty was analyzed. It was total number of young per mother with retarded or absence of ossification in sternal centres other than the fifth (Figures 24 and 25). A highly significant stomach tube effect was noted when the mean for the normal group (XXXI) was compared against the means for all of the other groups (I to XIX and XXII to XXX) that received various treatments on days eight to twelve, ten to twelve, and day ten only of pregnancy (R-40, Table XVII). Because there were no other significant observations in this response, this indicated that the stomach tube treatments administered on the various days were retarding or completely deleting ossification in the first to fourth and sixth sternebrae. The means (R-40, Table XVII) for all the treatments groups (I to XIX and XXII to XXX) were larger than the mean for the normal group (XXXI).

C. Sternal Centres One to Six Inclusive. The analysis was done on response forty-one; it was total number of young per mother with abnormal or absence of ossification in any of the six sternebrae. It was a summary of effects on the entire sternum and not an addition of the values in responses thirty-nine and forty.[1]

There was a highly significant increase in the overall mean (R-41, Table XVII) numbers of fetuses with sternebral effects in the groups that received treatments on day ten only when compared with the means for those groups that received treatments on days eight to twelve and ten to twelve of pregnancy. This seemed to indicate that treatments generally administered on day ten only gestation were more effective in retarding sternebral ossification.

A significant podophyllin (in alcohol) effect was observed when the drug was administered to groups I to VI on days eight to twelve of pregnancy (Graph 7). The means, indicating the number of fetuses with affected sternebrae in the podophyllin treated groups (I to VI) varied from 25.9 to 49.8, but the mean for the alcohol control group (XXII) was 27.7 (R-41, Table XVII). In addition, the podophyllin in alcohol dose response curve for groups I to VI had a significant cubic component. The means for groups I to VI were 38.0, 36.5, 25.9, 39.4, 49.8, and 40.1 respectively (R-41, Table XVII). These means suggested that the low and high podophyllin doses generally inhibited or delayed ossification

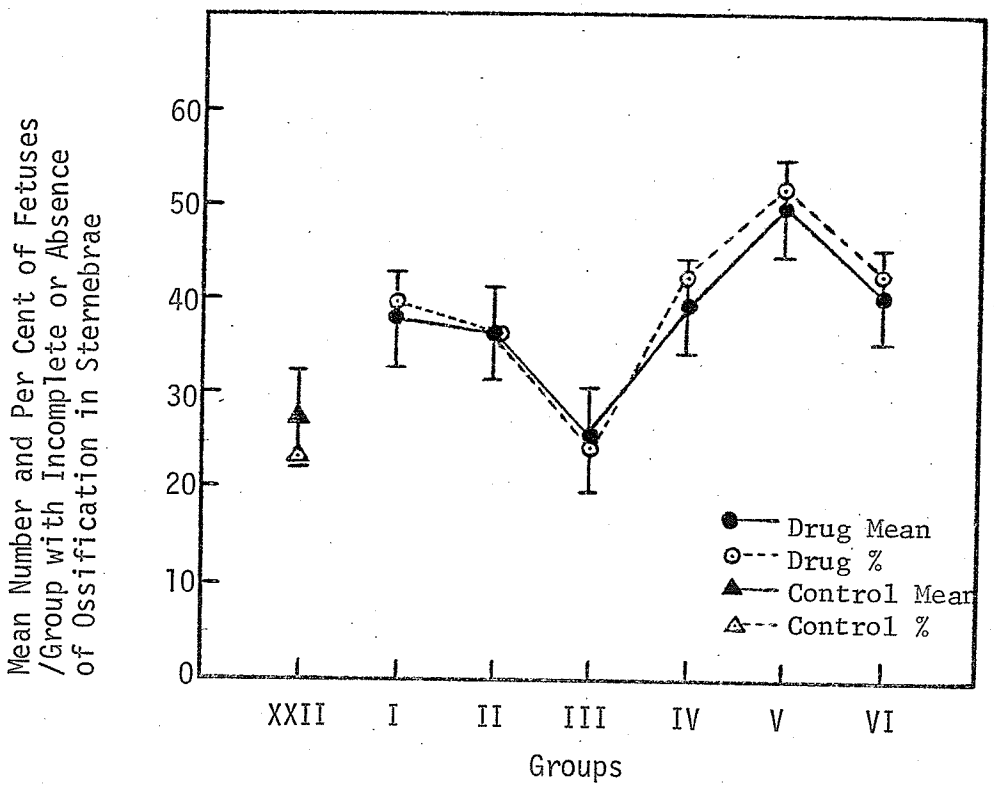
---

[1]When a fetus had an affected fifth and sixth sternal centre, this was counted as only one effect and not as two separate effects.

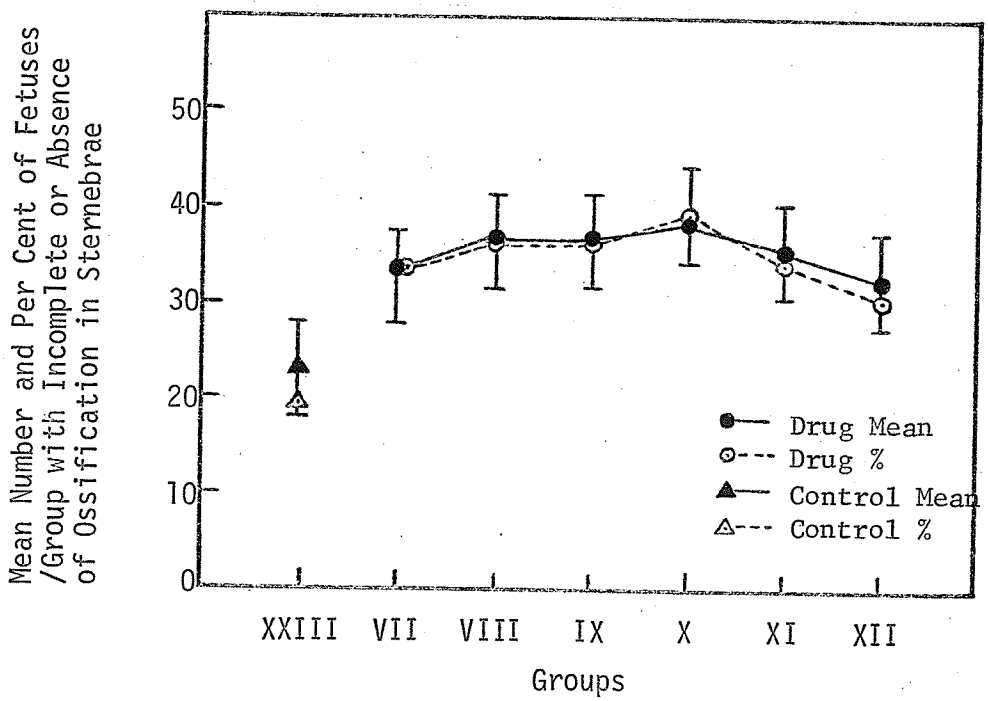
Graphs 7 and 8

7. There is a significant increase in the means for groups given podophyllin in alcohol on days eight to twelve of pregnancy (groups I to VI) when compared with the mean for the alcohol control group (XXII). The daily dosage of podophyllin administered to groups I to VI, as well as the mean values for each of the groups on this Graph, are shown in Table XVII, R-41 (refer to page 104). Standard deviation is also shown for each of the means, and the per cent values have been calculated so that comparisons can be made with the means, since the means are not arithmetic.

8. The means for groups given podophyllin in alcohol on days ten to twelve of gestation (groups VII to XII) are significantly higher than the mean for the appropriate alcohol control group (XXIII). The dosage of drug given per day to groups VII to XII, as well as the mean values for each of the groups on this Graph, are shown in Table XVII, R-41 (refer to page 104). Standard deviation is also shown for each of the means, and the per cent values have been calculated so that comparisons can be made with the means, since the means are not arithmetic.



Graph 7



Graph 8

in all sternal centres. The intermediate drug dose (0.05 milligram) either stimulated ossification or it was less effective since the mean was lowest (25.9).

A significant podophyllin in alcohol effect was also noted when the drug was administered to the animals in groups VII to XII on days ten to twelve of gestation (Graph 8). The means (R-41, Table XVII) for the podophyllin treated groups (VII to XII) ranged from 32.5 to 39.3, but the mean for the alcohol control group (XXIII) was 23.1. This observation further showed that podophyllin inhibited or delayed ossification in all sternebrae.

Once again, although a significant podophyllin effect was observed when animals were treated with it on days eight to twelve and ten to twelve of pregnancy, there was no significant drug effect when it was given to the animals on day ten only of gestation. Instead as already noted in responses thirty-six and thirty-nine, a highly significant water treatment effect was observed. Group XXX, which received the stomach tube treatment on day ten was compared with the groups (XXVII, XXIV, XIX, and XIII to XVI) which received the other forms of treatment on this day. The mean for the stomach tube group (XXX) was 54.9, but the means for the water control group (XXVII), alcohol control group (XXIV), drug in water group (XIX), and the podophyllin in alcohol groups (XIII to XVI) varied from 30.8 to 46.1 (R-41, Table XVII).

Effects on Pelvic Girdle and Coracoid Process. The analysis was done on responses forty-two and forty-three. Forty-two was total number

of young per mother with abnormal or absent ossification in bones of the pelvic girdle, and forty-three was total number of young per mother with an ossification centre in the coracoid process. There were no significant observations in either responses forty-two or forty-three.

Effects on Tarsal, Metatarsal, and Metacarpal Bones. Responses forty-four and forty-five were analyzed. Forty-four was total number of young per mother with presence of an ossification centre in the calcaneus or other tarsal bones; one foot or both feet (Figure 26). All tarsal bones were examined, but the calcaneus was the only bone which varied in its ossification. Response forty-five was total number of fetuses per mother with absence of ossification in metatarsal or metacarpal bones; one foot or both feet. There were no significant observations in the latter.

Analysis of the calcaneus (response forty-four) showed that there was a highly significant decrease in the overall mean numbers of fetuses which had an ossification centre in this bone of the groups that received treatments on days eight to twelve and ten to twelve when compared with the groups that received treatments on day ten only of gestation (R-44, Table XVIII). This indicated that treatments generally administered on days eight to twelve and ten to twelve were more effective in retarding or delaying ossification in this centre than those given on day ten only of pregnancy.

A significant podophyllin effect was observed when groups I to VI, which received the drug in alcohol on days eight to twelve, were compared

Table XVIII

Mean Number of Fetuses Per Group in Podophyllin Study with Presence of an Ossification Centre in the Calcaneus (R*-44)

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension 10% OH	Suspension H_2O	Stomach Tube	Mean/Group R-44
I	8-12	0.012	+	-	+	59.9
II	8-12	0.025	+	-	+	52.8
III	8-12	0.05	+	-	+	60.2
IV	8-12	0.1	+	-	+	52.9
V	8-12	0.2	+	-	+	41.8
VI	8-12	0.4	+	-	+	50.3
XVII	8-12	0.1	-	+	+	63.9
XXII	8-12	-	+	-	+	68.3
XXV	8-12	-	-	+	+	66.3
XXVIII	8-12	-	-	-	+	55.6
VII	10-12	0.012	+	-	+	72.8
VIII	10-12	0.025	+	-	+	56.9
IX	10-12	0.05	+	-	+	26.7
X	10-12	0.1	+	-	+	39.6
XI	10-12	0.2	+	-	+	32.5
XII	10-12	0.4	+	-	+	68.9
XVIII	10-12	0.1	-	+	+	53.7
XXIII	10-12	-	+	-	+	69.2
XXVI	10-12	-	-	+	+	36.4
XXIX	10-12	-	-	-	+	57.4
XIII	10	0.1	+	-	+	66.5
XIV	10	0.4	+	-	+	69.2
XV	10	0.8	+	-	+	65.3
XVI	10	1.6	+	-	+	67.6
XIX	10	0.1	-	+	+	45.8
XXIV	10	-	+	-	+	64.9
XXVII	10	-	-	+	+	69.7
XXX	10	-	-	-	+	62.9
XXXI	-	-	-	-	-	49.6

Explanation of Table XVIII and Key to Symbols.

Refer to pages 69 to 70.

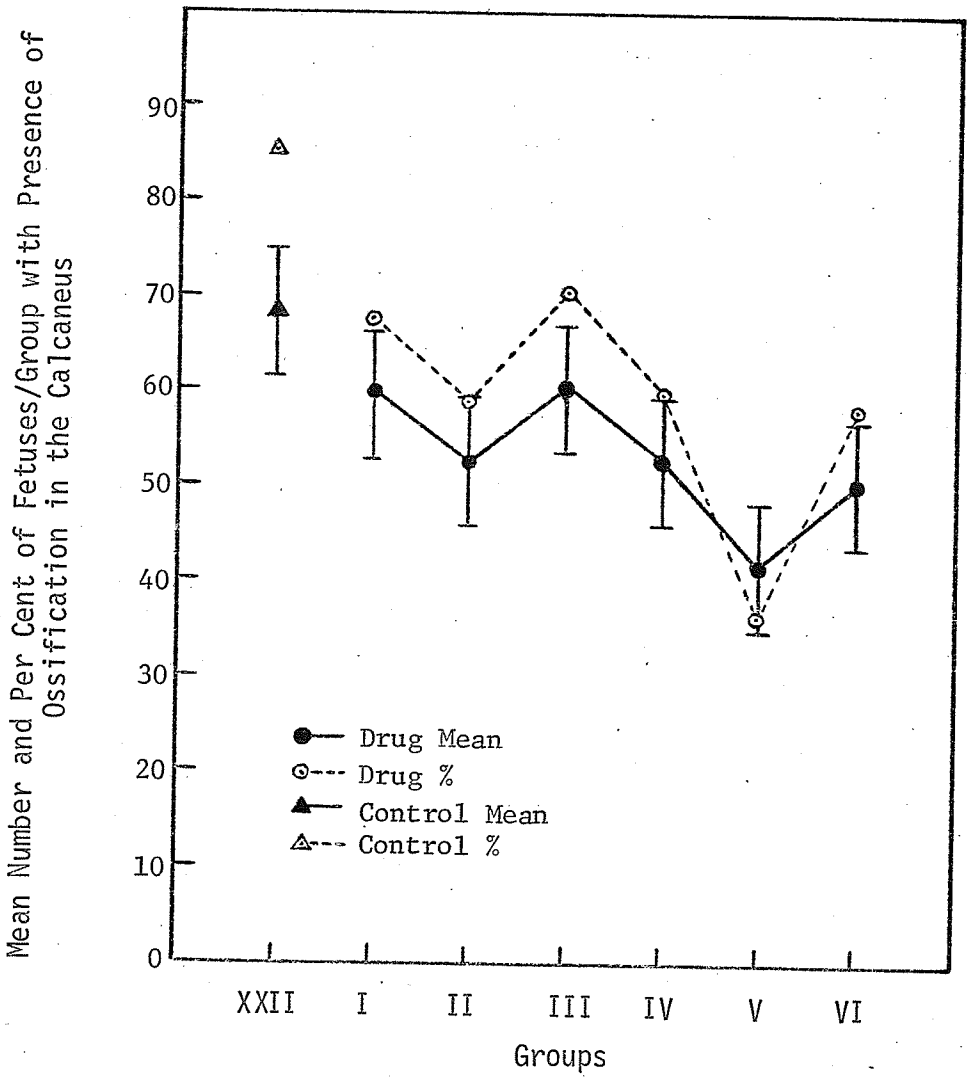
with group XXII, which received only 10 per cent ethanol on these same days of pregnancy (Graph 9). The means for the podophyllin treated groups (I to VI) ranged from 41.8 to 60.2, but the mean for the alcohol control group (XXII) was 68.3 (R-44, Table XVIII). This clearly demonstrated that podophyllin was either inhibiting or delaying ossification in the calcaneus since all the means for the drug treated groups were lower than the mean for the alcohol control group.

A highly significant drug effect was noted when the podophyllin alcohol mixture was administered to groups VII to XII on days ten to twelve of gestation (Graph 10). The means (R-44, Table XVIII) for the podophyllin treated groups (VII to XII) varied from 26.7 to 72.8 whereas the mean for the alcohol control group (XXIII) was 69.2. With the exception of the high mean in group VII (72.8) all other drug treated groups had means that were lower than the mean in the alcohol control group (XXIII). This also generally indicated that podophyllin delayed or inhibited ossification in the calcaneus.

The podophyllin in alcohol dose response curve for groups VII to XII in addition had a highly significant quadratic and significant fifth or higher order component. The means (R-44, Table XVIII) for groups VII to XII were 72.8, 56.9, 26.7, 39.6, 32.5, and 68.9 respectively. These means suggested that the low and high podophyllin doses either stimulated ossification or they were least effective because they were high. The intermediate drug doses (0.05, 0.1, and 0.2 milligram) either inhibited or delayed ossification in the calcaneus since these means were considerably lower than those at the low and high podophyllin doses.

Graph 9

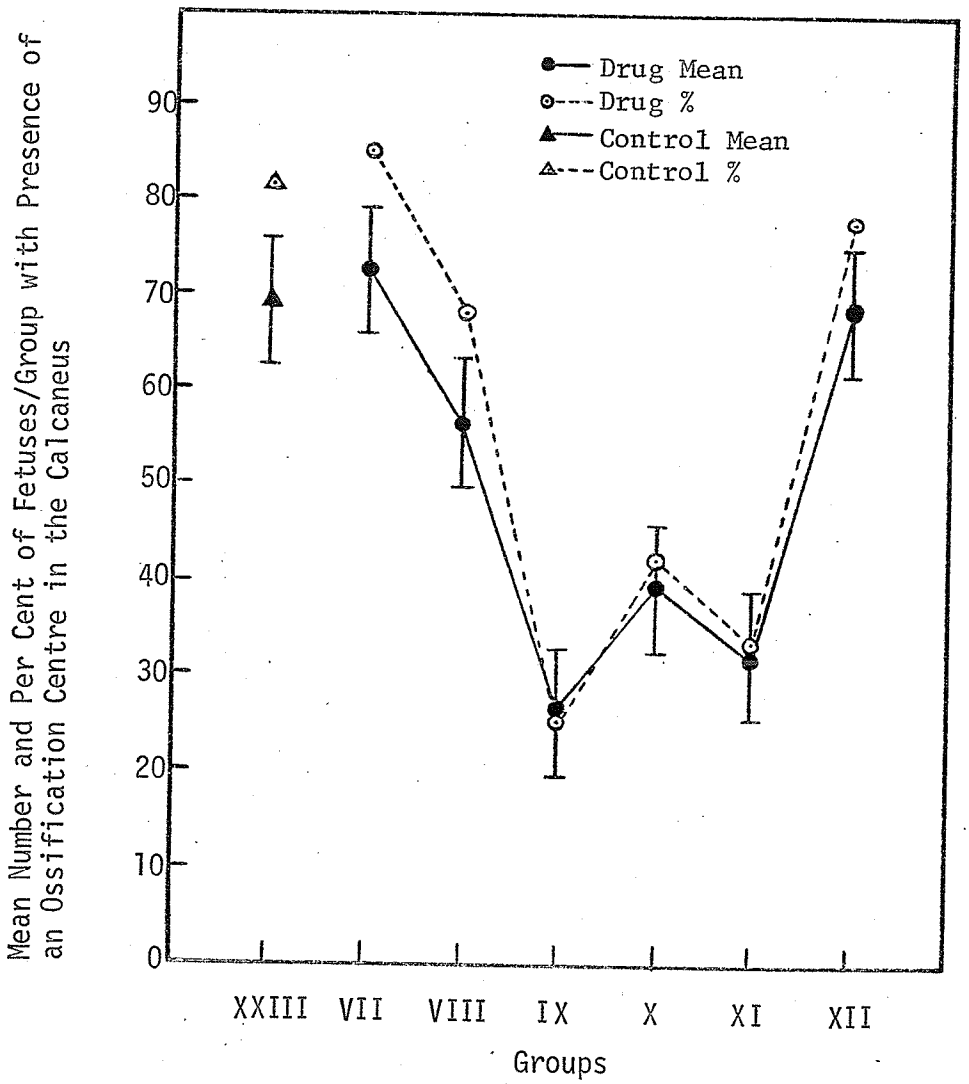
9. The means for groups given the drug in alcohol on days eight to twelve of pregnancy (groups I to VI) are significantly lower than the mean for the alcohol control group (XXII). The daily doses of podophyllin given to groups I to VI, as well as the mean values plotted for each of these groups on this Graph, are shown in Table XVIII, R-44 (refer to page 110). Standard deviation is also shown for each of the means, and the per cent values have been calculated so that comparison can be made with the means, since the means are not arithmetic.


Mean Number and Per Cent of Fetuses/Group with Presence of Ossification in the Calcaneus
90
80
70
60
50
40
30
20
10
0
Drug Mean
Drug %
Control Mean
Control %
XXII
I
II
III
IV
V
VI
Groups

Graph 9

Graph 10

10. There is a highly significant decrease in the means for groups given podophyllin on days ten to twelve of gestation (groups VII to XII) when compared with the mean for the alcohol control groups (XXIII). The dose of podophyllin administered per day to groups VII to XII, as well as the mean values for each of the groups on this Graph, are shown in Table XVIII, R-44 (refer to page 110). Standard deviation is also shown for each of the means, and the per cent values have been calculated so that comparisons can be made with the means, since the means are not arithmetic.


Drug Mean
Drug %
Control Mean
Control %
Mean Number and Per Cent of Fetuses/Group with Presence of an Ossification Centre in the Calcaneus
0
10
20
30
40
50
60
70
80
90
XXIII
VII
VIII
IX
X
XI
XII
Groups

Graph 10

A significant podophyllin (in water) effect was further noted when the drug was administered to group XIX on day ten only of gestation. The mean for group XIX was 45.8 whereas the mean for the water control group (XXVII) was 69.7 (R-44, Table XVIII). This once again indicated that podophyllin was delaying or inhibiting ossification in the calcaneus since the mean was lower in the drug treated group (XIX) than in the water control group (XXVII).

Effects in Groups which were Cross Sectioned. The results were obtained from twenty-one-day-old fetuses in groups XX and XXXII. The animals in group XX received 0.1 milligram of podophyllin in 10 per cent ethanol on days ten to twelve of pregnancy. The animals in group XXXII did not receive any treatment; they were the normal group. The number of liveborn fetuses in each group is shown in Table II of the Appendix.

In one instance only, second animal in group XX, the average placental diameter was 23 millimeters; there were two fetuses in the left uterine horn and two in the right. The podophyllin may have either stimulated growth of the placentas or it may be that the placentas were large due to the small litter size or both; that latter seems more likely than the former.[1]

Also in group XX, the ninth mother had one relatively small fetus which was 36 millimeters long; the weight of this animal was 3.1 grams.

---

[1]Placentas of this size were not observed in any of the podophyllin treated, treated control, and untreated control groups.

Fetuses of this length and weight were not observed in any of the normal mothers in group XXXII. Podophyllin may have probably been responsible for this small fetus.

Every second fetus in both groups was cross sectioned (approximately every 1 to 2 millimeters) through its entire length and examined under a dissecting microscope (7.5X). No apparent differences in fetal sections of treated and normal mothers were recognizable.

Effects on Postnatal Ossification. The results were obtained from ten-day-old survivors in groups XXI and XXXIII. The animals in group XXI received 0.1 milligram of podophyllin (in ethanol) on days ten to twelve of gestation. The animals in group XXXIII did not receive any form of treatment; they were the normal group. The total number of ten-day-old newborn survivors examined in each of these groups is shown in Table II of the Appendix. The fourth mother in group XXI had a litter of ten offspring; of these, five were stillborn and the remaining five died on the day of birth.

The average length of all animals in the podophyllin treated group (XXI) was approximately 68 millimeters; the average for animals in the normal group (XXXIII) was approximately 65 millimeters. The average weights of young in groups XXI and XXXIII were approximately 17 and 15 grams respectively. Although it is not known if podophyllin was responsible for these differences, this possibility can not be ignored.

The podophyllin may have had an effect on tooth development because in the drug treated group (XXI), 17.3 per cent of the young had

incisors (usually maxillary) of unequal length (Figure 27). All animals in the normal group (XXXIII) had incisors that were equal in length.

In group XXI, it was found that 7.4 per cent of the young had two separated ossification centres in the odontoid process; in group XXXIII, only 1.4 per cent of the animals had two separated centres. The podophyllin treatment may have delayed or retarded ossification in these centres.

The podophyllin may have also had an effect on ossification of vertebral centra. In group XXI, there were two animals which had slightly dumbbell vertebral centra and two others which had dumbbell centra (Figures 28 and 29); these effects were present in approximately 5 per cent of the animals. Effects of this type were not observed in the normal offspring.

In group XXI approximately 10 per cent of the young had retarded ossification in the sixth sternebra (Figure 30). This effect was not observed in the normal group (XXXIII). The podophyllin may have had an effect on sternebral ossification by delaying or retarding development.

Interestingly, one animal in group XXI had an unusual pair of thirteenth ribs. The rib on the left side appeared normal, but the rib on the right side was reduced in length (Figure 31).

