COPPER IN MAMMALIAN REPRODUCTION

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

July 29, 1976
date

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Abstract

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Part I Copper and Uterine Contractions

The introduction of the copper IUD and the subsequent finding that copper is released from the device in utero raises the question of what effect copper has on the myometrium. Initial clinical findings showed an expulsion rate of 0.6% for the copper IUD as compared to 5.9% for a similar device without copper. This statistically significant lowering of the expulsion rate shows that copper has an effect on uterine motility.

By way of introduction, uterine smooth muscle function and the role of copper in other reproductive processes are reviewed.

Smooth muscle, and particularly uterine smooth muscle is compared to skeletal muscle in a brief summary of the classical differences in contractile proteins, the arrangement of the contractile elements, the roles of sodium and calcium in the action potentials, as well as the similar role of calcium in activating the contractile proteins in all muscle. Recent findings of myosin thick filaments and their oblique orientation are also presented. Smooth and skeletal muscle are also compared in terms of their gross function--their similarity in load-shortening and length tension relationships and the ability of smooth muscle to accommodate to various lengths.

The overall function of the myometrium as an organ is also briefly reviewed. The cyclic pattern of uterine activity in the non-pregnant uterus--low activity at midcycle and high activity during menstruation is viewed with respect to expulsion of IUD's. The quiescence of the pregnant uterus and the emergence of activity with the onset of labor is summarized.

The emerging role of prostaglandins in initiation of labor is presented. Prostaglandins, released upon stretching the uterus, cause contraction and thus further prostaglandin release and contraction. A new point of view of the initiation of labor is presented whereby previously divergent theories may be incorporated into a larger framework using a systems analysis approach. Thus estrogens, corticoids, oxytocin, oxygen tension and feto-placental volume, all affect the myometrium, but probably not profoundly enough to individually initiate labor. Any one of these factors, as well as a drop in progesterone or an increase in prostaglandins can affect the dynamic balance between stretch, prostaglandins, and progesterone. A small change in any factor that affects these can, by the positive feedback nature of the system, have the final dramatic effect--the initiation of labor.

In 1969, Zipper and his associates found that copper wire has a contraceptive effect when placed in one uterine horn of the rabbit. These findings were extended to human use--a plastic T-shaped device with copper wire wound around its stem had an increased contraceptive effect in women compared to the "T" without copper. The clinical evidence to date indicates that the copper IUD has a pregnancy rate equal to or lower than that for conventional IUD's. Because of its smaller size its major advantage is that it can be used with fewer complications in nulliparous women.

It was shown that copper was released from the device at a rate of approximately 50 μg per day. Oster and Oster subsequently showed that this release of copper requires oxygen and chloride ions, and is augmented by amino acids or proteins which chelate cupric. A particularly striking finding was that this release of copper acts as a free radical generating system.

The mechanism of contraceptive action of the plastic IUD is unclear. Despite widespread current interest the contraceptive mechanism of the copper IUD also remains elusive.

The copper only exerts the effect locally, and fertilization per se is not affected. Plastic IUD's cause an infiltration of polymorphonuclear leukocytes and with the copper IUD this effect is more pro-

nounced. One as yet untested hypothesis of the contraceptive action of the copper IUD has been presented by Csapo, and is related to the effect of copper on smooth muscle as presented here for the myometrium. The hypothesis states that copper--perhaps via prostaglandin synthesis--contracts the vascular smooth muscle of the endometrium thus cutting off the circulation needed for successful implantation.

As shown in this thesis, cupric salts at low concentrations cause the in vitro rat uterus to contract. Exposure of the uterus to high concentrations of copper salts for long periods of time leaves it unable to develop full tension with either oxytocin, copper, or electrical stimulation.

Copper ions cause contractions of the uterus in the range of 10^{-6} to 10^{-5}M, as compared to 5×10^{-10}M to 1×10^{-8}M for oxytocin. The maximum tension achieved by copper is often 20% higher than that achieved with oxytocin. Copper-induced contractions are more sustained than the oxytocin-induced ones, and are harder to reverse by rinsing the uterus. The copper chelating agent, diethyldithiocarbamate, irreversibly depresses the uterine responses to oxytocin and copper. Copper does not seem to be acting on the oxytocin receptor of the uterus; this was shown by the lack of inhibition with the oxytocin antagonist, 2-alanine-deamino-dicarba-oxytocin. Copper-induced contractions are inhibited by theophylline. This implies that changes in cyclic adenosine monophosphate (cAMP) are involved in the action mechanism of copper stimulation. The possible role of prostaglandins is also discussed.

Exposure of the rat uterus to 10^{-4}M $CuSO_4$ for an hour causes an irreversible decrease in uterine tension. After such treatment neither

oxytocin nor electrical stimulation can contract the uterus to the tension it had achieved before exposure.

Copper salts administered into the uterine lumen also caused contractions, but higher concentrations were needed than when cupric ion was added to the outside bathing medium. A copper wire placed in the lumen of the *in vitro* uterus caused intense contractions for several hours, with a final inhibition of the uterus' ability to develop tension.

Very high doses of copper salts (1×10^{-1}M) infused into the uterine lumen *in situ* increased intrauterine pressure in pregnant and non-pregnant rats and rabbits.

The possible relevance of these *in vitro* and *in vivo* animal studies to the use of the copper IUD is discussed, especially in relation to expulsions of the device. In addition, the work of the thesis leads directly to certain hypotheses concerning the contraceptive action of the copper IUD. These are presented with a view towards achieving a basic understanding of the inhibition of implantation that occurs with intrauterine devices.

Part II Copper and the Interruption of Pregnancy

Our involvement in research on the copper IUD (Part I) stimulated our interest in the action of copper on other aspects of reproduction.

Copper is involved in many phases of reproduction. For example serum copper is raised in pregnancy, and injection of copper salts to rabbits causes ovulation. Copper administration or copper deficiency during pregnancy can lead to reproductive failure.

Livestock raised in copper deficient areas fail to reproduce. Rats fed a copper deficient diet conceive but the fetuses are resorbed.

It occurred to us that rather than feeding the rats a copper deficient diet, the same end result, namely resorption of the embryo, might be brought about by the administration of a copper chelating agent.

The experimental work of Part II consists of the administration of copper chelating agents and pharmacologically related drugs to pregnant rats and the documentation of the embryocidal and teratological effects of these drugs.

When pregnant rats given disulfiram, a copper chelating agent, orally at a dose of 100 mg per day from Day 3 of pregnancy were killed on Day 12 or 13, 83% of the conceptuses showed fetal resorption. In any rat, fetal resorption was uniform among all the conceptuses. The similarity of our results with those found by others for rats fed on a copper deficient diet suggests that disulfiram renders copper unavailable for the developing embryo.

Because disulfiram caused toxic symptoms in the mother rats, a

pharmacologically related drug, Win 18,446 was tried. N,N'-bis(dichloroacetyl)-1,8-octamethylenediamine (Win 18,446), previously used to suppress spermatogenesis in man and other mammals, is shown to be an effective oral abortifacient for rats. When administered at the appropriate dosage and time of gestation, the drug produces the resorption of 100 per cent of the embryos, with no toxic effect to the mother. The optimal regimen for embryocidal action is 200 mg daily for days 10 and 11 after mating. A mechanism is proposed involving mitochondrial function and replication to explain the action of this drug in the inhibition of embryonic development as well as the suppression of spermatogenesis.

At lower doses, or at administration schedules that excluded the critical period for embryocidal action Win 18,446 causes teratologies. There teratologies appear in as many as 100% of the offspring, and result from as little as a single dose of Win 18,446. Malformations seen include face defects, septal heart defects, diaphragmatic hernias and cryptorchism. The most sensitive time for generation of these teratologies is if the drug is given at day 11 of gestation. From various lines of evidence it is suggested that Win 18,446 is acting as a copper chelating agent and may be interfering with the availability of vitamin A.

Preface

Portions of the present thesis have been published. They are:

Part I:

1. Salgo, M.P. and Oster, G.: Copper stimulation and inhibition of the rat uterus. Fertility and Sterility 25: 113-120, 1974.
2. Oster G. and Salgo, M. P.: The copper intrauterine device and its mode of action. New England J. of Medicine 293(9): 432-438, 1975.

Part II:

3. Salgo, M. P. and Oster, G.: Fetal resorption induced by disulfiram in rats. J. Reproduction and Fertility 39: 375-377, 1974.
4. Oster G., Salgo, M. P., and Taleporos, P.: Embryocidal action of a bis-(dichloracetyl)-diamine: an oral abortifacient for rats. Amer. J. Obstetrics and Gynecology 119: 583-588, 1974.
5. Oster, G. and Salgo, M. P.: Copper in Mammalian Reproduction. Advances in Pharmacology and Chemotherapy 14, 1976. In press.
6. Taleporos, P., Salgo, M. P. and Oster, G.: Teratogenic action of a bis(dichloroacetyl)diamine on rats: patterns of malformations produced in high incidence at time-limited periods of development. In preparation.

The author appreciates the support of the Ford Foundation, grant No. 690-0106.

D E D I C A T I O N

Dedicated to the memory of

GISELA KALLMANN OSTER

1927-1972

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PART I

COPPER AND UTERINE CONTRACTIONS

I INTRODUCTION

A. THE COPPER INTRAUTERINE DEVICE AND UTERINE CONTRACTIONS: THE PROBLEM STATED

The present thesis arose from a practical problem associated with the copper intrauterine device. Our research developed into an investigation into some basic mechanisms of uterine contraction and smooth muscle function. Its implications bring it full circle through hypotheses on the mode of contraceptive action of the copper intrauterine device to a practical suggestion for new forms of contraception.

The development, in 1969, of an intrauterine contraceptive device wrapped with metallic copper raised the question of what effect the copper would have on uterine contractions. Preliminary clinical trials by Zipper et al. (1) found the copper intrauterine device to be not only more effective for contraceptions than the IUD without copper, but also less likely to be initially expelled.

The expulsion rate was 5.9 for 100 women for a year with the T-shaped IUD. When it was wrapped with copper wire of 120 sq mm surface

area, the expulsion rate dropped to 0.6. With 362 women in the group with the copper IUD and 605 in the group with the T without copper, the observed expulsion rate for the T with copper differs from that of the T without copper by 12 times the standard error (2).

Medel et al. (3) found a lower expulsion rate with copper wire than with a thin piece of polyethylene when these had been inserted into the lumen of the rat uterus. Several days after insertion, the copper wire was found to be higher in the uterus or into the fallopian tubes, while the polyethylene had been expelled. Apparently, then, copper metal has an effect on myometrial activity *in vivo*.

The copper metal in the uterus dissolves slowly, losing about 50 μg per day (4). Its dissolution in biological fluids involves the cleavage of disulphide bonds (5, 6) and the formation of free radicals (7) with the production of cupric ions. These reactions result *in vitro*, at least, in an inactivation of enzymes and the liquification of human uterine mucins (6,8).

It appears that the copper ions act on the myometrium. Indeed, several workers in studies of the mode of action of a variety of uterine stimulants, mention that copper ions induce rat uterine contraction *in vitro* (9,10,11).

The problem stated, then, is to investigate the effects of copper ions and copper metal on uterine contractions in laboratory animals. This thesis presents our findings that copper has profound stimulatory and inhibitory effects on the uterine muscle, that these effects may be relevant in women wearing the copper intrauterine device, and that

these actions of copper are useful as a tool in investigating the basic physiology of smooth muscle activity. In addition our findings lead directly to hypotheses on the contraceptive mode of action of the copper IUD.

To serve as a background for the work presented on copper and the myometrium, uterine smooth muscle function is reviewed.

B. UTERINE SMOOTH MUSCLE STRUCTURE AND FUNCTION

1. Structure and Biochemistry

Smooth muscle cells are small (50-250 μ x 5-10 μ diameter) and spindle-shaped (12,13). The cells are grouped in fiber bundles about 100 μ thick which unite in an elongated net-like structure surrounded by connective tissue and permeated by vascular areas (14).

a. Contractile elements

The distinguishing structural feature of smooth muscle is the conspicuous lack of the characteristic banding pattern seen in skeletal and cardiac muscle. Skeletal and cardiac muscle striations are determined by the regular positioning of action and myosin strands (13).

Actomyosin can be extracted from the uterus. It differs from striated muscle actomyosin in being soluble in low, as well as high ionic strength solutions. Extracted in low ionic strength solutions it is

referred to as tonoactomyosin (15). Actin-free myosin (16) and myosin-free actin (15) have been prepared. Smooth muscle myosin differs from striated muscle myosin by its amino acid composition and by its low ATPase activity. No difference has yet been found between smooth muscle and striated muscle actin (17). The basic similarity between contractile proteins of smooth muscle and those of striated muscle indicates that the contractile process, as envisioned in the sliding filament theory (13,18) is essentially the same (13).

Recent experiments have shown that vertebrate smooth muscle is controlled, not by troponin-tropomyosin on the actin, but directly by calcium sensitive myosin (19,20). If skeletal muscle actin, free of troponin and tropomyosin is added to smooth muscle actomyosin (from chicken gizzard), the calcium requirement for ATPase activity remains unchanged. If there were control of ATPase by troponin-tropomyosin, adding unregulated actin would free the ATPase of its calcium requirement; the fact that it does not indicates that control is on the myosin (15). There is also converse evidence showing that there is no troponin functioning in vertebrate smooth muscle (19). When skeletal muscle heavy mero-myosin (which has no calcium sensitivity) is added to smooth muscle actomyosin, the calcium sensitivity of the ATPase is lost; the unregulated skeletal heavy mero-myosin reacts with the unregulated smooth muscle actin (19). This myosin regulation should not be that much of a surprise--it is the usual type of regulation in many invertebrate muscles and it now seems that vertebrate skeletal

muscle, like some other invertebrate muscles, has both types of control (19).

The positioning of actin and myosin in smooth muscle has long been in question. There are no Z, I or A bands and usually only thin filaments are seen running longitudinally (12,13). These do not begin and end at discrete locations so no cross banding is evident. Thick filaments were not seen regularly in vertebrate smooth muscle. Hypotheses were presented that the myosin did not aggregate into thick filaments unless the muscle was contracting, or that the thick filaments were present but were destroyed in the histological processes. Needham and Schoenberg (22) then showed a process whereby thick filaments (130 Å wide) could be seen repeatably. It was later shown that the crucial step was a slight stretching of the smooth muscle cells (21). The exact shape and state of the myosin is still a matter of controversy, as the myosin appears differently depending on the fixative procedures used (for recent discussion, see 22).

Recently, J. V. Small has observed fibrils in smooth muscle under the phase contrast microscope (23). These fibrils, thought to be the "contractile units" of thin and thick filaments, are arranged obliquely with respect to the cell axis, forming a criss-cross pattern. These fibrils are seen when smooth muscle cells are in rigor--the stiff state caused by the lack of ATP when myosin crossbridges are firmly bound to actin. In contracted muscle, the angle that these fibrils subtend with respect to the cell axis increases from the 5^{o} seen in uncontracted

cells to 24^{o}, or even 40^{o} in supercontracted cells (23). This unique finding may be related to ability to accommodate to different lengths--as we shall discuss later.

b. Structures associated with excitation

Neuromuscular junctions in smooth muscle are usually not the discrete end plates seen in skeletal muscle, but rather small varicosities where the naked axon touches the smooth muscle fibers. These varicosities and neural branching allow one neuron to affect many muscle cells (12).

Although there is no definite triad system in smooth muscle, sarcoplasmic reticula are present, and these form appositions with the cell membranes and with caveoli (little coves) along the surface of the cell membrane. These structures are probably the site of transmission of the depolarization of the surface membrane of the sarcoplasmic reticulum, with their subsequent release of calcium. The sarcoplasmic reticulum, whether near the surface or central, will accumulate strontium, as will smooth muscle mitochondria, implicating these as well in the control of calcium in the cell (24). In addition, the cell membrane itself could be involved in the regulation of calcium (24,25).

Another aspect of smooth muscle morphology which is still unresolved is the type of junction between cells which allows for their functional coupling. Most smooth muscles act as functional syncytium-electrotonus spreads from one area to another (25). Close appositions

of membranes as well as gap junctions and bulbous processes from one cell into another, are often seen in smooth muscle, and may be the structures of electrical interaction (25). As we shall see below, such connections must play a vital role in the control of uterine activity, for the functional syncytial nature of the uterus varies with the hormonal state.

2. Function

a. Contraction

Beside the structural and biochemical similarities between skeletal and smooth muscle, functional comparisons also show that the basic contractile process is similar and that the sliding filament theory holds for smooth muscle. Just as with the frog sartorius (26) the product of the strength of electrical stimulus (V/cm) and duration of stimulation determines the threshold at which the muscle responds (27,28). The plot of velocity of shortening vs. load showing that the heavier the load, the slower the shortening, has the same shape for the frog sartorius (29) as for the rabbit uterus (27,28) although the time course for the uterus is about 10 times slower than for skeletal muscle. The length-tension relation plot for skeletal muscle (30) has similar shape to that for the uterus (27,28). A difference exists, however, in that smooth muscle has the ability to develop maximum tension at different lengths depending on its prior treatment. The ef-

fect is that the length-tension curve can be shifted back and forth along the length axis (13). This accommodation could be related to the oblique orientation of smooth muscle fibrils mentioned above. One can imagine that growth or changes in length of a muscle would be easier with this criss-cross arrangement of contractile elements than if the filaments were arranged in discrete sarcomeres with fixed ranges of actin myosin overlap.

b. Excitation

Skeletal muscle responds to nervous stimulation, and various smooth muscles are controlled by autonomic innervation to varying degrees (13). The uterus has only sparse sympathetic and parasympathetic innervation, and these are of doubtful functional importance since women with complete transverse lesions of the spinal cord deliver normally (31). Smooth muscle, like cardiac muscle, contracts spontaneously. As with the heart, this autorhythmicity is a consequence of spontaneous depolarizations.

This autorhythmicity is reflected in the behavior of smooth muscle undergoing electrical stimulation; a basic dissimilarity exists between striated and smooth muscle when stimulated electrically. Striated muscle contracts once and then relaxes as threshold is approached and passed. With smooth muscle, the muscle is spontaneously active, and this spontaneous activity increases toward threshold. The resting potential of striated muscle is -96 mv., while that of smooth muscle

is less and much more variable, from -22 to -66 mv. (12,28). The correlation between amount of spontaneous activity and membrane potential has been documented (27,28). Thus spontaneous activity of the uterus increases as the membrane potential decreases, as with increasing extracellular potassium. At a "critical potential" of about -25 mv. the uterus goes into a state of contracture. With membrane hyperpolarization of -60 mv. (as with 1 mM external potassium) spontaneous activity is completely suppressed (28,32). As we shall discuss later, membrane potential in the uterus is dependent on the hormonal state (28).

Mechanical activity is not initiated by a single depolarization in smooth muscle, as it is in skeletal or cardiac muscle, but with a train discharge of depolarizations. Potassium depolarization increases the spike frequency of the train discharge from a single cell, as well as the frequency of repetitions of the train discharges (28,33).

Although the intracellular increase in calcium seems to be the end result of electrical depolarization, the question of which ions are responsible for depolarizations (spike generation) as well as which are responsible for the resting potential in smooth muscle is still not resolved. (For recent review, see 34) Raising the external potassium depolarizes the cell membrane and increases the spontaneous activity, as already mentioned. The uterus is also very reactive to changes in the external calcium concentrations. In calcium-free media, electrical activity, spontaneous activity, and reaction to oxytocin are lost. Although electrical threshold is augmented when calcium

is removed, the uterine contractile apparatus is still functioning. This can be demonstrated by stimulating with a large electrical stimulation (10 V/cm) (28).

Evidence in the literature is now accumulating that different ways of stimulating smooth muscle result in different types of calcium movement. Stimulation by high external potassium results in a net influx of calcium; on the other hand, stimulation by noradrenaline or angiotensin causes a release of calcium bound inside the cell membrane (35). Under physiological conditions the major factor for contraction is release of calcium from intracellular stores, not via calcium influx (35). Relaxation of the muscle seems to be primarily by sequestration of calcium (35).

Although the role of calcium seems more clear in the contractile process, the role of various ions in maintenance of the resting potential and in carrying the charge in the depolarization spike, is still controversial for smooth muscle (34).

Tetrodotoxin, a sodium channel inhibitor (36) has no effect on spontaneous discharge of taenia coli of guinea pigs, and spike height and rate of rise are related to the external concentration of calcium, but not to the external concentration of sodium (37,38). In addition, uterine action potentials are not extinguished in sodium deficient solutions (28), and smooth muscle spontaneous activity can be maintained in sodium free calcium containing solutions (39). These three

experiments indicate that the smooth muscle action potential is brought about by an increase in calcium permeability, as in molluscan catch muscle, rather than sodium permeability, as is usually the case for skeletal muscle and nerve (13).

Other investigators, state, on the other hand, that an increase in external calcium does not influence the rate of rise of the spike, thus arguing against calcium's being the major charge carrier under normal conditions. With oxytocin and low external sodium however, the rate of rise of the action potential is enhanced by an increase in external calcium, suggesting that oxytocin mobilizes calcium to function as a current carrier when sodium is low (40). (For a recent discussion see 41.) An important item now emerging which may reconcile these divergent arguments is the finding of sodium-calcium exchange in smooth muscle membranes (39,42,43).

The ion movements responsible for the maintenance of the resting potential of smooth muscle differs somewhat from the classical case of nerve or skeletal muscle (13,44). Raising the external potassium depolarizes smooth muscle, but the plot of membrane potential vs. the log of potassium concentration is only a straight line at high potassium concentrations (32). If potassium permeability were the only factor determining resting potential, the plot would show a straight line throughout the range of potassium concentrations, following the Nernst equation (44). Recent evidence implicates an electrogenic sodium-potassium exchange pump as contributing to the resting potential (39,45).

In addition, it appears that calcium influences the potassium permeability (39).

In cardiac muscle potassium "leakiness" is thought to be the cause of spontaneous depolarizations (44). In autorhythmic smooth muscle, the involvement of the electrogenic sodium pump as well as calcium in the maintenance of the resting potential implies that these are involved in the instability of the resting potential. Whatever factors determine the autorhythmicity of smooth muscle, for the uterus they are in turn dependent on steroid hormones.

c. Effects of steroids on excitation and conduction.

The steroidal states of the uterus have profound effects on its excitation, and as we shall see later these effects are among the main determinants of uterine function.

When the estrogen output is low, as in the immature animal, the membrane potential of uterine cells averages around -30 to -35 mv. (28, 32). If the animal had been treated with estrogens for several days, the uterine cell membrane becomes more hyperpolarized to about -43 mv. (28,32). If the animal had been treated with estrogens and progesterone, the membrane potential goes more towards hyperpolarization, to about -50 mv. (28,32).

