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THE ACTION OF SOME NUCLEOTOXIC SUBSTANCES ON PREGNANCY

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In a previous investigation (Didcock, Picard, and Robson, 1952) it was shown that podophyllotoxin, which is a nucleotoxic substance with striking inhibitory effects on certain tumours, will interrupt pregnancy in mice and rabbits. The relation between the dose necessary to affect pregnancy and that toxic and lethal to the mother—what might be called in this connexion the therapeutic ratio—was, however, low, being of the order of 4. It was of interest to investigate the effect of other nucleotoxic substances on pregnancy, to determine whether there is any relation between nucleotoxic activity and effect on pregnancy, and also whether there are any nucleotoxic substances with a higher therapeutic ratio than podophyllotoxin.

METHODS

The experiments were performed on mice and rabbits. In the former the duration of pregnancy was dated from the finding of the vaginal plug, and in the latter from the observed mating. In mice injected between the sixth and tenth days of pregnancy a laparotomy was performed to establish that a normal pregnancy was present. After this time it is possible to confirm the existence of pregnancy by abdominal palpation. Before the sixth day, when implantation occurs, the effect of the drug was established by dividing a number of mice, in which vaginal plugs had been found, into two groups. One group was treated and the other served as control.

The intra-amniotic and intraplacental injections were performed on rabbits under ether anaesthesia with full sterile precautions. The abdomen was opened by a midline incision and the uterus exposed. A particular site was chosen for injection and its various parts identified by transillumination from a strong source of light. The material was then injected in 0.1 ml. into the amniotic fluid or in 0.05 ml. into the placenta. For the latter injection it was desirable to have a needle fitted with a collar some 3–4 mm. from the tip to ensure that there should not be too deep a penetration and that the material was actually injected into the placenta.

The effect on pregnancy was determined by looking for vaginal bleeding or for presence of foetal remains in the cage, by changes in the maternal weight, by abdominal palpation, and by examination at laparotomy or post-mortem.

RESULTS

The Effects of Colchicine and Some of its Derivatives on Pregnancy

Colchicine.—Colchicine was injected subcutaneously in aqueous solution. The results are shown in Table I. On the day following the injection there was usually blood in the vagina of the mice, showing that a toxic effect on the foetus had started; occasionally abortion quickly followed, but sometimes there was a slower loss in maternal weight indicating gradual reabsorption of the foetuses. Of the two rabbits injected one was dead the next day and showed early haemorrhagic reabsorption, whereas the other died after two days, with no obvious effect on the foetus. The lowest dose to interrupt pregnancy consistently in mice is 2 mg./kg. which kills some of the mothers. The "therapeutic ratio" is therefore even lower than that of podophyllotoxin.

TABLE I

THE EFFECT OF COLCHICINE ON PREGNANCY

Animals	Duration of Pregnancy at Time of Injection (Days)	Dose of Colchicine (mg./kg.)	Interruption of Pregnancy*	Maternal Mortality
Mice ..	11	2	4/4	1/4
	10	1·5	2/5	1/5
	11	1	1/5	0/5
Rabbits ..	14	2	1/2	2/2

* Number of pregnant females in which abortion or foetal death occurred out of total number of females studied.

Demecolcin.—Demecolcin is an alkaloid which, like colchicine, is extracted from *Colchicum autumnale* and was first described by Santavy and Reichstein (1950). In animal experiments it is some thirty times less toxic than colchicine and has been used in the treatment of chronic myeloid leukaemia (Moeschlin, Meyer, and Lichtman, 1953). This derivative of colchicine was tested for

TABLE II
THE EFFECT OF DEMECOLCIN ON PREGNANCY

	Route of Administration	Dose (mg./kg.)	Interruption of Pregnancy	Maternal Mortality
Mice 11–13 days pregnant	Subcutaneous	1	4/4	0/4
	,,	2	4/6	0/6
	,,	4	5/5	0/5
	,,	8	3/3	0/3
	,,	16	4/4	1/4
	Oral	1	0/5	0/5
	,,	2	2/5	0/5
	,,	4	3/4	0/4
	,,	8	5/5	4/5
	,,	16	3/3	2/3
Rabbits 13–16 days pregnant	Subcutaneous	1	0/2	0/2
	,,	2	2/2	0/2
	,,	4	2/2	0/2
	,,	8	2/2	1/2
	Oral	2	0/2	0/2

its effect on pregnancy, and the results are shown in Table II. The effect on pregnancy was manifest in from 1–2 days after injection. The "therapeutic ratio" of demecolcin administered subcutaneously to mice and rabbits is about 4, but the drug is less effective when given orally.