The ten-day-old animals had a number of additional ossification centres which were not included in the list of responses. It was observed that in group XXI, 41 per cent of the animals had an ossification centre present between the fifth and sixth sternebrae; only 25 per cent of the young in group XXXIII had this centre (Figure 32). This may have been a secondary ossification centre because it was present in both groups.

In group XXI, 7.4 per cent of the animals did not have an ossification centre in the epiphysis at the proximal end of the radius (bilaterally); in group XXXIII, 15.5 per cent of the animals did not have this centre ossified. In group XXI, 10 per cent of the animals did not have an ossification centre in the olecranon process (bilaterally), but in group XXXIII only 5.6 per cent of the young did not have this centre. In group XXI, 6.2 per cent of the offspring did not have any ossification in the medial and lateral femoral condyles (bilaterally); in group XXXIII, 11.2 per cent of the animals did not have these centres. Also in group XXI, 1.2 per cent of the animals did not have an ossification centre in the epiphysis at the distal end of the tibia (bilaterally) whereas in group XXXIII, 8.5 per cent of the young did not have this centre. In most instances it is noted that these percentages are usually lower in group XXI than in group XXXIII. Taking into account the difference in weight between these two groups, it may be that if the animals in group XXXIII were allowed to live another two or three days, that the above percentages would have been approximately equal. It is therefore possible that these differences are within normal limits and they were not directly due to podophyllin, however, the possibility that podophyllin had an effect on intrauterine development can not be ignored.

Part II: Temperature Study

The statistical analysis was done on structures in mothers and their twenty-one-day-old fetuses in groups ATE to HTE inclusive.[1] The overall results generally showed that, 1) there was less variability in skeletal ossification at the lower fixed temperatures (70, 75, and 80 degrees Fahrenheit) than at the higher gradient and varying temperatures (85, 90, and 65 and 90 degrees), 2) exposure of animals during pregnancy to 75 degrees Fahrenheit seemed to result in less variation in skeletal ossification than at other temperature, and 3) placental diameter, fetal length, and weight were reduced when animals were exposed to fluctuating temperatures during their pregnancies.

Results observed in each of the responses have been grouped together where possible and presented as effects on various structures. The text includes tables with means for each of those responses that contained significant or highly significant effects; all others were omitted. Once again the means in most instances are not arithmetic, but were derived according to the statistical method given on pages 50 and 51 of the podophyllin study. The alpha values in this study were the same as those in the podophyllin study (refer to page 66).

The temperature response curve for the fixed temperature groups (ATE to GTE) had a predominant linear component. An example of this

---

[1]A complete and detailed summary of the entire statistical analysis is shown in Table I of the Appendix; it follows contrast six of the podophyllin study. Table III in the Appendix contains the number of liveborn twenty-one-day-old fetuses examined in each of the above groups.

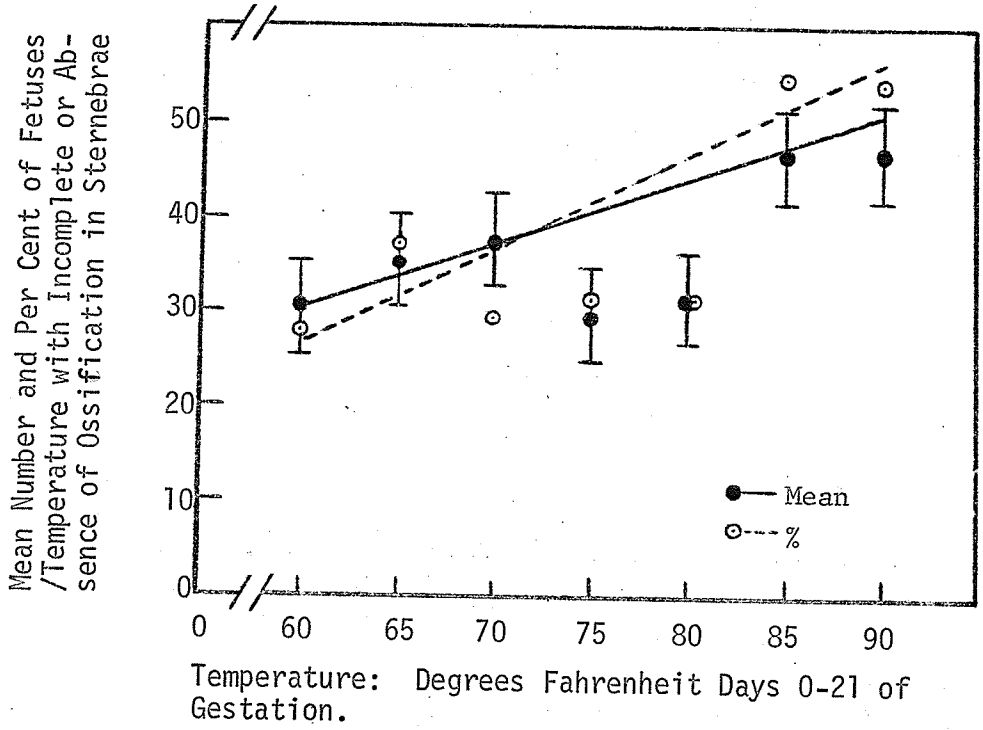
component is shown in Graph 11 using data in response forty-one. Examples of quadratic and cubic components are shown in Graphs 1 and 2 on page 67; these were constructed from data in the podophyllin study.

Effects on Implantation, Resorption, and Stillbirth Rate. The analysis was done on responses one, two, and three. Response one was total number of implantation sites per mother, two was total number of resorption sites per mother, and three was total number of stillborn animals per mother. There were no significant observations in any of these responses. It was nevertheless interesting to note that in response two (resorptions) the means for groups ATE to HTE were 10.9, 10.5, 10.2, 8.7, 8.0, 7.9, 4.7, and 5.7 respectively (R-2, Table XIX). The direction of these means suggested that temperature may have had an slight effect on resorption rates since a decrease in temperature seemed to cause a decrease in resorption rate.

Effects on Placental Development. Response four concerned average placental diameter (in millimeters) per mother. A highly significant difference was noted between the mean (R-4, Table XIX) in the varying temperature group (HTE) and the means in the fixed temperature groups (ATE to GTE). The mean for group HTE was 14.5, but the means for groups ATE to GTE varied from 15.0 to 16.4 millimeters (R-4, Table XIX). It appeared that an overall decrease in placental diameter occurred when temperature was varied throughout pregnancy.

Graph 11

11. This temperature response curve is a linear component. Note that the mean generally increases as temperature is increased. In this instance, this suggests that high temperatures are detrimental on sternebral ossification. Group GTE was exposed to a temperature of 60 degrees Fahrenheit, group FTE to 65 degrees, group ETE to 70 degrees, group DTE to 75 degrees, group CTE to 80 degrees, group BTE to 85 degrees, and group ATE to a temperature of 90 degrees Fahrenheit.


Mean Number and Per Cent of Fetuses /Temperature with Incomplete or Absence of Ossification in Sternebrae
50
40
30
20
10
0
0
60
65
70
75
80
85
90
Mean
%
Temperature: Degrees Fahrenheit Days 0-21 of Gestation.

Graph 11

Table XIX

Mean Resorption Rate (R*-2), Placental Diameter (R-4), Fetal Length (R-5), and Fetal Weight (R-6) Per Group in Temperature Study

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group			
			R*-2	R-4	R-5	R-6
ATE	90	0-21	10.9	15.0	43.1	5.3
BTE	85	0-21	10.5	15.3	44.6	5.7
CTE	80	0-21	10.2	15.3	43.0	5.5
DTE	75	0-21	8.7	16.4	44.2	6.1
ETE	70	0-21	8.0	15.5	42.5	5.2
FTE	65	0-21	7.9	15.8	43.4	5.4
GTE	60	0-21	4.7	16.1	44.3	5.4
HTE	65 and 90	0-21	5.7	14.5	42.1	5.1

Explanation of Table XIX.

The relative humidity for groups ATE to FTE remained constant at 50 per cent, but for groups GTE and HTE it varied from 50 to 75 per cent.

The animals in group HTE were exposed to an alternating temperature of 65 degrees Fahrenheit for eight hours and then 90 degrees Fahrenheit for sixteen hours.

*R means response; it is followed by the response number.

The temperature response curve for groups ATE to GTE had a significant linear component. The means for groups ATE to GTE were 15.0, 15.3, 16.4, 15.5, 15.8, and 16.1 respectively (R-4, Table XIX). These means indicated that there was a general increase in placental diameters as temperature was decreased. Interestingly, the placental mean was highest

in the group (DTE) exposed to a temperature of 75 degrees Fahrenheit.

Effects on Fetal Length and Weight. Responses five and six were analyzed. Response five was average length (in millimeters) of young per mother, and six was average weight (in grams) of fetuses per mother. In both of these responses highly significant differences were noted between the means (R-5 and R-6, Table XIX) in the varying temperature group (HTE) when compared with the means in the fixed temperature groups (ATE to GTE). The mean length of fetuses in group HTE was 42.1 millimeters, but the means for groups ATE to GTE ranged from 43.0 to 44.6 (R-5, Table XIX). The mean weight of young in group HTE was 5.1 grams whereas the means for groups ATE to GTE varied from 5.2 to 6.1 grams (R-6, Table XIX). Although the differences in these means were small, they illustrated that varying temperature may have had an effect on fetal growth because mean length and weight in group HTE was decreased when compared with means in the fixed temperature groups (ATE to GTE).

In response five (length) the temperature response curve for the fixed temperature groups (ATE to GTE) had a significant cubic component. The mean lengths for groups ATE to GTE were 43.1, 44.6, 43.0, 44.2, 42.5, 43.4, and 44.3 millimeters respectively (R-5, Table XIX). These means, though not widely divergent, generally suggested that fetuses were longer in those animals exposed to fixed temperatures than in the group exposed to a varying or fluctuating temperature. The means were largest in those groups (BTE, DTE, and GTE) exposed to temperatures of 85, 75, and 60 degrees Fahrenheit.

The temperature response curve in response six (weight) for groups ATE to GTE had a significant quadratic component. The mean weight for fetuses in groups ATE to GTE were 5.3, 5.7, 5.5, 6.1, 5.2, 5.4, and 5.4 respectively (R-6, Table XIX). From these means it appeared that 75 degrees Fahrenheit was the optimum temperature for maintaining pregnant rats since the mean weight of fetuses in group DTE was highest (6.1 grams).

Effects on Cranial Sutures. Total number of young per mother with wide cranial sutures or abnormal ossification in cranial bones that participate in forming the major sutures were analyzed (response seven). There were no significant observations.

Effects on Teeth. The analysis was done on response eight; total number of young per mother with uneven length of incisor teeth in the maxilla or the mandible (Figures 5 and 6). The temperature response curve for groups ATE to GTE had a highly significant linear and cubic component. The means (R-8, Table XX) for groups ATE and GTE were 54.1, 21.3, 11.4, 12.5, 25.7, 12.9, and 4.2 respectively. These means suggested that there was a general reduction in the number of fetuses that had uneven incisoral length as temperature was decreased.

Effects on Hyoid Bone. Number of fetuses per mother with retarded ossification in the hyoid bone was analyzed (response nine). There were no significant observations.

Table XX

Mean Number of Fetuses Per Group in Temperature Study With Uneven Length of Incisor Teeth (R*-8)

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group R*-8
ATE	90	0-21	45.1
BTE	85	0-21	21.3
CTE	80	0-21	11.4
DTE	75	0-21	12.5
ETE	70	0-21	25.7
FTE	65	0-21	12.9
GTE	60	0-21	4.2
HTE	65 and 90	0-21	23.6

Explanation of Table XX and Key to Symbol.

Refer to page 121.

Effects on Vertebrae. Responses ten to thirty inclusive were analyzed. These responses were organized, grouped together, and presented in the same manner as in the podophyllin study (refer to pages 77 and 78).

A. Anterior Arch of the Atlas. Responses ten, eleven, and twelve were analyzed. Ten was number of young per mother with uneven ossification in the anterior arch of the atlas (Figure 8), eleven was number of fetuses per mother with no visible ossification in the anterior arch, and twelve was total number of young per mother with uneven or absence of ossification in the anterior arch of the atlas. The latter was an addition of values in responses ten and eleven.

In response ten (unevenly ossified) the temperature response curve for groups ATE to GTE had a highly significant linear component. The means (R-10, Table XXI) for these groups were 44.0, 50.8, 49.8, 58.0, 52.6, 57.8, and 67.1 respectively. There was a general increase in the number of fetuses that had unevenly ossified arches as temperature decreased.

Response eleven (absence of ossification) contained a significant fifth or higher order component in the temperature response curve for groups ATE to GTE. However, because this component is too difficult to interpret when present by itself, no interpretation was made; the means are shown in Table XXI (R-11).

In response twelve (unevenly ossified or absent) the temperature response curve for groups ATE to GTE had a highly significant linear component. The means (R-12, Table XXI) for groups ATE to GTE were 47.0, 54.3, 53.0, 60.9, 67.1, 60.5, and 69.8 respectively. These means intimated that there was a general increase in the number of fetuses with these effects as temperature decreased.

B. Odontoid Process. Responses thirteen to seventeen were analyzed. Response thirteen was number of young per mother with one ossification centre in the odontoid process, fourteen was number with two separated ossification centres, fifteen was number of fetuses with two joined ossification centres in the dens, sixteen was total number of young with no visible ossification centre in the odontoid process, and response seventeen was total number of fetuses with one centre or two separated or joined centres in the odontoid process. The latter was an

Table XXI

Mean Number of Fetuses Per Group in Temperature Study With Uneven (R*-10), Absence (R-11), and Uneven or Absence of Ossification (R-12 in the Anterior Arch of the Atlas

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group		
			R*-10	R-11	R-12
ATE	90	0-21	44.0	8.5	47.0
BTE	85	0-21	50.8	6.4	54.3
CTE	80	0-21	49.8	7.2	53.0
DTE	75	0-21	58.0	5.2	60.9
ETE	70	0-21	52.6	22.2	67.1
FTE	65	0-21	57.8	5.4	60.5
GTE	60	0-21	67.1	6.8	69.8
HTE	65 and 90	0-21	49.2	14.0	55.9

Explanation of Table XXI and Key to Symbols.

Refer to page 121.

addition of the values in responses thirteen to fifteen. There were no significant observations in responses thirteen, sixteen, and seventeen.

In response fourteen (two separated centres) a significant difference was observed in the mean of the varying temperature group (HTE) when compared with the means in the fixed temperature groups (ATE to GTE). The mean for group HTE was 3.9, but the means for groups ATE to GTE ranged from 8.6 to 15.7 (R-14, Table XXII). The larger means indicated that there was a tendency for more fetuses to have two separated ossification centres at the higher temperature levels than at the lower temperatures. The

Table XXII

Mean Number of Fetuses Per Group in Temperature Study with Two Separated (R*-14), and Two Joined (R-15) Ossification Centres in the Odontoid Process

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group	
			R*-14	R-15
ATE	90	0-21	14.6	28.8
BTE	85	0-21	15.7	25.4
CTE	80	0-21	12.8	38.5
DTE	75	0-21	13.4	47.9
ETE	70	0-21	8.6	34.3
FTE	65	0-21	9.6	38.0
GTE	60	0-21	11.0	22.5
HTE	65 and 90	0-21	3.9	23.7

Explanation of Table XXII and Key to Symbols.

Refer to page 121.

effect of varying temperature in this response seemed comparable to the effects observed at the lower fixed temperatures (R-14, Table XXII).

In response fifteen (two joined centres) the temperature response curve for the fixed temperaturegroups (ATE to GTE) had a highly significant quadratic component. The means (R-15, Table XXII) for groups ATE to GTE were 28.3, 25.4, 38.5, 47.9, 34.3, 38.0, and 22.5 respectively. Because the largest mean (47.9) was in group DTE, exposed to a temperature

of 75 degrees Fahrenheit, it showed that this might be the optimum temperature at which ossification occurs in the dens.[1]

C. Cervical Centra (Number Present, Absent, and Sequence). Responses eighteen to twenty-one were analyzed. Response eighteen was number of young per mother with absence of either one or two cervical centra in consecutive order starting with the second cervical. There were no significant effects observed.

Response nineteen was number of young per mother with absence of three or more cervical centra in consecutive order starting with the second cervical (Figure 33). The temperature response curve for the fixed temperature groups ATE to GTE had a significant linear component. The means (R-19, Table XXIII) for these groups were 11.7, 17.0, 14.5, 9.3, 23.4, 19.3, and 22.2 respectively.

Response twenty was number of fetuses per mother with absence of cervical centra in nonconsecutive order starting with the second cervical (Figure 11). The temperature response curve for groups ATE to GTE also had a significant linear component. The means (R-20, Table XXIII) for these groups were 4.7, 9.0, 9.9, 9.7, 13.4, 12.8, and 16.7.

The means in the two previous responses (nineteen and twenty) generally indicated that the number of fetuses with absent cervical centra increase as temperature decreased. Interestingly, in response nineteen the mean was lowest in group DTE (75 degrees Fahrenheit), and in response

---

[1]Observations in this laboratory indicate that the odontoid process at first has one ossification centre, and later two separate centres which then become joined and these eventually fuse with the body of the axis vertebra.

Table XXIII

Mean Number of Fetuses Per Group in Temperature Study with Absence of Three or More Cervical Centra in Consecutive Order (R*-19), and One or More in Nonconsecutive Order (R-20), Starting the Count with the Second Cervical

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group	
			R*-19	R-20
ATE	90	0-21	11.7	4.7
BTE	85	0-21	17.0	9.0
CTE	80	0-21	14.5	9.9
DTE	75	0-21	9.3	9.7
ETE	70	0-21	23.4	13.4
FTE	65	0-21	19.3	12.8
GTE	60	0-21	22.2	16.7
HTE	65 and 90	0-21	20.4	11.3

Explanation of Table XXIII and Key to Symbols.

Refer to page 121.

twenty this group also contained a rather low mean when compared with the others. These deviations mildly suggested that a temperature of 75 degrees Fahrenheit may be an ideal one for ossification to occur in cervical centra.

Response twenty-one was total number of young per mother with absence of cervical centra irrespective of the number that were absent; this response was an addition of the values in responses eighteen to twenty. There were no significant observations.

D. Thoracic, Lumbar, and Sacral Centra (Number Absent).

Total number of young per mother with absent thoracic or lumbar or sacral vertebral centra were analyzed (response twenty-one). There were no significant observations.

E. Incidence of Dumbbell and Duplicated Centra (All Regions).

Responses twenty-three to twenty-six were analyzed. Response twenty-three was number of young per mother with slightly dumbbell vertebral centra in the cervical or thoracic or lumbar or sacral regions (Figure 12), twenty-four was number with dumbbell centra (Figure 13) in all regions, and twenty-five was number with duplicated vertebral centra in all regions. Response twenty-six was an addition of the values in responses twenty-three to twenty-five. There were no significant observations in responses twenty-five and twenty-six.

In response twenty-three (slightly dumbbell), the temperature response curve for the fixed temperature groups (ATE to GTE) had a highly significant quadratic component. The means (R-23, Table XXIV) for groups ATE to GTE were 4.6, 4.2, 10.7, 7.8, 11.6, 2.5, and 2.5 respectively. Because the means were highest at the intermediate temperatures (80, 75, and 70 degrees Fahrenheit), it appeared that these temperatures may have either had a detrimental effect on the ossification of the vertebral centra or these means may have represented an sample of groups varying only within normal limits.

In response twenty-four (dumbbell centra) there was a highly significant difference between the mean for the varying temperature group (HTE) and the means for the fixed temperature groups (ATE to GTE). The

Table XXIV

Mean Number of Fetuses Per Group in Temperature Study with Slightly Dumbbell (R*-23), and Dumbbell (R-24) Vertebral Centra in All Vertebral Regions

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group	
			R*-23	R-24
ATE	90	0-21	4.6	6.9
BTE	85	0-21	4.2	3.2
CTE	80	0-21	10.7	9.5
DTE	75	0-21	7.8	1.1
ETE	70	0-21	11.6	2.4
FTE	65	0-21	2.5	0.9
GTE	60	0-21	2.5	2.6
HTE	65 an 90	0-21	3.4	0.8

Explanation of Table XXIV and Key to Symbols.

Refer to page 121.

mean for group HTE was 0.8, but the means for groups ATE to GTE varied from 0.9 to 9.5 (R-24, Table XXIV). It is not known why the mean in the varying temperature group was so low.

Interestingly, though not significant in the above, means at the upper temperatures (90, 85, and 80 degrees Fahrenheit) were considerably higher than those in groups exposed to temperatures of 75 degrees Fahrenheit and lower. It is speculated that exposure of rats to temperatures over 75 degrees Fahrenheit may have cuased an increase in the number of fetuses that had dumbbell centra.

F. Tail Centra. Total number of tail centra per mother's litter was analyzed (response twenty-seven). A highly significant difference was noted in the mean for the varying temperature group (HTE) when compared with the means for the fixed temperature groups (ATE to GTE). The mean for group HTE was 6.4 whereas the means for groups ATE to GTE ranged from 6.8 to 7.6 (R-27, Table XXV). Although difficult to interpret because the means were not widespread, it appeared that fixed temperatures were probably more ideal for tail centra ossification than varying temperature since the means in the former were all higher than in the latter.

Table XXV

Mean Number of Tail Centra Per Fetus (R*-27) in Each Group in Temperature Study, and Mean Number of Fetuses Per Group with Bilateral Supernumerary Ribs (R-33)

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group	
			R*-27	R-33
ATE	90	0-21	6.8	5.5
BTE	85	0-21	7.0	11.3
CTE	80	0-21	7.0	10.6
DTE	75	0-21	7.6	7.8
ETE	70	0-21	7.7	17.3
FTE	65	0-21	6.9	13.5
GTE	60	0-21	6.8	16.2
HTE	65 and 90	0-21	6.4	10.0

Explanation of Table XXV and Key to Symbols.

Refer to page 121

G. Vertebral Arches. Responses twenty-eight, twenty-nine, and thirty were analyzed. Twenty-eight was number of young per mother with fusion of vertebral arches, twenty-nine was number of young per mother with absence of ossification in vertebral arches, and response thirty was total number of young per mother with abnormal vertebral arches; fusion, abnormal ossification or absence of ossification. The latter was an addition of values in responses twenty-eight and twenty-nine. There were no significant observations in any of these responses.

Effects on Ribs. The analysis was done on responses thirty-one to thirty-four. Response thirty-one was total number of young per mother with anomalous or absent ribs (excluding forms of supernumerary ribs). Response thirty-two was number of young per mother with unilateral cervical or lumbar supernumerary ribs, thirty-three was number of fetuses with bilateral cervical or lumbar accessory ribs, and response thirty-four was an addition of the values in responses thirty-two and thirty-three. There were no significant observations in responses thirty-one, thirty-two, and thirty-four.

In response thirty-three, bilateral cervical or lumbar supernumerary ribs, the temperature response curve for the fixed temperature groups (ATE to GTE) had a significant linear component. The means (R-33, Table XXV) for groups ATE to GTE were 5.5, 11.3, 10.6, 7.8, 17.3, 13.5, and 16.2 respectively. From these means it appeared that the number of fetuses with bilateral supernumerary ribs increased as temperature was decreased.

Effects on Sternebrae. Responses thirty-five to forty-one inclusive were analyzed. These responses were organized, grouped together, and presented in the same manner as in the podophyllin study (refer to page 98).

A. Fifth Sternal Centre. The analysis was done on responses thirty-five to thirty-nine. Thirty-five was number of young per mother with slightly retarded ossification in the fifth sternebra (Figure 20), thirty-six was number of fetuses with retarded ossification in this centre (Figure 21), thirty-seven was number of young with almost no ossification in the fifth sternal centre, and response thirty-eight was number with no ossification in this centre. Response thirty-nine was an addition of the values in responses thirty-five to thirty-eight. There were no significant observations in responses thirty-seven and thirty-eight.

The temperature response curve in response thirty-five (slightly retarded) had a highly significant linear component in groups ATE to GTE. The means for these groups were 29.5, 28.3, 19.0, 22.0, 15.5, 20.1, and 17.4 respectively (R-35, Table XXVI). The means showed that the number of fetuses with this effect generally decreased as temperature decreased. These results mildly intimate that exposure of pregnant rats to fixed temperatures might be more ideal for development of this centre.

In response thirty-six (retarded ossification), considered to be more serious than the previous response, the temperature response curve also had a highly significant linear component and a significant quartic component. The means (R-36, Table XXVI) for groups ATE to GTE were 28.3, 27.2, 17.9, 13.4, 20.2, 22.8, and 9.3 respectively. These means illustrated

Table XXVI

Mean Number of Fetuses Per Group in Temperature Study with Slightly Retarded (R*-35) and Retarded Ossification (R-36) in Fifth Sternebra, Abnormal or Absence of Ossification in the Fifth Sternebra (R-39), and Abnormal or Absence of Ossification in Any or All of the Six Sternal Centres (R-41)

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group			
			R*-35	R-36	R-39	R-41
ATE	90	0-21	29.5	28.3	46.4	46.9
BTE	85	0-21	28.3	27.2	45.9	46.7
CTE	80	0-21	19.0	17.9	30.7	31.6
DTE	75	0-21	22.0	13.4	28.2	29.9
ETE	70	0-21	15.5	20.2	32.2	37.5
FTE	65	0-21	20.1	22.8	34.9	35.8
GTE	60	0-21	17.4	9.3	21.9	30.4
HTE	65 and 90	0-21	18.1	15.5	26.4	30.4

Explanation of Table XXVI and Key to Symbols.