Progesterone also has a profound effect on spontaneous depolarizations. In the rabbit, electrical and mechanical activity of the uterus has been monitored at various times during pregnancy, and under va-

rious experimental conditions. During pregnancy only few spontaneous discharges are seen and train discharges are absent (28,46). If progesterone support is removed, as by removal of the ovaries and dislocation of the placentae, spontaneous electrical activity evolves in a matter of hours, and regular train discharges are seen (28,46). This evolution of electrical activity can be suppressed by exogenous progesterone (28,46).

Another membrane phenomenon effected by steroids is the conduction of the action potential from one segment of the uterus to another. In uteri from pregnant animals electrical stimulation of one segment of the uterus causes a train of action potentials and contraction only in that area. At term, or if progesterone support is withdrawn by ovariectomy and dislocation of the placentae, trains of action potentials are conducted from one part of the uterus to another (28). In other words, when progesterone support from the ovaries and/or placenta is withdrawn, the uterus acts as a functional syncytium.

Another profound effect of steroids on the uterus is the induction of uterine hypertrophy by estrogens. This field is currently receiving considerable attention because the mode of action of estrogen is being taken as a general model for steroid action.

Briefly, when estrogen acts on the uterus after about one hour there is an increase in glucose oxidation. After about three hours there is an increase in wet weight of the uterus, and starting six hours

after initial estradiol treatment there is an increase in RNA and protein synthesis (47). The estrogen, when it comes into contact with the uterine cell, enters the cytoplasm and binds to a specific cytoplasmic binding protein--the receptor. The estrogen induces a conformational change in the receptor protein causing or allowing the estrogen-receptor complex to migrate to the nucleus (47). The increase in wet weight of the tissue is associated with the appearance of the estrogen-receptor complex in the nucleus, and can be inhibited by actinomycin D (47). The receptor interacts directly with DNA and this results in the production of new RNA thought to be messenger RNA, and de novo protein synthesis (47).

Various workers (48,49,50,51) have found a rapid increase in cyclic adenosine monophosphate (cAMP) in the uterus upon estrogen administration. Other workers (52,53) on the other hand have found no change in cyclic AMP. Exogenous cAMP can mimic some estrogen-like effects such as the increases in RNA, de novo protein synthesis, and glycogen synthesis (50,51). However, these increases are not consistent and are not strictly similar to the increases seen with estrogen administration (51). The increase in cAMP induced by estrogen can be blocked by adrenergic blocking agents (50,51), indicating catecholamines in estrogen action (see action of adrenergic agents in section d below). The later more lasting effects of estrogen take place despite blockage by adrenergic blocking agents (50,54). This implies that catecholamines do not play an obligatory part in the estrogen stimulation sequence (50).

The overall view of estrogen action is considered to be in two phases (47). Phase I involves the permeability change, an increase in cAMP, and a release of histamine. This is a period of preparation for true growth (47). Phase II, changes occurring six hours or later after initial stimulation, consists of the RNA and protein changes controlled by the presence of the receptor-estrogen complex within the nucleus (47).

Progesterone has recently been shown to interact with target cells in a manner similar to that of estrogen. A progesterone binding protein has been isolated (47). In estrogen-primed chick oviducts the progesterone-receptor complex has been shown to interact with the genome resulting in the formation of a new species of RNA from the DNA template, and the subsequent de novo synthesis of avidin (47). Progesterone causes a delayed increase in adenylate cyclase to 250% of controls in 24 hours (51).

d. Uterine pharmacology

Drugs affecting the uterus have been known for over two thousand years. In the books of the Parsees, from 400 to 300 B.C., the abortifacient effect of ergot poisoning was noted. Eating rye contaminated with fungus which produces the ergot alkaloids would "cause pregnant women to drop the womb and die in childbed" (55). All the natural ergot alkaloids increase the activity of the uterus, ergonovine being the most active. Because of its tendency to produce sustained contractions its obstetrical use is presently limited to the third stage of

labor (delivery of the placenta) and to control postpartum hemorrhage (55).

As mentioned before, the uterus has both sympathetic and parasympathetic innervation (although denervation has little effect) (31,55). Both the catecholamines and acetylcholine cause changes in activity of the uterus.

Acetylcholine excites the uterus by depolorizing it, probably via increases in membrane conductance of sodium, potassium and calcium (56, 57,58). Smooth muscle cholinergic stimulation is mediated by muscarinic rather than nicotinic receptors (56,59).

The action of epinephrine and norepinephrine on the uterus varies with the species and with the hormonal state (41,56,58; for review see 41). Briefly, excitation is generally mediated by alpha receptors and relaxation by beta receptors. The uterus has both types of receptors so the final response depends on the degree to which each is being activated (41). In the estrogen-dominated uterus adrenergic stimulation is mainly excitatory--via alpha receptors, and can be blocked by the alpha blocking agent phentolamine. In the progesterone-dominated uterus adrenergic stimulation is mainly inhibitory--via beta receptors, and can be blocked by the beta blocking agent propranolol (41).

In vivo, autonomic stimulation plays a minor role in uterine activity (see Initiation of parturition, section f below). Autonomic blockers thus have little effect. Direct acting smooth muscle relaxing agents such as papaverine, nitroglycerin and caffeine will relax the

uterus, but in vivo their action lacks specificity (55). Their action probably results from inhibition of phosphodiesterase, increasing cyclic AMP levels (60) (see cyclic AMP, section e below).

Histamine contracts smooth muscle including the human uterus but it causes relaxation of the rat uterus (61). Its stimulating action is thought to be via calcium influx since it has an effect on K^+ depolarized smooth muscle (61).

Barium can stimulate smooth muscle including the uterus at concentrations around 10^{-4}M. It probably substitutes for calcium, but it also seems to cause an increase in sodium conductance (52).

Oxytocin, the natural uterine stimulant, acts on a specific oxytocin receptor where various oxytocin analogues will serve as inhibitors. Oxytocin will contract the K^+ depolarized uterus (36) but under physiological conditions its action is thought to be via depolarization--an increase in sodium permeability. Sodium is required for its action but the contraction is diminished in low calcium, so calcium permeability may also play a role (56) (see also Excitation, section b above). Oxytocin induced as well as spontaneous contractions are probably mediated via prostaglandin synthesis, as will be discussed later (see Initiation of parturition, section IB2f, and Discussion, section IVD).

Oxytocin is potentiated by magnesium and manganese. This potentiation could be a result of complexes it forms with these metals (56, (62).

The structure-activity relationships of neurohypophysial hormones

has been examined in great detail and now includes information on the three-dimensional conformation of oxytocin. (For review see 63, 64. 65.) The following discussion is, of necessity, confined to the most basic aspects of oxytocin structure-activity relationships.

Oxytocin is the octapeptide with the structure CyS-Tyr-Ile-Glu-Asn-CyS-Pro-Leu-GlyNH_2, with the half cysteins at 1 and 6 joined forming a pentapeptide ring (63,64). The natural neurohypophysial peptide hormones arginine and lysine vasopressin, arginine vasotocin, as well as other naturally occurring hormones found in birds, reptiles, amphibians and cartilaginous fish differ from osytocin only in positions 3, 4 and 8 (64).

The closed pentapeptide ring is required for activity, although the disulfide per se is not necessary as shown by the activity of analogues with a closed ring but with the two sulfur atoms replaced by selenium or methylene groups (63,66).

At position 1, removing the terminal amine group enhances oxytocin activity. In position 2 the aromatic ring is not critical, but a hydrophobic side chain favors activity (63). The presence of isoleucine in position 3 is critical for oxytocin activity--the naturally occurring analogues with phenylalanine here have reduced oxytocin and milk ejecting activities, but only a small increase in vasopressive and antidiuretic activities (63). In position 4, a 2 carbon side chain is optimal. Substituting threonine or other hydrophilic hydroxyl or carboxyamide groups adds considerably to oxytocin activity (63). Asparagine in position 5 is absolutely required for activity. It is proba-

bly critical for maintenance of the secondary structure in solution (65). Although substitutions for proline in position 7 have reduced activity this has not been investigated as fully as changes in other positions. Position 8 is the most varied among the natural neurohypophysial hormones. Substitutions of quite different types only slightly decrease oxytocin activity, while the presence of a basic amino acid is required for strong vasopressor and antidiuretic activity (63,64). Changes in position 9 reduce oxytocin activity (63).

e. Cyclic AMP

An increasingly universal picture is emerging concerning the interaction of cAMP and calcium in hormone responses in a wide variety of tissues (for review see 67,68). In most systems where hormone action is cAMP related the end reaction of the target tissue is dependent on the presence of calcium (67,68). It is thus not surprising that in muscle, a tissue totally dependent on calcium for its activation, cAMP-calcium interactions are being found. Many other cAMP mediated systems have contractile elements such as microtubules or microfilaments related to their end responses, such as secretion (67). Investigations of cAMP-calcium interactions in muscle thus have profound implications for the other contractile element systems. The findings of cAMP interaction with glycolysis in muscle brings the similarity full circle, to the first findings of cAMP dependent phosphorylations controlling liver glycolysis.

For the uterus and other smooth muscles actions of agonists and

antagonists on cAMP and calcium regulatory systems provide a model for explaining interactions of these active substances that was never before possible.

The most clear-cut case of cAMP involvement in uterine activity is with exposure to beta adrenergenic agents such as isoproterenol. Beta adrenergic effectors cause a clear increase in cAMP and this increase is related to the relaxing effect of these agents (60,69,70,71,72). The degree of relaxation and amount of increase in cAMP are related to the dose of isoproterenol (60). The order of potency of isoproterenol, adrenaline and noradrenaline is the same for the increase in cAMP as for the relaxing effect (73). Both the increase in the cAMP and the relaxing effect can be blocked with the beta blocking agent propranolol (71). The increase in cAMP, which occurs in other smooth muscle as well with beta adrenergic stimulation, is thought to be brought about by activation of adenylate cyclase, since phosphodiesterase is unaffected (60). The increase takes place even in CA^{++} poor muscle, so the action is not secondary to a change in calcium (60). Exogenous dibutryl cAMP causes a relaxation (60). With isoproterenol administration, the increase in cAMP is very rapid while the relaxation effect takes about 45 seconds (60). Isoproterenol increases the Ca^{++} binding of microsomal fractions, which is probably composed of sarcoplasmic reticulum (60). cAMP will also increase the Ca^{++} binding of microsomal fractions (60). Recently, it has been shown that cAMP causes a migration of the catalytic subunit of a cAMP dependent protein kinase to the microsomes (74).

The active kinase in the microsomes phosphorylates a protein which is thought to be responsible for calcium binding in the calcium pump (74). This would obviously account for the calcium sequestration and the relaxation.

An interesting corollary to the resequestration of calcium by the cAMP dependent beta adrenergic system is that oxytocin or other induced contractions subsequent to isoproterenol relaxation are more forceful (60). The hypothesis is that more calcium has been sequestered so that more can be released. This is the same mechanism which occurs in the heart, where beta adrenergic agents have a positive inotropic effect.

Another consequence of the increase in cAMP subsequent to beta adrenergic stimulation is an increase in phosphorylase a and the ensuing increase in glycolysis, generating ATP (60,75). This is the same cascade reaction seen in the liver with glucagon administration (76). In skeletal muscle during contraction calcium causes an activation of phosphorylase b kinase leading to ATP production (60). This probably occurs in smooth muscle as well during contraction. Both contraction and relaxation are energy-requiring events; isoproterenol relaxation will not occur in carbohydrate poor muscle (60). The cAMP and calcium dependent phosphorylase cascade thus stimulates glycolysis for contraction, where calcium is the main mediator, or relaxation, where cAMP is the main mediator.

The uterine stimulants oxytocin, prostaglandin E_2 and prostaglandin

$F_{2\alpha}$ inhibit the isoproterenol induced increase in cAMP (71). Oxytocin or acetylcholine induced contractions can be inhibited by isoproterenol, theophylline, or dibutryl cAMP (60). With stimulation by angiotensin II there is a contraction and no change in level of cAMP (72). With simultaneous administration of angiotensin II and adrenalin there is an increase in cAMP, but less than with adrenalin alone (72). There is a contraction, but the tension is less than with angiotensin II alone (74). These various uterine stimulant-cAMP interactions show that cAMP can act by causing a resequestration of calcium to interfere with or inhibit excitation-contraction coupling.

With uterine stimulants acting alone, the cAMP interaction is elusive. Many workers have documented that there is no increase in cAMP with oxytocin, angiotensin or electrical stimulation (60,72,77). Some workers have found a decrease in cAMP with oxytocin (69,70), as well as alpha adrenergic (69) stimulation of the rat uterus.

In other types of smooth muscle various pharmacological agents cause increases or decreases in cAMP (for review see 60.) Some of these changes in cAMP are thought to be brought about by calcium dependent inhibition or, less usually, a stimulation of phosphodiesterase. These may be opposing parts of the same mechanism (60). The theory for some of these actions is that calcium inhibits phosphodiesterase; there is a subsequent rise in cAMP which acts to bring about a resequestration of the calcium. This feedback mechanism would serve to turn a contraction off after a certain period of time (60). This may be the origin of the characteristic slow rhythm of contractions and relaxations seen in

smooth muscle.

Cyclic guanosin monophosphate, cGMP, levels are now also known to change in a variety of systems (78,79,80). In many of these systems changes in cGMP and cAMP levels are associated with opposing responses (78,79,80). In most of these systems, such as various smooth muscles, agents which increase cytoplasmic calcium also cause an increase in cGMP, and that increase is dependent on the presence of calcium (79). It thus appears that cytoplasmic calcium is the activator for guanylate cyclase (80).

Specifically, in the estrogen dominated rat uterus, oxytocin, serotonin, prostaglandin $F_{2\alpha}$ and methacholine, which all stimulate the uterus, all cause a two and one-half to fivefold increase in cGMP within forty-five seconds (80). In these conditions there is usually no change in cAMP (80). The role of cAMP in tissues where its level is found to change on stimulation is still unclear. The cAMP might be a mediator for further effects, perhaps via a cAMP dependent kinase. It may act only as a negative feedback system to restore calcium to prestimulus levels (79); cGMP could affect phosphodiesterase, thus causing a change in cAMP levels (80).

In summary then, in the rat uterus beta adrenergic stimulation leads to an increase in cAMP which causes a sequestration of calcium and relaxation. With stimulation by oxytocin and other uterine stimulants cytoplasmic calcium increases and at least with some of the stimulants, a fall in cAMP has been documented.

f. Initiation of parturition

Although the initiation of parturition has little to do with the copper IUD, a brief look at the mechanisms that control the uterus to initiate labor will afford a dynamic view of basic uterine function.

As we have seen, there are many humoral factors which influence uterine activity. Changes in various of these hormones have been implicated in different theories of the control of parturition. The activity of the fetal adrenals, especially in sheep, affects the onset of labor. Destruction of sheep fetal pituitary prolongs gestation (81) and administration of glucocorticoids to sheep induces premature parturition (82) Anencephaly, which leads to adrenal hypoplasia, leads to prolonged pregnancy in humans if there is no abnormal increase in volume of the uterine contents (83). Conversely, prematurity has been associated with high fetal adrenal weight (84). Although the corticosteroids have profound effects, especially in sheep, they are probably not controlling factors in humans since after fetal death, delivery occurs spontaneously after the demise of the fetus (85). It has been suggested that the fetal adrenal exerts its effect by altering the concentration of progesterone affecting the myometrium (86).

Although estrogen and progesterone have the dramatic effects discussed above on myometrial electrical and mechanical activity, the role that changes in these two hormones play in the onset of parturition has been a source of controversy. Estrogen levels reach a peak at parturition (87), and rats ovariectomized late in pregnancy fail to deliver

without estrogen replacement (88). Because the increase in estrogens is gradual over most of gestation its role in parturition is probably permissive rather than controlling. (See discussion of estrogen-prostaglandin interactions towards the end of this section.)

Progesterone, the "hormone of pregnancy" maintains the uterus in a quiescent state. The degree to which progesterone controls uterine activity has been disputed, primarily because administering progesterone during threatened abortion is not dramatically effective (89). Progesterone will, however, delay induced abortion (90) and recent evidence shows that progesterone levels do start to fall with the onset of labor (91).

Oxytocin is released from the pituitary during delivery by neural reflex from mechanical stimulation of the uterus, cervix, and vagina (92,93). The sensitivity of the uterus to oxytocin is dependent on estrogens and progesterone, the response to oxytocin is especially low in a progesterone dominated uterus (94). Oxytocin levels during labor, however, are not consistently high, and labor will still progress when the nerves of the afferent pathway of the oxytocin release reflex are cut (31). Oxytocin probably speeds labor if it is released, but is not essential for the onset of labor (95).

Prostaglandins, 20 carbon fatty acid derivatives with a cyclopentone ring, and a hydroxyl group on carbon 15, are found practically throughout the body and are biologically active in a myriad of processes (96). They have a particularly profound involvement in uterine and other

smooth muscle physiology. Prostaglandin $F_{2\alpha}$ is a strong uterine stimulant (97). It is found in amniotic fluid in increased levels during labor (98). Recently, prostaglandin $F_{2\alpha}$ was shown to be synthesized and released from the myometrium during stretching of the muscle (99). Prostaglandin synthesis is a prerequisite for normal uterine activity in that inhibitors of prostaglandin synthesis such as indomethacin block oxytocin induced contractions and delay or prolong parturition (100,101,102, 103). Prostaglandins, like oxytocin, are less active on the progesterone dominated uterus (104). Prostaglandins increase the 20α hydroxylation of progesterone at least in rat ovarian tissue (105); thus they may effectively decrease the concentration of the hormone.

An attractive theory has been presented (106) which can accommodate many of the older theories on the onset of parturition. The activity of the uterus is basically determined by the prostaglandin to progesterone ratio. Before labor progesterone predominates, the uterus is inactive and there is no prostaglandin synthesis. Any of a number of factors, or a combination of various factors can start the production of prostaglandins. Stretch of the uterus is probably the main factor, and has been shown to be critical and the determining step in induced abortion and induced labor (107). Fetal distress and the fetal adrenals could play a role via the production of corticosteroids since these have been shown to increase placental production of prostaglandins, at least in sheep (108). With the production of prostaglandins in the myometrium and/or placenta a positive feedback cycle commences. First, the prosta-

glandins cause contractions of the uterus and subsequent stretching of other areas of the muscle resulting in more stretch-induced prostaglandin production. In addition another positive feedback cycle may occur involving prostaglandin and estrogen production (109). In vitro estrogen inhibits the placental breakdown of some prostaglandins (110), and prostaglandins stimulate the placental production of estrogen (111). This could explain the estrogen requirement for the initiation of labor in rats mentioned before (88).

From a systems analysis point of view previous theories had a profound deficiency. Each theory stated that a specific parameter, either progesterone, estrogen, corticosteroids, oxytocin, oxygen, etc. changed, and that this brought about labor. Critics of each theory argued correctly, however, that the small change found in the level of the parameter in question (progesterone, estrogen...) was not sufficient to cause the effect seen--i.e., labor. None of the theories had any provision for the amplification or gain required so that a small change in one parameter would end up having a large effect. The progesterone-prostaglandin positive feedback theory provides this gain. The positive feedback systems not only make the theory tenable, but because older theories can be incorporated into it, it is particularly attractive. This discussion becomes important with regard to the copper IUD because, as we will present later, copper can increase the production of prostaglandins. (See Discussion, section IV D and IV G.)

g. Inert intrauterine devices and myometrial activity

Central to any investigation of the effect of copper intrauterine devices on uterine activity is the question of whether an inert IUD affects the myometrium. Although, as we shall see this question is not completely resolved, it is obvious that when an IUD is expelled the expulsion is the result of myometrial contractions, whatever the cause of the contractions is.

In non-pregnant women through the menstrual cycle, uterine activity is highest during menstruation. On day 2 rhythmic contractions average 75 mm Hg pressure with a frequency of less than one per minute (112,113). After menstruation the active pressure decreases drastically and the frequency increases. This midcycle pattern of small (10 mm Hg) irregular contractions of high frequency, most pronounced at midcycle, continues until 1 to 2 days before the onset of menstruation. At this time the activity evolves again to the high activity of menstruation. (For a detailed discussion of uterine activity measurement and physiology see 112,113).

An intrauterine thread increases uterine activity in rats when measured <u>in vitro</u> on day 4 of pregnancy after insertion before mating (114). This is reminiscent of stretch induced uterine stimulation discussed before. In sheep as well, uterine activity is increased by an IUD (115). In this species IUD's cause alterations of ovarian function via a "uterine luteolytic factor" (116).

In rhesus monkeys studies of intrauterine pressure are difficult due to the highly convoluted cervix of this species. Indirect measurements with a strain gauge on the surface of the uterus (117) or based on recovery of ova (117) indicate that there is no profound effect of the IUD on uterine motility. These methods however are obviously not very sensitive.

In women, different methods of recording activity have lead to divergent findings on the effect of IUD's on intrauterine pressure. When intrauterine pressure is measured with a strain gauge fitted to the intrauterine device itself no long-term difference is found in uterine activity as compared to controls without IUD's. There is however a stimulation of activity for a short time after initial insertion of the IUD (116,118). With an open catheter technique of measuring intrauterine pressure, others (119) have found "prelabor-like" activity around the time implantation would be expected. This high activity is thus found about 5 to 6 days earlier than the normal rise of activity seen premenstrually in women without IUD's (119,120). These authors hypothesize that this increase in activity inhibits implantation. The increase in activity could be due to the increased production of pros-

taglandins which is thought to occur with IUD's (121,122,123). More recent findings on prostaglandins and IUD's related to the work of this thesis have strong implications on the effect of inert and copper IUD's on uterine motility as well as on their contraceptive mode of action. These will be presented in the discussion of Part I of the thesis.

C. The Copper Intrauterine Device and Its Mode of Action

In the following four sections we first present some clinical aspects of the Cu-IUD. The contraceptive action and side effects of the Cu-IUD will then be approached from the pharmacological and biochemical points of view. Then we consider the corrosion chemistry of copper as it relates to the Cu-IUD. We have recently presented this material in more detail (124,125).