Trimethyl Colchicinic Acid Methyl Ether.—Trimethyl colchicinic acid methyl ether (TME) was first prepared by Ullyot and has been tested for its ability to damage sarcoma 37 in mice (Leiter, Downing, Hartwell, and Shear, 1952). These workers found that TME has a therapeutic ratio—maximum tolerated dose to minimum effective dose—of 50 whereas colchicine, under similar conditions has a therapeutic ratio of 2. It was tested for its effect on pregnancy in mice and rabbits, and the results are shown in Table III. The effect on pregnancy came on more gradually than with podophyllotoxin or demecolcin, the characteristic effect being a gradual loss in weight and abortion or reabsorption in 2–3 days. TME has a "therapeutic ratio" of about 12 in mice since a dose of 1.5 mg./kg. of the tartrate, equivalent to 1 mg. of the alkaloid, will interrupt pregnancy in all animals without producing any maternal deaths whereas a dose of 18 mg./kg. of the tartrate kills some of the mothers.

In rabbits a dose of 4 mg./kg. will consistently interrupt pregnancy without producing toxic effects on the mother, whereas doubling the dose is lethal to the mother. The therapeutic ratio in this species is therefore about 2.

Since TME has been used orally in patients, its effect by this route was also investigated in animals; it was less effective and more toxic to the mothers than when given by injection.

The drug was also given before implantation, i.e., before the sixth day. Pregnancy occurred in 18 out of 45 of the mice which received a dose of 4 mg./kg., whereas 22 out of 42 control mice became pregnant. With 8 mg./kg. TME 2 out of 32 of the treated mice became pregnant against 14 of the 19 untreated mice. The "therapeutic ratio" is therefore much lower at this stage of pregnancy.

Local Action of Trimethyl Colchicinic Acid Methyl Ether and Podophyllotoxin.—It seemed likely that the effects of the colchicine derivatives on pregnancy were due to a selective toxic action on the foetus, the foetal tissue being more sensitive to the drugs than the maternal organism. The drugs were therefore applied directly to the intrauterine tissues by intra-amniotic or intraplacental injection.

The rabbits were injected between the 15th and 21st days of pregnancy and laparotomies were performed 5–8 days after injection. The results are shown in Table IV. It was found that the injection of a control solution into either the amniotic fluid or the placenta produced no effect on the

TABLE III
THE EFFECT OF TRIMETHYL COLCHICINIC ACID METHYL ETHER (TME) ON PREGNANCY

Animal	Duration of Pregnancy (Days)	Route of Administration	Dose of TME* (mg./kg.)	Interruption of Pregnancy	Maternal Mortality
Mice	13	Subcutaneous	1·5	7/7	0/7
	14	,,	4	14/14	0/14
	14	,,	8	7/7	0/7
	13	,,	18	4/4	3/4
	12	Oral	2	3/5	0/5
	12	,,	4	6/9	0/9
	12	,,	8	4/4	3/4
	6	Subcutaneous	4	10/10	1/10
	6	,,	8	8/8	2/8
	7	,,	4	5/5	0/5
	8	,,	4	5/5	0/5
	9	,,	4	6/6	0/6
	15	,,	1·5	0/3	0/3
	15	,,	4	14/14	0/14
Rabbits	15	,,	1	0/2	0/2
	13	,,	2	2/4	0/4
	14	,,	4	5/5	0/5
	15	,,	8	1/1	1/1

* Tartrate used. The actual dose of alkaloid, therefore, is about two-thirds the stated dose.