Refer to page 121

once again that the lower temperatures might be more ideal for sternebral ossification. Interestingly, the mean was lower in group DTE than in most of the other groups.

Results in response thirty-nine were similar to those noted in responses thirty-five (slightly retarded) and thirty-six (retarded ossification).[1] The fixed temperature groups (ATE to GTE) had a highly significant linear component and a significant quartic component. The means

[1]Response thirty-nine was an addition of the values in responses thirty-five to thirty-eight.

(R-39, Table XXVI) for these groups were 46.4, 45.9, 30.7, 28.2, 32.2, 34.9, and 21.9 respectively. As previously stated, these means further suggested that the lower temperatures were more suitable for ossification of the fifth sternebra.

B. First, Second, Third, Fourth, and Sixth Sternal Centres. Response forty was studied, it was total number of fetuses per mother with other affected sternebrae; retarded ossification or absence. There were no significant observations.

C. Sternal Centres One to Six Inclusive. Total number of young per mother with abnormal or absence of ossification in sternebrae regardless of the sternal centre affected (response forty-one). The temperature response curve, as in responses thirty-five, thirty-six, and thirty-nine, had a significant linear component. The means (R-41, Table XXVI) for groups ATE to GTE were 46.9, 46.7, 31.6, 29.9, 37.5, 35.8, and 30.4 respectively. These means once more intimated that lower temperatures (80 degrees Fahrenheit and lower) were probably more ideal for sternebral ossification than higher temperatures (85 and 90 degrees Fahrenheit).

Effects on Pelvic Girdle and Coracoid Process. Responses forty-two and forty-three were analyzed. Response forty-two was total number of fetuses per mother with abnormal or absence of ossification in bones of the pelvic girdle, and forty-three was total number of young per mother with a visible ossification centre in the coracoid process. There were no significant observations in these responses.

Effects on Tarsal, Metatarsal, and Metacarpal Bones. The analysis was done on responses forty-four and forty-five. Response forty-four was total number of fetuses per mother with presence of an ossification centre in the calcaneus or other tarsal bones; one or both feet (Figure 26).[1]

The temperature response curve (in response forty-four) had a highly significant cubic component. The means for groups ATE to GTE were 48.2, 67.7, 65.6, 73.5, 47.5, 55.6, and 62.7 respectively (R-44, Table XXVII).

Table XXVII

Mean Number of Fetuses Per Group in Temperature Study with Presence of an Ossification Centre in the Calcaneus (R*-44), and No Visible Ossification in Medial Metacarpal and Metatarsal Bones (R-45)

Group	Temperature Fahrenheit	Exposure Day(s) of Gestation	Mean/Group	
			R*-44	R-45
ATE	90	0-21	48.2	6.9
BTE	85	0-21	67.7	0.8
CTE	80	0-21	65.6	2.5
DTE	75	0-21	73.5	1.1
ETE	70	0-21	47.5	7.4
FTE	65	0-21	55.6	0.9
GTE	60	0-21	62.7	0.9
HTE	65 and 90	0-21	47.1	2.4

Explanation of Table XXVII and Key to Symbols.

Refer to page 121.

[1]All tarsal bones were examined, but the calcaneus was the only bone which varied in its ossification. Therefore when response forty-four is mentioned, it refers to the calcaneus only.

These means suggested that exposure of pregnant rats to a temperature of 75 degrees Fahrenheit was probably ideal for maximum ossification to occur in the calcaneus; the mean was highest in group DTE (73.5).

Response forty-five was total number of young per mother with no visible ossification in metacarpal or metatarsal bones; one or both feet (Figure 34). The effect in this response was restricted to the medial metatarsal bone only; one or both feet. The temperature response curve for groups ATE to GTE had a significant quartic component. The means (R-45, Table XXVII) for these groups were 6.9, 0.8, 2.5, 1.1, 7.4, 0.9, and 0.9 respectively. With the exception of means in groups ATE and ETE that were relatively high, the other means did not show that temperature had much effect on metatarsal ossification. Furthermore, if temperature did have an effect, it was not apparent in this response. Because all means were quite low, it may be that ossification in these bones is variable and therefore these means may have represented an sample of variation within normal limits.

V DISCUSSION

Part I: Podophyllin Study

Introduction. The purpose of this series of experiments was to determine whether podophyllin has an influence on skeletal ossification in the Holtzman rat fetus.

A number of effects noted in this study are not considered to be true malformations. A preliminary study was undertaken, which compared fetuses of drug-treated and control animals with the newborn of drug-treated rats. This showed that centres which were absent or incompletely ossified in the twenty-one-day-old fetus, were usually well ossified in ten-day-old newborn rats of the podophyllin-treated mothers (group XXI). It was concluded that those centres not ossified in the newborn of rats in group XXI could be considered malformations, since all corresponding bones in the newborn of untreated control mothers (group XXXIII) were well ossified. These results suggest that there may be various degrees of delay in ossification, not amounting to malformations, that are due to several factors. It is evident that care is required before making any conclusions from fetal studies.

With this caution in mind, it is believed that some of the findings in this study, occurring in the fetuses of the various groups, may be considered abnormalities, or unusual variations. These are: 1) severly stunted viable fetuses, 2) duplicated and possibly dumbbell vertebral centra, 3) fused vertebral arches, 4) bizarre-shaped or shortened ribs, 5) cervical supernumerary ribs, and 6) severely scrambled sternebrae.

The reason for considering the aforementioned findings as possible abnormalities is because, 1) they have never been observed in fetuses of

untreated control mothers maintained at a relatively uniform temperature, and 2) other investigators have noted similar effects in their experiments and have considered them to be abnormalities; in some instances the frequency was much higher than observed in this study (Murphy, 1960; Klein Obbink and Dalderup, 1963; McColl, Globus and Robinson, 1963; DiPaolo, Gatzek and Pickren, 1964; Murphy, 1965; Runner, 1965).

Some of the above variations were initially observed in the fetuses of mothers in podophyllin-treated groups and were in most instances thought to be due to podophyllin. However, during this study a few of these effects also occurred in some of the alcohol, water, and stomach tube control groups. Considering the small amounts of ethanol and water administered, these latter implications were doubted. In particular, the effects attributed to water seemed patently absurd. Therefore another explanation was sought. Either the stomach tube, temperature variations in the animal quarters (especially during the winter months), or both were having an effect on skeletal ossification. The temperature was primarily suspected because MacFarlane, Pennycuik and Thrift (1957) observed high resorption rates in unacclimatized Wistar rats exposed to a temperature of 95 degrees Fahrenheit during pregnancy.

In this study, temperature in the animal quarters (78 degrees Fahrenheit, ± 7 degrees) varied considerably on occasion, and it was thought that this stress might influence skeletal ossification without producing high resorption rates. A few of the previously listed defects were observed in some of the fetuses in groups exposed to temperatures that were either fluctuating or that were lower or higher than normal

(normal was considered to be 78 degrees Fahrenheit). Accordingly, a temperature study was done which suggests that this parameter does in fact affect the rate of ossification.

Because the results suggest that podophyllin, the stomach tube, and temperature may independently influence the rate of ossification, it is believed that the stomach tube and, mainly, temperature fluctuations in the animal quarters that were probably responsible for most of the inconsistencies in the results of the statistical analysis. In addition, it is believed that the runting effects were also a contributing factor because these effects were included in the analysis of each of the forty-five responses. Part II of this study, concerning the effects of temperature on ossification, suggests that if it had been possible to conduct the podophyllin experiments under constant environmental conditions of temperature and, possibly, relative humidity, that the results might have been more uniform and consistent with one another. Some of the discrepancies in results will be considered later in this discussion.

To ensure clarity of understanding, I have broken down the discussion of my observations into a number of sections. These will now be considered under their various headings.

Is Podophyllin a Teratogen? The results of these experiments indicate that podophyllin may be a mild teratogen in the Holtzman rat, since evidence suggests that it is capable of producing severely runted viable fetuses (Figures 3 and 4). Although podophyllin did not produce bizarre skeletal anomalies, the results further indicate that this drug is capable of significantly altering the frequency or type of ossification

in some centres when given to pregnant rats on days eight to twelve or ten to twelve of gestation. These centres are:

a) the anterior arch of the atlas (Figures 8 and 9, Graphs 3 and 4),

b) vertebral centra (Figure 13, Graphs 5 and 6),

c) sternebrae (Figures 20 to 25, Graphs 7 and 8), and

d) calcaneus (Figure 26, Graphs 9 and 10).

In addition, postnatal skeletal ossification was delayed in

a) teeth (Figure 27),

b) vertebral centra (Figures 28 and 29), and

c) sternebrae (Figure 30)

when the drug was given to the pregnant rats on days ten to twelve of pregnancy.

The most noteworthy effect seems to be the serious retardation of fetal growth and development. In podophyllin-treated animals a number of small viable fetuses were noted (Figures 3 and 4). Fetuses of this size have not been observed in any animals of treated control or untreated control groups; nor were they observed in any groups exposed to variations in environmental temperature. During the past five years I have had the opportunity of examining some six hundred litters (approximately six thousand fetuses). Runts have been noted only in drug-treated animals.

Severe fetal runting was also noted by Thiersch (1963) following intraperitoneal administration of either podophyllin or one of its constituents, podophyllotoxin, to pregnant Long-Evans rats. He did not, however, observe skeletal defects in alizarin-stained specimens. Dwornik

and Moore (1965) noted fetal runts following the administration of thalidomide to Holtzman rats, as did Riddell (1967) using colchicine.

According to Cahen (1964) and Fraser (1964) drugs may produce adverse effects in some strains and species and not in others. These authors feel that before any definite conclusion as to teratogenicity can be reached, a drug must be tested in a number of strains and species of animal. Even then the results of animal experiments may not always be applicable to man. For example, Somers (1962) showed that the pregnant Wistar rat is not susceptible to the effects of thalidomide, but he demonstrated that it produces congenital defects in the offspring of rabbits that closely resemble those produced in humans. It is not yet clear whether the same relationship applies in the case of podophyllin. This question will require further investigation.

Another indication of the need for further studies is the fact that investigators have reported a variety of effects which are believed attributable to podophyllin and some of its compounds. This drug and some of its constituents have been shown to inhibit mitosis in various types of cells in a manner similar to colchicine, a known and established mitotic inhibitor. According to some workers podophyllin prevents spindle formation as does colchicine (King and Sullivan, 1947; Sullivan and Wechsler, 1947; Cornman, 1947; Cornman and Cornman, 1951; Padawer and Gordon, 1956; Spendlove et al., 1964; Karnofsky, 1965). Broomhead (1967) in our laboratory has clearly demonstrated the mitosis-inhibiting effect of podophyllin in the duodenum of the pregnant Holtzman rat.

Deleterious effects of podophyllin on various organs and systems in a variety of animals have also been noted (Viehoever and Mack, 1938; Sánchez Caballero and Ergueta Collao, 1949; Greenspan and Leiter, 1949; Miller, Davison and Smith, 1949; Kelly et al., 1951 b; Kelly and Hartwell, 1954).

Podophyllin and podophyllotoxin have also been shown to interrupt pregnancy, or cause an increase in resorptions when administered to rats, mice, and rabbits at various times after mating (Didcock, Picard and Robson, 1952; Wiesner and Yudkin, 1955; Didcock, Jackson and Robson, 1956; Thiersch, 1963). In this connection the following two human cases are worth mentioning in which it has been suggested that podophyllin may have been responsible for 1) the birth of a stillborn infant to a mother who approximately three hours earlier had been treated for fifty minutes with a 25 per cent solution of podophyllin for condylomata acuminata (Gorthey and Krembs, 1954), and 2) the multiple deformities in an infant born to a mother who had taken herbal "slimming tablets" containing podophyllin during the first trimester of her pregnancy, a critical period in organogenesis (Cullis, 1962). Whilst it is impossible to make any conclusions on the basis of two case reports, it is nevertheless interesting to note that some of the malformations observed by Cullis closely resemble those observed in the thalidomide syndrome (Lenz and Knapp, 1962; Taussig, 1962).

These findings would suggest that podophyllin is an undesirable drug for administration to either a pregnant woman, or a woman in the nubile period of life. In fact, The Medical Letter on Drugs and

Therapeutics (1962) states, "It is therefore an undesirable remedy for women who are, or might become, pregnant. Any one of numerous laxatives other than podophyllum can be chosen if a laxative is needed." The present findings would appear to support this conclusion.

The Most Effective Teratogenic Dose of Podophyllin. The results of these experiments suggest that the most effective dose is 0.1 milligram per 100 grams body weight. The primary reason for believing this to be true is because a litter, partly or wholly made up of runts, was observed once in each of groups X, XIII, and XVIII. Each animal in these groups had received 0.1 milligram of podophyllin in either 10 per cent ethanol or water per 100 grams body weight. By contrast, runting was not observed in any of the other drug-treated, treated control, or untreated control groups. In addition, runts were not observed in any of the animals exposed to various environmental temperatures during pregnancy. These observations suggest that the above dose was probably the most effective and in all likelihood may have caused runting, since fetuses of this size are outside the limits of normal variation.

Furthermore, fetal runting was also observed by Thiersch (1963) in Long-Evans rats following the administration of podophyllin suspended in water. Apart from noting stunted fetuses at the above dose, he also observed runted fetuses at other dosage levels. The other doses he administered and noted this effect at were 0.5, 1.0, 5.0, and 10 milligrams per kilogram; conversion of these doses to milligrams per 100 grams body weight shows that these were 0.05, 0.2, 0.5, and 1.0 milligram per 100 grams body weight. Although his results indicate a toxic effect of the

drug at these dose levels, similar responses were not observed in the present experiments.

Some of the reasons why Thiersch noted fetal runting and high resorption rates at different doses may be due to the following factors. First, route of administration. He administered the drug intraperitoneally whereas I administered podophyllin by stomach tube. Secondly, strain differences. He used Long-Evans rats, but I used Holtzman rats. Thirdly, different days of drug administration. He administered the podophyllin on various days from the sixth to twenty-first days of gestation, but I gave the drug on various days from the eighth to twelfth days of pregnancy. According to Cahen (1964), Wilson and Warkany (1965), and Robson, Sullivan and Smith (1965), these factors, and others can contribute to variation in results within different strains of the same species.

One other reason for believing that the 0.1 milligram per 100 grams body weight podophyllin dose is or may be the most effective dose in illiciting a response is that this dosage usually came close to agreeing with the results in the statistical analysis of the dose response curves. The quadratic dosage response curve was the most frequently observed (example, Graph 1). This curve in a number of instances suggested that the 0.1 milligram dose may be the most effective in delaying ossification, the 0.05 milligram dose appeared to be the second most effective, and the 0.2 milligram dose appeared to be the third most effective dose in delaying ossification.

In this same respect, the quadratic dose response curve usually suggested that the 0.012, 0.025, and the 0.4 milligram doses may be either the least effective, or in some instances may be stimulating ossification. The latter possibility will be discussed under, How Does Podophyllin Exert Its Effect on Fetal Development (page 160). Because it has been established that podophyllin is a mitotic inhibitor (King and Sullivan, 1947; Kelly and Hartwell, 1954; Karnofsky, 1965; Broomhead, 1967), one would have expected it to inhibit rather than stimulate ossification.

The Most Critical Days of Podophyllin Administration. If one uses only the presence of stunted viable fetuses to determine the critical days of drug administration, then days ten to twelve, and day ten only of pregnancy are the most critical. The number and percentage of fetal runts observed in groups X, XVIII, and XIII is shown in Table XXVIII. However, if one uses the results of the statistical analysis (Table I, Appendix), it is noted that ten of forty-five responses showed a possible podophyllin effect on ossification of various centres in groups treated on days eight to twelve of gestation. On the other hand, seven of forty-five responses showed that the drug appears to affect ossification when exhibited on days ten to twelve of pregnancy, but only one of forty-five responses showed an effect on ossification when the drug was given on day ten only of gestation. On the basis of the latter, one could conclude that days eight to twelve, and ten to twelve of gestation were

Table XXVIII

Percentage of Runted Fetuses Per Total Number of Implants in Podophyllin-Treated Groups as Compared with Alcohol and Water Treated Control Groups

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension*	Implants/ Group	Runts/ Group	% Runts/Total Implants/Group
X	10-12	0.1	OH	112	13	11.6
XVIII	10-12	0.1	H_2O	112	3	2.7
XIII	10	0.1	OH	113	1	0.9
XXIII	10-12	-	OH	119	0	0
XXVI	10-12	-	H_2O	123	0	0
XXIV	10	-	OH	115	0	0

Explanation of Table XXVIII.

All animals in groups X, XVIII, and XIII received the drug by stomach tube.

Animals in groups XXIII, XXVI, and XXIV were controls for groups X, XVIII, and XIII respectively. They received the alcohol or water by stomach tube. The amount given was 0.1 cubic centimeter per 100 grams body weight.

*This represents the suspension medium used for the drug; the OH represents 10 per cent ethanol and the H_2O distilled water.

the most critical, since the administration of the drug on these days delayed ossification in a greater number of centres than when given on day ten only of pregnancy.

Consideration of the above points leads me to conclude that days ten to twelve of pregnancy are probably the most critical days for administration of podophyllin because there were more stunted fetuses in groups treated on days ten to twelve of pregnancy, and there is little difference in the number of responses that showed delay in ossification between the groups treated with the drug on days eight to twelve and ten to twelve of gestation.

Thiersch (1963) observed various percentages of stunted fetuses when he administered the previously mentioned doses of podophyllin intraperitoneally on days six to sixteen (0.4 per cent), seven to twenty-one (5.7 per cent), eleven to twenty-one (1.6 per cent), eleven and twelve (9.9 per cent), and eighteen and nineteen (3.5 per cent). Although he observed runts in animals treated on various days of gestation, it is interesting to note that he observed the highest percentage of runts in the group treated on days eleven and twelve of pregnancy (9.9 per cent). He also observed that in this group, 90.1 per cent of the fetuses were completely resorbed; this further suggests a highly toxic effect of the drug on the developing embryo when given to the pregnant rat on these days of gestation.

The percentage of fetal runts observed by Thiersch, when he administered the drug on days eleven and twelve of gestation, compares favourably with the percentage of runts in group X (Table XXVIII) in the present study. This was not true in group XVIII. Surprisingly, there was not a significant number of resorptions in the present study that could be attributed to podophyllin. Therefore it was not possible to determine the critical days of administration from resorption rates.

It is believed that part of the difference between the results reported by Thiersch and those presented here may be due to differences in the route of administration and subsequent absorption of the drug. It seems likely that the drug would be more quickly absorbed when administered intraperitoneally than when given by stomach tube because the surface area for absorption may be larger. Furthermore, when the drug is administered intraperitoneally it is primarily excreted in the urine, but when given by stomach tube it is probably eliminated in the feces as well. In addition, it is also possible that the drug given intraperitoneally may be retained for a longer period of time and thereby be able to exert more profound toxic effects on the fetus than when it is administered by stomach tube.

Kelly et al. (1951 a), and Kelly and Hartwell (1954) administered podophyllotoxin (route not stated) to rats. Less than 50 per cent of the quantity given was recovered one hour later. At the end of four hours, less than 10 per cent of the original dose was recovered. This finding suggests that a moiety of the drug may have been metabolized to other substances. Kocsis, Walaszek and Geiling (1957) obtained further evidence of this supposition that this substance may be converted to other metabolites; one of them possibly picropodophyllin. The latter is another highly toxic substance (Viehoever and Mack, 1938). They noted that little or no C^{14}-podophyllotoxin could be recovered from the tissues and urine of mice that had four hours earlier been subcutaneously injected with biosynthetically labelled C^{14}-podophyllotoxin. They also noted that mice and hamsters are able to convert C^{14}-podophyllotoxin to

$C^{14}O_2$; this indicates oxidative degradation of administered podophyllotoxin. Although the above studies indicate that podophyllotoxin is metabolized to other substances, they still do not show what happens to the other constituents of podophyllin (Hartwell and Schrecker, 1958). Therefore additional biochemical studies are required.

Further support for my belief that days ten to twelve of gestation are the critical days for podophyllin administration is Wilson and Warkany's opinion (1965) that, "The onset of teratogenic susceptibility occurs at about the time the germ layers are formed." They state that this period begins about the eighth day of gestation in the rat. Administration of podophyllin, therefore, on days ten to twelve of pregnancy would be during the time of teratogenic susceptibility.

Do Ethanol and Water Influence Ossification? This is not believed to be so, even though the statistical analysis at first glance seems to suggest it. This possibility furthermore appears absurd when one considers that the amount of either of these solutions given at any one time did not exceed approximately three-tenths of a cubic centimeter (0.1 cubic centimeter per 100 grams body weight).

A further reason for questioning these effects lies in the fact that none of the following investigators observed adverse effects attributable to these agents, when administering podophyllin or one of its constituents to various pregnant and nonpregnant animals. Sánchez Caballero and Ergueta Collao (1949) intraperitoneally administered podophyllin in 10 per cent ethanol to nonpregnant rats. Didcock, Picard

and Robson (1952) intraperitoneally and subcutaneously administered podophyllotoxin in 10 per cent ethanol to pregnant mice and rats. They also administered podophyllotoxin to pregnant rabbits in absolute alcohol. Wiesner and Yudkin (1955) subcutaneously administered podophyllotoxin in alcohol (percentage not stated) to pregnant inbred Swiss mice. Thiersch (1963) used water as a suspension medium to administer podophyllin and podophyllotoxin intraperitoneally to pregnant Long-Evans rats.

Because the statistical analysis appears to suggest that the stomach tube may affect skeletal ossification in a few instances, and since temperature variations also seem to affect ossification, it is believed that these two factors were largely responsible for the inconsistencies in results. It is also possible that the water and alcohol may have been actually masking an effect more properly attributable to the stomach tube.

Does the Stomach Tube Have an Effect on Ossification? The results of the analysis of these experiments have in some instances suggested that the stomach tube may have been responsible for retarded or delayed ossification in the body of the hyoid bone, duplicated vertebral centra, fused vertebral arches, absence of ossification in the fifth sternebra, and absence or delayed ossification in the sternal centres other than the fifth. These observations will be separately evaluated as the literature does not incriminate the stomach tube in any experiments of a similar nature, and also because of some inconsistencies in present results apparently due to alcohol and water.

The delayed or retarded ossification in the body of the hyoid bone (Figure 7) is questioned because 1) there were fairly large differences between the number and percentage of fetuses with this effect in the podophyllin-treated groups, and the treated control and untreated control groups, 2) almost half of the affected fetuses in the podophyllin-treated groups were runts, a phenomenon which was apparently caused by podophyllin, and 3) there were some fetuses in the temperature study that also had delayed ossification in the body of this bone. The number and percentage of fetuses with this effect in the various groups were as follows: in the nineteen podophyllin-treated groups, each containing ten mothers (exception was in group XVI; one animal had complete resorptions), there were one thousand nine hundred and seventy-nine viable fetuses. Of these, thirty-five fetuses of ten mothers had delayed ossification in this bone. This represents 1.8 per cent of the fetuses. Furthermore, of these thirty-five fetuses, seventeen were runts and thirteen of these were in group X. In the one untreated and nine treated control groups, one thousand and forty-two fetuses were examined. One of the animals in the water control group had two fetuses with delayed ossification in the body of the hyoid. This represents 0.2 per cent of the fetuses. As stated, there were also some fetuses in the temperature study that had delayed ossification in the body of the hyoid bone. Eight hundred and ninety-three fetuses were examined in eight groups of mothers each containing ten animals. Six of the fetuses in two mothers had delayed ossification in this bone. This represents 0.8 per cent of the fetuses. From this it may be inferred that the rate of ossification in the body of the hyoid bone can be altered by various treatments.

Although the stomach tube at first glance appears capable of delaying ossification in the body of the hyoid bone, it is believed that this is not an exclusive stomach tube effect. Temperature variations in the animal quarters, as well as the presence of the runts in podophyllin-treated groups, were probably responsible for this unusual result in the statistical analysis. In most instances, except the runts, ossification was only delayed and not permanently retarded by the stomach tube or temperature treatments. The body of the hyoid bone was well ossified in all ten-day-old newborn rats born to podophyllin-treated mothers in group XXI, as well as in newborn rats in the untreated control group (XXXIII).

The significance of the stomach tube in producing duplicated vertebral centra (response twenty-five, Figure 14) is questioned further because the results of the analysis of dumbbell centra (response twenty-four, Figure 13) indicated that podophyllin was responsible for inducing this variation which is though to be a precursor of duplicated centra (compare Figure 13 with Figure 14). In turn, both of these effects may be questioned because in the temperature study, a few fetuses had dumbbell and only one fetus had duplicated centra.