1. Development and clinical aspects of the Cu-IUD

In 1969, Zipper et al. found that copper wire when placed in the uterus of the rabbit has a contraceptive effect (126). This discovery was applied to humans (127). A plastic T-shaped device with copper wire wound around its stem had an increased contraceptive effect in women compared to the "T" without copper. For plastic IUD's the greater the size, that is, the greater the endometrial surface contacted, the lower the pregnancy rate but the greater the incidence of pain and bleeding (128,129). Bleeding induced by IUD's averages more than twice that of normal menstrual bleeding (130). Pain and bleeding, along with spontaneous expulsion and infection, are the main reasons for discontinua-

tion of use of IUD's (129,130). Adding copper to the plastic IUD permits the use of a small device thereby reducing the incidence of pain and bleeding. Because of its small size, the Cu-IUD can be used for nulliparous women, perhaps the most important advantage over the other IUD's (129). Adding copper to the device seems to lower the expulsion rate (1,131) and decreases the excessive bleeding associated with IUD's (132,133,139). Clinical studies (135) showed that with a surface area of 200 mm^2 of copper wire the device has a one-year's continuation rate as high as 89% with 1.5% pregnancies while conventional plastic IUD's (e.g., Lippes Loop) have a continuation rate around 75%. On the other hand, more recent studies involving greater numbers of women, the Cu-IUD does not differ appreciably in performance with the Lippes Loop (129). The pregnancy rate for the "T" is inversely proportioned to the surface area of the copper and is 18% with no copper, 5% with 40 mm^2, and 1% with 200 mm^2 (135). The contraceptive efficacy and its low incidence of side effects are comparable to those of oral contraceptive steroids. IUD's have the obvious advantage that once inserted they require no daily attention. The copper wire on the two devices most studied, namely the Cu-T and the Cu-7, lasts for about two years. In a more recent development the wire is replaced with copper sleeves since the wire has a tendency to fragment with time. These newer Cu-IUD's may have a lifespan of from four to five years (135). The original T-shaped Cu-IUD

has been further modified by addition of copper to the arms of the device (135). This brings the metal in closer contact with the fundus of the uterus where implantation usually occurs.

One hazard with any intrauterine device is uterine perforation usually occurring with faulty insertion. The presence in the peritoneal cavity of the inert plastic device may not be of concern but with the Cu-IUD inflammation and adhesions may form around the metal (136). Here, obviously surgical removal of the Cu-IUD is indicated.

When pregnancy occurred with an IUD present it was often the practice to leave the device in place. With the Cu-IUD, however, removal of the device has been advised because of possible teratological effects of copper (see Part II, Sec. IC). So far, only a few cases of births occurred for women who retained the Cu-IUD through pregnancy. The babies appear normal (1, 135).

A side effect of IUD's of increasing concern is infection. In one disease, at least, namely gonorrhea, the Cu-IUD might actually have a therapeutic effect. In vitro, copper has a very powerful gonococcicidal effect (137). A Cu-IUD will not protect the wearer from gonorrheal infection (138). On the other hand, copper could prevent the spread of the disease to the uterus. Studies are now under way (139) to determine the incidence of pelvic inflammatory disease in women wearing IUD's (with and without copper) who contract gonorrhea.

2. Nature of the contraceptive action

The mechanism of the contraceptive action of the plastic IUD is un-

clear (130). As for the Cu-IUD, one can consider three factors, namely 1) the action of an inert foreign body in the uterus, 2) the effect of chemical reactions taking place during the dissolution of the metal and 3) the effect of the released metal ions. The Cu-IUD is only effective locally. In rats and rabbits, where the uteri are bicornate, copper inserted in one horn has no contraceptive effect on the other horn (126, 140). When the lumen of the two horns of the rabbit (141) or of the rat (142) are connected surgically a copper wire in one horn prevents pregnancy in both horns.

It has long been known that copper prevents fertilization of sea urchin eggs (143). Cupric salts at 2×10^{-5}M concentration stop fertilization, and at 2×10^{-4}M stops cleavage of the fertilized egg in sea water. For laboratory animals, the metal does not affect fertilization *per se*. Fertilized eggs recovered from rats (144) with copper wires *in utero*, on transplantation to recipient control animals, develop normally. If recipient rats have copper wires *in utero* and the donors do not, implantation does not occur (144). Although copper metal has some spermicidal action (145), and copper salts inhibit the lysis of the semen coagulum (146), the fact that fertilization takes place in rabbits with copper wires shows that neither of these phenomena is an important mechanism for the contraceptive action of the Cu-IUD. Copper metal will kill mouse blastocysts *in vitro* (147) but here again this may not be important *in vivo* judging from the transplantation experiments. On the other hand, in these transplantation experiments the blastocysts

Fertilized eggs have also been recovered from rabbits with intrauterine copper (141).

might not have been exposed long enough to the metal or might not have been in a sensitive stage. In any case, all animal experiments show that with the copper metal implantation does not occur (148).

Plastic IUD's cause an infiltration of polymorphonuclear leucocytes, characteristic of inflammation, into the uterine lumen in humans (130, 149). With the Cu-IUD this effect is considerably more pronounced (150). It is also known that cupric salts increase the inflammation infiltrate around a scratch on the skin (151). These facts favor the hypothesis that the contraceptive action of IUD's is a result of a sterile inflammation which is enhanced by copper. It is of interest that when indomethacin, an anti-inflammatory agent, is given to animals with IUD's at least in some cases the contraceptive action of the IUD is lost (152) (see Discussion, Part I. sec. IV F).

With the 200 mm^2 Cu-IUD in women, the metal is gradually dissolved at a rate of 50 micrograms per day (153) and appears as cupric ions, free or complexed. Tracer studies in rats show that a certain amount of copper from the wire inserted <u>in utero</u> exchanges with systemic copper and appears throughout the body (154). In women the Cu-IUD causes no measurable increase in serum copper (153) and in animals, no increase in copper in a variety of tissues (155,156). The copper levels in the endometrium as well as in the endometrial aspirants and in cervical mucus are increased with the Cu-IUD and are of the order of 10^{-5}M cupric ion (153,157). Menstrual blood collected from women wearing the Cu-IUD

contains about half of the total copper lost from the device during one month (158). Even if all of the remaining copper released became systemic it would only amount to 1% increase in the serum copper on a daily basis assuming that copper is not accumulated in the blood.

Any contribution by the Cu-IUD to copper levels in the blood would be difficult to follow because of fluctuations in serum copper in normals as discussed above.

The copper released daily from the IUD is equal to about 1% of the copper taken orally per day (159). On the other hand, the route of administration of copper can be important. It has long been known that intravenous injection of copper salts will cause ovulation in rabbits or pseudopregnancy in rats, as will be discussed in Part II, sec. LB3. No observations on animals or women indicate that the Cu-IUD acts at the hypothalamic level. Ovulation is normal and it appears that the contraceptive action is entirely within the uterus.

3. Effect of the Cu-IUD on the endometrium

The Cu-IUD causes biochemical changes in the endometrium. Thus, in women with the device alpha amylase activity is depressed and the normal cyclic increase in glycogen synthetase is eliminated (160). The changes in endometrial enzyme levels in both the proliferative and secretory phases of conventional and copper IUD's have been recently reviewed in detail (161). In vitro, cupric salts are inhibitors of certain enzymes but only at high concentrations (greater than about 10^{-4}M Cu^{++}). At these high concentrations the cupric ion causes precipitation of many

proteins, but at the low concentrations associated with the Cu-IUD, namely 10^{-5}M Cu^{++} or less (153,157), inhibition of activity for most enzymes does not occur. As is shown below, copper metal can inhibit enzyme activity even though the cupric ions produced by the metal are at very low concentrations.

The endometrium in the vicinity of a copper wire placed in the rat uterus undergoes histological changes (126). There is intense proliferation in the mucosal stroma, especially where the endometrium touches the metal; such changes are characteristic of a local estrogenic effect. Consistent with this observation is the finding (162,163) that estrogen uptake, presumably by the specific estrogen-binding protein, is greater in the rat uterine horn containing the copper wire than that of the control having a nylon IUD or none at all. The fact that the inert IUD causes no increase in estrogen binding, while the Cu-IUD does, demonstrates a qualitative difference in the actions of the inert and the Cu-IUD. The measurable differences in uptake of estrogen occur one week after insertion of the copper wire (162) but the contraceptive effect occurs within twelve hours (148). In women, however, no endometrial proliferation has been observed with the Cu-IUD (164).

For humans, there seems to be no influence of the Cu-IUD on endometrial DNA (157), while RNA levels are decreased (165). In rats, the incorporation of labeled thymidine into endometrial DNA is inhibited by copper metal (161,166).

In the uterus the incorporation of sulphate into mucoproteins is de-

creased by conventional IUD's but much more so with the Cu-IUD (161, 166). Copper also changes the fucose-sialic acid ratio of glycoproteins (165). Thus, copper may suppress the synthesis of mucins, but copper metal also causes the liquification of mucins (5,6). Both processes might be expected to inhibit implantation.

The effect of copper on uterine muscle, the theme of this thesis, suggests new models for the contraceptive mode of action of the Cu-IUD. These will be presented in the discussion of the thesis.

4. Corrosion chemistry of copper in biological fluids

Although the cupric ions, as produced in the dissolution of the metal, have biological activity, the chemical reactions at the surface of the copper metal may cause more profound or even qualitatively different effects than the same concentration of cupric ions. For example, in serum, cupric sulphate has only a slight inhibitory effect on sperm motility after six hours at 10^{-3}M copper. Incubation with copper metal is spermacidal after four hours even when the cupric ions produced by the metal are less than 10^{-3}M (145). Similarly in the killing of mouse blastocysts the metal is much more effective than the addition of cupric ions alone at a concentration equal to that which the metal would produce over the same time (147). The mode of contraceptive action of the Cu-IUD is probably based on the chemical reactions associated with the dissolution of the metal.

Despite the widespread use of copper over some four thousand years, the science of the corrosion of <u>pure</u> copper is poorly developed. Studies on the corrosive action of sea water on copper have been confined to its

alloys, namely bronzes and brasses (167) but not to the pure metal which is well known to be unstable in contact with saline solutions.

In order to understand the corrosion chemistry of the pure copper in the Cu-IUD, Drs. G. K. Oster and G. Oster undertook studies of the chemical reactions of electrolytically pure copper metal under conditions which simulate the physiological condition (5,6,7).

In solutions of saline and serum albumin a strip of copper 200 mm^2 will dissolve at a rate comparable to that of the Cu-IUD in women (6). Although amino acids (notably glycine and histidine) or proteins which can complex with cupric ions greatly enhance the rate of copper released, the presence of saline is a prime factor in the dissolution of the metal. In distilled water no measurable cupric ions are produced (5,6,7). The dissolution of copper metal in saline requires oxygen. Although cupric salts are the end product in the dissolution of pure copper in saline, cuprous ions are formed as an intermediate. In the absence of oxygen and/or saline no cuprous is formed (7).

Copper metal incubated with a saline solution of a disulphide will cleave the S-S bond when oxygen is present (5,6). With copper metal in saline, serum albumin undergoes a conformational change as indicated by alteration in its solubility and availability of sulphydryl groups (5).

Incubation of uterine aspirant with copper metal causes liquification of the viscous mucoid (5,6). Cervical mucus also liquifies when incubated with metallic copper (5,6,168). The liquification ef-

fect may involve conformational changes of the albumin component of the viscous albumin-mucopolysaccharide complex, thereby weakening the interaction of the albumin with the sialic acid side groups of the polysaccharide. Carbonic anhydrase and alkaline phosphatase, enzymes thought to be important in the implantation process, are inactivated on incubation with copper metal in saline (6). Cupric sulphate at a concentration equal to the cupric concentration produced by the dissolution of the metal during the incubation period does not inhibit these enzymes. The liquification of mucins and the cleavage of disulphide bonds does not occur even at high concentrations of cupric ion. Cuprous salts stabilized by chloride ions will cleave disulphide bonds and produce all the above biochemical changes which copper metal causes (7).

Copper metal incubated in saline in the presence of oxygen produces free radicals (7). This system initiates the free radical polymerization of acrylamide. The free radicals produced on the dissolution of metallic copper will convert benzene into phenol (7). The reaction does not proceed in the absence of saline or of oxygen. For a 200 mm^2 sheet of copper metal 28 micromoles of phenol are produced in the first hour.

The polymerization of acrylamide and the conversion of benzene to phenol can also be carried out with freshly prepared cuprous chloride (7). Cuprous ions are formed in the dissolution of the metal and are complexed with chloride ions. Free radicals are formed in the autoxidation of cuprous ions. The overall picture is that on the surface of

pure copper metal in the presence of oxygen a layer of cuprous oxide is formed; the reducing action of the metal keeps the oxide as cuprous. In contact with saline the well known water-soluble cuprous chloride complex ($CuCl_2$) is formed and diffuses away from the metal. The oxygen dissolved in this aqueous medium rapidly oxidizes the cuprous complex to yield cupric ion and free radicals. The free radicals may be hydroxyl radicals or, as has been recently proposed for many biological autoxidations, superoxide free radicals (169). If there is a paucity of substrate with which the radicals can react, the radicals will combine with each other, especially in the presence of superoxide dismutase, to form hydrogen peroxide. The hydrogen peroxide will not accumulate, however, because of the presence of cupric ions. When cupric ions are added to hydrogen peroxide, cuprous ions are formed and oxygen is evolved. From these considerations it appears that copper metal in saline is an efficient source of free radicals. Ultimately, it is probably the free radicals formed by the metal which are responsible for the contraceptive action of the Cu-IUD.

The rate of production of free radicals by copper metal in saline is prodigious (7). It is equivalent to that produced by 14,000 rads of X-rays per hour (7). The significance of this high initial rate of free radical production by metallic copper to the long-term safety of the Cu-IUD is unclear. There are many substances, notably naturally occurring reducing compounds, in the endometrial fluid which could consume free radicals without deleterious effect. In addition, intrauterine

copper stimulates the activity of catalase in the rat endometrium in excess of that produced by a nylon thread (170). A direct comparison of the copper production of free radicals with that for ionizing radiation may not be justified since in the latter free radicals can be generated intracellularly, whereas with the Cu-IUD one might expect the free radicals to be produced extracellularly. On the other hand, fine copper metal fragments have been found around the Cu-IUD in the endometrial fluid (171), and these could conceivably enter cells. Still further, the cuprous chloride produced might diffuse into cells and likewise on autoxidation form free radicals intracellularly. There is no evidence linking endometrial cancer with use of the Cu-IUD (135,171) or, for that matter for any IUD (128).

II MATERIALS AND METHODS

A. THE IN VITRO RAT ISOMETRIC UTERINE TENSION ASSAY

Estrous rats were used for the study, the uterine assay being most sensitive at this time (172). Their selection was based on vaginal smears (173).

Virgin Sprague-Dowley rats, 150-175 gm (from Marland Farms), were kept under 14-hour light and 10-hour dark cycles at 22°C with free access to water and Purina Lab Chow. The rats were sacrificed by decapitation and their uteri quickly removed and placed in van Dyke-Hastings solution (see below). Adipose tissue and mesometrium were carefully removed, and a silk 000 thread attached to each end of the uterine horn, with a loop tied in the bottom thread. In each case, we measured one uterine horn with the contralateral horn serving as the control. Each uterine horn was hung in a 10 ml muscle bath container (Metro Scientific, Farmingdale, NY) with a constant temperature (32.5°C) water circulation. A gas mixture of 95% oxygen and 5% carbon dioxide provided constant bubbling of the bathing solution.

Isometric rather than isotonic tension was measured because with

isotonic recording a partial stimulation can lead to a full contraction (174). To measure isometric tension, the top thread was attached to a force displacement transducer (Grass FT03c) without spring, connected to a polygraph recorder (Grass, model 7) and a DC amplifier (Grass). Resting tension of 1 gm was kept on the uterus by adjusting length with a thumb screw. The recorder was calibrated daily so that a 1 cm deflection equals 2 gm tension.

The van Dyke-Hastings solution consists of: 115.0 mM NaCl; 6.2 mM KCl; 0.5 mM $CaCl_2$; 30.0 mM $NaHCO_3$; 0.8 mM Na_2HPO_4; 0.2 mM NaH_2PO_4 (all salts were analytical grade, Fisher Scientific). The pH was 7.0-7.3. The low temperature and the low calcium ion concentration of the medium eliminated spontaneous contractions of the rat uterus (175).

Cupric ions are precipitated by the phosphate and carbonate ions of the van Dyke-Hastings solution but only at cupric concentrations exceeding 10^{-4}M. Because it was found that copper sulphate induced uterine contractions at concentrations of 10^{-6}-10^{-5}M, this precipitation was not of serious consequence. We deliberately avoided the use of Tham (tris hydroxymethyl amino methane) buffer since we found that it complexes cupric ions.

All compounds being tested for their action on the uterus were first dissolved in 0.9% NaCl.

The oxytocin standard was the synthetic nonapeptide (176)(provided by the Department of Physiology and Biophysics, the Mount Sinai School of Medicine). This oxytocin at a concentration 1 x 10^{-9} molar has an oxytocin

activity equivalent to 0.5×10^{-6} milliunits/ml, i.e., 1 mg is equivalent to 500 units.

The oxytocin analogue, 2-alanine-deamino-dicarbaoxytocin (177), which acts as a competitive inhibitor to oxytocin, was similarly obtained. Other compounds tested included cupric sulfate ($CuSO_4$), cupric chloride ($CuCl_2$), Barium chloride ($BaCl_2$) and sodium sulphate (Na_2SO_4) (all were analytical grade, Fisher Scientific), copper wire (0.020" electrolytically pure, Baker Chemical), ascorbic acid (Fisher Scientific), ouabain (Lilly), theophylline (Mallinckrodt), indomethacin (Merck, Sharp and Dohme). The copper chelating agent, diethyldithiocarbamate (sodium salt, Fisher Scientific) was used both as a test substance as well as for the colorimetric determination of copper (optical density at 466 nm - 0.2 for 2×10^{-5} molar Cu^{++} and linear up to 5×10^{-5} molar) (6).

The dose-response curve was obtained by adding increasing amounts of the test solution (without washing out) in the 1/4 log 10 increment cumulative dose-response procedure of van Rossum (178). Incremental amounts of test solution were added as follows: for example, 0.1 ml of 10^{-8}M into the 10 ml bath, then 0.08 ml of 10^{-8}M, then 0.14 ml of 10^{-8}M; then 0.24 of 10^{-8}M, then 0.44 of 10^{-8}M, then 0.08 of 10^{-7}M, etc. This results in 1/4 log 10 increments in final concentrations in the 10 ml bath of, respectively, 1.0×10^{-10}M, 1.8×10^{-10}, 3.2×10^{-10}M, 5.6×10^{-10}, 1.0×10^{-9}M, etc. The volume of the bath was maintained at 10 ml by an overflow drain.

B. MODIFICATIONS

In contrast to the experiments where test substances were added to the bath, a few experiments were carried out adding the test substance to the lumen of the uterus. As shown in Figure 1, a thin polyethylene tube (PE50 Intramedic, Clay Adams) was inserted into the top and bottom of the uterine horn. The top and bottom of the uterus were tied securely with a thread, sealing the system. The test solutions were injected slowly into the bottom of the tube and allowed to drain from the tube at the top, which was left open. The open end of the top tube was secured to the bath container at 3 cm below the bathing solution level. In this way, the injected test solutions would drain easily and not subject the horn to undue stretch, which by itself causes contractions. The tubes were attached so as not to interfere with the measurement of tension.

C. COPPER METAL IN THE UTERINE TENSION ASSAY

Experiments were also conducted with a piece of copper wire in the uterine lumen. A copper wire, 2.5 cm long, was placed in the lumen of one uterine horn, and the horn suspended in the bath as before. The

Fig. 1. Apparatus for measuring uterine tension, showing polyethylene tubes for administering test substances into the lumen of the uterus.

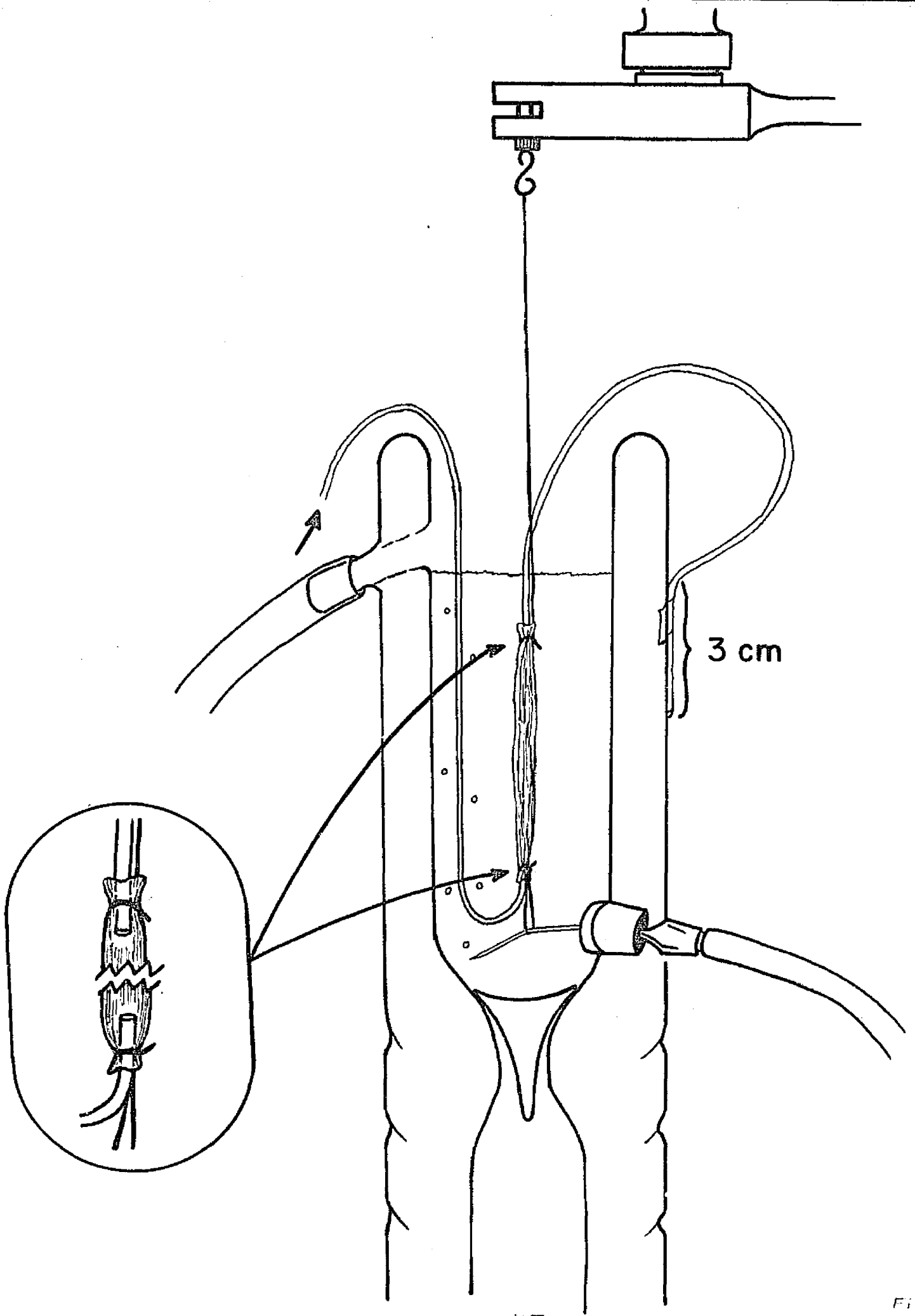


Fig. 1

control horn had a similar piece of copper wire, but the wire was encased in polyethylene. The encasing of the wire was made by placing it in a polyethylene catheter (PE 50-Intramedic), sealing the ends with heat, and carefully smoothing the ends so as to leave no rough edges.