TABLE IV
THE EFFECT ON PREGNANCY OF TRIMETHYL COLCHICINIC ACID METHYL ETHER AND PODOPHYLLOTOXIN INJECTED INTO THE AMNIOTIC FLUID OR PLACENTA IN RABBITS

The first numeral shows the total number of sites in which the pregnancy was interrupted; the second shows the total number of sites injected with that particular dose

Dose of TME (μg.)	5	10	25	50	100	200	500	1,000
Intra-amniotic ..	0/1	3/5	7/9	5/5				
Intraplacental ..			0/3	0/5	1/4	1/3	2/2	1/1
Dose of podophyllotoxin (μg.)	**0·2**	**1**	**2**	**5**	**10**	**20**	**100**	
Intra-amniotic ..	0/3	0/5	0/6	4/8		3/3	2/2	
Intraplacental ..				0/2		2/9	3/6	

foetus. When pregnancy was interrupted following intra-amniotic injection, a small dead foetus was usually found, but at a few sites a reddish brown gelatinous mass was all that remained of the conceptus. The following examples illustrate these effects. The uterus of a rabbit 21 days pregnant contained 11 foetuses. Four of these had 25 μg. or 50 μg. TME injected into the amniotic fluid, the remaining 7 serving as controls. The mother was killed 5 days after injection and the uterine contents removed and examined. All the untreated foetuses were alive and appeared normal. The weights of these seven foetuses varied from 16 to 20 g. and the weights of the placentae from 2.3 to 4 g. Where the TME had been injected into the amniotic fluid all the foetuses were dead. The weights of the dead foetuses ranged from 4–6 g., the corresponding placentae weighing from 1–3.5 g. With intraplacental injection a similar effect was seen, and again there was little effect on the placental weight even though a very much larger dose was administered by this method. In a 22-day pregnant rabbit there were 5 foetuses. One of these was given an intraplacental injection of 1 mg. TME and the others remained as controls, 2 of them receiving injections of normal saline. The mother was killed 5 days after injection and the foetuses and placentae removed for examination. At the injected site the foetus was dead; it weighed 7 g. and the placenta 9 g. All the controls were alive and appeared normal, the average foetal weight being 43 g. and the average placental weight 10 g. There was no apparent difference between the untreated controls and the controls injected with saline.

The intra-amniotic injection of 10 μg. TME killed 3 out of the 5 foetuses injected. As the volume of the intra-amniotic fluid at this stage of pregnancy—around 20 days—was about 5 ml. this dose is roughly equivalent to a subcutaneous dose of 2–4 mg./kg. to the doe. With intraplacental injection the dose required to kill the foetus is about ten times greater, suggesting that the toxic effect is primarily on the foetus and not on the placenta.

Podophyllotoxin was also investigated by this method (at a slightly earlier stage of pregnancy, around 17 days) and the results are shown in Table IV. This drug also appears to exert its toxic effect directly on the foetus and not on the placenta.

The Action of p-*(Di-2-chloroethylamine)-phenyl Butyric Acid on Pregnancy in Mice*

The aromatic nitrogen mustard *p*-(di-2-chloroethylamine)-phenyl butyric acid (CB 1348; chlorambucil) synthesized by Everett, Roberts, and Ross (1953) inhibits the transplanted Walker Rat Tumour 256 (Ross, Davis, Roberts, and Everett, 1952) and is effective against certain malignant lymphomas (Galton, Israels, Nabarro, and Till, 1955). It was tested for its effect on pregnancy, and the results are shown in Table V. The sodium salt of CB 1348 was prepared by dissolving the mustard in N/50 sodium hydroxide in methanol and evaporating *in vacuo* in a tepid water bath. The residual gum was dissolved in saline and injected within an hour of preparation, the total dose being given in a single injection.

TABLE V

THE EFFECT OF *p*-(DI-2-CHLOROETHYLAMINE)-PHENYL BUTYRIC ACID ON PREGNANCY IN MICE

Duration of Pregnancy	Route of Administration	Dose (mg./kg.)	Maternal Mortality	Effects on Foetus
12–13 days	Intraperitoneal	10	0/12	35 live (average weight 1·66 g.) and 18 dead (average weight 1·0 g.) mice delivered on 20th day
	,,	20	0/13	Dead litter at term. Average weight 0·59 g.
	Intravenous	20	0/2	Dead litter at term
	Intraperitoneal*	20	0/2	Dead litter at term. Average weight 0·63 g.
	,,	30	0/2	Dead litter at term. Average weight 0·65 g.
	,,	40	0/3	Dead litter at term
7	,,	20	0/5	Reabsorption of foetuses
1	,,	20	0/12	Pregnancy prevented in all animals
1	—	Nil	0/10	8 out of 10 had normal litters

* Ovariectomized on the 12th day of pregnancy and treated with progesterone 1·5 mg./day.