The foregoing indicates that various factors can influence ossification, the frequency depending on the nature of the treatment. It may be that slightly dumbbell and dumbbell centra occasionally occur and are within the limits of normal variation. This, however, can also be doubted since some of the ten-day-old newborn animals born to drug-treated mothers in group XXI had slightly dumbbell (Figure 28) and

dumbbell (Figure 29) centra, whereas, this was not observed in any rats born to untreated control mothers in group XXXIII. Therefore it is possible that duplicated, and perhaps dumbbell centra, though occurring in a few fetuses of treated control animals, will occur more frequently if 1) the animal's external environment is altered (temperature, relative humidity or both), 2) its eating habits change due to variations in external environment or administration of different types of treatment, and 3) it is given drugs that alter the maternal environment.

The latter possibilities seem likely if one considers the following percentage of fetuses with duplicated centra in the various groups examined. In the temperature study (least severe treatment) there were eight hundred and ninety-three fetuses. Of these, one had duplicated vertebral centra. This represents 0.1 per cent of the fetuses. The mother had been exposed to a temperature of 65 degrees Fahrenheit for the full twenty-one days of her pregnancy. In the podophyllin study, the one untreated control and nine treated control groups contained one thousand and forty-two fetuses. Two treated control mothers (group XXV and XXIX) each had one fetus that had duplicated centra. This represents 0.2 per cent of the fetuses. However, in the podophyllin-treated groups (most severe treatment) there were one thousand nine hundred and seventy-nine fetuses. Seven mothers had eight fetuses with duplicated centra. This represents 0.4 per cent of the fetuses. Here it is noted that the percentage of fetuses with duplicated centra increases slightly as the treatment becomes more severe.

A reason for believing that an increased incidence of duplicated, and possibly dumbbell, centra may be abnormal is that other investigators have noted similar effects in rats and mice exposed to other teratogens. DiPaolo (1963), and DiPaolo, Gatzek and Pikren (1964) observed skeletal effects in fetuses of mice treated with thalidomide, and Dwornik and Moore (1965) observed this in fetuses of Holtzman rats given thalidomide. Ionizing radiation has also been shown to produce such anomalies in rats (Hicks and D'Amato, 1966). More recently, Riddell (1967) noted these abnormalities in fetuses of Holtzman rats given colchicine (doses and days of administration were the same as those in the present study).

The apparent stomach tube effect on sternebral ossification is also doubted. Although some of the reasons for this are similar to those just expressed, the major one being as follows: when various degrees of ossification in the fifth sternebra were analyzed separately (responses thirty-five to thirty-eight), the results suggested that the stomach tube treatment may be responsible for absence of ossification in this centre (response thirty-eight), and also for abnormal ossification in other sternal centres excluding the fifth (response forty). However, a significant podophyllin, instead of stomach tube, effect was observed when various degrees of ossification in the fifth sternebra were combined and analyzed (response thirty-nine). In addition, a podophyllin effect was noted when the six sternal centres were analyzed together (response forty-one).

The above, apart from suggesting that the stomach tube may actually have little or no effect on sternebral ossification, also illustrates that 1) two effects attributed to different factors may be

obtained, depending on the manner in which the responses (observations) are analyzed, 2) as a result of the former one should be careful in deciding how one will analyze the raw data, and 3) that the sternum should be analyzed as a complete unit rather than as individual sternal centres and degrees of ossification in these centres. The reason for being so thorough in examining the sternum is that Klein Obbink and Dalderup (1963) noted considerable variation in its ossification in their study of thalidomide on the rat.

Whether or not the stomach tube actually had an effect on ossification in sternal centres other than the fifth is even less certain. In the ten-day-old newborn animals of podophyllin-treated mothers in group XXI, it was noted that ossification in the sixth sternebra was retarded in 10 per cent of the young (Figure 30), but this was not observed in any of the young of untreated control mothers in group XXXIII.

As previously stated, the results of the statistical analysis suggested that the stomach tube was responsible for fusion of vertebral arches (Figure 15). Although this seems somewhat doubtful, it is possible. In the nineteen podophyllin-treated groups there were only two mothers (group IV and IX) that had three fetuses with fused arches whereas in the one untreated control and nine treated control groups, one animal in the stomach tube control group (XXX) had one fetus with fused vertebral arches. These results suggest that the stomach tube may be responsible for fusion of vertebral arches, however, no definite conclusion can be made due to the small number of affected fetuses.

If the stomach tube does mildly influence ossification in some of these instances, then this may occur as follows: possibly the blunt end of the polyethylene tube causes trauma to the animal's esophagus. This may result in a decrease in the animal's food consumption, in turn causing a temporary deficiency or reduction in essential compounds necessary for a metabolic pathway or pathways in the pregnant animal that may affect skeletal ossification.

Supporting this concept are Runner's (1959) experiments in which he fasted pregnant mice for twenty-four hours, beginning on the ninth day of gestation. He noted that 22 per cent of the fetuses had either cranioschisis or deformed ribs. Supplements of glucose, ketone-body or a variety of amino acids protected the fetuses from the fasting effect. He suggested that the protective compounds acted by supplying substrate for the citric acid cycle. Shortly thereafter, Runner and Dagg (1959) studied the comparative effects of fasting, hypoxia, trypan blue, iodoacetate, 9-methylfolic acid, and X-rays on the mouse exposed to these treatments during the ninth day of gestation. Each of these treatments produced a variety of skeletal anomalies, but the one common to all treatments was abnormal thoracic vertebrae. On the basis of the previous study (Runner, 1959) they stated, "The array of treatments causing deformed vertebrae has suggested that a common feature at the metabolic level is that they interfere with energy production from carbohydrate oxidation." More recently, Runner (1965) on the basis of the previously mentioned investigations further stated, "I believe we have shown that normal development in mouse embryos, and probably all mammalian embryos, is dependent on carbohydrate metabolism."

Because the blunt polyethylene stomach tube may in some instances influence skeletal ossification, future experiments will be done with a commercially prepared intubation needle (Popper & Sons, New York) or a small rubber catheter. If the foregoing hypothesis is correct, either of these should be less traumatic to the animal's esophagus and thereby result in fewer variations in fetal ossification.

How Does Podophyllin Exert Its Effect on Fetal Development? A number of hypotheses can be proposed concerning the mode of action of podophyllin in the experiments reported here. The historical review has shown that the effects of this drug or its compounds on various systems in animals and humans are numerous. It has been shown that podophyllin contains at least twelve compounds; some of which are known to be mitotic inhibitors (Kelly and Hartwell, 1954; Hartwell and Schrecker, 1958). Some of the more likely hypotheses are discussed below.

First, podophyllin may affect the vascular supply in either the uterus or the placenta of the pregnant rat. It will be recalled that the experimental results reported here have suggested that podophyllin may on occasion be responsible for severely runted fetuses. It is suggested that in these instances the intrauterine growth retardation may be due to a drug effect on the vessels of the uterus or placenta. Decreased blood supply, and consequently reduced oxygen tension might also delay, but not permanently inhibit ossification in the fetuses. Thus the variability observed in ossification, could have been increased in this way without producing clearly defined anomalies.

Algire, Legallais and Anderson (1954) subcutaneously injected various doses of podophyllin in olive oil into a number of different strains of mice with transplanted sarcomas. Results of microscopic studies *in vivo* indicated that within one hour after podophyllin injection, blood flow was slower in the arteries, veins, and capillaries of normal tissue as well as in vessels of the tumour. This was followed by stasis or occlusion of blood flow in many vessels. In addition, when they injected agents (type not stated) known to depress peripheral blood pressure, the observations were identical to those noted with podophyllin. On the basis of these results, these workers stated that podophyllin induced hypotension on the peripheral circulation of the host and that this seemed to be sufficient to account for the tumour damage. It is likewise possible that podophyllin might have similar effects on uterine and placental vessels.

Other possible supporting evidence for this hypothesis is the work by Gorthey and Krembs (1954). They treated condylomata acuminata of the vulva in a young woman who was in her eighth month of pregnancy. The podophyllin (25 per cent) was applied to the lesion in a hydrophilic base for fifty minutes. Prior to the podophyllin application fetal heart tones were good, but approximately three hours after cessation of treatment, the woman complained of perineal pain. Shortly thereafter a caesarian section was performed and a stillborn infant delivered. Autopsy findings on the infant revealed no other cause of death than anoxia. These authors speculated that the anoxia may have been caused by arteriolar spasm of the decidua basalis from the systemic absorption of the drug.

Yet again, Thiersch (1963) injected podophyllin in distilled water intraperitoneally into pregnant rats on various days of gestation. He observed great variation in surviving placental remnants of rats treated on the eleventh and twelfth days of pregnancy. This was not apparent in the present study. Some of Thiersch's specimens showed varying states of intraplacental hemorrhage and marginal necrosis. He also observed quite frequently a network of stoma filled with blood and varying numbers of relatively well-preserved giant cells in the placental periphery.

By contrast, however, Didcock, Jackson and Robson (1956) injected podophyllotoxin, a constituent of podophyllin, directly into the placentas of pregnant rabbits between the fifteenth and twenty-first days of pregnancy. The drug had little effect on placental weight. These workers stated that the toxic effects of podophyllotoxin appeared to be exerted directly on the fetus and not on the placenta.

Secondly, podophyllin and some of its compounds have also been shown, like colchicine, to inhibit division in cells that are undergoing mitosis by preventing spindle formation (King and Sullivan, 1947; Kelly and Hartwell, 1954; Karnofsky, 1965; Broomhead, 1967). Consequently podophyllin may induce anomalies in the fetus due to its effects on various cells in either the mother, the fetus or both. Preliminary studies in this laboratory have indicated that some doses of podophyllin in alcohol will inhibit mitosis in the duodenum of the pregnant Holtzman

rat (Broomhead, 1967).[1]

Therefore it may be possible that the fetal runts observed in some podophyllin-treated groups occurred as a result of a drug effect on the intestinal absorptive cells, and thereby absorption of essential nutrients was decreased. As stated previously, Runner (1959) observed that fasting of mice for twenty-four hours during the ninth day of pregnancy produced either cranioschisis or deformed ribs in 22 per cent of fetuses. Supplements of glucose, ketone-body or a variety of amino acids protected the fetuses from the fasting effect. He suggested that the protective compounds acted by supplying substrate for the citric acid cycle. It may be that even without fasting, a decrease in food absorption might cause some defects, or variations, to occur in a similar manner.

Thirdly, podophyllin or one of its compounds might also produce congenital malformations by various effects on a system(s) or metabolic pathway in either the mother, the fetus or both. Any one or a combination of these effects might also be responsible for the occurrence of fetal runts in drug-treated animals.

Supporting this concept in part, Greenspan and Leiter (1949)

---

[1]Podophyllin (0.05, 0.1, 0.2, and 0.4 milligrams per 100 grams body weight) administered to pregnant Holtzman rats on days eight to twelve, and ten to twelve, inhibits mitosis at the higher doses in a manner similar to colchicine; spindle formation seems to be impaired and chromatin material is at times dispersed throughout the cell. Similar observations were noted by King and Sullivan (1947). They topically applied podophyllin and colchicine to human skin and noted certain cellular changes. The most characteristic observation was enlarged, swollen cells with finely reticulated pale basophilic cytoplasm and dispersed chromatin material.

parenterally administered podophyllotoxin, alpha-peltatin, and beta-peltatin to mice, rats, rabbits, and dogs. One-half to two hours after injection of either of these substances, a leukopenia developed that was followed by leukocytosis with degenerating neutrophiles and lymphocytes. Waterman (1950) administered podophyllotoxin to an inbred strain of mice which developed leukosis. He observed cellular damage in the spleen and lymph nodes.

In another instance, Miller, Davison and Smith (1949) noted that podophyllotoxin inhibited respiration *in vitro* in a number of tissues taken from rats and mice. Furthermore, inhibition became more pronounced with time. When several enzymes were tested, podophyllotoxin did not inhibit any of them. Thiersch (1963), on the basis of observations by Miller and his colleagues, speculated that this inhibition, especially in rat homogenates of spleen, thymus, and lymph nodes, may have accounted for the action of podophyllin on fetuses in his study; he noted stunted fetuses and high resorption rates.

More recently, Freedberg (1965) studied the effects of podophyllin and podophyllotoxin on cell-free amino acid incorporation; according to Hartwell and Detty (1949), the percentage of podophyllotoxin in podophyllin is approximately 7 per cent. A cell-free amino acid incorporating system was prepared from mice that were intraperitoneally given podophyllin (3 and 15 milligrams per 100 grams body weight) and podophyllotoxin (5 milligrams per 100 grams body weight) fifteen hours earlier. The preparations made from animals receiving the 3 and 15 milligrams of podophyllin stimulated amino acid incorporation whereas

those preparations made from the podophyllotoxin-treated mice caused inhibition. When the dose of podophyllotoxin (given to mice) was lowered to 1 milligram per 100 grams body weight, stimulation was also observed.

Because Freedberg noted opposite effects between comparable high doses of podophyllin and podophyllotoxin, but observed similar effect with the low podophyllotoxin dose and the high podophyllin dose, it may be that podophyllin is capable of producing a variety of effects in the mother, the fetus, or both, depending on the dosage. Furthermore it is possible that podophyllin may be capable of stimulating ossification at low and high doses, and delaying or inhibiting ossification at intermediate doses, since the percentage of podophyllotoxin present in the podophyllin doses would vary according to the amount of the administered dose.

This concept seems to be further supported when the podophyllin dose response curves are analyzed for the groups receiving the drug on days eight to twelve and ten to twelve of gestation. It was noted that the most prominent curve was the quadratic component (example, Graph 1). It occurred thirteen times in eleven of the forty-five responses analyzed and suggests that low and high podophyllin doses are either ineffective, or they stimulate ossification. In contrast, the shape of this curve suggests that the intermediate doses will usually inhibit ossification.

Conclusions. The most noteworthy podophyllin effect observed in this study appears to be severe runting of fetuses (intrauterine growth retardation). Thiersch (1963) also noted severely runted fetuses

in rats given podophyllin and one of its constituents, podophyllotoxin. Karnofsky (1965) believes that both these drugs are possible teratogens in animals and humans. He bases this conclusion on two facts: first, that podophyllin and podophyllotoxin are metaphase inhibitors, and, secondly, Thiersch's observation noted above. Although the present study indicates that podophyllin is a weak teratogen in the Holtzman rat, additional studies need to be done to ascertain the possible teratogenicity of this drug in other mammalian species.

In most instances described herein, podophyllin does not produce bizarre skeletal anomalies, but does increase the incidence of normal skeletal variations. In view of this, the drug should not be given to pregnant humans during gestation. Although the drug may have no effect on human embryos, the present study suggests the possibility that it might be involved in the production of intrauterine growth retardation and of minor skeletal variations.

Part II: Temperature Study

Does Varying Room Temperature Have an Effect on Skeletal Ossification? Although varying temperature was shown to influence 1) placental diameter, 2) fetal length and weight, 3) ossification in the odontoid process, 4) incidence of dumbbell centra, and 5) ossification of tail centra, it is believed that in most instances the differences in means were so small that no firm conclusion can be made. Some measurements (placental diameter, fetal length, and weight) were not made as accurately as the means indicate, however, it is thought that because the samples were large, there is a suggestion that varying temperature may be detrimental in some instances on ossification. This study also suggests a general trend or pattern of events which might occur.

A reason why more variations were not observed may be due to an ability of these rats to acclimatize partially to the varying temperature. It will be recalled that temperature in the animal quarters, although usually near 78 degrees Fahrenheit, did vary ± 7 degrees. Furthermore, since animals were kept in this room for a period of seven to ten days before mating, partial acclimatization probably occurred.

Slightly supporting this concept are MacFarlane, Pennycuik and Thrift's (1957) experiments in which they exposed unacclimatized pregnant Wistar rats to a temperature of 95 degrees Fahrenheit. They noted that 58 per cent of the fetuses were resorbed, whereas at normal temperatures, 72 to 82 degrees Fahrenheit, they found that only 7 per cent of the fetuses were resorbed. They also noted a highly significant decrease in fetal loss when the rats were acclimatized for two to ten

weeks at a temperature of 95 degrees Fahrenheit prior to mating. This indicates that animals should be acclimatized before mating and attempting to study the effects of various drugs on the fetus. Furthermore, this work suggests that some of the variations noted in the podophyllin experiments may have been produced by temperature variation.

What is the Optimum Room Temperature at Which the Number of Variations Occurring in Skeletal Ossification will be Constant? The effects of temperature on fetal ossification was studied in seven groups of rats (each containing ten animals) exposed to fixed temperatures of 60, 65, 70, 75, 80, 85, and 90 degrees Fahrenheit from days zero to twenty-one of gestation. The results generally indicate that there is less variation in fetal ossification in rats exposed to temperatures of 70, 75, or 80 degrees Fahrenheit than in animals exposed to temperatures of 60, 65, 85, or 90 degrees Fahrenheit.

The above conclusion seemed apparent from the analysis of the temperature response curves for the groups exposed to the above temperatures. It was noted that a linear component was present in seven of the forty-five responses analyzed (Table I, Appendix). In some responses (observations) recorded, the slope (Graph 11) of this component was rising, and in others it was declining, with increase in temperature. Therefore this suggests that the degree of variation in ossification of most centres will be more constant at the intermediate (70, 75, or 80 degrees Fahrenheit) temperatures than that at either the low (60 or 65 degrees Fahrenheit) or high (85 or 90 degrees Fahrenheit) temperatures.

It is possible that the intermediate temperatures do not affect the physiological state of the pregnant animal, whereas high and low temperatures may. It also seems that ossification in some centres is more vulnerable to the effects of heat on the mother, whereas ossification in other centres seem to be more susceptible to the effects of cold. These findings lead one to conclude that fetuses exposed to temperatures of 70, 75, or 80 degrees Fahrenheit are likely to have more uniformly ossified centres, and that fewer skeletal variations are likely to occur than in fetuses exposed to higher or lower temperatures.

Supporting the concept that high temperatures may indirectly influence fetal ossification are MacFarlane, Pennycuik and Thrift's (1957) experiments. They noted high resorption rates (58 per cent), associated with small litter size in pregnant Wistar rats exposed to a temperature of 95 degrees Fahrenheit between the sixth and twelfth days of gestation. In another study, MacFarlane et al. (1959) observed that 74 per cent of the fetuses were resorbed in rats exposed to the above mentioned temperature (days of exposure not stated). Because high temperature causes increased resorptions, it is possible that exposure of pregnant rats to comparable temperatures may also affect ossification, and likely accounts for the skeletal variations observed in the present study.

Although there appear to be no experiments to show that lowering of room temperature, in the range studied here (60 and 65 degrees Fahrenheit), can have an effect on the physiological state of the pregnant rat, it has been shown that extreme cooling of body temperature in pregnant

rats results in deleterious effects on embryonic development. Vidovic (1952 and 1956; cited by Smith, 1957) cooled the deep body temperature of pregnant rats in a range from 59 to 68 degrees Fahrenheit on various days of gestation. He observed that fetal sensitivity to cooling increased after the fourteenth day and particularly after the sixteenth day of pregnancy; the litter size was small and the proportion of stillborn animals increased. One rat cooled on the seventeenth day of gestation gave birth to three malformed animals. Courrier and Marois (1953 and 1954; cited by Smith, 1957) observed uterine hemorrhage in rats cooled to body temperatures between 61 and 68 degrees Fahrenheit on the twelfth to eighteenth days of pregnancy. Most of the fetuses died and were resorbed. By comparison, implantation was delayed, development retarded, and parturition was postponed when rats were cooled daily to the same temperatures during the first eleven days of gestation. Because their results show that extreme cooling of the rat results in some severe fetal and maternal effects, it is possible that a low room temperature of 60 or 65 degrees Fahrenheit may have a mild effect on rat fetuses resulting in some variation in skeletal ossification observed in the present study.

Because high and low temperatures, in the range studied here, appear to influence skeletal ossification, it is concluded that ideal room temperature for fetal rat development is between 70 and 80 degrees Fahrenheit, and that the variation should not be more than ± 2 degrees.

In What Way Does Temperature Exert Its Effect on the Fetus?

A number of hypotheses can be proposed concerning the mode of action of high temperatures (85 and 90 degrees Fahrenheit) on fetal ossification since it has been shown that high temperature (95 degrees Fahrenheit) indirectly exerts its effects on various organs and possibly systems in pregnant Wistar rats (MacFarlane, Pennycuik and Thrift, 1957; MacFarlane et al., 1959). However, practically no reasonable hypothesis can be made concerning the mode of action of low temperatures (60 and 65 degrees Fahrenheit) on fetal ossification. According to Smith (1957) comparatively little is known about the effects of cold on fetal development; this also seems to be true for low room temperature.[1]

First, it became apparent that food consumption in pregnant rats exposed to high temperatures in the present study was less than in those animals maintained at intermediate temperatures of 70, 75, and 80 degrees Fahrenheit. These animals seldom ate and were relatively inactive in comparison with animals living at intermediate temperatures. This decrease in food, though not great, may result in a reduction of nutrients required for a metabolic pathway in the pregnant rat which in turn may influence ossification. This may partly account for increased variations in skeletal ossification in fetuses of animals exposed to high temperatures throughout gestation.

Supporting the hypothesis that a decrease in food intake by pregnant animals kept at temperatures of 85 and 90 degrees Fahrenheit

---

[1]The cold referred to by Smith was probably extreme. He studied the effects of freezing on fetal development in the pregnant hamster.

resulted in a partial reduction in essential nutrients for a metabolic pathway, are MacFarlane, Pennycuik and Thrift's (1957) observations that food consumption was approximately 43 per cent lower in rats living at 95 degrees Fahrenheit than in animals kept at room temperatures from 73 to 82 degrees Fahrenheit. They further observed, that pregnant animals maintained at this temperature resorbed 58 per cent of their fetuses. They were able to reduce the resorption rate to 25 per cent (not significantly) by administering supplements of protein and vitamins to these animals. The resorption rate at normal room temperature was found to be 7 per cent.

In a more extensive study on pregnant rats exposed to elevated room temperature, MacFarlane et al. (1959) also administered various supplements of vitamins in group B and vitamins A and E to the diets of rats. They speculated that pyridoxine was probably the most important vitamin. On the basis of these experiments, it is suggested that a decrease in food intake by pregnant rats, in the present experiments due to high temperature, may have an effect on a metabolic pathway in the animal and produce a variability in skeletal ossification.

Supporting the hypothesis that a metabolic pathway may be involved, are the fasting experiments done by Runner (1959), and Runner and Dagg (1959) in which they showed that complete fasting of pregnant mice for twenty-four hours during the ninth day of gestation results in severe fetal deformities.[1] These workers also subjected pregnant

---

[1]For additional details refer to Does the Stomach Tube Have an Effect on Ossification, page 153.

mice to a number of other teratogenic substances during the ninth day of pregnancy and noted similarities in effects, such as malformed thoracic vertebrae. Because these effects were similar for all treatments, Runner (1965) postulated that normal development in mouse embryos, and probably in all mammalian embryos, is dependent on carbohydrate metabolism. If carbohydrate metabolism is severely affected by complete fasting, it seems probable that a reduction in food intake may only mildly affect carbohydrate metabolism. This derangement may partly account for the increased variations noted in skeletal ossification of fetuses in rats exposed to high temperatures in the present study.

It is also possible that the increased variability in skeletal ossification in fetuses of animals exposed to high temperatures may be due to effects of temperature on the normal physiology of one or more organs of the pregnant animal. Although there is no evidence that temperatures of 60 and 65 degrees Fahrenheit alter the physiology of the pregnant animal, such disturbances may be possible and therefore should not be excluded as a cause of skeletal variations in rats.

Further support for this concept are MacFarlane, Pennycuik and Thrift's (1957) observations of high resorption rates (58 per cent) in rats exposed to a temperature of 95 degrees Fahrenheit during their pregnancies. By administering progesterone and thyroxine to these rats, they were able to reduce the resorption rate to 32 and 30 per cent respectively. However, when they administered cortisone to pregnant rats kept at this temperature, the resorption rate was 60 per cent. By comparison, rats given cortisone and maintained at a temperature of 73 degrees Fahrenheit had only 22 per cent resorptions.

These workers noted that if they first acclimatized rats for twenty-five to seventy days at 95 degrees Fahrenheit and then mated them, that only 7 per cent of the fetuses were lost. They stated, "It is possible that the common pathway of action of proteins, vitamins, thyroxine and progesterone in saving fetuses runs through the adrenal cortex in its effects on uterine muscle cells." In another instance they stated, "All the factors considered probably act upon the uterus rather than the fetus." Shortly thereafter, MacFarlane et al. (1959) in another report state, referring to the pregnant rat kept at elevated room temperature, "The evidence indicates that uteroplacental function was improved by supplements to the diet (probably pyridoxine is important) and by long-term acclimatization taking place over generations." Their results suggest that numerous factors could be involved. Therefore in the present study, the variations observed in groups exposed to high, and maybe low, temperatures may have been due to the effects of heat and possibly cold on a number of biochemical systems in the pregnant rat.