D. ELECTRICAL STIMULATION OF THE ESTROUS RAT AND POSTPARTUM RABBIT UTERUS

A small number of uterine tension measurements were made using electrical stimulation according to the method of Csapo (179):

Estrous rat uteri are removed as described above, or alternately, postpartum rabbit uterine strips are used. The rabbit is sacrificed by a blow on the back of the head and sections of the uteri are quickly removed. Strips are cut about 3 cm long, 0.5 cm wide from the uterus on the side opposite to the site of placental attachments. The rat uteri or rabbit uterine strips are placed in a muscle bath consisting of glass tube of 10 ml capacity, with rubber stopper in the bottom end. Through the stopper are a polyethylene tube for changing the bathing solution and an 18 gauge needle bent in a hook which serves to anchor the bottom of the uterus or uterine strip and through which 95% O_2 - 5% CO_2 is bubbled through the bathing solution. In the case of the rat uterus the top of the uterus is attached to a thread which in turn is attached to a tension transducter and Grass recorder as noted above. With the rabbit uterine strips, the top of the strip is held with a platinum

hook connected to a fine platinum chain which in turn connects to a Grass FTO3C transductor with spring and to a Grass Model 5 polygraph recorder with low level DC preamplifier Model 5PI and driver amplifier Model 5E: with this set up, 1 cm deflection--10 gm tension.

Electrical stimulation is via 2 ring electrodes of platinum wire formed into 1.2 cm-diameter circles. The rings are held by an insulated wire support keeping the rings parallel to each other and 5 cm apart. The electrodes are connected to a tandem recycling timer stimulator (Industrial Timer Corp. Model CU-A) and rheostat (Powerstat, type 116B, Superior Electric Co.). Stimulation was from 10-14 volts (as described below), 60 cycles per sec, A.C. for 4 sec once per minute. This technique is described elsewhere (174).

The bathing solution is mammalian Krebs solution consisting of the following final salt concentrations in g/L: NaCl, 6.87; KCl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.14; $CaCl_2$, 0.28; NaH_2PO_4, 0.14; $NaHCO_3$, 2.1; glucose, 2.0 (180).

As with the rat uterine assay, calcium is low to prevent spontaneous contractions.

E. INTRAUTERINE PRESSURE MEASUREMENTS

In vivo recording of intrauterine pressure (IUP) was carried out by the microballoon technique, previously used in non-anesthetized patients (181), rabbits (182) and rats (183). The pressure "sensor" was a sau-

sage-shaped rubber balloon of 0.8 ml capacity, when unstretched. The balloon was fastened on a polyethylene catheter (O.D. 0.06") by loops of silk and filled with distilled water. Several holes, placed around the inside tip of the catheter, insured free fluid communication between balloon and catheter. Before placement the recording system was evacuated and the catheter was subsequently sealed with heat.

The sterile (air free) pressure sensor was placed inside the uterus through a small slit at the ovarian end, so as to cause a minimum disturbance in normal physiology. The slit was closed by a "purse string" suture. Before closing the abdominal layers, the external end of the catheter was passed through the peritoneum and through a tunnel, prepared under the skin, with an exit at the neck of the animal. After the filling of the balloon, the tip of the catheter was again sealed by heat. The exteriorized end of the catheter being accessible, the IUP could be recorded sequentially for several days, without anesthesia. During the recording, the animals were restrained in movement by a plexiglass cage of appropriate dimensions.

The IUP was measured every day (by a Sanborn transducer #267, and recorder #321) as long as the condition of the animal indicated good general health and the design of the study demanded it. In each recording session, both spontaneous activity, oxytocin response and response to intrauterine copper sulfate were measured, for periods of 20 minutes each. The uterine response to a single i.m. dose of 100 mU

oxytocin constituted the standard oxytocin response test. Copper sulfate solutions (in saline) were introduced into the lumen of the uterus via a catheter parallel to the sensing balloon catheter, but open to the uterus at its end. In between recording sessions, the balloon volume was kept constant (at $0.8^{\wedge}_{ml.}$) by sealing the external end of the catheter. In terminating the study, the animals were sacrificed, the balloon, the catheter, and the abdominal and uterine cavities examined, so as to recognize artifacts due to balloon and catheter leakage, infection, adhesion, uterine rupture, etc. Only those studies were considered which were conducted throughout the observation period without the development of any of these artifacts.

III RESULTS

A. COPPER INDUCED CONTRACTIONS

In all uteri from 56 rats where uterine tension was measured *in vitro*, $CuSO_4$ induced uterine contractions at a concentration of between 1×10^{-6}M to 1×10^{-5}M; oxytocin initiated them at 5×10^{-10}M to 1×10^{-8}M. In Figure 2 is shown a typical recording of the time course of uterine contraction as incremental amounts of oxytocin or of copper sulphate are added. The maximum tension achieved by copper was often higher (sometimes by 20%) than that achieved by oxytocin. The contractions induced by copper tended to be more sustained than those of oxytocin, as seen in Figure 2. After doses of copper slightly higher than threshold, there was a sustained contraction of the uterus, whereas with oxytocin this condition was achieved only with doses far above the threshold value. Figure 3 shows representative dose-response curves for copper and for oxytocin from records such as those in Figure 2. As also seen in Figure 2, copper-induced contractions persisted even after multiple rinsings with fresh van Dyke-Hastings solution. These contractions often persisted as long as a half-hour. Oxytocin-induced

Fig. 2. Tracings from typical recordings of uterine tension. A. oxytocin, B. $CuSO_4$. Arrows indicate doses of agonist. Dose for first contraction oxytocin: 1×10^{-9}M, copper ion: 5×10^{-6}M.

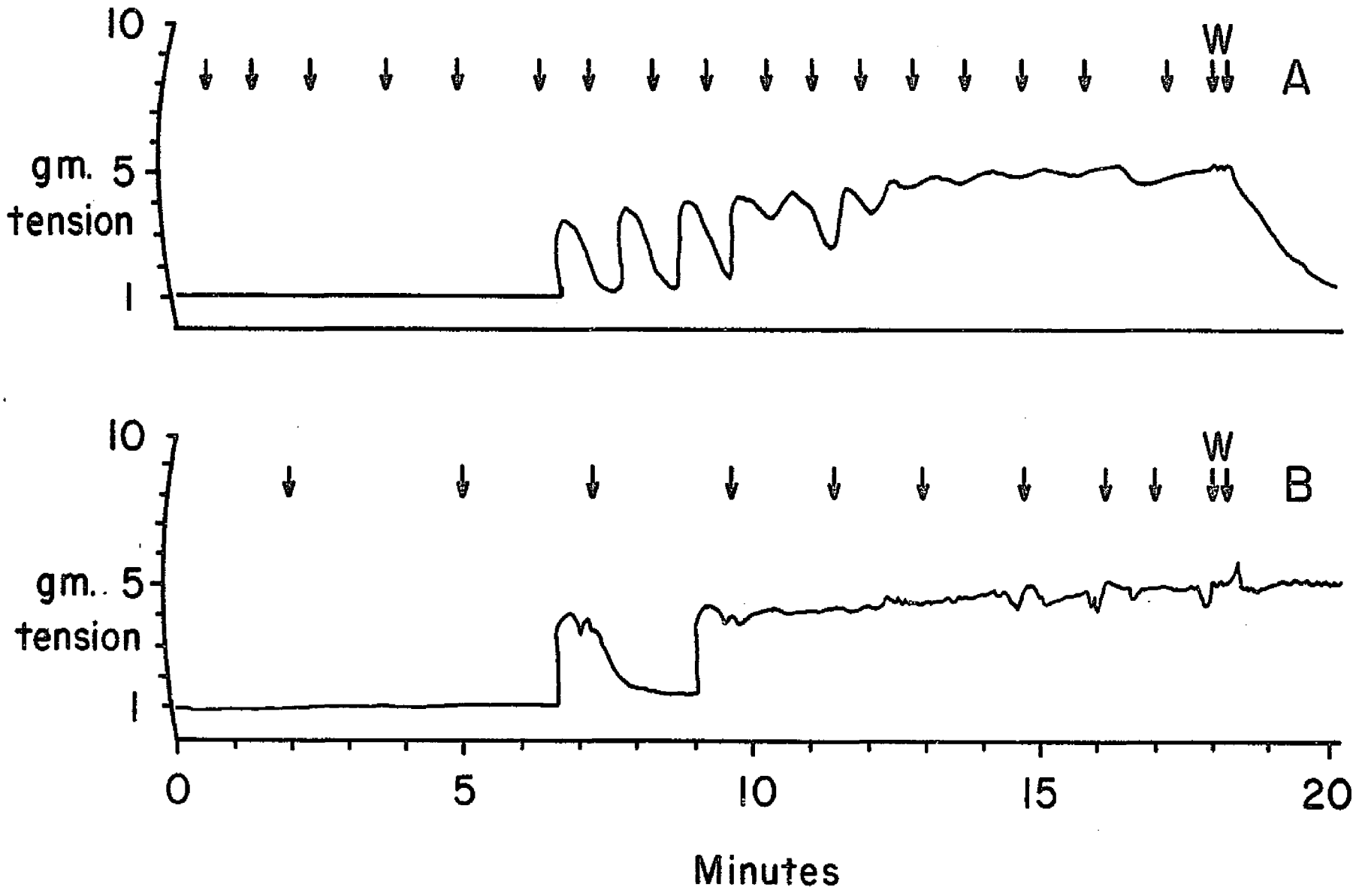


Fig.2

Fig. 3. Typical dose-response curves for oxytocin (circles) and $CuSO_4$ (triangles) induced contractions.

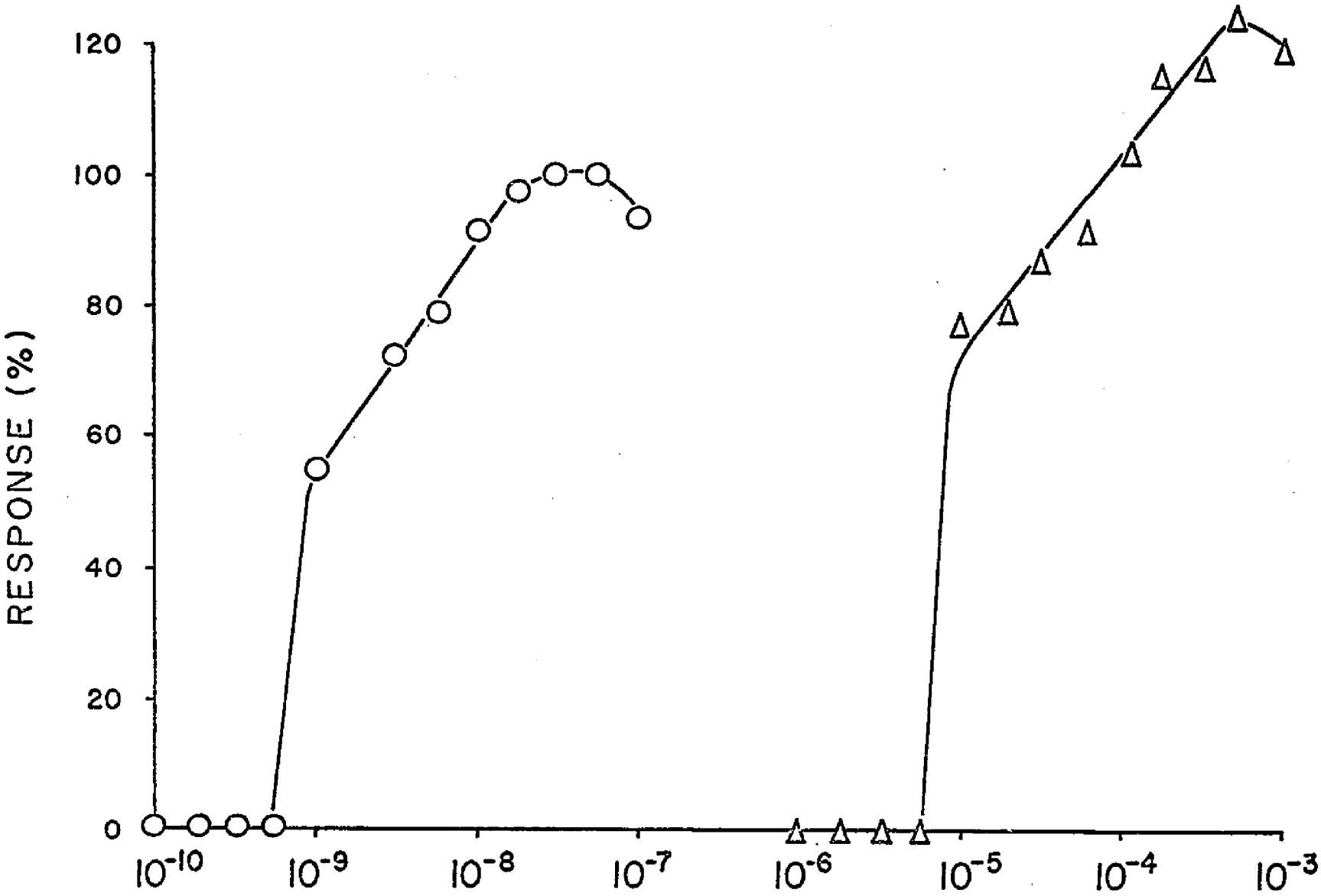

120
100
80
60
40
20
0
RESPONSE (%)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
DOSE (MOLES/LITER)

Fig.3

contractions disappeared within minutes after rinsing.

The $CuSO_4$ induced contractions are caused by the cupric ion; cupric chloride is equally effective, and sodium sulfate has no effect.

By contrast, barium, also known to cause uterine contractions (see uterine pharmacology, section I B 2 d) was active starting only at concentrations above 5.6×10^{-5}M. Potassium induced depolarization and contracture occurred at 10^{-3}M KCl.

B. COPPER INDUCED INHIBITION

When uteri (from six animals tested) were exposed to $CuSO_4$ at higher concentration (10^{-4}M) for 1 hour, they became less active. The tension dropped to about half of its earlier value for this copper concentration and was sustained despite repeated washings with van Dyke-Hastings solution. The copper-treated uterus was then practically unresponsive to oxytocin. Even at high oxytocin concentrations (10^{-6}M), the tension was only 50% of that which it had been initially. On the other hand, the control horns which had been undergoing the same magnitude of contractions, but induced by oxytocin during the 1-hour period, responded fully. Similarly, control horns which had been kept active by periodic electrical stimulation responded normally to oxytocin. However, the contralateral horns, which had been exposed to prolonged $CuSO_4$ treatment, showed diminished response (Figure 4) (see also sec. J).

Fig. 4. Prolonged electrical stimulation of the uterus in the presence of 10^{-3}M $CuSO_4$ (top) and without $CuSO_4$ (bottom). Isometric tension was measured from strips of post-partum rabbit uterus in Krebs-Ringer's solution, with electrical stimulation (14 v. 60 cycles A.C.) from ring electrodes above and below the strips. Stimulation was once per minute for 4 seconds. Length of this recording is 2 hours 40 minutes.

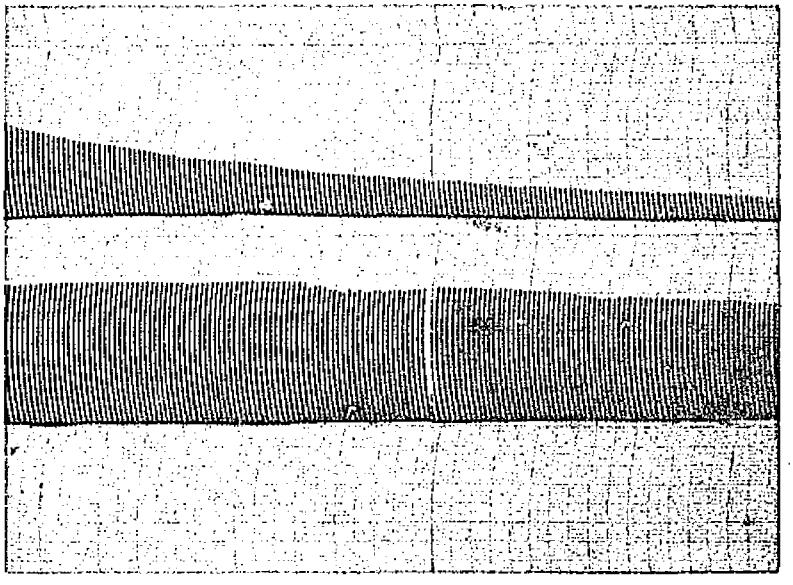


Fig. 4

C. COPPER OXYTOCIN INTERACTIONS

Subthreshold levels of copper and oxytocin applied initially to fresh uteri augmented each other's response. This was shown in 20 repeated experiments with uteri from six animals. A dose of oxytocin just below the threshold, i.e., the dose which would give any response, given together with a dose of copper just below that which (if given alone) would give any response, caused a contraction. However, higher doses of oxytocin and copper, when given together, resulted in contractions only slightly greater than when each was given separately.

The oxytocin analogue (2-alanine-deamino-dicarba-oxytocin), when added to the bath at a final concentration of 5×10^{-6} M did not cause contractions. In the presence of the analogue, a higher dose of oxytocin was required to achieve a given degree of contractions than with oxytocin alone. The analogue was a competitive inhibitor to oxytocin. Thus, the analogue caused the oxytocin dose-response curve to be displaced to the right. On the other hand, when $CuSO_4$ was added in the presence of the analogue, the dose of copper needed for a given contraction was less than that for copper alone. Consequently, the copper dose-response curve was shifted to the left. Typical curves are shown in Figure 5 and the trend in the displacements was seen in four trials.

Fig. 5. Dose-response curves showing the effect of the oxytocin analogue, 2-alanine-deamino-dicarba-oxytocin, on oxytocin induced contractions (left) and on $CuSO_4$ induced contractions (right). Oxytocin (opened circles), oxytocin and 2-alanine-deamino-dicarba-oxytocin 10^{-4}M (filled circles), $CuSO_4$ (opened triangles), $CuSO_4$ + 2-alanine-deamino-dicarba-oxytocin (filled triangles).

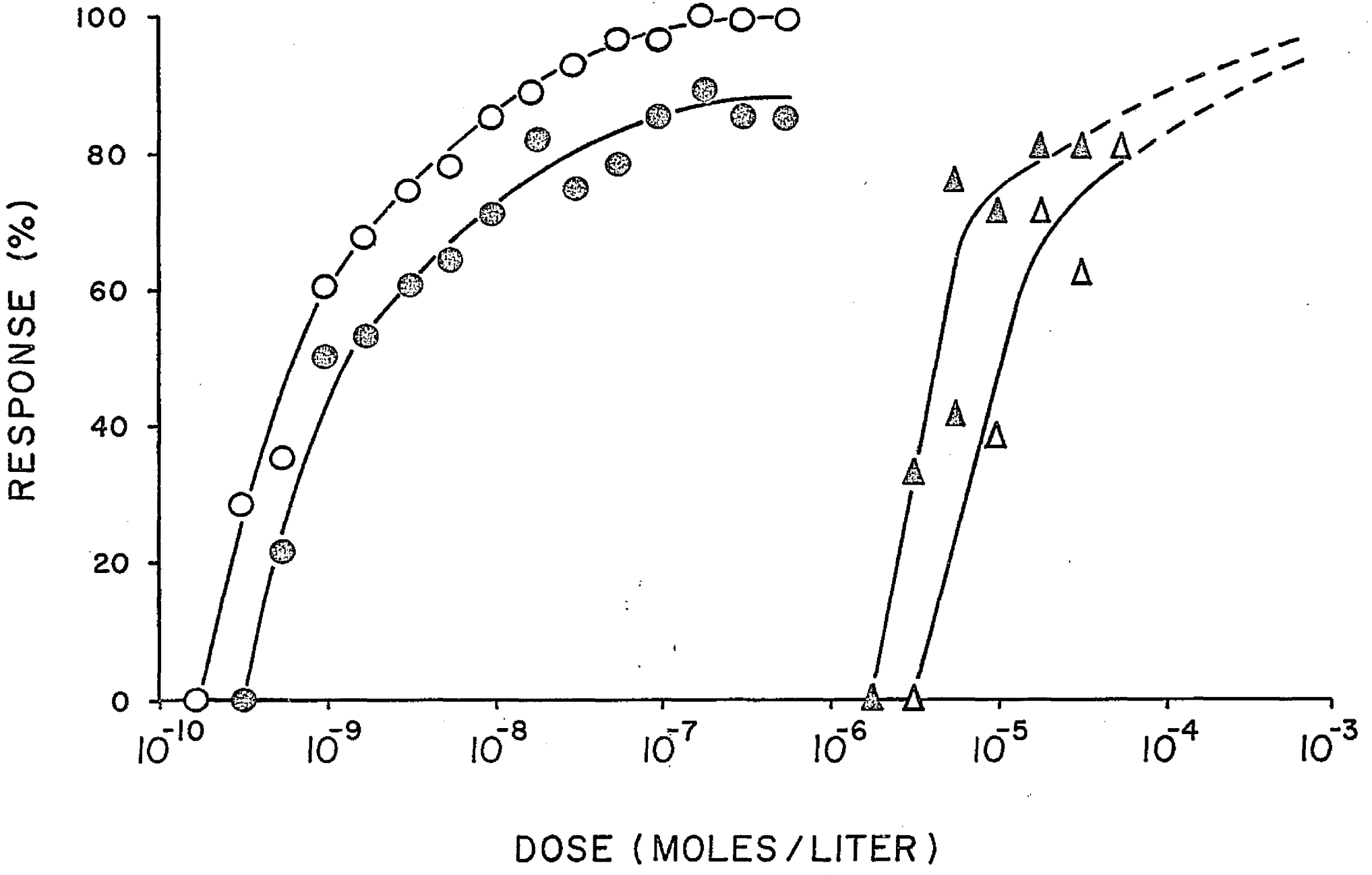

100
80
60
40
20
0
RESPONSE (%)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
DOSE (MOLES/LITER)

Fig. 5

Diethyldithiocarbamate irreversibly suppressed uterine activity. Treatment with this chelating agent at concentrations as low as 10^{-5}M (uteri from five rats), even with repeated rinsings, decreased the sensitivity of the uterus to subsequent oxytocin or $CuSO_4$. The maximum tension developed by the uterus at high oxytocin or copper concentrations was less with treatment with diethyldithiocarbamate than without. Indeed, after exposure to a high concentration (10^{-3}M) of the chelating agent, the rinsed uterus was totally unresponsive to either oxytocin or copper.

We wished to determine whether the sensitivity to copper ion is different in the vaginal end, as opposed to the ovarian end of the uterus. Such a difference might result in changes of the peristaltic actions of the uterus in the presence of copper. The uterine horns were cut transversely and the copper sulphate and the oxytocin responses were measured for the top and bottom halves. No differences were found between the two halves.