The characteristic effect of this mustard, when administered on the 12th or 13th day, is to produce a dead litter at term. This occurs with doses of 20 mg./kg. whereas a dose of 10 mg./kg. results in both normal and stillborn offspring.

The LD50 was 45 mg./kg., in an experiment with 90 mature non-pregnant mice, and therefore the therapeutic ratio is low, being of the order of 2. During this experiment it was noticed that, whereas a dose of 40 mg./kg. produced no maternal deaths, a dose of 48 mg./kg. resulted in a 100% mortality.

Two mice were ovariectomized on the 11th day and pregnancy was maintained by the daily administration of 1.5 mg. progesterone. This is ample to maintain gestation in ovariectomized mice (Robson, 1938). The first dose of progesterone was given on the day preceding ovariectomy and the CB 1348 on the day following ovariectomy. Removal of the ovaries did not alter the effect produced by the mustard.

When CB 1348 was administered on the 7th day absorption of pregnancy occurred. Consideration of the weights shows that development ceased soon after injection of the mustard. Laparotomy performed on the 16th day showed complete reabsorption of all foetuses. If a dose of 20 mg./kg. is given before implantation has occurred, i.e., before the sixth day, pregnancy is prevented.

Effect of CB 1348 on the Foetus.—The dead foetuses were all well below the size of normal live offspring and all showed various degrees of arrested development. Not all the foetuses were obtained for examination, as many were eaten by the mothers soon after delivery.

Examination showed cleft palate and non-expansion of the lungs to be the two most constant features. Abdominal hernias, enlargement of the spleen, absence of buccal opening, and the presence of limb buds instead of hind legs were also found.

Of the 30 live mice from the two groups in which the mothers received 10 mg./kg., five were eaten by the mother within the first 10 days of life. The remainder have been observed for three months, animals being killed and examined at various times. With two exceptions, all have developed normally, and have been interbred, with the production of normal litters. The two exceptions were both from the same mother and were very small at birth, 1.04 g. and 0.98 g. The smaller one was placed with a foster-mother, but still failed to develop. It died 25 days after birth, but post-mortem showed no abnormality. The other mouse has grown slowly and only weighed 10 g. when 60 days old as compared with 16 g. for other mice in this group.

As closure of the palate occurs on the 14th–15th day of gestation in the mouse, two animals were injected with CB 1348 20 mg./kg. on the 16th day of pregnancy. Each produced a live and normal litter at term.

Effect of Other Compounds on Pregnancy

Various other compounds were tested on mice 11–13 days pregnant and on rabbits 14–15 days pregnant. The substances used were D-glucosamine hydrochloride (Quastel and Cantero, 1953), 4-amino-N^{10}-methyl pteroyl glutamic acid, 2:4:6-tris-ethylene-imino-1:3:5-triazine (triethylene melamine) (Hendry, Homer, Rose, and Walpole, 1951), Actinomycin C, 6-mercaptopurine and four dimethane sulphonyloxyalkanes $CH_3SO_2O(CH_2)_nOSO_2CH_3$ (Haddow and Timmis, 1953). Those tested were the compounds where $n=3$, 4, 6, and 8. The compound where $n=4$ is known as Myleran. None was more than slightly active at this stage of pregnancy, and then only at a dose level which caused maternal deaths, except triethylene melamine and 6-mercaptopurine. Triethylene melamine interrupted pregnancy in 3/8 mice treated with 0.8 mg./kg. of this compound, with no maternal deaths; a dose of 1.5 mg./kg. interrupted pregnancy in 6/8 mice, but killed 2 of the mothers. 6-Mercaptopurine interrupted pregnancy in 6/6 mice treated with doses ranging from 150–250 mg./kg. There were no maternal deaths with these doses, but a dose of 300 mg./kg. killed some of the mothers.