Conclusions. It is believed that the effects of temperature (below 70 and especially above 80 degrees Fahrenheit) on the pregnant untreated rat are probably sufficient to account for physiological disturbances in the animal. These in turn likely account for the considerable variations that were observed in skeletal ossification of the fetuses. It will be recalled that considerable variation was similarly noted in the podophyllin experiments, and that it was usually almost impossible to make definite deductions due to possible environmental

influences. Therefore it is concluded that all future teratogenic studies, especially those involving drugs, should be done under rigidly controlled room temperature, and possibly relative humidity. This would enable one to evaluate more accurately the effects of a drug on fetal development.

In accordance with this conclusion, Wilson (1954), and Wilson and Warkany (1965), with reference to embryonic environment state, "Consequently, the physiologic state of the mother is of considerable importance to the embryo." In addition, Cahen (1964) emphatically stresses the importance of maintaining a constant environmental room temperature and a well-balanced diet during teratogenic studies in order to obviate the introduction of unexpected variables into an experiment.

VI SUMMARY OF RESULTS

Part I: Podophyllin Study

1. Pregnant Holtzman albino rats received podophyllin by stomach tube daily in doses ranging from 0.012 to 1.6 milligrams per 100 grams body weight of the animal for various periods from the eighth to twelfth days of gestation; this is considered to be the critical period in rat development. All animals, except those in groups XXI and XXXIII, were killed on the twenty-first day of pregnancy. This was done to prevent cannibalism of the offspring. All litters, except those in groups XX and XXXII, were cleared in 1 per cent potassium hydroxide. The skeletons were stained with alizarin red S and examined to determine if the drug has any effect on skeletal formation.

2. Considerable variation was noted in ossification, however, there were no major skeletal malformations. It was not feasible to conclude that podophyllin was responsible for these variations because time as a variable, was not incorporated into the experimental design. Changes have been proposed for similar experiments of the future in order to make studies more meaningful, even in the absence of major skeletal malformations.

3. This study suggests that podophyllin is capable of severely stunting fetal growth; viable runts were observed in a few instances (Figures 3 and 4). This occurred in litters of three of seventy mothers (4.2 per cent) that had received 0.1 milligram of podophyllin per 100 grams body weight on the tenth to twelfth, and

day ten only of gestation. This was never observed in fetuses of treated or untreated (normal) control animals.

4. Widespread variation in ossification was noted in fetuses of animals in the various treated control groups (XXII to XXX) and the one untreated control group (XXXI). Although there are a number of factors that may account for this variability, fluctuating environmental temperature is believed responsible, since it was not possible to maintain a constant temperature throughout the entire course of this investigation.

5. No visceral variations were detected in razor-blade cross sections of fetuses of mothers (group XX) that had been treated with podophyllin (0.1 milligram per 100 grams body weight) on days ten to twelve of gestation.

6. The skeletons of ten-day-old prenatally-podophyllin-treated newborn rats (group XXI) showed skeletal variation, namely, slightly dumbbell vertebral centra (Figures 28 and 29). It cannot be proved that this was a podophyllin effect.

7. There is suggestive evidence which indicates that temperature fluctuation in the animal quarters may have an indirect influence on skeletal ossification; it appears to cause widespread biological variation. Future studies of this type should be done in a constant environmental temperature, and possible per cent relative humidity.

8. The skeletal variations noted in this study were not observed during gross examination and became visible only after the

tissues had been cleared in potassium hydroxide and the bones stained with alizarin red S. Therefore it is concluded that this technique should be a part of all experiments aimed at detecting the teratogenicity of drugs.

9. The computer analysis used in this study, though useful in screening for the effects of podophyllin on ossification of the fetal skeleton, presented some difficulties of interpretation. A modified use of the computer in teratogenic studies is likely to be more useful in future experiments.

Part II: Temperature Study

1. Using the alizarin technique it was observed that fetuses of mothers exposed to temperatures of 70, 75, or 80 degrees Fahrenheit had fewer skeletal variations in ossification than fetuses of mothers exposed to temperatures of 60, 65, 85, or 90 degrees Fahrenheit.

2. This study indicated that there was no apparent difference in skeletal ossification of fetuses in mothers exposed to gradient (fixed) temperatures and to fluctuating temperatures throughout pregnancy.

3. This study indicated that temperature has an indirect effect on fetal skeletal ossification. It should therefore be closely regulated in all future teratogenic studies.

APPENDIX

In Table I the contrasts for the podophyllin study, numbers one to six, are listed down the left-hand side followed by contrasts T-1 and T-2 for the temperature study.[1] Contrasts one to three were general and were used to determine which treatment days were most effective. Contrasts four (a) to (e) concerned only those animals treated on days eight to twelve of pregnancy. Contrasts five (a) to (e) concerned only those animals treated on days ten to twelve of gestation. Contrasts six (a) to (f) concerned only those animals treated on day ten only of pregnancy. Contrast T-1 compared the varying temperature group with the fixed temperature groups (ATE to GTE). Contrast T-2 was used to determine the temperature response curve for the fixed (gradient) temperature groups (ATE to GTE).[2] The responses, numbers one to forty-five, are across the top of the table.[3]

A double asterisk (**) shows that alpha was less than 0.01 (highly significant). A single asterisk (*) indicates that alpha was less than 0.05, but greater than 0.01 (significant). A single dash (-) shows that alpha was greater than 0.05, but less than 0.1; this suggested that the effect might have been significant (borderline). When alpha was 0.1 or

---

[1]In the podophyllin study the analysis was done on groups shown in Table II of this Appendix. In the temperature study the analysis was done on groups shown in Table III of this Appendix.

[2]Additional details of the contrasts in the podophyllin study are shown on pages 52 to 54. Details of contrast in the temperature study are shown on page 59.

[3]A description of the individual responses is given on pages 46 to 50.

greater, indicated by a double dash (--), this was taken to mean that the observation was definitely not significant.

The interpretation of the significance levels for each response in Table I was as follows:

1. If contrast one was highly significant or significant, but contrasts two to six were not significant, this was interpreted as a stomach tube effect.
2. If contrast four (a) was highly significant or significant, but contrasts four (b) to (e) were not significant, this implied a water treatment effect. This same interpretation was used in contrasts five (a) and six (a).
3. If contrast four (b) was highly significant or significant, but contrasts four (c) to (e) were not significant, this implied an alcohol effect. This same interpretation was used in contrasts five (b) and six (b).
4. If in contrasts four (a) to (e), contrast four (c) or (d) showed a highly significant or significant podophyllin effect, explanations of contrasts four (a) and (b) were omitted (if significant) because contrasts four (a) and (b) were then a reflection of the effects in contrast four (c) or (d). This same interpretation was used in contrast five (a) to (e) and six (a) to (f).
5. If a podophyllin in alcohol effect was not significant, but a podophyllin in alcohol dose response curve had a highly

significant or significant component(s), this was interpreted as meaning that podophyllin was least effective at some dose levels and either directly or indirectly delayed, retarded, or inhibited ossification at other dose levels.

Table I

Summary of Statistical Analysis

Contrasts	Response Numbers 1	2	3	4	5	6	7	8	9
1	--	--	-	--	**	--	--	--	*
2	--	--	--	--	**	**	--	--	--
3	--	-	--	--	--	--	*	--	-
4(a)	--	--	--	--	**	--	--	--	--
(b)	--	--	--	--	**	--	--	--	--
(c)	-	--	-	--	-	-	--	**	--
(d)	--	--	--	--	--	--	--	--	--
(e-i)	-	--	--	--	--	--	--	--	--
(e-ii)	--	--	--	**	*	--	--	--	-
(e-iii)	--	--	--	**	-	--	--	**	--
(e-iv)	--	--	--	--	--	--	--	--	--
(e-v)	--	--	--	-	--	--	--	--	--
5(a)	--	--	--	--	--	--	--	*	-
(b)	--	--	--	--	--	--	--	--	--
(c)	--	--	--	*	--	--	--	--	-
(d)	--	--	--	--	*	--	-	--	--
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	--	--	--	**	--	*	--	**	--
(e-iii)	--	--	--	--	--	--	--	--	--
(e-iv)	--	--	--	--	--	--	--	-	--
(e-v)	--	-	--	--	--	--	--	--	--
6(a)	--	--	--	--	--	-	--	**	-
(b)	--	--	--	--	--	-	--	-	--
(c)	--	--	--	--	--	-	--	--	--
(d)	-	--	--	-	--	--	--	-	--
(e)	--	-	--	--	--	--	--	--	--
(f-i)	-	--	-	--	--	--	--	--	--
(f-ii)	-	--	--	--	--	--	--	--	--
T-1	--	--	--	**	**	**	--	--	--
T-2(i)	--	--	--	*	--	--	-	**	--
(ii)	--	--	--	--	--	*	--	-	--
(iii)	--	--	--	--	*	-	--	**	--
(iv)	--	--	--	--	--	--	-	--	--
(v)	--	--	--	--	--	--	--	--	--

Table I Continued

Contrasts	Response Numbers 10	11	12	13	14	15	16	17	18
1	**	--	**	--	--	--	--	--	-
2	**	**	**	*	--	**	--	--	**
3	--	--	--	--	--	--	--	--	--
4(a)	**	**	**	**	--	*	--	--	--
(b)	**	**	**	--	--	--	--	--	*
(c)	--	--	--	--	--	--	--	--	--
(d)	*	**	*	--	--	--	--	--	*
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	--	*	--	--	--	--	--	--	--
(e-iii)	--	--	--	--	-	--	--	--	--
(e-iv)	-	--	-	--	--	--	--	--	--
(e-v)	--	--	--	--	--	*	--	--	--
5(a)	*	-	**	--	-	--	--	--	--
(b)	**	**	**	--	--	--	--	--	**
(c)	-	--	--	--	--	--	--	--	--
(d)	*	--	*	--	--	--	--	--	--
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	--	--	--	--	--	--	--	--	--
(e-iii)	*	--	--	--	--	--	--	--	--
(e-iv)	--	--	--	--	--	--	--	--	--
(e-v)	--	--	--	*	--	--	--	--	--
6(a)	--	--	--	--	--	--	--	*	--
(b)	*	--	*	--	--	-	--	--	--
(c)	--	--	--	--	--	--	--	--	--
(d)	--	--	--	-	--	--	--	--	--
(e)	--	--	--	-	--	--	--	*	--
(f-i)	*	--	**	**	--	--	--	--	--
(f-ii)	--	-	--	--	--	--	--	--	--
T-1	--	--	--	--	*	--	-	-	--
T-2(i)	**	--	**	--	--	--	--	--	--
(ii)	--	--	--	--	--	**	-	-	--
(iii)	--	--	--	--	--	--	--	--	--
(iv)	--	--	--	--	--	--	--	--	--
(v)	--	*	--	--	--	--	-	--	--

Table I Continued

Contrasts	Response Numbers 19	20	21	22	23	24	25	26	27
1	--	--	--	--	--	**	*	--	-
2	--	--	**	--	--	--	--	--	-
3	--	--	--	--	--	-	--	--	--
4(a)	--	--	--	--	--	*	-	--	--
(b)	--	-	--	--	--	*	-	-	--
(c)	--	--	--	--	--	--	--	--	--
(d)	--	--	--	--	--	*	-	--	--
(e-i)	--	--	*	--	--	--	--	--	--
(e-ii)	--	--	*	-	*	--	--	--	--
(e-iii)	--	--	--	--	-	--	--	--	--
(e-iv)	--	--	--	--	--	--	--	--	--
(e-v)	--	--	-	--	--	--	--	--	--
5(a)	--	--	--	--	--	--	--	--	--
(b)	--	--	--	--	--	--	--	--	--
(c)	--	--	--	--	--	--	--	--	--
(d)	--	--	--	--	--	--	--	--	--
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	*	--	**	--	--	--	--	--	**
(e-iii)	--	--	--	--	--	--	--	--	--
(e-iv)	--	--	--	--	-	--	--	--	--
(e-v)	--	--	--	--	--	--	--	--	--
6(a)	--	--	--	-	--	--	--	--	*
(b)	--	--	--	-	--	--	--	--	--
(c)	--	*	-	--	--	-	--	--	-
(d)	--	--	--	-	--	**	--	--	--
(e)	--	*	--	--	--	--	--	--	--
(f-i)	--	-	--	--	--	-	--	-	-
(f-ii)	--	--	--	--	--	--	--	--	--
T-1	--	--	--	--	--	**	--	-	**
T-2(i)	*	*	-	--	--	--	--	--	--
(ii)	--	--	--	--	**	--	--	-	--
(iii)	--	--	--	--	--	--	--	--	--
(iv)	--	--	--	--	--	--	--	--	--
(v)	--	--	--	--	--	--	--	--	--

Table I Continued

Contrasts	Response Numbers 28	29	30	31	32	33	34	35	36
1	*	--	-	--	*	--	--	--	-
2	--	--	--	--	-	--	--	--	**
3	--	--	--	--	--	--	--	--	*
4(a)	--	--	--	--	--	--	--	--	--
(b)	--	--	--	--	--	--	--	-	--
(c)	--	--	--	--	--	--	--	--	--
(d)	--	--	--	--	*	--	--	--	*
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	--	-	--	-	-	--	-	--	**
(e-iii)	--	--	--	--	--	--	--	--	-
(e-iv)	--	--	--	--	--	--	--	--	--
(e-v)	--	--	--	--	--	-	--	--	--
5(a)	--	--	--	--	--	--	--	--	--
(b)	--	--	--	--	--	--	--	--	--
(c)	--	--	--	--	--	--	--	*	--
(d)	--	--	--	--	-	--	-	--	*
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	--	--	--	--	--	--	--	--	--
(e-iii)	--	--	--	--	--	*	--	--	--
(e-iv)	--	--	--	--	--	--	--	-	--
(e-v)	--	--	--	--	--	--	--	--	--
6(a)	--	-	--	--	--	--	--	*	**
(b)	--	-	-	--	--	-	--	--	--
(c)	--	--	--	--	--	--	-	--	--
(d)	--	-	-	--	--	--	--	--	--
(e)	--	--	--	--	--	-	--	--	*
(f-i)	--	--	--	--	--	--	--	--	--
(f-ii)	--	--	--	--	--	--	--	-	--
T-1	--	--	--	--	--	--	--	--	--
T-2(i)	--	--	--	--	--	*	--	**	**
(ii)	--	--	--	--	--	--	--	--	--
(iii)	--	--	--	--	--	--	--	--	--
(iv)	--	--	--	--	--	--	--	--	*
(v)	--	--	--	--	--	--	--	--	--

Table I Continued

Contrasts	Response Numbers 37	38	39	40	41	42	43	44	45
1	**	*	-	**	*	--	-	--	--
2	*	--	**	--	**	--	--	**	--
3	--	-	--	--	--	--	--	-	--
4(a)	*	--	--	--	-	--	--	--	--
(b)	*	--	*	--	-	--	--	-	-
(c)	--	--	--	-	--	--	--	--	--
(d)	-	--	*	--	*	--	--	*	--
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	-	-	--	--	--	-	-	--	--
(e-iii)	--	--	--	--	*	--	--	--	--
(e-iv)	--	--	--	--	-	--	--	--	--
(e-v)	--	--	--	--	--	--	--	--	--
5(a)	-	--	-	--	-	--	--	--	--
(b)	-	--	--	--	--	--	--	--	--
(c)	--	--	--	--	--	--	--	-	--
(d)	--	--	*	--	*	--	--	**	--
(e-i)	--	--	--	--	--	--	--	--	--
(e-ii)	--	--	--	--	--	--	--	**	--
(e-iii)	--	--	--	--	--	--	--	--	--
(e-iv)	--	--	--	--	--	--	--	--	--
(e-v)	--	--	--	--	--	--	--	*	--
6(a)	--	--	**	--	**	-	--	--	-
(b)	--	--	--	--	--	-	--	--	-
(c)	--	--	--	--	--	--	--	*	--
(d)	--	--	--	--	--	-	--	--	--
(e)	--	--	-	--	-	--	--	--	--
(f-i)	--	--	--	--	--	--	--	--	--
(f-ii)	--	--	--	--	--	--	--	--	--
T-1	-	--	--	--	--	--	--	-	--
T-2(i)	--	--	**	--	*	--	--	--	--
(ii)	--	--	--	--	--	--	--	--	--
(iii)	--	--	--	--	--	--	--	**	-
(iv)	--	--	*	--	--	--	--	--	*
(v)	--	--	--	--	--	--	--	--	--

Table II

Number of Young Examined in the Podophyllin Study

Group Number	Treatment Day(s) of Gestation	Dose/Day Mg./100 Gm.	Suspension		Stomach Tube	Number of Young Examined /Group
			10% Ethanol	Water		
I	8-12	0.012	+	-	+	112
II	8-12	0.025	+	-	+	122
III	8-12	0.05	+	-	+	91
IV	8-12	0.1	+	-	+	102
V	8-12	0.2	+	-	+	91
VI	8-12	0.4	+	-	+	105
VII	10-12	0.012	+	-	+	100
VIII	10-12	0.025	+	-	+	104
IX	10-12	0.05	+	-	+	96
X	10-12	0.1	+	-	+	108
XI	10-12	0.2	+	-	+	115
XII	10-12	0.4	+	-	+	114
XIII	10	0.1	+	-	+	111
XIV	10	0.4	+	-	+	79
XV	10	0.8	+	-	+	102
XVI	10	1.6	+	-	+	102†
XVII	8-12	0.1	-	+	+	108
XVIII	10-12	0.1	-	+	+	106
XIX	10	0.1	-	+	+	111
XX	10-12	0.1	-	+	+	98
XXI	10-12	0.1	-	+	+	81
XXII	8-12	-	+	-	+	94
XXIII	10-12	-	+	-	+	114
XXIV	10	-	+	-	+	104
XXV	8-12	-	-	+	+	91
XXVI	10-12	-	-	+	+	121
XXVII	10	-	-	+	+	109
XXVIII	8-12	-	-	-	+	105
XXIX	10-12	-	-	-	+	91
XXX	10	-	-	-	+	111
XXXI	-	-	-	-	-	102
XXXII	-	-	-	-	-	103
XXXIII	-	-	-	-	-	71

Explanation of Table II.

In groups I to XVI, the podophyllin-alcohol suspension was administered by stomach tube.

In groups XVII to XXI, the podophyllin-water suspension was administered by stomach tube.

In groups XXII to XXIV, no podophyllin was given, only alcohol by stomach tube.

In groups XXV to XXVII, no podophyllin or alcohol was given; only water by stomach tube.

In groups XXVIII to XXX, no podophyllin, alcohol or water was given. The stomach tube was inserted into the animal's esophagus and then withdrawn.

Groups XXXI to XXXIII, the untreated controls (normal) were not subjected to any treatment.

The twenty-one-day-old fetuses in groups I to XIX and XXII to XXXI were cleared, stained with alizarin, examined and the observations were statistically analyzed.

The twenty-one-day-old fetuses in groups XX and XXXII were cross sectioned and studied. The observations were not statistically analyzed.

The ten-day-old newborn rats in groups XXI and XXXIII were cleared, stained with alizarin, and examined. The observations were not statistically analyzed.

†This was the number of fetuses examined in nine mothers. The third animal in this group had entire litter resorption; there were twelve resorption sites.

Table III

Number of Young Examined in the Temperature Study

Group	Exposure Day(s) of Gestation	Temperature in Degrees Fahrenheit	Number of Young Examined/Group
ATE	0-21	90	103
BTE	0-21	85	119
CTE	0-21	80	111
DTE	0-21	75	93
ETE	0-21	70	125
FTE	0-21	65	113
GTE	0-21	60	114
HTE	0-21	65 and 90	115

Explanation of Table III.

The twenty-one-day-old fetuses in groups ATE to HTE were cleared, stained, examined, and the observations were statistically analyzed.

The relative humidity for groups ATE to FTE remained constant at 50 per cent, but for groups GTE and HTE it varied from 50 to 75 per cent.

The animals in group HTE were exposed to an alternating temperature of 65 degrees Fahrenheit for eight hours and then 90 degrees Fahrenheit for sixteen hours.

BIBLIOGRAPHY

Algire, G. H., Legallais, F. Y., and Anderson, B. F.: Vascular reactions of normal and malignant tissue *in vivo*. VI. The role of hypotension in the action of components of podophyllin on transplanted sarcomas. J. nat. Cancer Inst. 14:879-893, 1954.

Bain, W.: The action of certain drugs and mineral waters on the secretion and composition of human bile; an experimental investigation. Brit. med. J. 1:1646-1650, 1898.

Baldi, D.: Recherches expérimentales sur la marche de la sécrétion biliare. Arch. ital. Biol. 3:389-397, 1883. (cited by Kelly and Hartwell, 1954).

Bartek, J., Potěšilová, H., Mašinová, V., und Šantavý, F.: Isolierung einiger Substanzen aus *Resina podophylli* (*Podophyllum peltatum* L.) und Beitrag zu ihrer Konstitution. Chem. Listy 49:1550, 1955; Collect. Czech. Chem. Communs. 21:392-404, 1954. (cited by Hartwell and Schrecker, 1958).

Bauer, J., and Spiegel, E.: Über das Bilirubin im Blute und seine pharmakologische Beeinflussbarkeit. Dtsch. Arch. klin. Med. 129:17-40, 1919.

Bertalanffy, F. D.: Personal communication, 1965.

Boyd, L. J.: The pharmacology of the homeopathic drugs. J. Am. Inst. Homeop. 21:312-323, 1928.

Broomhead, F. A.: A study of the antimitotic effects of podophyllin on gastrointestinal cells in pregnant rats. B. Sc. Med. Thesis, The University of Manitoba, 1967.

Cahen, R. L.: Evaluation of the teratogenicity of drugs. Clin. Pharmacol. Therap. 5:480-514, 1964.

Case, J. T., and Powers, K. V.: Do resin cathartics cause gall-bladder contraction? Gastroenterology 6:182-184, 1946.

Chatard, H.: Traitement d'un epithelioma basocellulaire par applications locales de podophylline. Bull. Soc. franç. Derm. Syph. 64:324-326, 1957.

Clark, A. N. G., and Parsonage, M. J.: A case of podophyllum poisoning with involvement of the nervous system. Brit. med. J. 2:1155-1157, 1957.

Cornman, I.: Inhibition of Arbacia egg cleavage by podophyllotoxin and related substances. (Abstract.) Biol. Bull. 93:192, 1947.

Cornman, I.: The effects of podophyllin on the maturation and cleavage of the starfish egg. Acta Un. int. Cancr. 6:606-608, 1949.

Cornman, I., and Cornman, M. E.: The action of podophyllin and its fractions on marine eggs. Ann. N.Y. Acad. Sci. 51:1443-1481, 1951.

Courrier, R., and Marois, M.: Action de l'hypothermie expérimentale sur la gestation chez le rat. C.R. Soc. Biol. Paris, 147:1922-1924, 1953. (cited by Smith, 1957).

Courrier, R., and Marois, M.: Retard de la nidation et du developpement foetal chez la rate en hypothermie. Ann. Endocr. 15:738-745, 1954. (cited by Smith, 1957).

Cullis, J. E.: Congenital deformities and herbal "slimming tablets." Lancet 2:511-512, 1962.

Culp, O. S., and Kaplan, I. W.: Condylomata acuminata, two hundred cases treated with podophyllin. Ann. Surg. 120:251-256, 1944.

Dawson, A. B.: A note on the staining of cleared specimens with alizarin red S. Stain Technol. 1:123-124, 1926. (cited by Wright et al., 1958).

Didcock, K. A., Jackson, D., and Robson, J. M.: The action of some nucleotoxic substances on pregnancy. Brit. J. Pharmacol. 11:437-441, 1956.

Didcock, K. A., Picard, C. W., and Robson, J. M.: The action of podophyllotoxin on pregnancy. J. Physiol. (Lond.) 117:65-66, 1952.

DiPaolo, J. A.: Congenital malformation in strain A mice. J. Am. med. Ass. 183:139-141, 1963.

DiPaolo, J. A., Gatzek, H., and Pickren, J.: Malformations induced in the mouse by thalidomide. Anat. Rec. 149:149-156, 1964.

Disque, L.: The constitution and action of podophyllin. Sbr. Abh. naturf. Ges. Rostock. N.P. 5:1-35, 1913. (cited by Kelly and Hartwell, 1954).

Dixon, W. E.: Hypodermic purgatives. Brit. med. J. 2:1244-1247, 1902.

Dudley, W. H.: Fatal podophyllin poisoning. Med. Rec. (N.Y.) 37:409, 1890.

Dunstan, W. R., and Henry, T. A.: A chemical investigation of the constituents of Indian and American podophyllum (*Podophyllum emodi* and *Podophyllum peltatum*). J. chem. Soc. 73:209-226, 1898.

Dwornik, J. J., and Moore, K. L.: Skeletal malformations in the Holtzman rat embryo following the administration of thalidomide. J. Embryol. exp. Morph. 13:181-193, 1965.

Excerpta Medica Foundation: Side effects of drugs. 4th ed. Ed., L. Meyler. The Hague: Mouton and Company, 1964. p. 215.

Extra Phramacopoeia, The. (Martindale, W.). The Council of the Pharmaceutical Society of Great Britain. 22nd ed., Volume 1. London: The Pharmaceutical Press, 1941. pp. 858-859.