D. ASCORBIC ACID

Ascorbic acid increased the sensitivity of the uterus to copper. In the presence of 1×10^{-4}M freshly prepared ascorbic acid, a typical uterus horn repeatedly contracted upon administration of 1×10^{-6}M $CuSO_4$. Without the ascorbic acid, this uterus was sensitive only to

1×10^{-5}M $CuSO_4$. Similar results, with a ten-fold increase in the uterine sensitivity to copper in the presence of ascorbic acid, were found over eight trials with four uterine horns.

Very high concentrations of ouabain (1×10^{-3}M) caused uterine contractions, but their magnitude was only about 20% of the peak tension caused by oxytocin or copper (six trials from four uterine horns).

E. THEOPHYLLINE

Theophylline inhibited copper-induced contractions, and to a lesser extent, oxytocin-induced contractions. Incubating the uterus in the presence of theophylline (5×10^{-3}M) for 10 minutes resulted in the horn being completely unresponsive to $CuSO_4$, even at concentration of 5×10^{-4}M. Before theophylline was added, this uterus reacted to 1×10^{-5}M $CuSO_4$. The sensitivity of the uterus to oxytocin was only slightly decreased with theophylline incubation. The horns reacted to 3×10^{-10}M oxytocin before theophylline, and to 1×10^{-9}M oxytocin after theophylline. All theophylline effects were reversible with rinsing of the uterus and were repeatable in four trials with two uterine horns.

F. COPPER SALTS INTRALUMINALLY

So far we have shown that $CuSO_4$, applied to the outside of the rat uterus, caused contractions. Introduction of $CuSO_4$ into the lumen, via the method described above, also caused contractions. However, a much higher concentration (about 1,000 times) of $CuSO_4$ was required. A similar result was found for oxytocin introduced into the lumen; about 10^{-6}M oxytocin was required for 50% contraction. A longer time (1 to 10 minutes) was required for the uterus to react to either oxytocin or $CuSO_4$ via this route.

Introducing fluids into the lumen by the method employed causes an initial stretch while the solutions are being injected. This stretch causes a small contraction (about 10%) which subsides rapidly (within 10 sec) after the injection is completed. The doses of copper and of oxytocin used elicit their effect at 1-10 minutes after the initial stretch-induced contraction, a time at which the saline-injected uterine horn is completely inactive. The system was checked for leakage of copper ions to the outside bathing medium by colorimetric determination of copper. This was done by analysis of the bathing medium in which uteri had been contracting for several minutes as a result of the administration of $CuSO_4$ into the lumen. No copper ion (limit of detection 10^{-6}M) was found in the outside bathing medium. For this uterus the threshold for contraction for copper in the outside bath-

ing solution is 10^{-5}M and hence, the contraction was induced entirely from the $CuSO_4$ in the lumen.

G. COPPER WIRE INTRALUMINALLY

Copper wire in the lumen of the uterus also caused contractions. Figure 6 shows typical activity at hourly intervals. Contractions began after about 1 hour. This increased to maximum contractions which were regular and had complete relaxation after each contraction. After about 4 hours, contractions were followed by incomplete relaxation, they became weaker, and their frequency increased. By the sixth hour, contractions were much less regular and the peak tension was about half that for the third hour. During these 6 hours, the control horn (i.e., with the polyethylene-encased copper wire) showed no activity. At the end of 6 hours, both horns were tested with oxytocin. The control horn responded as it had 6 hours before. However, the horn containing the bare copper wire could not be stimulated to develop more than 50% of the tension it had developed when tested with oxytocin at the beginning of the experiment. The results were essentially the same for two animals.

Fig. 6. Effect of copper wire placed in the lumen of the uterus in the *in vitro* assay, as described in the text. Each segment is a tracing of 5 minutes of typical activity, taken at hourly intervals. The control horn, with a polyethylene coated copper wire in its lumen, was completely inactive throughout the six hours of the experiment.

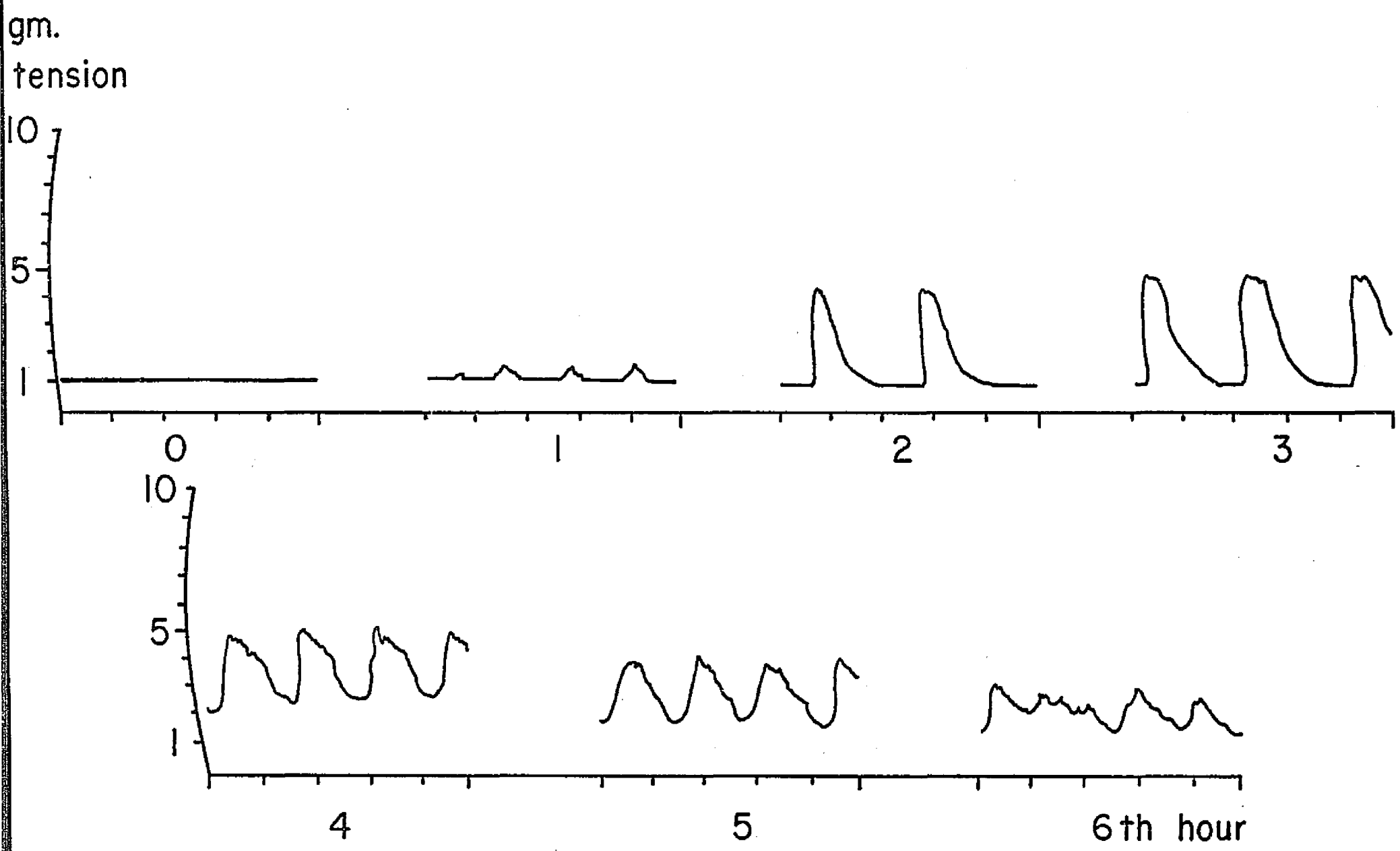


Fig. 6

H. INDOMETHACIN

After indomethacin pretreatment oxytocin-induced responses are inhibited. A five-fold higher oxytocin concentration is required to cause contraction with 10 μg/ml indomethacin present than without (four horns from 2 rats). A ten-fold higher concentration of oxytocin is required for contraction if 100 μg/ml indomethacin is present (2 horns from 1 rat). This confirms the findings of others (see Introduction, section I B 2 F).

Copper-induced contractions are much less affected by indomethacin. Even at 100 μg/ml indomethacin the same concentration of copper sulfate as was effective without indomethacin, or at most one dose higher (1.8-fold increase), would contract the uterus.

I. CALCIUM

Copper, like oxytocin, was found to have no effect on the uterus in calcium-free van Dyke-Hastings solution. The calcium free solution caused the uteri to go into contracture at about 50% of the maximum tension possible with oxytocin and normal calcium. Copper or oxytocin have no additional effect on this contracture. If a uterus which had been exposed to copper or oxytocin in normal van Dyke-Hastings is then rinsed with calcium free van Dyke-Hastings solution the muscle

will not relax. Upon rinsing with normal van Dyke-Hastings solution relaxation takes place (see Discussion, section IV A).

J. ELECTRICAL STIMULATION

Electrical stimulation of copper treated and control uterine horns was done to insure that both horns underwent the same total amount of contraction. In this way any long-term inhibitory effects could be attributed to the copper and not simply to fatigue of the muscle because of overstimulation. With 10^{-3}M copper sulfate the inhibitory action of copper is dramatic (Figure 4). After two hours and forty minutes the copper treated horn only contracts to 25% of the tension it had initially, while the control horn has decreased only slightly to 85% of its initial tension. Even at 10^{-5}M cupric, an inhibitory effect is seen, but only after a longer time. The copper treated horn first shows a decrease in tension after 2 hours, and after 7 hours is down to 80% of its initial value. The control horn of this pair was still contracting to 100% of its initial tension after 7 hours, after which it too started to degenerate.

K. IN VIVO INTRAUTERINE PRESSURE

In vivo intrauterine pressure measurements were made in pregnant

rats and pregnant and non-pregnant rabbits while copper sulfate or saline (control) was administered into the lumen of the uterus. This was an extension of the *in vitro* intraluminal copper administration, but with the critical difference that the intact endometrium with active circulation separated the uterine lumen from the myometrium. It was also thought that copper solutions might be able to abort the pregnant animals.

Intrauterine pressure was found to increase consistently only with very high copper sulfate doses; 1 ml of 10^{-1}M $CuSO_4$ for rats (3 rats) and 5 ml of 10^{-1}M $CuSO_4$ for rabbits (3 rabbits). Lower doses gave only equivocal results. Attempts to abort two pregnant rats (day 15 and day 18 of pregnancy) and one pregnant rabbit (approximately day 30) by administration of copper salts into the lumen of the uterus proved unsuccessful. An increase in intrauterine pressure was seen at the high doses mentioned previously, however. The fetuses in the horn receiving the copper were invariably killed. The higher doses used were also toxic to the mothers.

IV DISCUSSION

Our basic finding that copper causes uterine contractions has since been confirmed by the laboratory of J. Zipper (184). In addition they have found that copper induced contractions are inhibited by zinc. Other workers have found that intrauterine copper wire of surface area comparable to the copper IUD (140 or 280 mm^2 surface area) would cause an increase in uterine activity in the rabbit, as measured electrically (185). They also found that infusions of cupric salts would cause an increase in uterine activity. In two animals with low doses of copper (0.1 and 0.3 mmoles), however, they found a decrease in activity. The doses at which they found an increase in activity (0.5 to 1 mmole) are comparable to the doses at which we found increased intrauterine pressure in rabbits (see Results, section K).

A. COPPER AND OXYTOCIN CONTRASTED

Although both copper and oxytocin induce uterine contraction, the response differs in two ways. First, oxytocin leads to rhythmic con-

tractions; copper tends to produce a sustained contraction. Second, prolonged treatment with high concentrations of copper ions renders the uterus unresponsive, while with oxytocin there is no long-term inhibitory effect. In calcium free solutions copper and oxytocin seem to act similarly. The contractile machinery is inoperative. Copper cannot substitute for calcium.

Our finding that an oxytocin analogue which inhibits oxytocin induced activity does not inhibit copper induced activity probably indicates that different receptor sites are involved, or at least that there is a fundamental difference between the two responses.

B. MEMBRANE EFFECTS

Daniel suggested that copper stimulates the uterus by inhibiting Na^{+}-K^{+} ATPase, depolarizing the muscle, and thus causing a contraction (11). The finding of Shild (62) that copper is inactive on the potassium-depolarized uterus is consistent with Daniel's suggestion. However, Daniel's observation, which we have confirmed, that very high concentrations of ouabain (10^{-3}M) are required to cause a contraction in the rat uterus, tends to weaken this argument. In addition, ouabain induced contractions produce only 20% of the tension obtainable with oxytocin, while copper induced contractions can reach 120% of the oxytocin maximum tension.

The fact that copper is inactive in calcium free solutions indicates that copper is not simply substituting for calcium.

Sandow and coworkers (186) have studied the effects of various metal salts on twitch tension in the frog sartorius. They found that Zn^{2+}, Be^{2+}, Ba^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Pt^{4+} and especially UO_2^{2+} potentiate twitch tension by slowing the falling phase of the action potential. Given the differences between skeletal and smooth muscle (see Smooth Muscle Function, section I B 2 b), an effect on the falling phase of the action potential in skeletal muscle could manifest itself as a change in resting potential or spontaneous activity in smooth muscle. Both effects are related to K^+ permeability (44).

Sandow et al. noted (186) exclusively with copper that although there was an initial potentiation (10^{-4}M Cu^{++}), after 40 minutes the potentiation was reduced. The tension that the muscle could then develop during tetanus was also reduced. This is very similar to the long-term inhibitory effect that we found with copper on the uterus.

Mercaptans have been implicated in the mechanism of oxytocin activity since N-ethylmaleamide, which covers mercaptans, inhibits uterine response to oxytocin (187). Copper ions form tightly bound complexes with mercaptans. The fact that copper ions and oxytocin augment each other's response is compatible with two types of mercaptan sites; perhaps those for oxytocin are at the uterus surface since oxytocin is a large molecule. The uterine inactivation might be caused by the mercaptyl group of diethyldithiocarbamate. Mercaptans, such as glutathione, have been shown to diminish oxytocin response (187), perhaps by acting on the contractile. rather than (74) the excitatory apparatus of the muscle. On the other hand, the principal action of diethyldithiocarbamate could be to tie up trace amounts of copper which might be

necessary for proper uterine functioning. Diethyldithiocarbamate is a specific chelating agent for copper with some affinity for zinc and slight affinity for iron (see sec. II B of Part II).

Sandow eliminates the possibility that the metal cations are potentiating the twitch tension by binding to sulfhydryls (186). At least for zinc, the effect is not inhibited by various sulfhydryl blocking agents.

Another approach to the problem is the possibility that copper is acting on the lipids of the membrane. Lipid peroxidation has been found on incubating erythrocytes in the presence of copper sulfate (188). Prior incubation with cupric ions causes red cells to become more susceptible to isotonic lysis by progesterone (188).

Shen (10) has found that rat uteri show contractions with low levels of copper ion when ascorbic acid is added. Our observation that ascorbic acid induces contractions even for a subthreshold copper concentration confirms his finding. Shen also found ascorbic acid augmentation of copper for other smooth muscles, including bronchus and small intestine of rat, rabbit, guinea pig, and pig. Copper-catalyzed oxidation of ascorbic acid produces hydroxyl free radicals (7). and hence peroxides. The ascorbic acid potentiation of cupric induced contractions suggests that the cuprous ion may be playing an active role.

C. PROSTAGLANDINS

The action of copper could be via prostaglandin synthesis. Copper salts have been shown to increase the biosynthesis of prostaglandin $F_{2\alpha}$ at copper concentrations in the range found to stimulate the uterus, around 10^{-5}M (189,190). Prostaglandin $F_{2\alpha}$ causes pronounced uterine contractions (191). The endogenous production of prostaglandins may be critical for normal uterine activity (100,101,102), as mentioned previously (Initiation of parturition, section I B 2 f). Hence, administration of copper could stimulate the uterus by causing the production of prostaglandin $F_{2\alpha}$. High concentrations of cupric salts (10^{-3}M), on the other hand, inhibit the production of prostaglandin $F_{2\alpha}$ (190). The inhibition could be the origin of the suppressive effect of high concentrations of cupric on uterine contractions. Dithiols, which can chelate copper, inhibit the biosynthesis of prostaglandins (189). Diethyldithiocarbamate inhibits uterine activity (11) (see Results, section III B) perhaps by inhibiting the production of prostaglandins.

Our finding that indomethacin has little effect on copper induced contractions while it severely inhibits oxytocin induced activity would seem to imply that prostaglandin synthesis is not a step in copper induced contractions. The situation is more complicated, however, since Maddox (190) has found that only the synthesis of the E series

of prostaglandins is inhibited by indomethacin. Considering that cupric stimulates the conversion of arachidonic acid to prostaglandin $F_{2\alpha}$ and decreases the synthesis of prostaglandin E_2 (190), the limited effect of indomethacin on copper induced contractions is not surprising. The question of prostaglandin involvement in copper induced uterine activity thus remains open.

D. CYCLIC AMP

Our finding that theophylline inhibits copper induced contraction implies that copper may act by causing changes in cyclic adenosine monophosphate (cAMP). cAMP levels decrease with oxytocin stimulation of the rat uterus (70) (see Uterine function, section I B 2 e). If copper also causes a decrease in cAMP leading to contraction, then theophylline which causes a build-up of cAMP would have the inhibitory effect which we observed. In the toad bladder, copper inhibits oxytocin action (192). In the bladder, however, oxytocin stimulation and the resulting increase in water permeability is associated with an increase in cAMP (192). This evidence is compatible with the hypothesis that, in both the bladder and the uterus, copper leads to a decrease in cAMP. This could explain why copper and oxytocin have similar stimulating effects on the uterus, with the stimulation (by either copper or oxytocin) inhibited by theophylline. Also a decrease in cAMP by cop-

per could explain the opposition of effects that copper and oxytocin have on the bladder, as well as the copper inhibition of theophylline-induced increase in water permeability in the bladder (192). A more detailed understanding of the nature of copper, as well as oxytocin and prostaglandin stimulation of the uterus, is most likely dependent on the clarification of the role of cyclic nucleotides in uterine contractions, and their interactions with endogenous prostaglandin production.

F. IMPLICATIONS FOR EXPULSION OF THE COPPER INTRAUTERINE DEVICE

Our studies have implications for the effect of copper on copper IUD expulsion. Copper ions are produced by copper dissolution in the lumen of the uterus (6). We have shown that copper salts, as well as copper metal, cause uterine contractions *in vitro*. The dose applied to the lumen is only quantitatively different, for the same response, from that supplied outside the uterus. Presumably higher doses are required in the lumen because the ions must first pass through the endometrium. These results may differ from the *in vivo* situation where the endometrium has an active circulation. Direct measurement of *in vivo* uterine activity with an intrauterine pressure monitoring balloon in pregnant and non-pregnant rats and rabbits has shown that acute application of high doses of copper sulphate (1 to 5 ml of 10^{-1}M) directly

into the uterus causes uterine contractions. This has been confirmed by others (185) measuring uterine electrical activity. Figure 3 might explain why the copper T IUD has a lower expulsion rate than the T IUD without copper. The lower rate of the copper IUD might be related to the irregular contractions which diminish in amplitude after a few hours. On the other hand, the initial stimulatory effect of the copper metal could cause an early expulsion, but if the device were retained initially, its chances of being expelled later would diminish.

Hagenfeldt (153) found concentrations of copper as high as 3.3×10^{-5}M in cervical mucus taken in the proliferative stage of women with copper IUD's. Although the concentrations of copper in the uterine lumen fluids are unknown, amounts in the endometrium are lower than those of the cervical mucus (153). We have shown that although 3×10^{-5}M Cu^{++} is more than enough to stimulate the rat uterus if put in the bathing medium, higher concentrations are necessary if copper salts are put into the lumen of the in vitro rat uterus. Whether the copper released from a copper IUD in women can cause changes in uterine motility would depend on how much finally reaches the myometrium. This would depend on thickness of the endometrium, uptake by the blood, exposure time, and other factors which make comparisons to the in vitro results for rats presented here difficult. In addition, as Hagenfeldt points out, it is not known to what degree released copper is bound by albumin and other substances in the uterine fluids.

Some workers attribute low expulsion of the Cu-IUD to mechanical factors rather than to the copper metal *per se* (131,193). These authors did not consider the effect of copper on uterine contractions.

F. IMPLICATIONS FOR THE CONTRACEPTIVE MODE OF ACTION OF THE COPPER INTRAUTERINE DEVICE.

The possible role of copper in prostaglandin biosynthesis with the Cu-IUD could be related in several ways to its contraceptive effect. Inflammatory reactions are mediated via prostaglandins (194). Thus, if the copper causes increased prostaglandin production, this could account for the increased inflammatory reactic seen with Cu-IUDs (150) as mentioned earlier. Anti-inflammatory drugs such as indomethacin inhibit the production of prostaglandins (190,194). Indomethacin counteracts the contraceptive action of inert IUDs in the rabbit (152), but apparently not in the mouse (195) or the rat (196). Alkalating agent immunosuppressants do reduce the contraceptive effect of IUDs in the rat, however (197).

Copper-induced prostaglandin production might also exert a contraceptive effect via increased uterine motility. This has been hypothesized to explain the decrease in the number of ova that can be recovered from rabbits with intrauterine copper (198). As mentioned, meas-

urements of uterine electrical activity in rabbits show an increase with intrauterine copper wire (185). No studies of uterine activity have been done on women wearing the Cu-IUD. With inert IUD's, abnormal pre-labor-like contractions have been observed around implantation (119,120). Presumably these contractions are induced by the increased production of prostaglandins which most likely occurs with IUD's (121, 122,123).

The lowered bleeding for the Cu-IUD (132,133,134) as compared to inert IUD's suggests that copper is affecting the vascular system of the endometrium. Copper salts cause the contraction of not only uterine muscle, but also of a wide variety of other smooth muscles (10), probably including those of the arterioles of the uterus. Csapo has recently suggested that the Cu-IUD could exert its contraceptive action by inducing uterine production of prostaglandins which, in turn, would cause uterine vasoconstriction, suppressing the blood supply necessary for successful implantation (199).

G. PROPOSED FUTURE RESEARCH

The hypothesis that prostaglandin production induced by the copper IUD is the source of the contraceptive effect is still unproved. The obvious experiment is to attempt to suppress the contraceptive effect of the copper IUD with inhibitors of prostaglandin synthesis such as

indomethacin or aspirin. (Compare to inert IUD's; see section I C 2.)

Another approach is to follow the hypothesis that the copper device inhibits implantation by constricting the vasculature of the endometrium. Micro-photographic techniques are available to study vasoconstriction in muscle or in the omentum (200). One could test the effects of copper salts or copper metals as well as of any inhibitory action of indomethacin. More directly, radioactive indium has been used to measure uterine circulation in women (201); thus one could measure the possible vasoconstrictive effect of a copper IUD directly.