6-Mercaptopurine and Actinomycin C were also given by intra-amniotic and intraplacental injection. The former compound only produced an effect with doses of 1 mg. and then not all the foetuses were affected. Actinomycin interrupted pregnancy at 5/5 sites with a dose of 10 μg. injected into the amniotic fluid, smaller amounts only occasionally producing the effect. Doses up to 700 μg. into the placenta had no effect on pregnancy.

Discussion

Nucleotoxic substances, given orally or by injection, differ considerably in their ability to interrupt pregnancy, and this difference bears some relation to their mode of action. By far the most active are the spindle poisons podophyllotoxin and the colchicine derivatives, all these substances being consistently capable of interrupting pregnancy in doses which are not toxic to the mother. The therapeutic ratio, however, varies considerably with the different substances.

The chromosomal poisons *p*-(di-2-chloroethylamine)-phenyl butyric acid (CB 1348), tris-ethylene-imino-1:3:5-triazine, and the dimethane sulphonyloxyalkanes are much less active and, with the exception of CB 1348, only produce an effect with doses which are also lethal to the mother.

The antimetabolites 6-mercaptopurine, D-glucosamine and 4-amino-N^{10}-methyl glutamic acid were almost completely inactive when the drugs were given to mice between the 11th and 13th days of pregnancy. This is supported by the work of Thiersch (1954) with 6-mercaptopurine; he found that when the drug was given to rats on the 12th–13th days there was little effect on the pregnancy although stunted foetuses were produced. However, the foetus was much more sensitive at the time of implantation—7th–8th day—when 90% foetal deaths were produced.

Similarly aminopterin, a folic acid antagonist, has been shown by Thiersch and Philips (1950) to

interrupt pregnancy in rats and mice in doses which are not lethal to the mother. The embryos were much more sensitive to the drug before the 10th day of pregnancy. Thiersch (1952) has also demonstrated a similar effect in the human subject. Abortion can be produced during the first 3 months of pregnancy with doses which have only a mild transitory effect on the haemoglobin and white cell count of the mother.

With the spindle poisons the therapeutic ratio in the pregnancy tests—ratio of dose which will consistently interrupt pregnancy to dose which kills some of the mothers—bears some relation to the ratio of the maximum tolerated dose to the minimum effective dose when the drugs are used in the treatment of neoplasms. The values of the former ratio with colchicine, TME, and demecolcin are respectively 1, 12, and 4, and for the latter ratio 2, 50, and 10. There is no such relationship with the other substances tested.

Our results indicate that a more satisfactory test for the cytotoxic effect of these substances is that of injection directly into the amniotic fluid. The effect is produced by a direct action on the foetus and not by an indirect effect through the placenta, as both intra-amniotic and intraplacental injections have very little effect on placental weight. Furthermore, to interrupt pregnancy by intraplacental injection a very much larger dose is needed than that which is effective by the intra-amniotic route.

As has already been seen the intra-amniotic injection of TME produces results which are comparable to those obtained by systemic injection, and the same applies to podophyllotoxin. Actinomycin C and 6-mercaptopurine are also effective by this method, whereas parenteral administration of the former produces toxic effects on the mother before producing a demonstrable effect on pregnancy.

All these experiments, therefore, suggest that the therapeutic ratio is essentially of value only in the testing of drugs which are spindle poisons, whereas the intra-amniotic injection method may also provide information about other cytotoxic drugs.

Summary

1. The action of various nucleotoxic drugs on pregnancy in mice and rabbits has been investigated.

2. The ability of these drugs to interrupt pregnancy bears a definite relation to their mode of action and to the stage of pregnancy at which they are administered.

3. By far the most effective are the spindle poisons, podophyllotoxin and the colchicine derivatives. With these drugs there is a definite relation between the therapeutic ratio as determined by the pregnancy test and that found by testing the anti-tumour activity of these compounds.

4. Intra-amniotic and intraplacental injection of various nucleotoxic drugs was performed to determine the site of action of these nucleotoxic substances. They probably act directly on the foetus and not via the placenta.

5. Intra-amniotic injection seems to be a simple and reliable method of testing a drug for cytotoxic properties.

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