Extra Pharmacopoeia, The. (Martindale, W.). The Council of the Pharmaceutical Society of Great Britain. 23rd ed., Volume 1. London: The Pharmaceutical Press, 1952. pp. 677-678.

Eyestone, W. H.: The effects of beta peltatin and other podophyllin components on the tissues of laboratory animals. (Abstract.) Am. J. Path. 29:603, 1953.

Farris, E. J., and Griffith, J. Q., Jr., Ed.: The Rat in Laboratory Investigation. 2nd ed. New York: Hafner Publishing Company, 1962. pp. 55-58.

Firstater, M.: Podophyllin in the treatment of vesical papillomas. J. Urol. (Baltimore) 86:565-567, 1961.

Fraser, F. C.: Experimental teratogenesis in relation to congenital malformations in man. In: M. Fishbein, Ed.: Second International Conference on Congenital Malformations. New York: The International Medical Congress, Limited, 1964. pp. 277-287.

Freedberg, I. M.: Effects of podophyllin upon macromolecular metabolism. J. invest. Derm. 45:539-546, 1965.

Fullerton, P. M., and Kremer, M.: Neuropathy after intake of thalidomide (Distaval). Brit. med. J. 2: 855-858, 1961.

Gardner, H. L., and Kaufman, R. H.: Condylomata acuminata. Clin. Obstet. Gynec. 8:938-945, 1965.

Goldberg, E. D., and Black, H. M.: The effect of podophyllin on regeneration in planaria. (Abstract.) Anat. Rec. 101:708-709, 1948.

Gorthey, R. L., and Krembs, M. A.: Vulvar condylomata acuminata complicating labor. Obstet. Gynec., N.Y., 4:67-74, 1954.

Grainger, T. H., Jr.: The effect of podophyllin on *Eberthella typhosa*. J. Bact. 54:759-760, 1947.

Greenspan, E. M., and Leiter, J.: Toxicity and hematologic changes produced by alpha-peltatin, beta-peltatin and podophyllotoxin. (Abstract.) Cancer Res. 9:626, 1949.

Greiner, G. F., Klotz, G., and Lang, G.: Résultats du traitement des papillomes de larynx chez l'enfant par le triéthylène-glycol associé à la résine de podophylle. Ann. Oto-laryng. (Paris) 76:484-488, 1959.

Gruber, C. M., Richardson, L. K., and Bryan, W. T. K.: The intact intestine in nonanaesthetized dogs as influenced by colocynth and podophyllin. J. Pharmacol. exp. Ther. 45:77-84, 1932.

Gurr, E.: Staining Animal Tissues. Practical and Theoretical. London: Leonard Hill (Books) Limited, 1962. pp. 125-126.

Hartwell, J. L.: α-peltatin, a new compound isolated from *Podophyllum peltatum*. J. Amer. chem. Soc. 69:2918, 1947.

Hartwell, J. L., and Detty, W. E.: β-peltatin, a new component of podophyllin. J. Amer. chem. Soc. 70:2833, 1948.

Hartwell, J. L., and Detty, W. E.: Isolation, properties and chemistry of alpha and beta-peltatin. (Abstract.) Cancer Res. 9:597, 1949.

Hartwell, J. L., and Schrecker, A. W.: The chemistry of podophyllum. Fortschr. Chem. Org. Naturst. 15:83-166, 1958.

Hazelton, L. W.: The influence of aloe and podophyllum on the flow of hepatic bile in the dog. J. Amer. pharm. Ass., sci. Ed. 31:53-56, 1942.

Hicks, S. P., and D'Amato, C. J.: Effects of ionizing radiations on mammalian development. In: D. H. M. Woolam, Ed.: Advances in Teratology. Volume One. Dublin: Hely Thom Limited, 1966. pp. 195-200.

Ingalls, T. H., Curley, F. J., and Prindle, R. A.: Experimental production of congenital anomalies. Timing and degree of anoxia as factors causing fetal deaths and congenital anomalies in the mouse. New Engl. J. Med. 246:758-768, 1952.

Ivy, A. C., Annegers, J. H., and Atkinson, A. J.: The effect of aloes and podophyllum and Carter's Little Liver Pills on bile formation. Quart. Bull. Northw. Univ. med. Sch. 16:197-202, 1942.

Ivy, A. C., DeHoog, H., and Gutmann, M. S.: The effect of aloes and podophyllum (resin) (Carter's Little Liver Pills) on the output of bilirubin and cholic acid in canine duodenal drainage fluid. Quart. Bull. Northw. Univ. med. Sch. 19:102-104, 1945.

Ivy, A. C., Roback, R. A., and Stein, J. F., Jr.: Do the ingredients of Carter's Little Liver Pills cause the gall bladder to contract, and stimulate the flow of bile by the liver? Quart. Bull. Northw. Univ. med. Sch. 16:298-301, 1942.

Ivy, A. C., Roth, J. A., and Gutmann, M.: Do aloes and podophyllum (Carter's Little Liver Pills) increase the flow of bile into the duodenum? Gastroenterology 5:27-33, 1945.

Jakobi, H.: Podophyllinbehandlung der Larynxpapillome. H.N.O. (Berl.) 5:361-362, 1956.

Kaplan, I. W.: Condylomata acuminata. New Orleans med. surg. J. 94:388-390, 1942.

Karnofsky, D. A.: Drugs as teratogens in animals and man. Ann. Rev. Pharmacol. 5:447-472, 1965.

Keim, H. L.: Abstract of discussion. In: Sullivan, M., and King, L. S.: Effects of resin of podophyllum on normal skin, condylomata acuminata and verrucae vulgares. Arch Derm. Syph. (Chic.) 56: 30-47, 1947.

Kelly, M. G., and Hartwell, J. L.: The biological effects and the chemical composition of podophyllin. A review. J. nat. Cancer Inst. 14:967-1010, 1954.

Kelly, M. G., Leiter, J., Bourke, A. R., and Smith, P. K.: Fate and distribution of podophyllotoxin in animals. (Abstract.) Cancer Res. 11:263, 1951a.

Kelly, M. G., Leiter, J., Gosh, O., and Smith, P. K.: Role of the adrenal glands in the hematopoietic and thymicolymphatic response of the rat to podophyllotoxin. J. nat. Cancer Inst. 12:1177-1201, 1952.

Kelly, M. G., Leiter, J., MacCardle, R. C., and Smith, P. K.: Effect of podophyllotoxin on hematopoietic system of adrenalectomized rats. (Abstract.) Cancer Res. 11:263-264, 1951 b.

Kelly, M. G., Ligon, E. W., Jr., Davison, C., and Smith, P. K.: A comparison of the toxicity and pharmacological actions of podophyllotoxin and picropodophyllin. (Abstract.) Cancer Res. 9:555-556, 1949.

Kelly, M. G., Truant, A. P., and Smith, P. K.: Effects of podophyllotoxin and picropodophyllin on intact intestine and salivary secretion of non-anesthetized dogs. (Abstract.) Fed. Proc. 8:306-307, 1949.

King, L. S., and Sullivan, M.: The similarity of the effect of podophyllin and colchicine and their use in treatment of condylomata acuminata. Science 104:244-245, 1946.

King, L. S., and Sullivan, M.: Effects of podophyllin and of colchicine on normal skin, on condyloma acuminatum and on verruca vulgaris. Arch. Path. 43:374-386, 1947.

Klein Obbink, H. J., and Dalderup, L. M.: Effects of thalidomide in the rat foetus. Experientia (Basel) 19:645-646, 1963.

Kocsis, J. J., Walaszek, E. J., and Geiling, E. M. K.: Disposition of biosynthetically labelled C-14 podophyllotoxin in normal and tumor-bearing animals. Arch. int. Pharmacodyn. 111:134-144, 1957.

Kofod, H., and Jørgensen, C.: Dehydropophyllotoxin, a new compound isolated from *Podophyllum peltatum* L. Acta chem. scand. 8:1296-1297, 1954.

Kofod, H., and Jørgensen, C.: Desoxypodophyllotoxin, isolated from podophyllin. Acta chem. scand. 9:346-347, 1955.

Kozakiewicz, J.: Leczenie nabłoniaków skóry podofiliną w świetle własnych postrzeżeń. Przegl. derm. Warsz. 8:53-63, 1958.

Lane, C. W.: Abstract of discussion. In: Sullivan, M., and King, L. S.: Effects of resin of podophyllum on normal skin, condylomata acuminata and verrucae vulgares. Arch. Derm. Syph. (Chic.) 56:30-47, 1947.

Leiter, J., Paradis, A. D., and Waravdekar, V. S.: Enzyme changes induced in normal and malignant tissues with chemical agents. II. Effect of various compounds on the cytochrome oxidase activity of transplanted tumors. J. nat. Cancer Inst. 14:177-188, 1953.

Lenz, W., and Knapp, K.: Thalidomide embryopathy. Arch. Environ. Health (Chicago) 5:100-105, 1962.

Liška, J.: Lechenie kozhnoga raka preparatom podofillin. Čs. Onkol. 3:61-69, 1956.

Long, J. A., and Burlingame, P. L.: The development of the external form of the rat, with observations on the origin of the extraembryonic coelom and fetal membranes. Univ. California Pub. Zoöl. 43:143-183, 1938-1944.

Long, J. A., and Evans, H. M.: The oestrus cycle in the rat and its associated phenomena. Memoirs University of California, 6:1922. (cited by Farris and Griffith, 1962).

MacCardle, R. C., and Perrault, A.: Degeneration of cerebellar Purkinje cells in tumor-bearing animals after subcutaneous injection of podophyllin. Acta Un. int. Cancr. 6:539-541, 1949.

MacFarlane, W. V., Pennycuik, P. R., and Thrift, E.: Resorption and loss of foetuses in rats living at 35°C. J. Physiol (Lond.) 135:451-459, 1957.

MacFarlane, W. V., Pennycuik, P. R., Yeates, N. T. M., and Thrift, E.: Reproduction in hot environments. Proceedings of the conference held in Syracuse, New York, June 9-12, 1958. Quoted by Lloyd, C. W.: Recent Progress in the Endocrinology of Reproduction. New York: Academic Press, 1959. pp. 81-96.

McColl, J. D., Globus, M., and Robinson, S.: Drug induced skeletal malformations in the rat. Experientia (Basel) 19:183-184, 1963.

Medical Letter on Drugs and Therapeutics, The. (Drugs and Therapeutics). New York. Vol. 4, no. 26, issue 103, Dec. 21, 1962. p. 106.

Miller, C. H.: Demonstration of the cartilagenous skeleton in mammalian fetuses. Anat. Rec. 20:415-419, 1921. (cited by Wright et al., 1958).

Miller, Z. B., Davison, C., and Smith, P. K.: The effect of podophyllotoxin on tissue metabolism and enzyme systems. J. exp. Med. 90:525-541, 1949.

Murphy, M. L.: Teratogenic effects of tumour-inhibiting chemicals in the foetal rat. In: G. E. W. Wolstenholme and C. M. O'Connor, Ed.: Ciba Foundation Symposium on Congenital Malformations. London: J. & A. Churchill Ltd., 1960. pp. 78-107.

Murphy, M. L.: Factors influencing teratogenic response to drugs. In: J. G. Wilson and J. Warkany, Ed.: Teratology Principles and Techniques. Chicago: The University of Chicago Press, 1965. pp. 145-184.

Monash, S.: Fungicidal activity *in vitro* of podophyllotoxin. Arch. Derm. Syph. (Chic.) 65:610, 1952.

Neuberger, J.: Ueber die Wirkungen des krystallisirten Podophyllotoxins. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 28:32-40, 1891.

Ogle, C.: Adaptation of sexual activity to environmental stimulation. Amer. J. Physiol. 107:628-634, 1934.

Padawer, J., and Gordon, A. S.: Effects of colchicine and podophyllotoxin on mast cells. (Abstract.) Anat. Rec. 124:427, 1956.

Petrovsky, G. A., and Pavlenko, D. S.: Effect of drugs on bile secretion. Bull. biol. méd. expér. 7:49-51, 1939.

Philips, F. S., Chenoweth, M. B., and Hunt, C. C.: Studies on the toxicology of podophyllotoxin and related substances. (Abstract.) Fed. Proc. 7:249, 1948.

Pincus, G.: The eggs of mammals. Exp. Biol. Monographs. Macmillan, New York, 1936. (cited by Farris and Griffith, 1962).

Pitini, A., and Fernandez, G.: Influenza di alcune sostance sulla secrezione della bile. Arch. internat. pharmacodyn. 24:135-151, 1914-1918. (cited by Kelly and Hartwell, 1954).

Podwissotzki, V., Pharm. J., III, 12, 217, 1011 (1881-1882); Arch. Pharm. Exper. Pathol., 13, 29 (1880); Pharm. Z. Ruszland, 12, 44 (1881); Ber., 15, 377 (1882). (cited by Viehoever and Mack, 1938).

Podwissotzki, V.: Pharmakologische Studien über Podophyllum peltatum. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 13:29-52, 1881.

Reiss, F., and Doherty, D. D.: Fungous infections of the skin and scalp: a new approach to their treatment. N. Y. St. J. Med. 49:1939-1943, 1949.

Reiss, F., and Doherty, D. D.: Podophyllum resin in treatment of tinea capitis. J. Amer. med. Ass. 147:225-226, 1951.

Remington's Practice of Pharmacy. Ed., Martin, E. W., and Cook, E. F., 11th ed. Easton: The Mack Publishing Company, 1958. pp. 1015-1016.

Richardson, E. J.: The use of podophyllin in the treatment of papillomatosis of the bladder. J. Urol. (Baltimore) 82:234-235, 1959.

Riddell, D.: Evaluation of colchicine as a possible teratogen in rats. B. Sc. Med. Thesis, The University of Manitoba, 1967.

Robbins, M. L., Bourke, A. R., and Smith, P. K.: The effect of certain chemicals on *Rickettsia typhi* infections in chick embryos. J. Immunol. 64:431-446, 1950.

Robson, J. M., Sullivan, F. M., and Smith, R. L., Ed.: Embryopathic Activity of Drugs. Boston: Little, Brown and Company, 1965. pp. 1-151.

Rosner, R. S.: Corneal insult from podophyllin. Amer. J. Ophthal. 29: 1448-1450, 1946.

Runner, M. N.: Inheritance of susceptibility to congenital deformity. Metabolic clues provided by experiments with teratogenic agents. Pediatrics 23:245-251, 1959.

Runner, M. N.: General mechanisms of teratogenesis. In: J. G. Wilson and J. Warkany, Ed.: Teratology Principles and Techniques. Chicago: The University of Chicago Press, 1965. pp. 95-103.

Runner, M. N., and Dagg, C. P.: Metabolic mechanisms of teratogenic agents during morphogenesis. National Cancer Institute Monograph No. 2, 1960. pp. 41-54.

Sánchez Cabellero, H. J., and Ergueta Collao, J.: Action of podophyllin on the number of blood leucocytes. Experientia (Basel) 6:250-251, 1949.

Schubert, M.: Screening of drugs in experimental schistosomiasis mansoni in mice. Amer. J. trop. Med. 28:137-156, 1948a.

Schubert, M.: Effect of drugs during earliest stages of experimental schistosomiasis mansoni in mice. Amer. J. trop. Med. 28:157-162, 1948b.

Sentein, P.: Sur les déviations de l'axe mitotique au cours de la segmentation de l'oeuf traité par la colchicine et leur signification. C. R. Soc. Biol. (Paris) 145:97-99, 1951a.

Sentein, P.: La podophylline, inhibiteur du clivage et de la polarité fusoriale dans l'oeuf de triton. C. R. Soc. Biol. (Paris) 145:667-668, 1951b.

Smith, A. U.: The effects on foetal development of freezing pregnant hamsters (*Mesocricetus auratus*). J. Embryol. exp. Morph. 5:311-323, 1957.

Smithells, R. W.: Drugs and human malformations. In: D. H. M. Woollam, Ed.: Advances in Teratology. Volume One. Dublin: Hely Thom Limited, 1966. pp. 251-278,

Somers, G. F.: Thalidomide and abnormalities of development. Orv. hetil. 103:2456-2459, 1962.

Spendlove, R. S., Lennette, E. H., Chin, J. N., and Knight, C. O.: Effect of antimitotic agents on intracellular reovirus antigen. Cancer Res. 24:1826-1833, 1964.

Steel, R. G. D., and Torrie, J. H.: Principles and Procedures of Statistics with Special Reference to the Biological Sciences. New York: McGraw-Hill Book Company, Inc., 1960. pp. 213-229 (a).

Steel, R. G. D., and Torrie, J. H.: Principles and Procedures of Statistics with Special Reference to the Biological Sciences. New York: McGraw-Hill Book Company, Inc., 1960. pp. 156-159 (b).

Steinmetzer, K.: Experimentelle Untersuchungen über Chologoga. Wien. klin. Wschr. 39:1418-1422, 1926. (cited by Kelly and Hartwell, 1954).

Stewart, W. H., and Ryan, E. J.: Cholecystographic studies after the administration of some of the popular cholagogues. Amer. J. Roentgenol. 19:341-343, 1928.

Sullivan, B. J., and Wechsler, H. I.: The cytological effects of podophyllin. Science 105:433, 1947.

Sullivan, M.: Podophyllotoxin. Arch. Derm. Syph. (Chic.) 60:1-13, 1949.

Sullivan, M., Follis, R. H., Jr., and Hilgartner, M.: Toxicology of podophyllin. Proc. Soc. exp. Biol. (N.Y.) 77:269-272, 1951.

Sullivan, M., and King, L. S.: Effects of resin of podophyllum on normal skin, condylomata acuminata and verrucae vulgares. Arch. Derm. Syph. (Chic.) 56:30-47, 1947.

Sullivan, T. D.: Somatic chromosomes of pedigreed hybrid petunia. Bull. Torrey Botanical Club 74:453-475, 1947. (cited by Kelly and Hartwell, 1954).

Sundstroem, E. S.: The physiological effects of tropical climate. Physiol. Rev. 7:320-362, 1927.

Taussig, H. B.: A study of the German outbreak of phocomelia. J. Am. med. Ass. 180:1106-1114, 1962.

Taylor, J. D.: Unpublished data. In: Kelly, M. G., and Hartwell, J. L.: The biological effects and the chemical composition of podophyllin. A review. J. nat. Cancer Inst. 14:967-1010, 1954.

Thiersch, J. B.: Effect of podophyllin (P) and podophyllotoxine (PT) on the rat litter *in utero*. Proc. Soc. exp. Biol. (N.Y.) 113:124-127, 1963.

Tomin, R.: [Favourable response of advanced ovarian cancer to treatment with a podophyllin derivative (SP-1 and SP-G).] Srpski Arkh. tselok. Lek. 93:973-979, 1965.

Vidovic, V.: Kälte-und Hypoxie Toleranz von Ratten Embryonen. Experientia (Basel) 8:304-306, 1952. (cited by Smith, 1957).

Vidovic, V.: Effect of hypothermia on the oestrus cycle, the mature egg, pregnancy and lactation of the white rat. Thesis: University of Belgrade, 1956. (cited by Smith, 1957).

Viehoever, A., and Mack, H.: Biochemistry of May apple root (*Podophyllum peltatum*) I. J. Amer. pharm. Ass., sci. Ed. 27:632-643, 1938.

Walker, D. O., and Wirstschafter, Z. T.: The Genesis of the Rat Skeleton. Springfield, Illinois: C. C. Thomas, 1957.

Waravdekar, V. S., Dominque, A., and Leiter, J.: Effect of podophyllotoxin, alpha-peltatin and beta-peltatin on the cytochrome oxidase activity of sarcoma 37. J. nat. Cancer Inst. 13:393-407, 1952.

Waravdekar, V. S., and Leiter, J.: Effect of peltatins on cytochrome oxidase activity of mouse tumors and organs. (Abstract.) Cancer Res. 9:625, 1949.

Ward, J. W., Clifford, W. S., Monaco, A. R., and Bickerstaff, H. J.: Fatal systemic poisoning following podophyllin treatment of condyloma acuminatum. Sth. med. J. (Bgham, Ala.) 47:1204-1206, 1954.

Waterman, N.: On some chemotherapeutic experiments with podophyllotoxin. Acta physiol. pharmacol. neerl. 1:638-644, 1950.

Wiesner, B. P., and Yudkin, J.: Control of fertility by antimitotic agents. Nature (Lond.) 176:249-250, 1955.

Wilson, J. G.: Influence on the offspring of altered physiologic states during pregnancy in the rat. Ann. N. Y. Acad. Sci. 57:517-525, 1954.

Wilson, J. G., and Warkany, J., Ed.: Teratology Principles and Techniques. Chicago: The University of Chicago Press, 1965. pp. 267-277.

Wright, H. V., Asling, C. W., Dougherty, H. L., Nelson, M. M., and Evans, H. M.: Prenatal development of the skeleton in Long-Evans rats. Anat. Rec. 130:659-672, 1958.

Young, C. J.: Podophyllotoxin treatment of *Microsporum audouini* scalp infections. Arch. Derm. Syph. (Chic.) 64:607-610, 1951.

Zakon, S. J.: Discovery of podophyllum resin. Arch. Derm. Syph. (Chic.) 65:620-622, 1952.

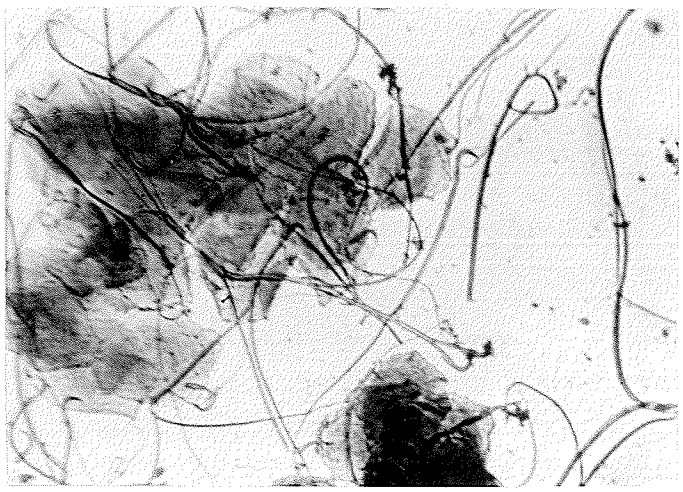
FIGURES

Figures 1 and 2

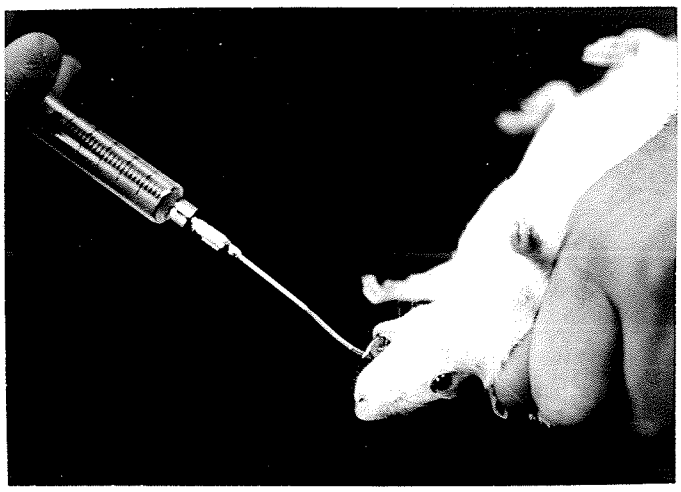
* Unless otherwise indicated, all Figures are of twenty-one-day-old fetuses. Skeletal structures are demonstrated by the alizarin technique.

1. Vaginal smear from a rat following mating. Note epithelial cells and spermatozoa (1200X).

2. The stomach tube apparatus and method of holding the animal.



1

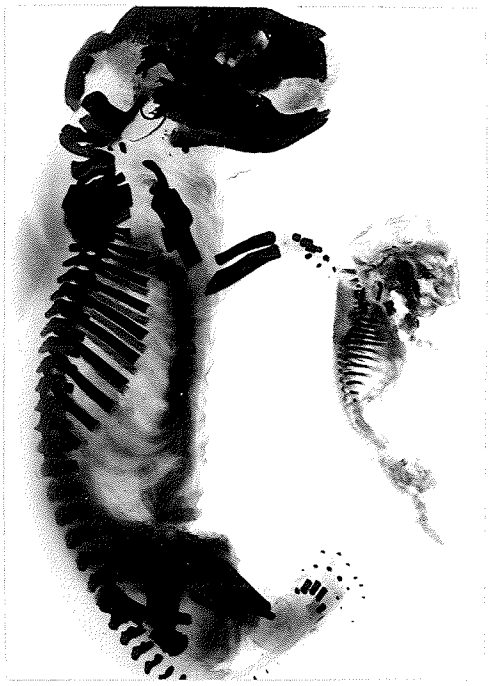


2

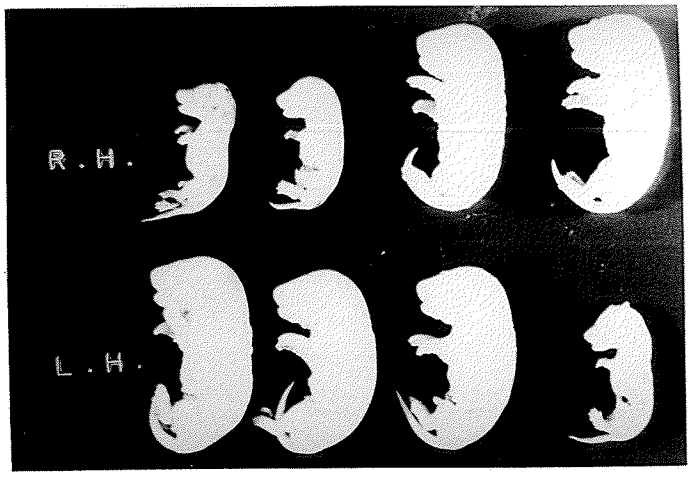
Figures 3 and 4

3. Normal twenty-one-day-old fetus on the left compared with a stunted fetus of the same gestational age on the right.

4. A litter from a mother who received 0.1 milligram of podophyllin (in water) per 100 grams body weight each day from the tenth to twelfth days of gestation. Note the two runts from the right uterine horn and from the left uterine horn.