The possibility that the copper IUD exerts its contraceptive effect through the biosynthesis of prostaglandins suggests a new type of IUD. An IUD which continuously delivered a small dose of prostaglandin might have the same end result as the copper IUD without the risks that copper itself introduces, such as free radicals or copper toxicity. (See I C 4.) A device made of silastic can be made to deliver a small continuous dosage of a hydrophobic substance. This is the basis of the progesterone IUD currently being tested clinically (202). Although our consideration of uterine function indicates that progesterone and prostaglandin serve more or less antagonistic roles, each might work in an IUD but for a different reason. The progesterone device is thought to act by changing the cervical mucus and the endometrial environment so that sperm transport and/or implantation is inhibited. The prostaglandin IUD might be practical if it circumvents side effects

found with the progesterone IUD. Academically, if a prostaglandin IUD has an increased contraceptive effect over an inert one, this would imply that inert and copper IUD's may be acting via prostaglandin synthesis. The prostaglandin IUD could thus serve as a tool for gain-insight into the the process of implantation.

Part II

COPPER AND THE INTERRUPTION OF PREGNANCY

I INTRODUCTION

A. COPPER AND THE INTERRUPTION OF PREGNANCY: THE PROBLEM STATED

The development of the copper intrauterine device stimulated our interest in the action of copper in reproductive systems generally. Searches through the literature revealed a considerable number of interactions between copper metabolism, copper deficiency or copper excess and reproductive function. These interactions occur in both directions; reproductive state can change copper metabolism and copper state can change reproductive function. Thus, serum copper levels are drastically increased during pregnancy as first found by H. A. Krebs in 1928 (203). Copper administration can lead to ovulation in rabbits (204). Often either copper excess or copper deficiency have similar end results. Thus copper salts are spermaticidal (205, 206) as are copper chelating agents. Indeed copper chelating agents are among the most powerful spermaticidal agents known (207). Excess copper (208) or copper deficiency (207) during pregnancy can result in reproductive failure. This failure of copper-deficient animals to carry their pregnancy to term lead directly to our findings described in Part II of this thesis.

It has long been known that livestock raised on fodder from copper-deficient soil exhibit reproductive failure (209). Dutt and Mills showed that rats fed on a copper-deficient diet conceive but the pregnancy is not maintained (210). Hall and Howell found that rats fed on a copper-deficient diet did not produce litters but when copper was supplied, the litters developed normally (211, 212). Implantation occurred but the fetuses had been resorbed by Day 13.

It occurred to us that rather than feeding the animals a copper deficient diet prior to mating, the same end result, namely demise of the embryo might be effected by the administration of a copper chelating agent. The initial question then is whether a copper chelating agent can bring about embryonic demise. Part II of this thesis documents the development of the use of copper chelating agents and pharmacologically related drugs as embryocidal agents.

By way of introduction the action of copper in reproductive systems is reviewed; the consideration of these interactions of copper and reproduction lead to the formulation of the thesis problem. In addition, it is hoped that the presentation of these interactions of copper and reproduction in a unified setting will help to elucidate some of the basic mechanisms by which these interactions occur.

B. COPPER AND REPRODUCTIVE PHYSIOLOGY

1. Copper metabolism

a. Copper homeostasis

Copper is an essential dietary trace element (214, see also 209, 213, 215). Copper is required for hematopoesis, myelination, osteogenesis, elastin formation, pigmentation and keratinization, as we shall see specifically when we look at copper deficiencies. The normal intake of about 5 mg/day (159) is roughly ten times that required so deficiencies rarely occur.

Of the dietary copper from 0.6-1.6 gm is absorbed, a bit by the stomach and mainly via the small intestine (216). The absorption is via an energy dependent system (213). In the mucosa copper is bound to the copper binding protein metallothionein (213). Chelation with L amino acids is probably an important part of copper uptake (213). Upon release from metallothionein copper enters the blood either free or chelated to amino acids; there it binds to serum albumin or free amino acids. This "direct reacting" copper is bound loosely to serum albumin and amino acids, though only about 5 to 7% of the copper in the serum (the rest being bound to ceruloplasmin) is the source of copper for the liver, bone marrow and other tissue (216).

All amino acids form stable complexes with copper, as well as other transition metal ions (for review see 217). They form coordinates through the amino and carboxy groups. In addition, cysteine is thought to coordinate through the amino and the mercaptan, as does penicillamine (dimethylcysteine). Cystine binds cuprons, but not cupric. The complexing with the imidazole of histidine is especially important in copper complexing to proteins, and a number of different complexes are possible (217).

About 8 mg of copper is stored in the adult liver. Newborn liver is particularly high in copper, having about seven times higher concentration copper (218, 219, see also 209, table 9). Milk is especially low in copper (159, p.378) and the newborns high liver copper probably serves to tide him over until it starts taking other foods. By several months of age the liver copper levels approach those of the adult. The few cases of copper deficiency which have occurred in humans have been found in infants who were born prematurely and were subsequently given milk formulas or parenteral alimentation for long periods (220, 221) see below.

Aside from storing copper, the liver synthesizes and secretes the copper protein ceruloplasmin into the blood; about 0.5 mg of copper being incorporated into ceruloplasmin per day. As we shall discuss later serum ceruloplasmin levels are influenced by many factors, including steroid hormones.

The copper in ceruloplasmin is thought not to exchange with the copper loosely bound to serum albumin or amino acids in the serum (216).

Copper is also taken up by the bone marrow to make erythrocuprein, which accounts for over half of the copper in red blood cells (216). This protein has recently been called superoxide dismutase (see below)

Copper is excreted mainly through the biliary system, although some is lost directly to the intestines, and a small amount via the urine (216).

There are two inherited disorders of copper metabolism, Wilsons disease and Menkes's kinky hair syndrome.

Wilsons disease, or hepatolenticular degeneration is a progressive fatal disease inherited as an autosomal recessive. It is characterized by basal ganglion degeneration with associated tremor, ataxia and incoordination, cirrhosis of the liver, and formulation of a brownish ring at the edge of the cornea - the Kayser-Fleischer ring (see 222, 223, 224). Tissue copper levels in patients with Wilsons disease are tremendously elevated, especially in the liver and brain. Often copper levels are ten-fold higher than normal (222, 223, 224, 225). The neural and liver damage is correlated with the elevated copper levels in these tissues (213, 222, 223, 224). There is almost always a decrease in ceruloplasmin and an increase in direct reacting CU in serum .

One theory that accounts well for the various findings of Wilsons disease, and especially also account for asymptomatic homozysons in patients with Wilsons disease is that there is abnormally high binding of copper in the liver (see 213). A protein similar to metallothionein has been isolated from Wilsons disease patients that binds copper more than that of controls (see 213).

The increased copper binding of this protein, it is thought, would shift copper metabolism in the hepatocyte making less available for biliary excretion or ceruloplasmin synthesis. Saturation of the copper binding sites would lead to the excess being taken up by liver lysosomes, and less being taken up from the blood -- so that a higher level of direct reacting copper would remain in the serum. In some advanced cases copper is released from the liver and a hemolytic crisis ensues (223, 224, 225). This is similar to the course of severe Cu toxicity in animals (see below).

The treatment for Wilsons disease is oral penicillamine (β, β, dimethyl cysteine) (226). Treatment with this copper-chelating agent mobilizes tissue Cu and results in increased Cu excretion in the urine (226, 223). The mode of action of penicillamine is not sure, for although it is a copper chelating agent, it is a poor one. Amino acid metabolism is changed with administration of this drug, and this may be related to its mode of action.

Patients treated with penacillamine often lose their senses of taste and smell (227). Administration of copper sulfate will reverse this (227). Certain spontaneous losses of taste have also been reversed with administration of copper salts (227). There is a condition, the Morisier syndrome, in which anosmia is found in conjunction with gonadal hypoplasia. In these patients there are deficiencies in the olfactory lobe together with underdeveloped gonads (228, 229, 230). In rats there is severe anorexia when high levels of the copper chelating agent disulfiram are given (213) see below. Sex steroids can also influence the perception of taste and smell. Thus a woman's ability to detect the odor of the synthetic musk, exaltolide, is greatest at midcycle (232, 233). A related phenomenon is the change in salivary mucoids over the menstrual cycle, the sialic content of saliva being at a minimum at midcycle (234).

Menkes kinky hair syndrome is a metabolic disorder inheret-ed as an X linked recessive (235, 236). It is marked by twisted hair, slow growth, tortuosity of cerebral arteries, cerebral degenera-tion and early death. Patients with Menkes' syndrome are severely copper deficient with low serum, hepatic and brain copper levels.

Oral copper administration is ineffective, but intravenous reverses the copper deficiency, localizing the defect to the intestinal absorption of copper (237, 238, see also 239, 240). Duodenal mucosa from patients with the disease have abnormally high concentrations of copper due to failure to transport copper across the serosal cell membrane (241).

b. Copper proteins and biochemistry

Many copper proteins, as we shall see later, influence, or are influenced by reproductive changes. The present section will deal however, with some generalities about copper proteins leaving the tie in to reproduction in other sections.

Copper proteins include ceruloplasmin, uricase, erythrocuprein, amine oxidase, dopamine β-hydroxylase and cytochrome oxidase. Copper enzymes are dehydrogenases with high oxidation potentials. They oxidize using oxygen as the acceptor as at the end of the respiratory chain (see 242).

The copper bound in ceruloplasmin accounts for about ninety-five percent of the copper in the serum (216). The ceruloplasmin copper is tightly complexed and can only be removed with chelating agents such as diethyldithiocarbamate after treatment with strong acid (216). Ceruloplasmin has recently been called ferroxidase I because of its role in iron metabolism (243-247). Ceruloplasmin oxidases ferrous to ferric allowing the ferric to be incorporated into the iron transport protein transferrin, making iron available to the blood forming tissues.

Sixty percent of copper in the red cell is bound to erthrocuprien, also called superoxide dismutase. This enzyme catalyzes the breakdown of superoxide free radicals.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

It probably serves as a form of protection from the potentially toxic superoxide radical.

Other copper enzymes include tyrosinase, which oxidizes tyrosine in the first step of melanin production for skin pigmentation (250, 251). The copper enzyme peptidyl lysyl oxidase is necessary for collagen synthesis (252, 253). This enzyme oxidizes lysine after the amino acid has been incorporated into the polypeptide. The aldehydes thus produced form collagen crosslinks by aldol condensation (252, 254, 255). If copper is deficient or withdrawn by chelating agents the resulting defectively-formed collagen has fewer crosslinks and is structurally weaker.

c. Copper toxicity and deficiency

Acute copper poisoning is rare, probably because of the metalic taste of copper salts and their emetic property. This emesis may be related to smooth muscle contractions caused by cupric as discussed in part I of this thesis.

When acute poisoning does occur it causes intestinal ulceration, hepatic necrosis, nausea, diarrhea and hemoglobinuria (213).

Chronic toxicity, often seen in animals given copper rich foods, results in liver and kidney demise and jaundice (213). Sometimes death results from an acute hemolytic crisis (209). These symptoms are quite similar to some of those of Wilson's disease.

In human adults copper poisoning is rare, but has occurred, particularly as attempted suicides (256), and recently a rash of chronic poisonings have come from the use of artificial kidney machines. It turns out that the dialysis membranes of the responsible machines were made of a polymer, cuprofam, made with copper (257). Infants are more sensitive to copper poisoning, and cases have appeared where the copper came from copper plumbing. The family involved happened to use hot tap water to make up the baby's formula, the hot water had even more copper than the already rich cold water (258).

One investigator claims that excess copper especially from tap water in houses with copper plumbing can result in zinc deficiency in humans (259). Copper-zinc nutritional antagonisms are common in animals (260, 261). It is claimed that the copper-induced zinc deficiency can lead to white spots on the fingernails and various psychological changes (259, 262).

Copper deficiency in livestock is characterized by reproductive failure, and neonatal ataxia, as will be discussed below. In addition, in sheep wool will not crimp, and in black sheep, the wool will come out white. Alternating copper containing and copper deficient fodder will result in wool with black and white bands (209, pp.87-90). This lack of pigmentation shows copper's role in tyrosinase, which is involved in producing the pigment melanin (250, 251).

Copper deficiency can occur in human infants. As mentioned above newborns have high liver levels of copper and milk is particularly deficient in copper. Premature infants whose livers presumably have not been able to store as much who are then kept on milk formulas or parenteral alimentation for a long period of time can

develop an overt copper deficiency, characterized by low copper levels, anemia and a failure to thrive (220, 221, 263, see also 264, 265, 266). Oral copper administration quickly reverses the symptoms. Copper deficiency is also seen in protein and protein-calorie malnutrition (267-272).

2. Serum Copper Levels Influenced by Hormonal Factors

Returning to reproductive biology, there are significant changes in copper metabolism related to hormonal state. Serum copper rises dramatically during pregnancy (203) (for additional references see 213). The rise takes place during the first and second trimesters and remains high at about three times the non-pregnant value. The serum copper level falls off slowly *post-partum* (273). A drop in the copper level during pregnancy has been proposed as an indicator of impending fetal death (274), and as an index of placental insufficiency (273). A rise in serum copper levels above the normal pregnancy levels is found in toxemia of pregnancy, and the copper levels are correlated with the severity of the clinical symptoms (273). When contraceptive steroids are administered to non-pregnant women the serum copper level rises about 50% above normal (275). The rise in serum copper is due, primarily, to the estrogens, which cause a *de novo* synthesis of ceruloplasmin (128). Progesterone causes a less drastic increase in serum copper (276), and cortisol causes a decrease (213). Diurnal fluctuation in ACTH and corticosteroids are reflected in diurnal variations in ceruloplasmin levels (213). In the normal menstrual cycle serum copper levels change only slightly (273,277,278), if at all (157). The increase in

ceruloplasmin accompanying increases in estrogen affect iron metabolism. Thus, with roosters it was shown that estrogen administration first causes an increase in ferroxidase and subsequently an increase in total serum iron (279). Transferrin mobilizes iron for erythropoiesis. Thus the high estrogen levels of pregnancy which induce production of ceruloplasmin, and thence transferrin, probably increase iron mobilization needed for red cell production in the mother and fetus.

Ceruloplasmin has ascorbic acid oxidase activity (280) which may be related to the pronounced changes in urinary ascorbic acid over the menstrual cycle (281). Oxidation of ascorbic acid has been implicated in iron metabolism in the reduction of ferric to ferrous, thereby releasing iron from transferrin for use in erythropoiesis (282).

Cloasma, a darkening of the skin ultimately produced by a copper-containing enzyme, is often a manifestation of high estrogen levels. This phenomenon, sometimes called "the mask of pregnancy," appears as dark splotches on the face. Cloasma is noted not only in pregnancy but also with women taking high estrogen oral contraceptives; the splotches are enhanced by sunlight (283). Topical application of estrogens on the nipple will cause a darkening of the nipple and areolae (284). Skin pigmentation is a result of oxidation of tyrosine to melanin by tyrosinase, a copper-containing enzyme (250,251). Cloasma thus can be regarded as another manifestation of the interrelation of estrogens and copper.

3. Hormone Levels Influenced by Copper

Both copper deficiency or administration of exogenous copper can cause profound changes in reproductive function. Such changes include induction of ovulation, with associated changes in hormonal levels, and induction of abortion. In many cases, either a deficiency of copper or an excess of copper leads to the same end result.

Either copper loading or dietary copper deficiency in rats results in a reduction of liver microsomal hydroxylating enzyme activity (235). Since these enzymes are responsible for the metabolism of sex steroids, one would expect that copper would influence reproductive function.

Copper can also cause changes in levels of gonadotrophic hormones. It has long been known that intravenous injection of copper salts will cause ovulation and pseudopregnancy in the rabbit (204). The rabbit is an induced ovulator, i.e., vaginal stimulation provokes the release of LH and FSH which causes ovulation as well as pseudopregnancy a state of prolonged life of the corpus luteum (286). In order to produce ovulation in the rabbit by intravenous injection of cupric salts, high doses of copper (3 mg per Kg) must be used (287). When cupric sulphate is applied directly into the hypothalamus of rabbits only $0.75^{\wedge}$ micrograms per Kg or 1/4,000 of the intravenous dose of copper are required (287). Copper administration has been shown to produce an increase in LH and FSH (288) to cause ovulation. Hypothalamic extracts are particularly rich in copper and inorganic residues from these extracts cause a release of various pituitary hormones including LH when injected into the hypothalamus (289). Cupric salts alone

at concentrations less than 1 microgram per ml (i.e., less than 10^{-5}M) will also cause this release of LH (289). The hypothalamic LH-FSH releasing hormone is a decapeptide containing histidyl and arginyl residues (290). These are strong copper complexing groups. As the copper complex, the decapeptide would be more lipid soluble. In this way copper might act synergistically with the releasing hormone to cause the release of LH and FSH (see also 204,288 and 289).

Copper administration leads to gonadotropin changes in the rat as well. Here intravenous injection of copper at estrus causes pseudopregnancy (291). The rat is a spontaneous ovulator, and after ovulation, vaginal stimulation causes pseudopregnancy. This condition is brought about in the rat by the release of prolactin. Prolactin is the primary luteotrophic agent in the rat (286), and with its release there is a consequent inhibition of the release of LH and FSH (286). To produce pseudopregnancy in the rat by intravenous injection of copper salts, doses of 16 mg per Kg must be used (292). These approach the lethal dose of 18 mg per Kg (292). Similarly to the induction of ovulation in the rabbit, when cupric sulphate is applied directly into the hypothalamus of rats much lower doses of copper are required to induce pseudopregnancy (293). An increase in prolactin and a decrease in LH and FSH have been shown with copper-induced pseudopregnancy (294). Since LH is the main luteotrophic hormone in the rabbit and prolactin serves this function in the rat (286), in both species copper administration causes the release of hormones that maintain the corpus luteum. It is perhaps significant that during early pregnancy, when maintenance of the corpus luteum is crucial for the continuation

of the pregnancy, serum copper levels are increased, as we discussed earlier.

C. COPPER ADMINISTRATION: ABORTION AND TERATOLOGIES

With the introduction of the copper intrauterine device concern arose as to the possible teratogenicity of the intrauterine copper. Teratological testing of intrauterine copper was effected by inserting copper wire into the uterus after implantation in rats, hamsters and rabbits (295). No teratologies were evident in any of the offspring (F 1) and their subsequent offspring (F 2). All reproductive tissues in the F 1 and F 2 appeared normal (295).

No teratologies have been reported in 25 human pregnancies where the pregnancy occurred with the copper device in place (296). There is an increased risk that such a pregnancy will end in spontaneous abortion, but the occurrence of spontaneous abortions in pregnancies with inert devices in place seems equally high (296).

The small amounts of copper released from intrauterine copper, about 50 µg/day, for the copper IUD (296) or 2.75 µg/day and 5.50 µg/ day for the rat and rabbit devices respectively does not produce teratologies. Larger amounts however, injected into animals, early in pregnancy is definitely harmful. Subcutaneous injections of large doses (total dose 15 mg/kg) of copper acetate from day seven to day ten of pregnancy in rats results in fifty percent of the pregnancies being interrupted (297). Injection of progesterone at the same time as the copper has a protective effect, all the pregnancies are maintained (297). This progesterone protection and examination of the ovaries of the rats treated with

copper alone indicate that copper administration causes a luteal insufficiency (297). In addition there is precocious mammary gland development, implying prolactin production. The abortifacient action of copper in rats is probably via the hypothalamus, reminiscent of its action causing pseudopregnancy in estrous rats (297) (See above).

In rabbits, subcutaneous injection of copper during early pregnancy, even at high doses, had no effect (297). The rabbits delivered normally.

In hamsters, intravenous copper salts cause fetal death and teratologies. Doses as low as $2.13 \overset{mg.}{\wedge} Cu^{++}$/kg produce an increase in the number of embryos dead (26%) as compared to controls (8%) and teratologies in 6% of the embryos (208). Complexing the cupric with citrate results in the copper being even more toxic in terms of embryocidal action as well as teratologies. (For a comparison of the embryocidal and teratological action of copper salts to those achieved via copper chelating agents and related drugs, see the 125).

D. COPPER DEFICIENCY AND REPRODUCTIVE FAILURE

1. Livestock

Copper deficiency in animals has long been known to cause reproductive failure or abnormal offspring (for review see 209, Chapt. 3) Aside from being required for hemoglobin regeneration, copper is required for proper reproduction in rats (298). Lambs born in certain pastures have a neonatal ataxia called swayback. The pastures in these cases are low in copper, and the ewes and

lambs are copper deficient. Supplying additional copper to the ewes during pregnancy prevents the disease (299). Cattle have reproductive disturbances and low fertility on copper-deficient pastures (300). The deficiency is thought to cause delayed or depressed estrus (see 209, p. 90). Copper deficiency also induces infertility in sheep (see 209). Other dietary factors, notably molybdenum, zinc, sulfate and vitamin C can influence the severity of copper deficiency (see 209). Zinc deficiency can lead to teratologies (301, 302).

2. Laboratory animals - Fetal Resorption and Teratologies

Rats fed on a copper-deficient diet have normal estrus cycles and conceive, but the pregnancy ends in fetal death and resorption (210). When a copper-deficient diet is started six weeks before mating, ovulation and implantation occur during the 22-day gestation but fetal necrosis is seen by day 13 and placental necrosis by day 15 after mating. If copper is supplemented the fetuses develop normally (211, 212). The copper deficient-diet had no effect on the estrous cycle, as seen by daily vaginal smears, and furthermore, no lesions were seen in the maternal mammary glands, ovaries and pituitary glands (211, 212). It would appear from these experiments that copper is necessary for the development of the implant. Copper deficiency also induces similar infertility in guinea pigs (303). With less severe copper deficiency the young rats are born anemic and non-viable and/or have teratologies. Many are edematous and have subcutaneous hemorrhagic lesions and abdominal hernias (304). Neural lesions are also seen if the live babies are kept on a copper deficient diet (305). The hemorrhages seen

with copper deficiency are probably a result of defective collagen synthesis. Chick embryos from hens receiving a copper-deficient diet show similar hemorrhages, as well as anemia and skeletal deformities (for review see 306). The hemorrhages are the result of defective collagen formation. Copper deficiency in growing animals or adults causes anemia. In addition, there are several other symptoms that can be attributed to defective collagen synthesis. These include bone deformaties, cardiovascular disorders and, especially in poultry, rupture of the aorta (304,307,308, for review see 209, Chapt. 3). The defect in collagen synthesis with copper deficiency is now understood to be a deficiency of the amine oxidase called peptidyl lysyl oxidase (252,253). This copper enzyme oxidizes lysine after it has been incorporated into the polypeptide. The aldehydes thus produced form collagen cross-links by aldol condensation (252,254,255). If copper is deficient or withdrawn by chelating agents the resulting defectively-formed collagen has fewer crosslinks and is structually weaker.

Another way that copper deficiency leads to fetal death could be because of a decline in protein synthesis. Rats fed on a copper-deficient diet have a lower rate of protein synthesis and this is attributed to a decrease in mRNA formation (309). Iron deficiency does not produce this.