3



4

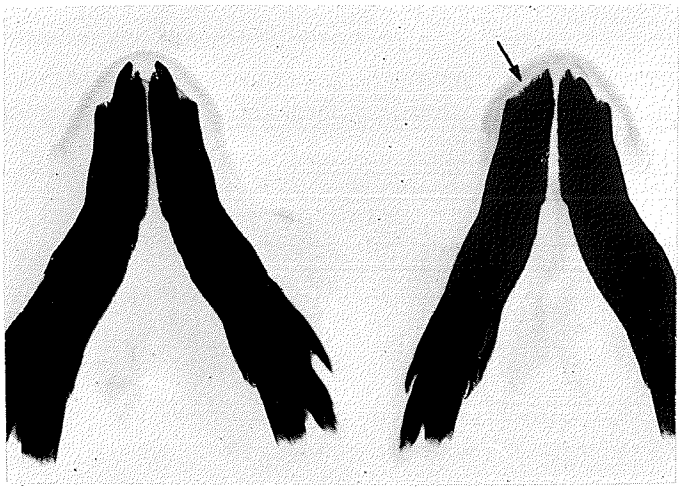
Figures 5, 6, and 7

5. Note even length of the maxillary incisors on the left. The arrow indicates a shortened incisor on the right.

6. Note even length of the mandibular incisors on the left. The arrow shows a shortened incisor on the right.

7. On the left, a well ossified body of the hyoid bone; on the right a poorly ossified body.

5



6

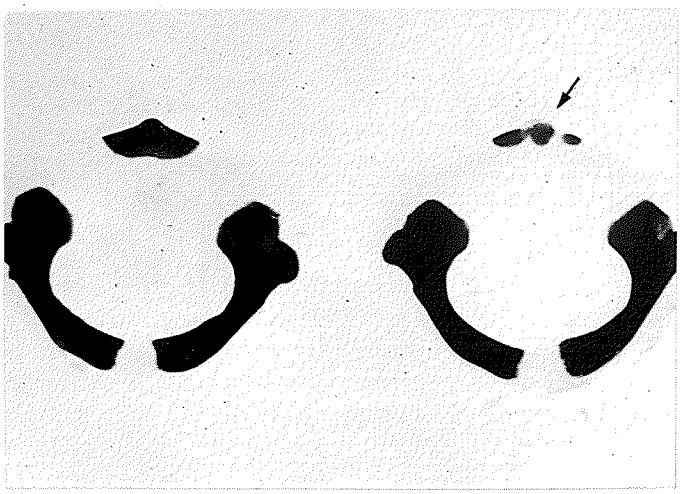
7

Figures 8, 9, and 10

8. On the left, a well ossified anterior arch of the atlas. The arrow on the right, shows an unevenly ossified anterior arch.

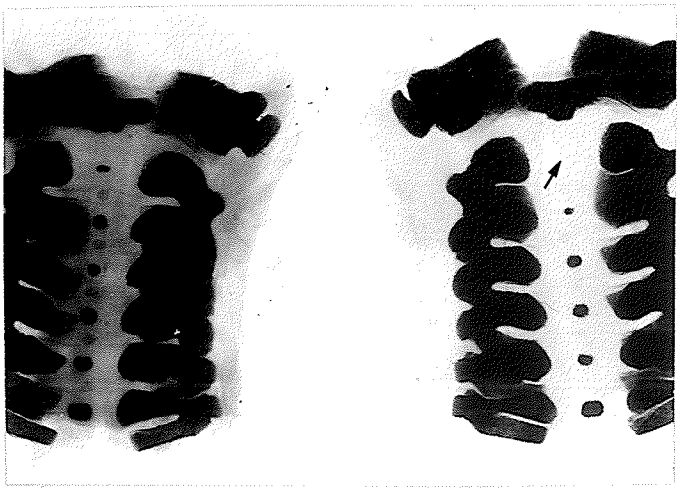
9. An atlas with a well ossified anterior arch. The arrow indicates the area where the anterior arch failed to ossify.

10. All the cervical centra are present on the left. On the right, the arrow shows the region of an absent centrum for the axis vertebra.



8

9

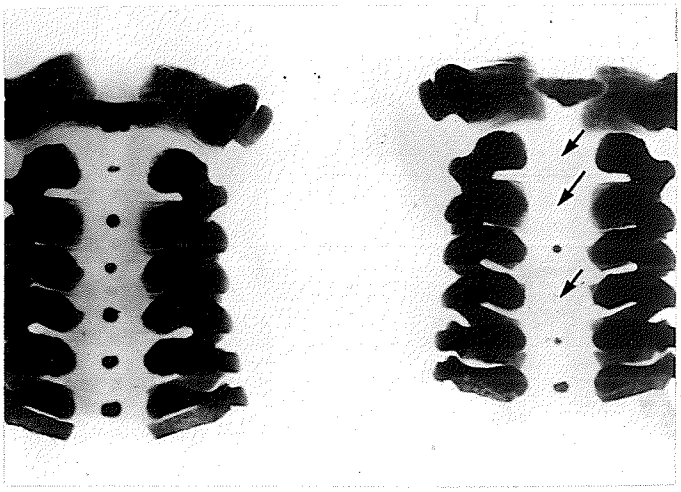


10

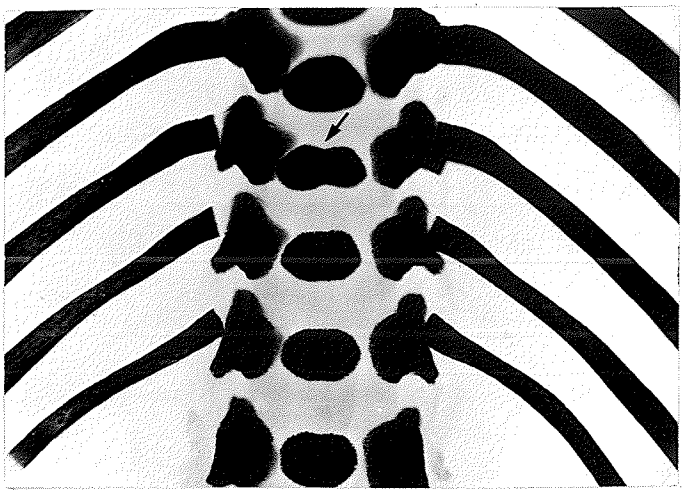
Figures 11 and 12

11. All the cervical centra are present on the left. On the right, the arrows indicate areas where cervical centra are absent in nonconsecutive order.

12. A slightly dumbbell vertebral centrum in the thoracic region. Note the concave central area of this centrum superiorly and inferiorly.



11

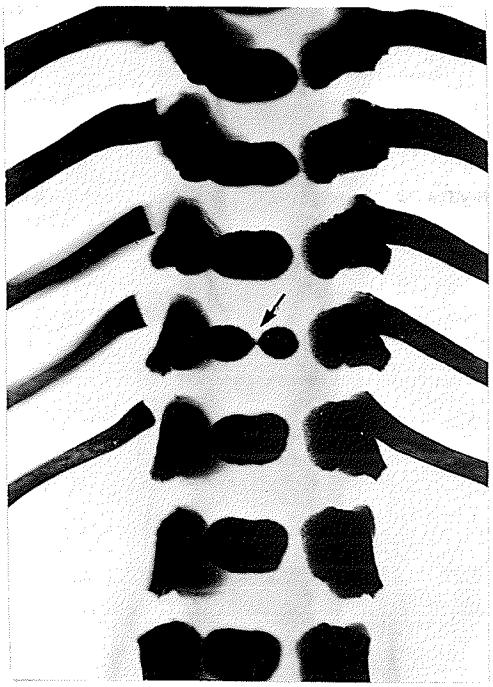


12

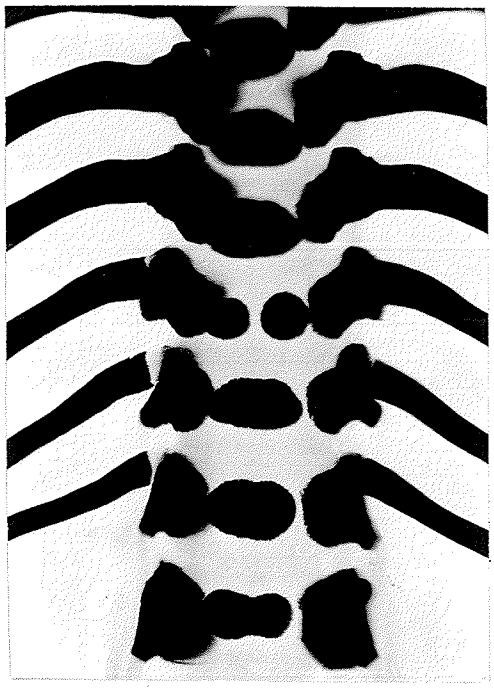
Figures 13 and 14

13. The arrow shows a dumbbell vertebral centrum in the thoracic region.

14. A duplicated vertebral centrum in the thoracic region.



13

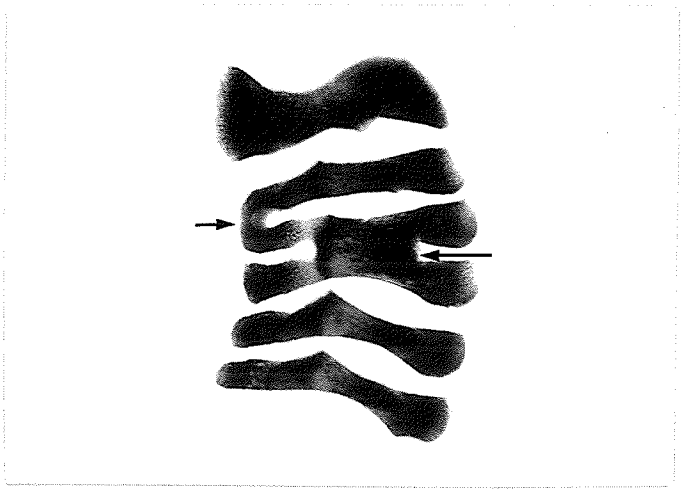


14

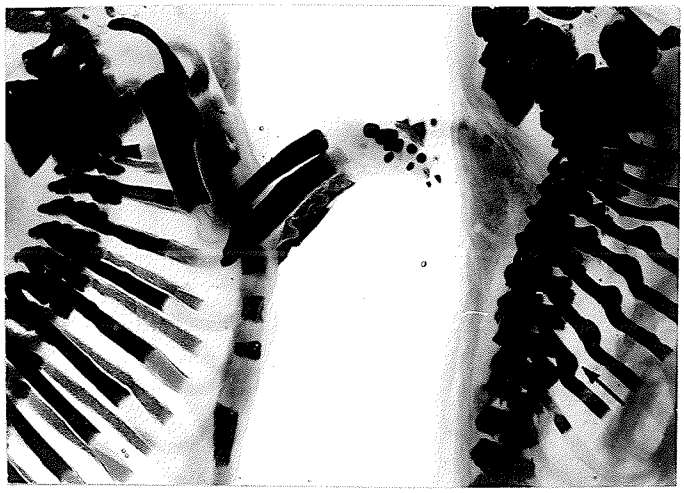
Figures 15 and 16

15. The arrows show fusion of adjacent vertebral arches in the cervical region. This also occurred in the lumbar region.

16. Smoothly curved ribs are observed on the left. The arrow on the right indicates a prominent protruberance in the middle area of each rib.



15

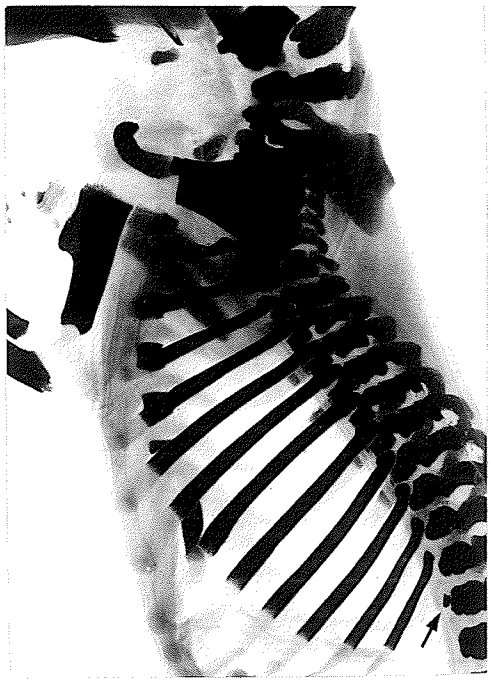


16

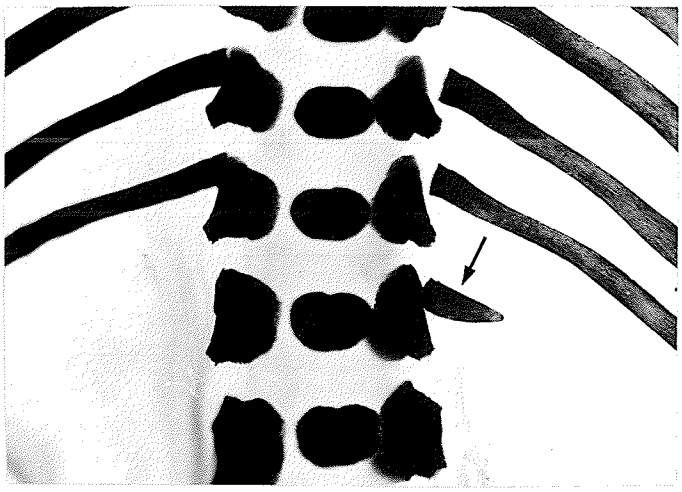
Figures 17 and 18

17. The thirteenth rib (indicated by the arrow) is retarded.

18. The arrow shows a unilateral supernumerary rib in the first lumbar position. Some of these ribs are smaller as shown in Figure 19.



17

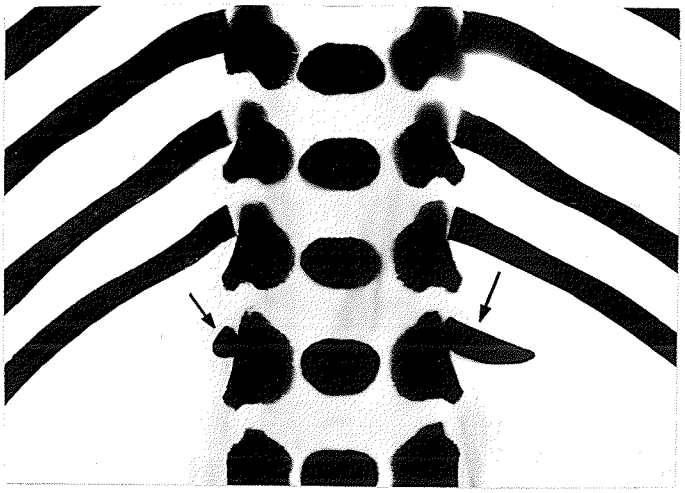


18

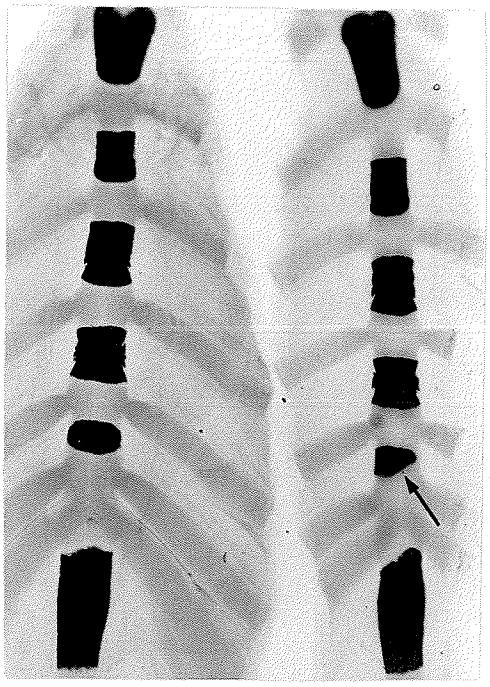
Figures 19 and 20

19. The arrows indicates a pair of supernumerary ribs. Note the variation in size.

20. The normal appearing sternum on the left has six ossification centres. On the right, note that the fifth sternal centre (shown by the arrow), is slightly retarded.



19

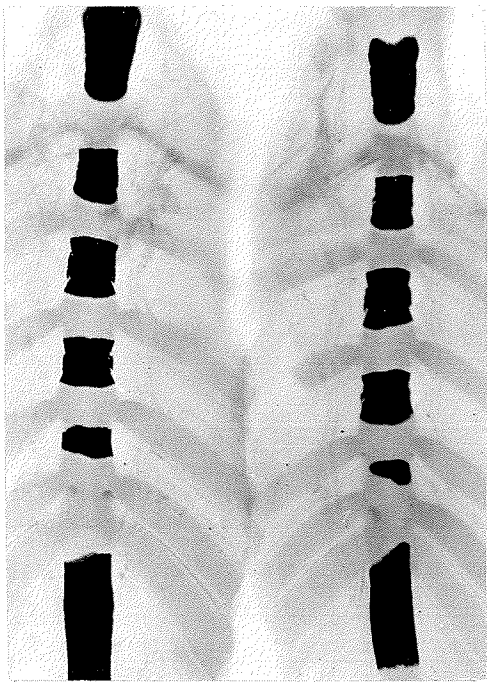


20

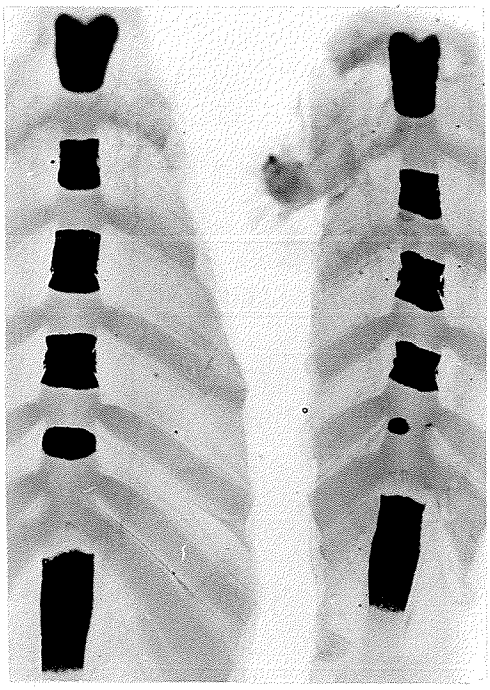
Figures 21 and 22

21. The normal sternum is on the left. Retarded ossification of the fifth sternebra is noted on the right.

22. The sternum on the left is normal. On the right, the equivalent fifth sternal centre is poorly ossified.



21

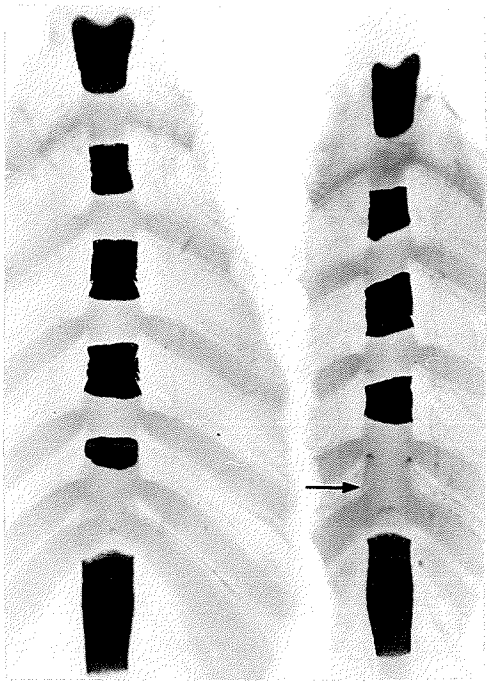


22

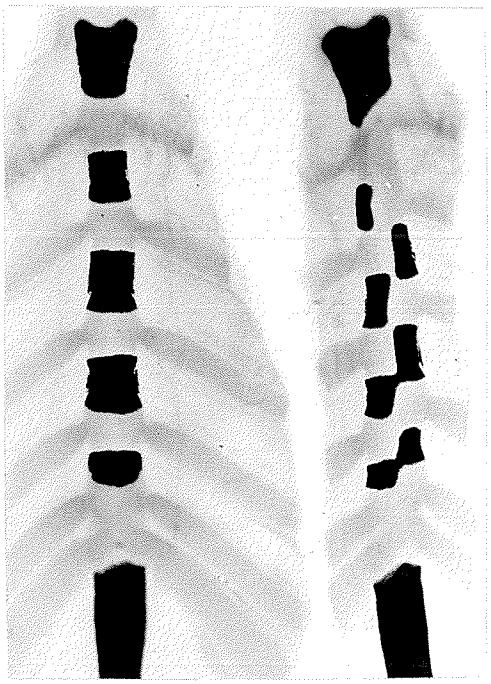
Figures 23 and 24

23. The normal sternum is on the left. Note the sternum on the right; there is no ossification in the area (indicated) where the fifth sternal centre should be present.

24. The sternum on the left is normal. On the right, observe the poorly ossified second, third, fourth, and fifth sternebrae.



23

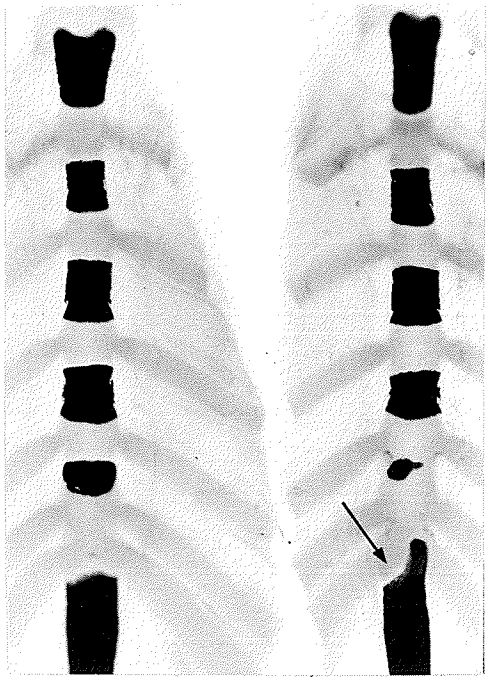


24

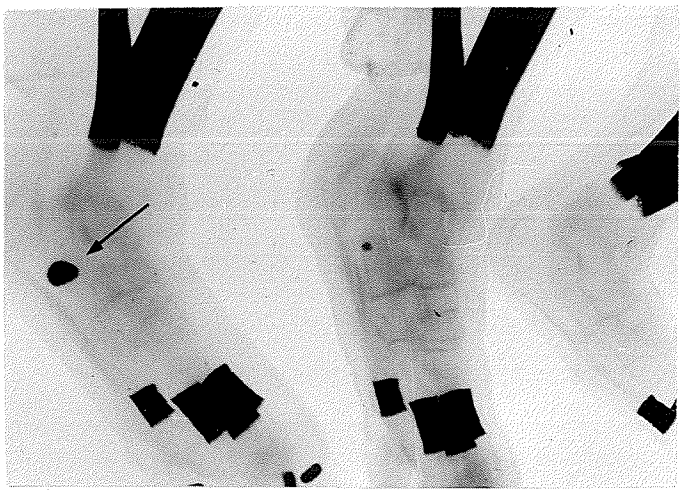
Figures 25 and 26

25. Normal sternum on the left. The arrow indicates retarded ossification in the sixth sternal centre.

26. The arrow shows an ossification centre in the calcaneus. In the middle, a small ossification centre is still present while on the right there is no ossification in the calcaneus.



25



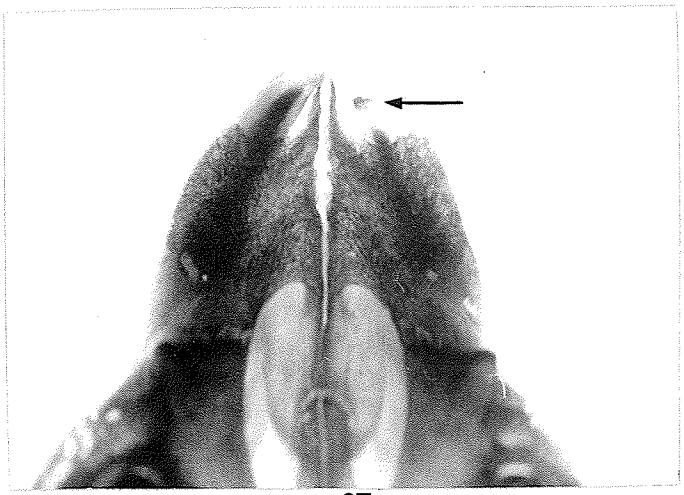
26

Figures 27, 28, and 29

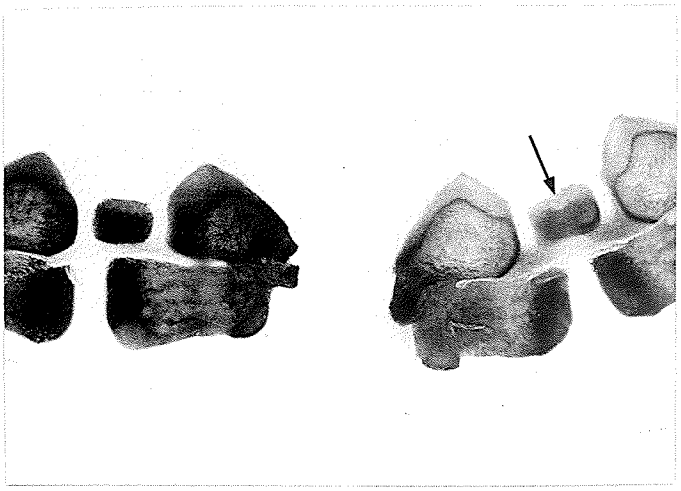
27. The arrow indicates a shortened left maxillary incisor in a ten-day-old newborn rat.

28. On the left, a typical cervical vertebra from a ten-day-old newborn animal. The arrow on the right indicates a slightly dumbbell vertebral centrum.

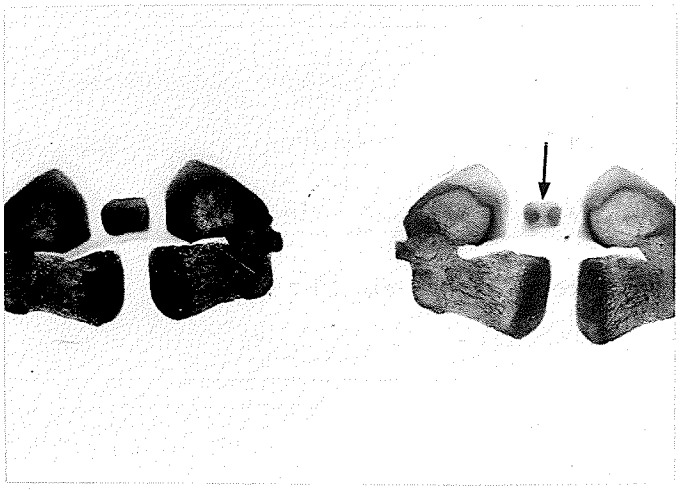
29. The cervical centrum on the left is normal. The arrow shows a dumbbell cervical centrum in a ten-day-old animal. This centrum and others like it, were shaped almost like a horse-shoe. Observe the two darker areas on both sides of the point.



27



28

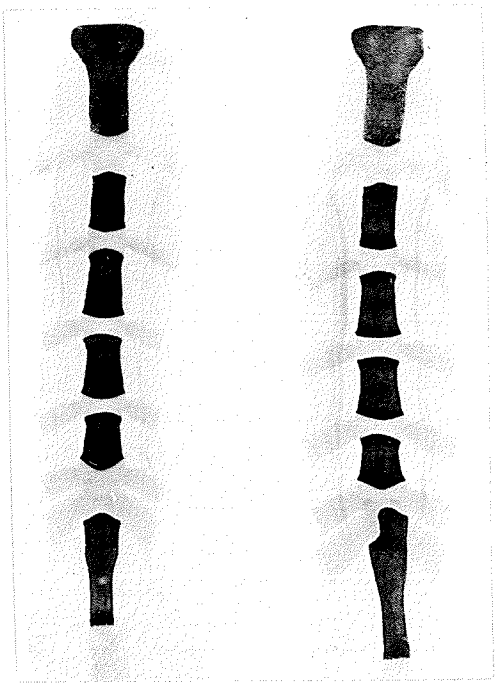


29

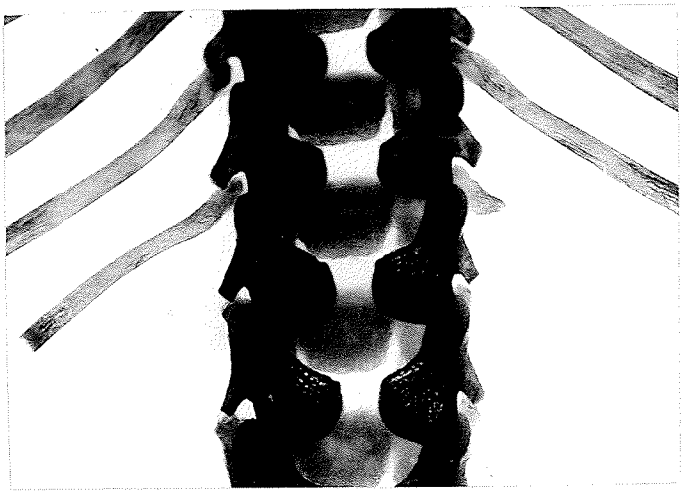
Figures 30 and 31

30. A normal sternum from a ten-day-old rat on the left. Note retarded ossification in the sixth sternebra on the right.

31. A ten-day-old rat with a stunted thirteenth rib on the right side. The rib on the left side is normal.



30

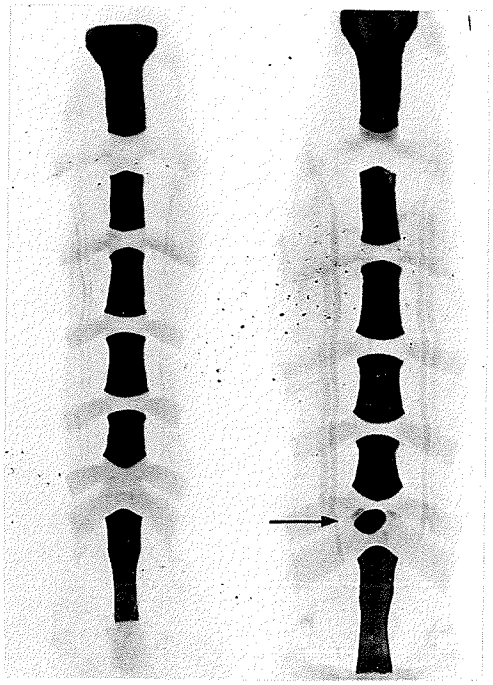


31

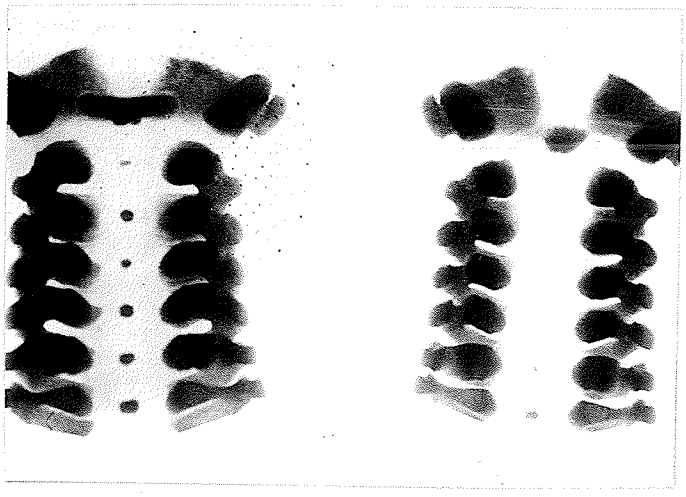
Figures 32 and 33

32. On the right, note the presence of an ossification centre between the fifth and sixth sternal centres (shown by the arrow) in a ten-day-old newborn rat.

33. Normal cervical vertebrae on the left. On the right, observe the absence of four cervical centra in consecutive order.



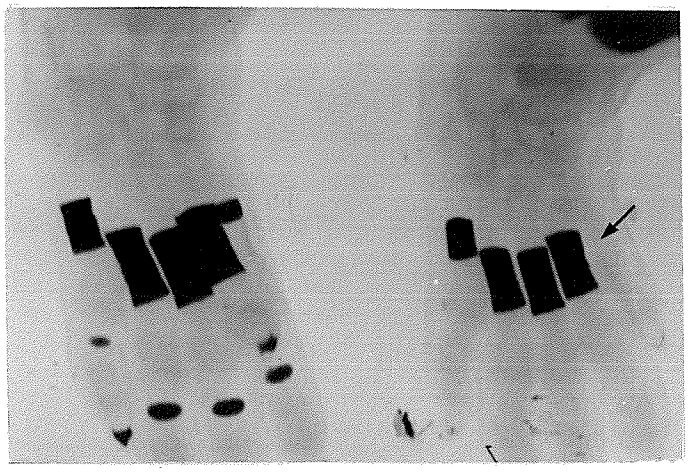
32



33

Figure 34

34. Note the presence of five metatarsals on the left. The foot on the right does not have an ossification centre in the medial metatarsal area (shown by the arrow).



34

ADDENDUM

This addendum is being written because it has been brought to my attention, since the writing of this thesis, that most of the unusual results and subsequent conclusions made after statistically analyzing the data in the podophyllin study, are invalid and unwarranted even though the statistical method was appropriate.[1] It has also been pointed out to me that the unusual U-shaped dose-response curves may be reconciled when one looks at the times (Table I, Addendum) during which the various groups of animals were studied over the two years of this investigation.[1] The inappropriate conclusions in this part of the study are because all animals in the groups given podophyllin (in either 10 per cent ethanol or water) on the eighth to twelfth days of gestation and the proper control groups, were not mated, treated and killed at the same approximate time (month); this is also applicable to groups of animals and the controls given the drug and/or suspension medium on days ten to twelve and day ten only of pregnancy.[2]

Because time on a seasonal or long term basis was not considered in the experimental design, it was initially suggested that I should attempt to salvage at least some of the data by re-analyzing those groups that were done at approximately the same time (Table II, Addendum) and amenable to statistical analysis.[1] However, after considerable deliberation with Dr. LaBella, and also Mr. Vivian, it was indicated that it

---

[1]Dr. Frank S. LaBella, in the Department of Pharmacology at the University of Manitoba, kindly directed my attention to these facts and made suggestions.

[2]The reasons this was not done are discussed on page 226.

Table I

Time Period Required to Mate, Treat, and Kill the Ten Animals in Each Group

Group Number*	Breeding Date of First Animal in Group	Killing Date of Last Animal in Group
I	May 21, 1966	July 28, 1966
II	May 22, 1966	July 28, 1966
III	Dec. 21, 1965	Jan. 27, 1966
IV	Dec. 21, 1965	Feb. 21, 1966
V	Jan. 31, 1966	July 18, 1966
VI	May 21, 1966	June 30, 1966
VII	May 20, 1966	July 27, 1966
VIII	Feb. 24, 1966	June 28, 1966
IX	Sept. 22, 1965	Oct. 19, 1965
X	Sept. 22, 1965	Oct. 17, 1965
XI	Sept. 22, 1965	Oct. 20, 1965
XII	May 23, 1966	July 26, 1966
XIII	Oct. 5, 1966	Nov. 17, 1966
XIV	Oct. 5, 1966	Nov. 17, 1966
XV	Oct. 5, 1966	Nov. 30, 1966
XVI	Dec. 1, 1966	Dec. 23, 1966
XVII	Mar. 29, 1967	May 31, 1967
XVIII	Feb. 13, 1967	Mar. 29, 1967
XIX	Mar. 28, 1967	June 13, 1967
XX	May 22, 1967	July 29, 1967
XXI	May 16, 1967	June 20, 1967
XXII	Jan. 3, 1966	Feb. 22, 1966
XXIII	Dec. 21, 1965	Feb. 22, 1966
XXIV	Oct. 5, 1966	Nov. 15, 1966
XXV	Mar. 28, 1967	June 13, 1967
XXVI	Feb. 13, 1967	Mar. 29, 1967
XXVII	Mar. 29, 1967	June 12, 1967
XXVIII	Dec. 28, 1965	Jan. 28, 1966
XXIX	Dec. 28, 1965	Feb. 22, 1966
XXX	Oct. 5, 1966	Nov. 17, 1966
XXXI	Dec. 21, 1965	Mar. 25, 1966
XXXII	June 28, 1967	July 31, 1967
XXXIII	Mar. 15, 1967	June 11, 1967

Explanation of Table I.

*For an account of the treatments administered to animals in each group refer to Table I, page 38.

Table II

Animal Groups in the Order of Time That They Were Mated, Treated, and Killed During the Two Years of This Study

Order	Group(s) Done at the Same Approximate Time
1	IX, X, XI
2	III, IV, V, XXII, XXIII, XXVIII, XXIX, XXXI
3	VIII
4	I, II, VI, VII, XII
5	XIII, XIV, XV, XXIV, XXX
6	XVI
7	XVIII, XXVI
8	XVII, XIX, XXV, XXVII, XXXIII
9	XX, XXI
10	XXXII

Explanation of Table II.

The time required to mate, treat, and kill all animals in each of the groups is shown in Table I of this Addendum.

Data in groups XX, XXI, XXXII, and XXXIII were not statistically analyzed. Fetuses in groups XX and XXXII were cross sectioned with a razor-blade. Animals in groups XXI and XXXIII were the ten-day-old newborn rats.

The various treatments administered to animals in each group are shown in Table I of the text, page 38.

would not be worthwhile to re-analyze even the data that could be analyzed in a valid manner. The amount of new information would still likely be either confusing, since there was considerable variation in fetuses of control animals (groups XXII to XXXI) studied at the same approximate time,[1] or the amount of new information gained might be little or none and not warrant the work required for re-analysis of the data.

I have since that time discussed my problem with Mrs. Kathryn Shotts at the Medical Computer Centre at the University of Louisville School of Medicine. She, too, is of the same opinion as Dr. LaBella and Mr. Vivian.

This now raises the question of why wasn't time, as a variable, carefully considered and incorporated into the experimental design in order to prevent the occurrence of the present situation? The major reasons are as follows:

1. In reviewing the literature on similar experiments conducted by other investigators, there appeared to be no emphasis on the importance and utmost necessity to include this factor in the design. Furthermore, their data did not clearly indicate that this factor had been considered.

---

[1]Table II of the Addendum contains the order of time in which the various groups were done. Tables VII to XVIII in the text contain data on responses analyzed in different control, as well as drug-treated, groups. Table I of the Addendum contains the actual time required to mate, treat, and kill the ten animals in each of the groups.

2. It was therefore believed that once values of variations noted in ossification and development of fetuses from different treated and untreated (normal) control animals had been obtained, that this information could be used to make comparisons with variations in fetuses of the different control animals and also the drug-treated animals.

Despite the fact that it is inappropriate to compare control groups with drug-treated groups separated in time by years or months, I still believe that I have learned a considerable amount of knowledge during the course of this investigation. I want to discuss 1) an observation that may be significant and possibly attributable to podophyllin, 2) variations present in fetuses of treated control and untreated control (normal) animals and factors that might influence biological variability, and 3) what has been learned from this study and improvements proposed for future experiments of a similar nature.

Observation That Suggests a Podophyllin Effect. As mentioned in the text (page 143), the most noteworthy observation in this study was the severe runting of fetuses (Figures 3 and 4) that occurred in a few instances (Table XXVIII, page 149). This was noted after the mothers had received podophyllin on either days ten to twelve or day ten only of their pregnancies. The following reasons are offered in support of the belief that podophyllin was responsible for the occurrence of these runts rather than thinking that they occurred either spontaneously or that they were caused by other factors.

1. In this study, fetuses of this size were never observed in any animals of treated control or untreated control (normal) groups. In addition they were not observed in any of the animals exposed to various environmental temperatures. Furthermore, during the past five years I have examined some six hundred litters (approximately six thousand fetuses) in this strain of rat. During that time I have only observed severe runting of fetuses in drug-treated animals; in all instances the incidence was low.
2. Though quite unusual, unexplainable at present, and maybe coincidental, the fetal runting in this study occurred at only one dose level, namely 0.1 milligram per 100 grams body weight of the animal.
3. Thiersch (1963) observed fetal runts following intraperitoneal administration of podophyllin, or one of its components, podophyllotoxin, to pregnant Long-Evans rats on various days of their pregnancy. Administration of podophyllin (5 milligrams per kilogram) to eleven rats on the eleventh and twelfth days of pregnancy produced 90 per cent fetal resorption, with stunting of all survivors (approximately 10 per cent). He also noted stunting of fetuses in rats given other doses of podophyllin and similar effects in rats given podophyllotoxin. The possible reasons he observed increased resorptions, and I did not, are discussed on page 147 of the text.

4. Karnofsky (1965) had an article published in the Annual Review of Pharmacology entitled "Drugs as Teratogens in Animals and Man." He states, "The plant alkaloids, colchicine, podophyllotoxin, vinblastine, and vincristine, have all shown teratogenic activity in animals." He appears to make this statement partly on the basis of the following, 1) the report by Cullis (1962) of multiple congenital defects in a baby whose mother had taken "slimming tablets" containing podophyllin during the fifth to ninth weeks of her pregnancy, and 2) Thiersch's study (1963) in which he noted increased resorption rates in pregnant rats that were given podophyllin or podophyllotoxin. It should be pointed out that though I did not observe an increase in resorptions, I did nevertheless note some fetal runting as did Thiersch. Although Karnofsky (1965) does not appear to make his conclusions about podophyllin on the basis of fetal runts observed by Thiersch, he does classify this drug as a *teratogen* in man and the rat. An increased resorption rate is a fairly good indication that a substance is affecting intrauterine growth. Furthermore, the fact that Thiersch and I in different strains of rat, noted runting, suggests that this is more than mere coincidence and quite likely due to podophyllin.
5. Riddell (1967), in this laboratory, also noted a few severely runted fetuses in Holtzman rats that had received

colchicine on various days of gestation. He did not, however, observe runting in any of the control animals. Riddell in his study used one hundred fifty animals, this representing approximately one thousand five hundred fetuses. I had the privilege of verifying these observations and was most interested because it has been shown by a number of investigators that podophyllin and some of its constituents inhibit mitosis in various types of cells in a manner similar to colchicine, a known and established mitotic inhibitor (King and Sullivan, 1947; Sullivan and Wechsler, 1947; Spendlove et al., 1964; Karnofsky, 1965; Broomhead, 1967).

Variations Observed in Treated and Untreated (Normal) Groups. When one looks at the means for most of the responses examined and analyzed (Tables VII to XVIII) it is noted that there is considerable variation in the means for animals of different control groups (XXII to XXX), and also the means for the treated control groups when compared with the one untreated control (normal) group (XXXI). In a number of instances, this variability appears too widespread to be considered within limits of normal variation.

According to Cahan (1964) and Fraser (1964), there are a number of factors that have been shown or are suspected by either them or others to have an effect on either the pregnant animal, the fetus or both. Some of these factors that may account for some of this variation are as follows: 1) fluctuating temperature and possibly relative humidity

in the animal quarters, 2) sudden noises in the animal room, 3) the presence of endemic viral, bacterial or parasitic infections, 4) the smell of a strange animal, 5) variations in composition of the diet during the period of investigation, 6) slight physiological differences between animals of the same strain, 7) differences in weight and age of animals at time of mating, treating and killing, 8) seasonal variations, and 9) interaction between any combination, or all, of these factors.

Although one or more of the above may have been responsible for the variation noted in some instances in the groups under discussion, I want to discuss the possible influence of temperature on the pregnant rat and her litter, since it was impossible to control this variable during the course of this investigation.

The results of the temperature study suggest that there may be an association between the degree of biological variation and the temperature to which pregnant animals are exposed. This is illustrated in Graph 11 on page 120. Here it is observed that the percentage of fetuses with incomplete or absence of ossification in sternebrae is generally more widespread in groups exposed to the higher and lower temperatures than in groups exposed to intermediate ones. Similar observations are also noted in responses four, eight, ten, twelve, nineteen, twenty, thirty-three, thirty-six, thirty-nine, and forty-one (pages 121, 123, 125, 125, 128, 128, 133, 134, 135, 136 respectively). Although these are suggestive observations, they are not conclusive evidence.

A similar, and slightly suggestive trend also emerges when one looks at the mean resorption rate for groups ATE to GTE (Table XIX, R-2; page 121). It is observed that there is a slight decrease in resorption rate with decreasing temperature. Though this is not significant, it may be associated with temperature. The reason for thinking this is that in reviewing the studies of MacFarlane, Pennycuik and Thrift (1957), it is noted that they observed a significant increase in the resorption rate (58 per cent) in groups of pregnant Wistar rats exposed to a temperature of 95 degrees Fahrenheit. In animals exposed to temperatures between 73 to 82 degrees Fahrenheit, the resorption rate was only 7 per cent. This suggests that there might be a critical temperature between 90 and 95 degrees Fahrenheit where there is a sharp increase in this rate.

Because an increase in resorption rate is a fairly good indication that an agent is directly or indirectly influencing embryonic development, it is not unreasonable to believe that a fluctuating temperature during pregnancy might not result in a significant increase in the resorption rate, but it could possibly result in the variations observed in treated and untreated control groups in the present study. A more detailed account on the effects of temperature is found on pages 171 to 174 of the text.

As previously mentioned, although temperature is not the only factor that might account for the widespread variations observed in different control groups, it seems to be the most reasonably explanation at present. It was not possible to accurately control temperature, and

relative humidity, in the animal room. The temperature though usually near 78 degrees Fahrenheit, ± 7 degrees, did on one occasion vary considerably more. A number of control animals, as well as drug-treated animals, were exposed to this latter extreme (refer to page 40). According to Cahan (1964), a constant environmental temperature is of utmost importance when undertaking a study concerning the teratogenic action of drugs. It may be that Cahan's statement is based on the fact that considerable variation can occur if this factor is not kept constant. As a consequence, this might make interpretation of results most difficult.

What Has Been Learned From This Study and Improvements Proposed for Future Experiments. The results in the podophyllin experiments suggest that time, and factors associated with it, were in all likelihood responsible for the observed variations (primarily in ossification) rather than the drug, suspension media or the stomach tube. Although the statistical technique was appropriate, because time was not taken into account, it has been very difficult to make any firm conclusions from this prodigious body of largely negative and confusing data obtained in this investigation. As a result of this, the statistical significance should not have been belaboured in the face of many illogical inconsistencies especially when there were logical indications to the contrary. Although the results do not significantly modify or enlarge existing knowledge in this area they do, however, confirm and enlarge ideas concerning the analysis of the problem of biological variability. This study has also shown that it is probably not wise to

use computer analysis to analyze biological variations in the absence of knowledge about assorted environmental conditions that may alter the parameters examined. Even at present it is difficult to distinguish normal from abnormal in biology. An attempt to correlate this with experimental variables has produced a very complex problem.

It is now believed that valid results might have been obtained and warranted conclusions could have been made if in the beginning I had determined all the groups to be examined, randomized them and then done them one at a time. This likely would have taken into account, and corrected for, variability due to time. Another method that could have been used, and possibly better, would have been to mate, treat, and kill all animals in groups (drug-treated, control, and normal) constituting an individual experiment (of a series) within as short a time period as possible. In the future my experiments will be done using one of these procedures and thereby correct for biological variability due to time.

Two other equally important facts have been learned from this investigation. First, it is important to consult with a biostatistician prior to undertaking any experiments. The objectives are as follow: 1) to make him or her aware of your problem, the experimental approach, and suggest improvements, 2) determine the correct statistical approach, 3) determine that the statistical method adequately tests the parameters to be analyzed, and 4) have confirmed that valid conclusions can be made on the basis of the analysis.

Secondly, although randomization of groups of animals before starting an investigation would likely correct for variations in

temperature, and relative humidity, it is believed that an experiment of this type could be improved on by keeping these two variables constant and thereby keep biological variability at a minimum. As previously pointed out, results of the temperature study suggest that intrauterine biological variation appears to be indirectly influenced by the temperature to which the pregnant rat is exposed. Cahan (1964) stresses the importance of a constant environmental temperature when conducting teratogenic studies.

The problems in teratology are vast and many times it is difficult, if not impossible, to arrive at appropriate conclusions on the basis of animal experiments. To assist in making more accurate conclusions all environmental, and other variables known or suspected of influencing embryonic development should be carefully considered and controlled in experimentation with animals. Even then it may be inappropriate, if not dangerous, to extrapolate information learned from animal studies and apply it to man. Fraser (1964) states, "a drug that is not demonstrably teratogenic in experimental animals may be so in man. A drug that is demonstrably teratogenic in animals may not be so in man. Therefore the final proof of whether a drug is likely to be teratogenic in man must be sought in man."