For humans, copper deficiency in adults as contrasted with infants (see discussion) is unknown. The diet for adults in most cases far exceeds the daily requirement for copper (216).

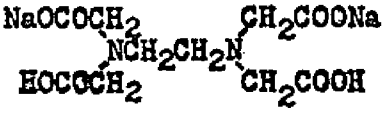
II MATERIALS AND METHODS

A. CHELATING AGENTS USED

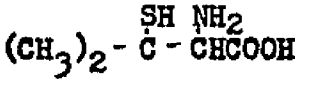
Initial trials consisted of giving copper chelating agents mixed in the feed to pregnant rats as described below. The chelating agents used were disodium ethylenediamine tetra-acetic acid (Na_2EDTA, Fisher Scientific) Penicillamine (Cuprime, Merck, Sharp and Dohme) and disulfiram (Antabuse, Ayerst or tetraethylthiuram disulfide, Aldrich).

Disodium ethylenediamine tetra-acetic acid chelates most divalent metals, especially calcium but including zinc, nickel, lead and copper (59, p. 949).

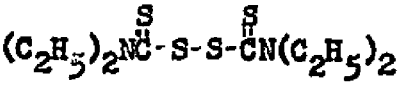
Penicillamine, β,β dimethyl cysteine is a chelating agent for copper (59, p. 953) and is the treatment used in Wilson's disease (226,221,222). It will also chelate other metal ions including mercury, zinc and lead and has been used in lead and mercury poisoning (59, p. 954). Although the pattern of amino acid synthesis in patients on penicillamine is altered, and this may play a role in the drug's effectiveness in Wilson's disease (59, p. 954). It has been shown that tissue copper is mobilized by penicillamine and that copper excretion is enhanced (225).

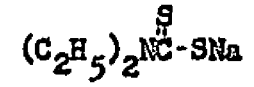


EDTA



PENICILLAMINE





DISULFIRAM DIETHYLDITHIOCARBAMATE

B. DISULFIRAM

Disulfiram, tetraethylthiuram disulfide is strictly speaking not a chelating agent, but the disulfide dimer of the chelating agent diethyldithiocarbamate.

Disulfiram is reduced *in vivo* to diethyldithiocarbamate (310). The carbamate is a well known chelating agent for copper; it has weaker chelation for zinc and is used in cupric assays (311). It also chelates ferric ions but to a much lesser degree than cupric. Disulfiram is used rather than the diethyldithiocarbamate because the latter decomposes in the acid of the stomach to produce H_2S (310). In rats, the copper-containing enzyme dopamine β-hydroxylase is inhibited *in vivo* with a dosage of disulfiram of about 100mg/day (312). It will also inhibit the enzyme *in vitro* (313). This strongly implies that it is chelating copper *in vivo*.

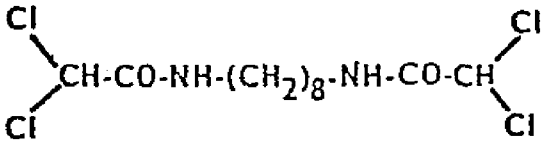
Disulfiram, under the trade name Antabuse is used to cause alcohol intolerance in the treatment of alcoholism. When a patient on antabuse takes alcohol, there is vasodilatation, respiratory difficulty, nausea and vomiting and hypotension (59, p. 147). Severe reactions, with respiratory depression, cardiovascular

collapse, heart failure and death can occur (59, p. 147). The most prevalent theory of the alcohol intolerance caused by disulfiram is that it inhibits the oxidation of ethanol. Alcohol is normally oxidized to acetaldehyde which is then rapidly further oxidized by aldehyde dehydrogenase, so that the acetaldehyde does not accumulate.

With disulfiram treatment, aldehyde dehydrogenase is inhibited and acetaldehyde accumulates resulting in the toxic symptoms seen (see 314). In addition, the acetaldehyde causes norepinephrine release and the disulfiram, by inhibiting dopamine β hydroxylase, prevents the resynthesis of norepinephrine (315,316). Others attribute the disulfiram-alcohol reaction to alcohol inhibition of the metabolism of the disulfiram, and thus increased toxicity from the disulfiram itself (310).

C. RELATED DRUGS

Our initial findings with disulfiram, (231, see below) led us to look for a related drug which might have the same embryocidal action. One drug which drew our attention was the experimental drug Win 18,446. This drug, which had been used experimentally as an inhibitor of spermatogenesis, had been abandoned for that use because the drug causes an alcohol intolerance similar to that of disulfiram (317) Win 18,446 is N,N'-bis(dichloroacetyl)-1,8-octamethylenediamine:



WIN 18,446

Win 18,446 and disulfiram also both inhibit mitochondrial oxidation and electron transport in similar ways (318).

Win 18,446 was generously given to us by Dr. A. R. Surry of the Sterling-Winthrop Research Institute, Rensselaer, N.Y. (Lot #R-010-TK). A further coverage of the pharmacology of Win 18,446 and disulfiram will be found in the discussion of the thesis (sec. IVA and B).

D. EXPERIMENTAL ANIMALS AND ADMINISTRATION OF DRUGS

Rats were chosen as the experimental animal because the embryotoxic effect of dietary copper deficiency had been demonstrated in these animals (210,211,212). In addition, they have a reasonably short gestation length (twenty-two days). Rats lack the vomiting reflex, an advantage when giving possibly noxious or emetic drugs orally.

Primapara Sprague-Dawley rats weighing from 200 to 250 grams were obtained from Marland Farms. The rats were of dated pregnancy as judged by the presence of sperm in the vaginal smear after overnight mating. The day after mating is considered day 1 of

pregnancy. The rats were kept under 14-hour light and 10-hour dark cycles at 22°C with free access to water and, except in the one paired feeding experiment described below, to Purina Laboratory Chow. The chow was powdered in experiments where agents were mixed with the feed, and in the paired feeding experiment. To eliminate the factor of reduction of food intake caused by disulfiram, the paired feeding experiment was conducted. In this experiment the amount of food that each disulfiram-treated rat consumed in a day was noted and this amount of food was given to the paired untreated control rat the following day.

Initial trails consisted of giving the copper chelating agents in the feed from day 3 of pregnancy on. Disodium ethylenediamine tetra-acetic acid, penicillamine and disulfiram were mixed with the feed at weight concentrations of 3%, 1% and 1% respectively. Because disulfiram caused anorexia it was given by gavage in subsequent experiments. Water suspensions of disulfiram at 100mg/ml were made by homogenizing Antabuse pellets in a laboratory blender. Control rats received equivalent volumes of water via gavage. In some experiments pure disulfiram (tetraethylthiuram disulfide, Aldrich) was dissolved by heating to 65°C with stirring in Mazola Corn Oil, also at 100mg/ml. The solution was cooled to room temperature just before administration. In these experiments, controls were given equivalent volumes of corn oil via gavage.

Win 18,446 was suspended (100 mg/ml) in 1% gum tragacanth in water with a laboratory blender. The Win 18,446 suspensions were administered via gavage once a day at noon, at various doses and schedules during gestation (see below). Control rats received

equivalent volumes of 1% gum tragacanth in water by gavage at equivalent schedules.

Dosages and administration schedules are detailed in Tables 1-5.

E. EXAMINATION OF PRODUCTS OF CONCEPTION

Rats were sacrificed on the day 21 of pregnancy except as noted, by decapitation for disulfiram and chelating agent studies, and by excess ether anesthesia for the Win 18,446 studies.

Day 21 fetuses were considered alive if there was any movement on gentle tapping. Live term fetuses and dead term fetuses were examined grossly for teratologies by the procedure of Wilson (319) (examination of head, limbs, lips, palate, etc.). The term "resorption button" is used in the present study to signify the remains of embryonic tissue found at the implantation sites, that have a weight one twentieth or less than that of term fetuses. Fetuses, resorption buttons, and placentae were weighed. Representative samples were fixed in Zambonis solution (320) for subsequent morphologic and histologic examination and (for term fetuses) clearing and bone staining (321). Data was evaluated using the Student t test (see 2).

F. PARTIAL HEPATECTOMY AND BIOCHEMICAL STUDIES

Because the literature indicated that Win 18,446 effects mitochondrial enzymes, (318) certain biochemical studies were done. Partial hepatectomy is an especially useful technique because one can see the effects of drugs on rapidly growing and differentiat-

ing tissue. Partial hepatectomy increases the toxic effects of disulfiram (see 310). Cell division and differation are, of course, accelerated in the situations where Win 18,446 has its most dramatic effects, spermatogenesis and embryogenesis. In addition the structurally related drug, chloramphenicol has profound effects on mitochondria during regeneration following partial hepatectomy (322).

Partial hepatectomies were performed according to the method of Higgins and Anderson (323). Male Sprague-Dawley rats, weighing 150-200 gm. were operated under ether anesthesia with clean, but not sterile procedure. A midline incision 4 cm long was made down from the sternum. The liver was exposed and a loop of double thickness 000 silk suture was slipped around both sections of the median lobe as well as the left lateral lobe. The loop was tightened while slipping it up to the bases of the enclosed lobes, and a knot tied. The median and left lateral lobes were excised with a cut just distal to the suture, and the animal examined for any bleeding. The incision was closed in two layers, first the peritonium and musculature and then the skin, using 000 silk suture. The animals were given 20% dextrose ad lib., the first 24 hours after the operation, as well as normal food and water. A few animals were lost during the operation because the suture was placed too high and too tight and interfered with the circulation of the animal. Some were lost because the suture was too loose or too low. In this case the loop would come off as the lobes were cut away and a large scale bleeding would result. No infections were seen. In later experiments mortality was under 10%.

The removed median and left lateral lobes, consisting of about 70% of the liver (see 323), was weighed and samples taken for histology and biochemical studies (see below).

Starting immediately after the operation, the animals were treated with either disulfiram 100 mg/day, Win 18,446 100 mg/day or Gum Tragacanth 1 ml/day (controls) for three days. At 72 hours the animals were sacrificed by excess ether. By this time the remaining right lateral and the two parts of the small caudate lobe had hypertrophied (in controls) from 30% to 70% of the original weight of the whole liver (see 323). These remaining hypertrophied lobes were quickly excised and weighed and samples were taken for histology and biochemical studies.

The biochemical studies consisted of measurements of cytochrome oxidase. In addition mitochondrial protein synthesis was measured in vitro with mitochondria isolated from normal rat livers.

Cytochrome c oxidase was measured spectrophotometrically (by Drs. N.G. Ibrahim and D.S. Beattie), measuring the decrease in absorbance of reduced cytochrome c at 550 nm (324).

Mitochondria were isolated (325) and incorporation of radioactive leucine into mitochondrial protein measured (326,327) (also by Ibrahim and Beattie).

III RESULTS

A. COPPER CHELATING AGENTS

The initial trials consisted of giving the disodium ethylene-diamine tetra-acetic acid (EDTA) penicillamine and disulfiram mixed with the feed at weight concentrations of 3%, 1% and 1% respectively. Each chelating agent was given to three pregnant rats from day 3 through day 21. A fourth group of three rats served as controls.

As seen in Table I two out of three rats in the EDTA, penicil-lamine and control groups were found to have complete live litters at day 21. In the disulfiram group one rat died on day 11 and at autopsy was found to have resorbed embryos. The other two had empty uteri on examination at day 21. All disulfiram treated rats drastically reduced their food intake shortly after starting.

The fetuses from the EDTA and penicillamine treated rats were smaller (means 3.0 ± 0.6 and 3.7 ± 0.5respectively than those of the controls, mean 4.6 ± 0.7 ($p. \leq .0001$) (Student t test, see 2). The fetuses from both the EDTA and penicillamine treated rats appeared normal on gross examination but seemed to bruise easily on handling.

Control rats which were pregnant gained an average of 167 ± 1.5 gm (day 21 weight - day 3 weight), which was more or less linear over gestation. The non-pregnant control gained 29 gm over the same time. EDTA and penicillamine treated rats which proved pregnant on day 21 gained an average of 56 ± 30.5 gm (although one, an EDTA treated rat, lost 26 gm). Disulfiram treated rats

TABLE I

CHELATING AGENTS MIXED IN FEED AND GIVEN DAYS 3 TO 21 INCLUSIVE

Agent	% of feed	No.of rats	Pregnancies	implants (total)	live fetuses (total)	av. fetal weight
Na_2EDTA	3	3	2	30	29	3.0 ± 0.6[b]
Penicillamine	1	3	2	28	27	3.7 ± 0.5[c]
Antabuse	1	3	1[a]	11	0	-
-----(Control)		3	2	30	29	4.6 ± 0.7

a. from examination of rat dead at day 11, b. and c. compared to controls ($p \leq 0.001$) Evaluation by Student t test (2).

lost an average of 36 $\pm$ 15 gm; the one that died on day 11 lost 70 gms by that time. The EDTA and penicillamine rats not pregnant at day 21 gained an average 9 $\pm$ 11 gm over the same period. Not unexpectedly, EDTA and penicillamine treated rats which lost weight or gained very little weight had smaller fetuses than those that gained more weight. The day to day weight changes in the control, EDTA and penicillamine treated rats which proved not pregnant at day 21 gave no indication that pregnancy had occurred and been terminated.

Our findings with EDTA and penicillamine implied that these drugs might have more damaging effect on the fetus than restricting their growth, if the drugs were given at higher doses. Indeed, other investigators have since found EDTA induced teratologies in rats and penicillamine induced teratologies in animals and humans (see 125) (see discussion).

Our search at the time, however, was for a copper chelating agent that could cause fetal resorption early in pregnancy as seen in copper deficient rats. We therefore concentrated our efforts on disulfiram. Because disulfiram caused anorexia the intake of disulfiram mixed with food was highly variable. Further experiments were done giving disulfiram by gavage with an esophageal tube.

B. DISULFIRAM

Further experiments with disulfiram were done with oral intubation of the drug. Rats were killed by decapitation on day 12 or 13 so that resorbed embryos could be seen. On occasion rats were killed on day 21 to note possible teratological effects.

Results for rats treated with 100 mg disulfiram/day from day 3 on are summarized in Table II. For rats treated with 100 mg/day disulfiram suspended in water 83% of the 124 implantation sites from the eleven rats showed resorption on examination at day 12 or 13. Resorption of the implant is characterized not only by a small fetus (one-fifth of the controls) but also by haemorrhage and disintegration. The placenta is also resorbed. A few rats were sacrificed at day 10 (not included in Table II). A typical uterus from a disulfiram treated rat on day 10, as well as one from a disulfiram treated rat at day 12 are shown in Fig. 7, with corresponding control uteri on the left. It is clear that while growth is marked between days 10 and 12 for the controls (left), in disulfiram treated rats (right) the uterine contents shrink drastically and indeed the uterus approaches the size for normal non-pregnant rats.

Control rats, either fed ad lib. or pair fed showed no fetal resorption. Weights of the uterus and its contents of control or pair fed controls were highly significantly higher than for disulfiram treated rats (Table II).

Also shown in Table II is the findings that of the 3 rats treated with 100 mg/day disulfiram in corn oil, there was 30% resorption, and no resorption in the 3 pair fed controls. There is thus more fetal resorption when the drug is given as the water suspension than for the oil solution. The water suspension is also more toxic to the mother (see below).

TABLE II

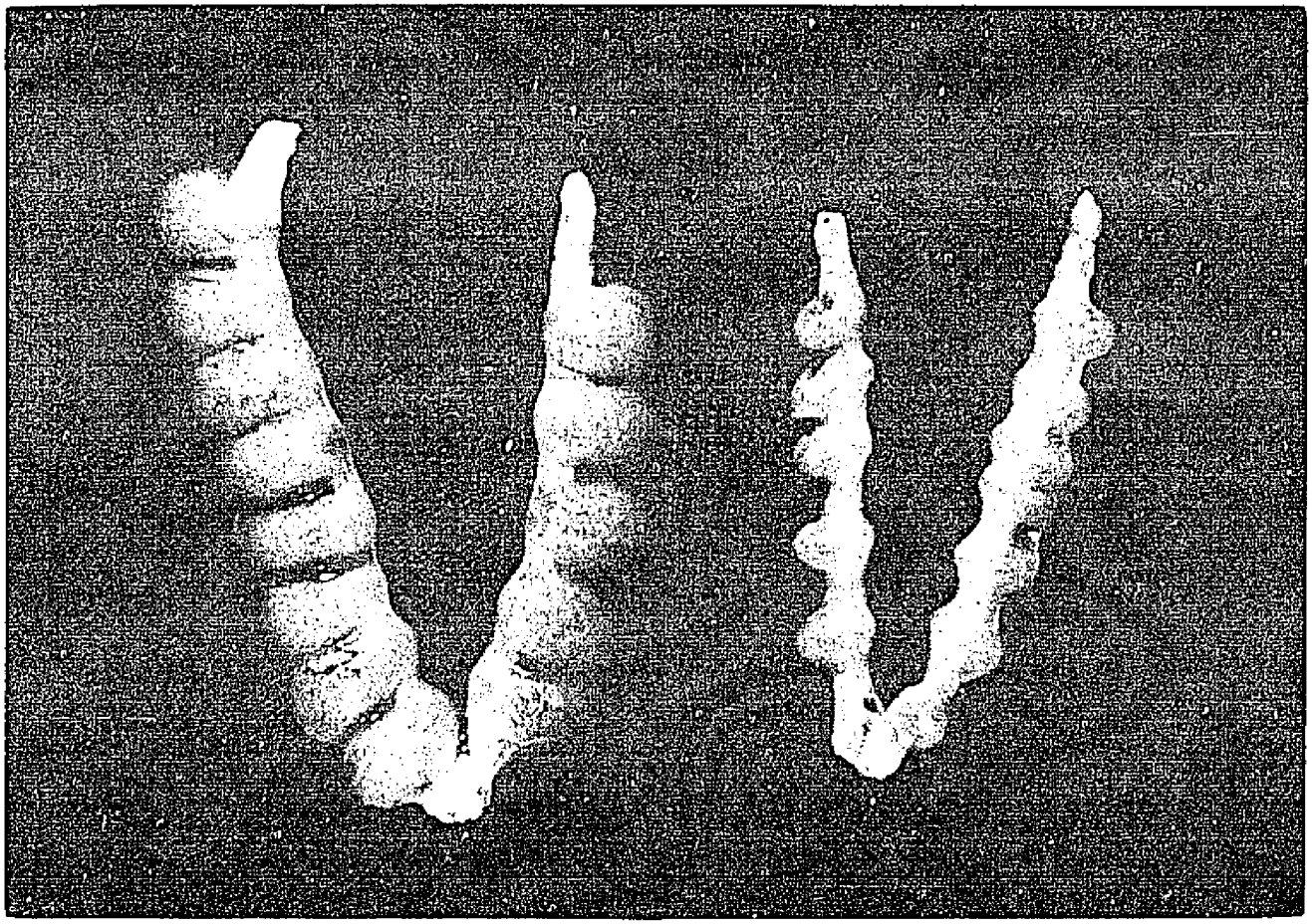
DISULFIRAM 100 mg/day ADMINISTERED VIA ESOPHAGEAL TUBE

	No.of rats	day sacrificed	No. or pregnancies	implants	live fetuses	av.weight of uterus & fetuses	% resorption
A. Water Suspension:							
Disulfiram	8	12[a]	8	87	10	0.5*	88
Control	4	12	4	52	52	2.7	0
Disulfiram	3	13[b]	3	37	11	2.8*	70
Control (pair-fed)	3	13	2	25	25	6.8	0
B. Oil Solutions							
Disulfiram	3	13	2	20	14	2.6*	30
Control (pair-fed)	3	13	3	40	40	7.3	0

died from treatment: a. one at day 7 and three at day 10; b. one at day 10.

* compared to controls ($p \leq 0.001$)

Fig. 7 Uteri of pregnant rats. Effect on the implant of oral administration of disulfiram, 100 mg. per day starting on day 3 after mating. Controls on left. Disulfiram-treated on right. Top, Day 10, bottom, Day 12.



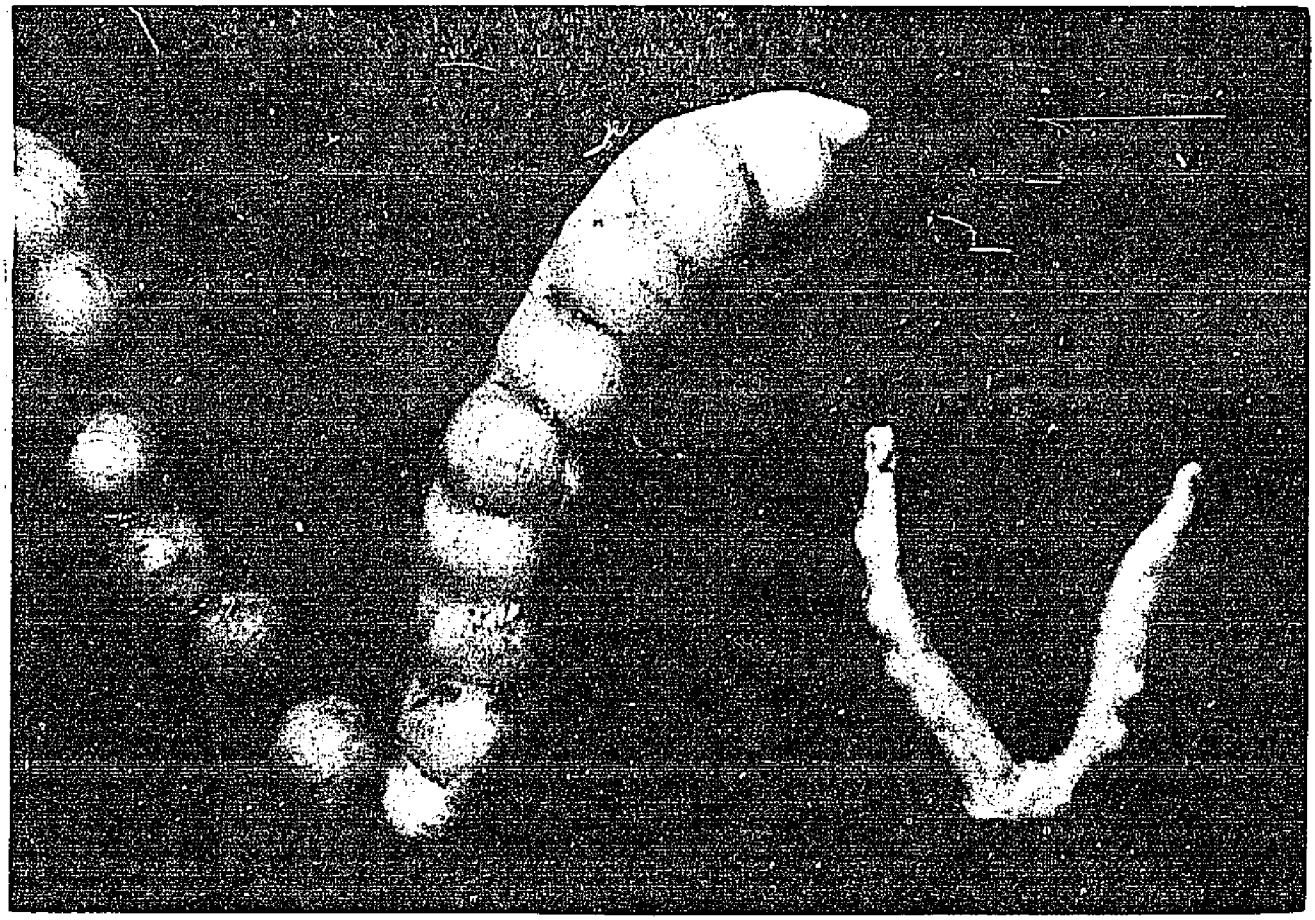


Fig. 7

Not indicated in Table II is the variability of the effect of disulfiram on resorption of embryos. Some animals sacrificed on day 10 already showed extensive resorption indicating that embryo demise had occurred a day or two before. Other rats showed evidence of embryo demise and subsequent resorption only at about day 12, while still others sacrificed at day 13 had intact live but smaller fetuses than the control rats. Although there was variability between litters, embryos within a litter were always similarly effected.

In cases where there was no resorption, there was no evidence of any gross malformations in the live fetuses. Four rats were given 50 mg/day disulfiram in water from day 3 through day 21, and sacrificed on day 21. There was no fetal resorption at this dose level. All these fetuses were alive and free of any malformations as judged by gross examination. Their weights, however, were low as compared to controls. Similarly, six rats given 100 mg/day on regimens started later than day 8 showed neither fetal resorption nor gross teratologies.

Disulfiram invariably caused a marked decrease in food intake and a consequent weight loss. Typically, the animal would eat about half its normal intake during the first 5 days of drug administration. With prolonged administration some rats would stop eating altogether. Control rats in paired feeding showed no fetal resorption and at day 13 had lost about 7% of their initial weight (at day 3). The disulfiram treated rats, on the other hand, lost an average of 17% of their initial weight, but in these rats the fetuses were resorbed.

Some of the rats showed toxic reactions to disulfiram at a dosage of 100 mg/day, especially with longer administration of the drug. These reactions ranged from lethargy, seen in most animals after about 5 days

of treatment, to coma and death, in about one-third of the rats, after a week or more on disulfiram. Symptoms of the sicker animals include ruffled fur, foul smelling yellow discharge appearing in the fur surrounding the genital area, occasional dried blood at the nostrils, less frequently shrunken appearance of one or both eyes, and, for two cases, swollen salivary glands. Autopsies of expired or very sick rats which were sacrificed often showed discoloration of the liver and hemorrhages in the lungs. Reducing the dosage to 50 mg/day greatly reduced the toxic effects, but no fetal resorption occurred in four rats examined.

Because of the toxic symptoms seen with disulfiram at doses that were effective for embryocidal action, we shifted our attention to the pharmacologically related drug Bis(dichloroacetyl)1,8 octamethylene-diamine, (Win 18,446).

C. BIS(DICHLOROACETYL)-1,8-OCTAMETHYLENEDIAMINE WIN 18,446

In the initial studies a fixed dosage of Win 18,446 was administered daily from day 3 to day 21 as in Table III (see also 328). With dosages equal to or greater than 100 mg. per day all the implants are resorbed although the implantation sites are clearly visible. At a dose of 50 mg. per day 90 percent of the embryos are resorbed, the remainder being alive but deformed. With dosages of 100 mg. per day, or greater, the resorbed embryos are less than one twentieth the weight of the control live fetuses and the placentas are about one half the weight of the controls.

T a b l e III

Win 18,446 administered from day 3 through day 21

Dosage mg/day	# rats	total implants	live normal fetuses		live deformed fetuses		resorption buttons		% resorption
			#	av.wt.(gm)	#	av.wt.(gm)	#	av.wt.(gm)	
0 (control)	2	25	25	3.7	0	--	0	--	0
50	4	49	0	--	5	2.9	44	0.09	90
100	4	49	0	--	0	--	49	0.03	100
150	3	35	0	--	0	--	35	0.05	100
200	2	24	0	--	0	--	24	0.01	100

To establish the most efficient schedule of drug administration for embryocidal action, a total dose of 200 mg. was given for various periods during gestation. Specifically, 50 mg. per day was applied for four days, 100 mg. per day for two days, and 200 mg. for one day at the days given in Table IVA and summarized in Fig. 8, A and B. Administration of 50 mg. per day applied from day 10 to day 13 gives the same results as treatment with 50 mg. per day given from day 3 to day 21. It appears that a four-day regimen at this critical period is optimal. When a total dose of 200 mg. is given for two days, or for one day around this period, the embryocidal efficacy is diminished and there is a concurrent rise in teratology. Outside of the critical period administration of the drug has practically no effect. A regimen of 50 mg. per day given on days 6 through 9 or on days 14 through 17 had no embryocidal, fetocidal, or gross teratologic effects, although the fetal weights were slightly (15 per cent) below normal. Table IVA summarizes the results for a total dosage of 200 mg (see also 328).

In order to insure complete embryocidal action, and to attempt to shorten the duration of treatment, a total of 400 mg. was administered either over four or over two days during gestation. As shown in Table IVB and summarized in Fig. 8, C and D, when the drug at this higher total dosage is given at the appropriate time, over four days or over two days, all the embryos are resorbed (Fig. 8). The effective period is days 10 through 13 for 100 mg. per day and days 10 and 11 for

T a b l e IVA*

Win 18,446 administered at different times with a total dose of 200mg

Days of drug administration	6	7	8	9	10	11	12	13	14	15	16	17
controls (7 rats, 89 implants)	(gum tragacanth only)				0, 0							
50mg/day for 4 days (9 rats, 114 implants)	0 , 0				10 , 90				0 , 0			
100mg/day for 2 days (11 rats, 128 implants)	7,63				81,19							
					20,80							
200mg/day for 1 day (8 rats, 105 implants)	10,72				96,4		0, 7		8, 0			

*The number on the left is the %live, deformed fetuses and the number on the right is the % dead fetuses and resorbed embryos

T a b l e IVB

Win 18,446 administered at different times with a total dose of 400mg

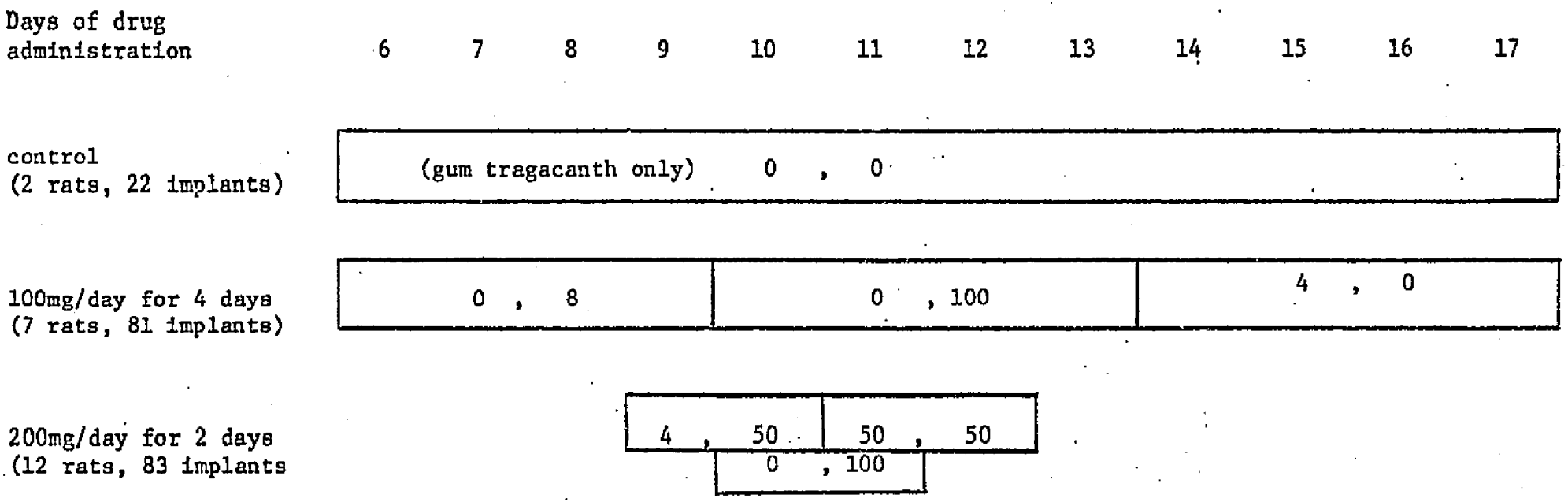


Fig. 8. Results for Win 18,446 for various regimens. A, 50 mg. per day for four days; B, 100 mg. per day for two days; C, 100 mg. per day for four days; D, 200 mg. per day for two days. Black, per cent fetal destruction; striped, per cent live but deformed fetuses.

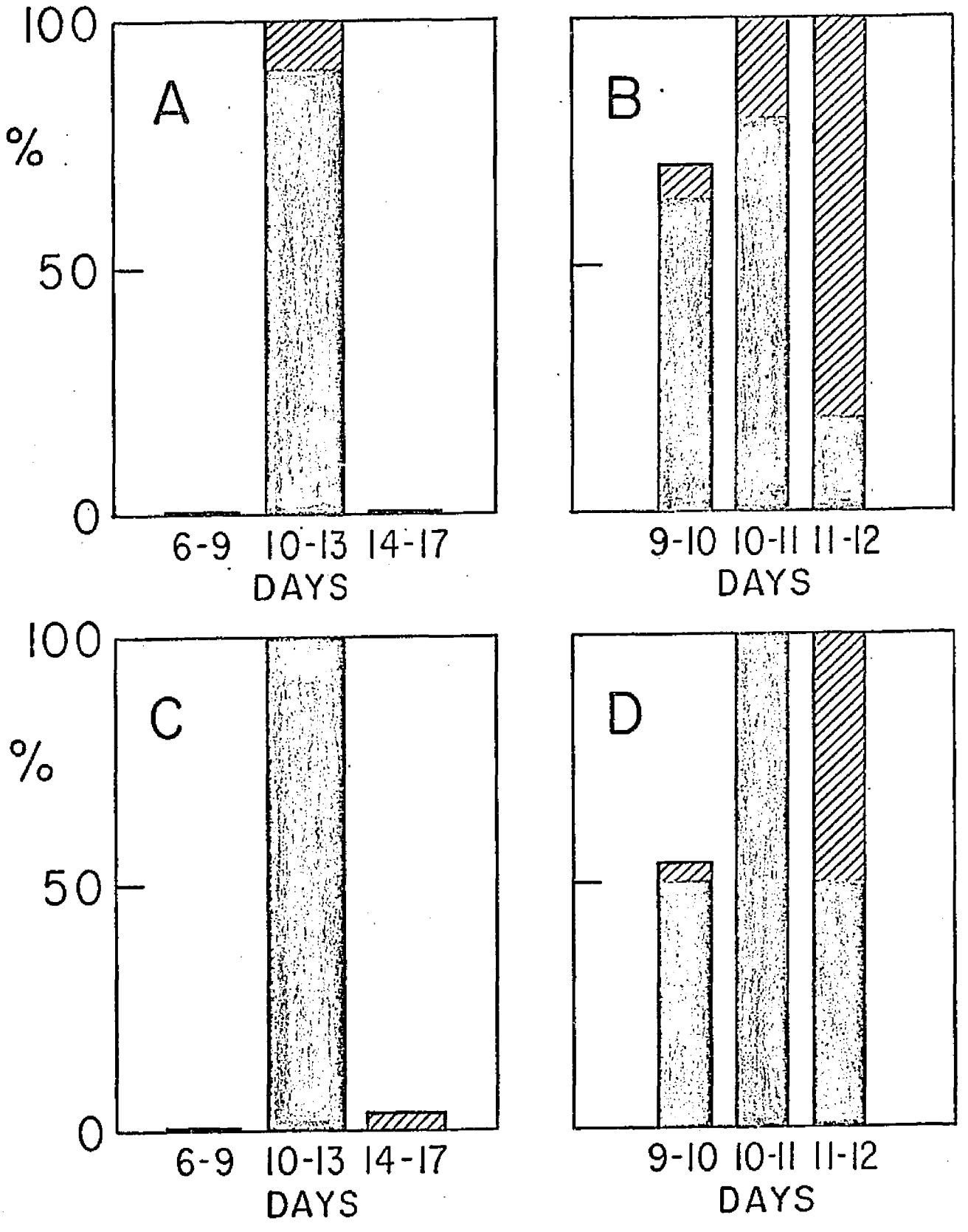


Fig. 8

200 mg. per day. When the drug is administered for four days either at days 6 through 9 or at days 14 through 17, no embryocidal action takes place. Of these two regimens, in the former there were no gross malformations, but in the latter regimen one fetus of the 24 live fetuses appeared small and slightly edematous. With 200 mg. per day given on days 9 and 10, in one pregnancy 13 fetuses out of 14 implants were alive and apparently normal, while in another pregnancy 13 embryos out of 14 implaints were destroyed but with one live microcephalic fetus. When 200 mg. per day are given on days 11 and 12, one half of the embryos were resorbed and the remainder were alive and clearly malformed. Evidently the crucial day for administering the drug to cause the resorption of all the embryos is day 10.

The term "resorption button" is used in the present study to signify the remains of embryonic tissue found at the implantation sites, that have a weight one twentieth or less than that of term fetuses (Fig. 9).

Resorption buttons from rats treated with the most effective doses during the sensitive period are typically flattened, bean-shaped, reddish-brown masses, 4 to 8 mm. long and 1 to 2 mm. thick (Fig. 9). Sometimes the embryonic material has degenerated bo a small, soft shapeless pulp. Preliminary histologic examination of typical resorption buttons shows almost total destruction of the tissue architecture and extensive regions of necrosis. In contrast, when the regimen is below optimum, i.e., when some live malformed fetuses are present, the resorption buttons tend to be larger.

Fig. 9. Typical implants at day 21. Left, control fetuses and placentas; right, resorption buttons and placentas produced by the administration of 200 mg. per day for two days of Win 18,446.

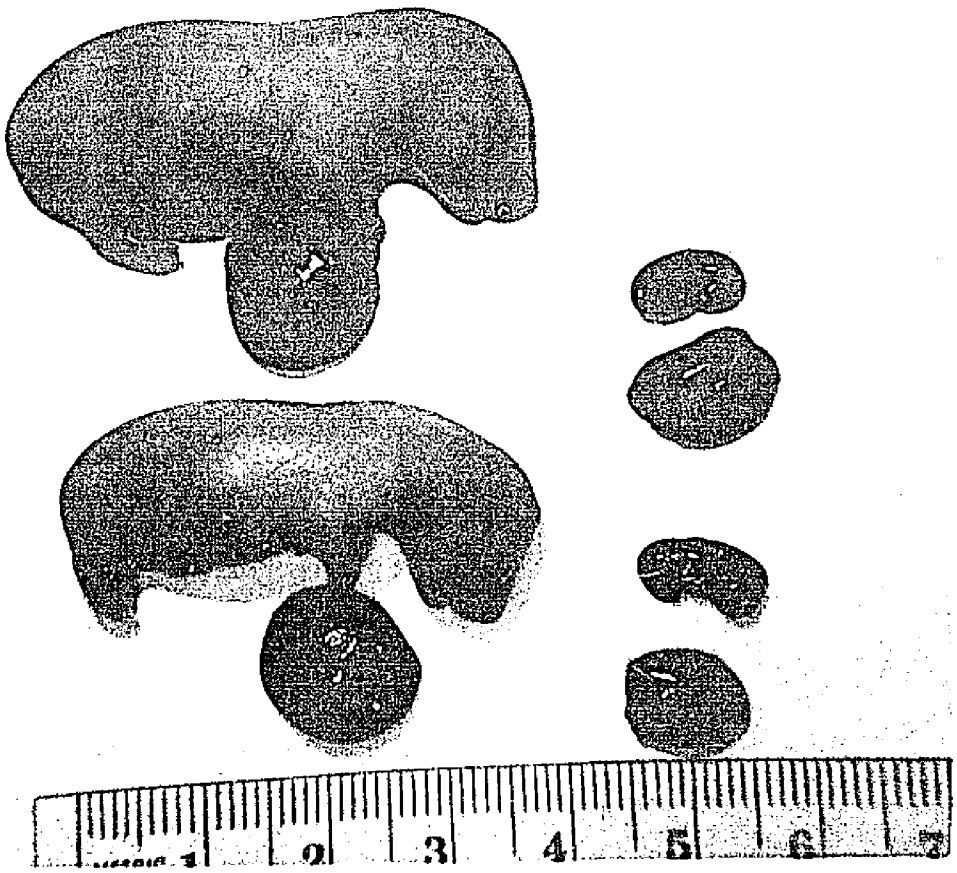


Fig. 9

When the fetal membranes of these buttons are removed, some of the grosser developing structures can be discerned. These include compressed and distorted head features, limb buds, and tail. Typical live malformed fetuses show a characteristic syndrome for Win 18,446 treatment. This consists of general edema and developmental abnormalities of the anterior portion of the body; the latter include microcephaly and cleft lip. Of particular interest is the fact that with a dosage of 200 mg. per day for days 11 and 12 all of the fetuses exhibited bilateral cleft lip. We have carried out a detailed morphologic study of these teratologies as well as a histological examination of the resorption buttons and their placentas. (See sec. III D).

With all the regimens employed, the placentas appear to be less affected by the drug than are the embryos. Even with the optimal regimen the placentas are about one half the normal weight whereas the embryonic masses are about one twentieth the weight of normal fetuses (Fig. 9). These placentas are roughly the weight of normal placentas at day 17. Gross examination of the placentas from treated rats revealed little or no necrosis.

Win 18,446 is not toxic to the mother. Only occasionally in the high dosages administered from day 3 through day 21 was a side effect found and this consisted of corneal lesion associated with dryness of the eye. In contrast to disulfiram treatment, the rats treated with Win 18,446 show no loss of appetite. Indeed, rats under the optimal regimen typically gain almost as much weight as the control pregnant rats, if the difference in the weight of uterine contents is considered. Typically, a treated rat with 12 implantation sites

gains about 90 Gm. from day 3 to day 21, while a control rat with 12 live fetuses gains 160 Gm. The uterine content of the treated rats is about 10 Gm., and of the control about 60 Gm., so that on subtraction, the weight gain is 80 and 100 Gm., respectively. A nonpregnant rat gains 30 Gm. during this period.

D. TERATOLOGIES INDUCED BY WIN 18,446

As mentioned in Sec. III C, administration of Win 18,446 at times and dosages which are not quite optimal for embryocidal action (Fig. 8) often resulted in malformations in the surviving fetuses. The surving fetuses ranged from grossly normal to severely malformed, as seen in Fig. 10. These fetuses, produced by administration of 200mg of Win 18,446 on days 11 and 12, are characterized by severe edema, deformed blunted snout and bilateral cleft lips.

A further study of Win 18,446 induced teratologies (329) indicate that the drug is capable of producing characteristic major congenital malformations in as many as 100% of the offspring with various dosage schedules, even by as little as a single treatment. The results summarized in this section will appear elsewhere in a detailed study by Taleporos et al. (329).

A total of over 50 pregnant rats were given Win 18,446 at various dosages and schedules as shown in Table V. Fifteen controls were given gum tragacanth alone at corresponding schedules. A total of 398 live fetuses from Win 18,446 treated rats were examined at day 21, as well as 183 controls. All were examined for gross teratologies and most were also preserved and sectioned for internal examination.

Fig. 10. External form of a control (A) and an experimental (B) fetus, the latter produced by the administration of Win 18,446 at 200 mgs per day on gestation days 11 and 12. Enlarged front(C) and side views (D) of the head illustrate the typical blunting of the snout with bilateral cleft lip. All fetuses from this litter were identical and, in addition to the facial malformations, displayed a characteristic edema with an overall reduction in body size and weight.

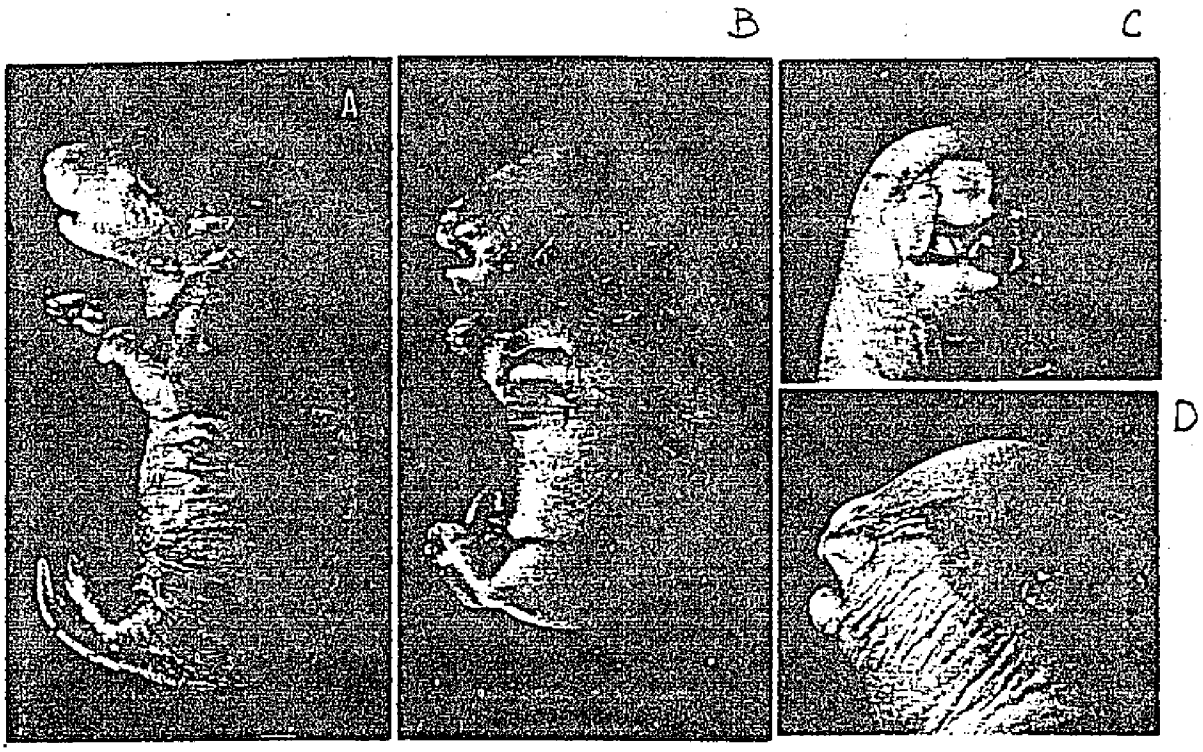
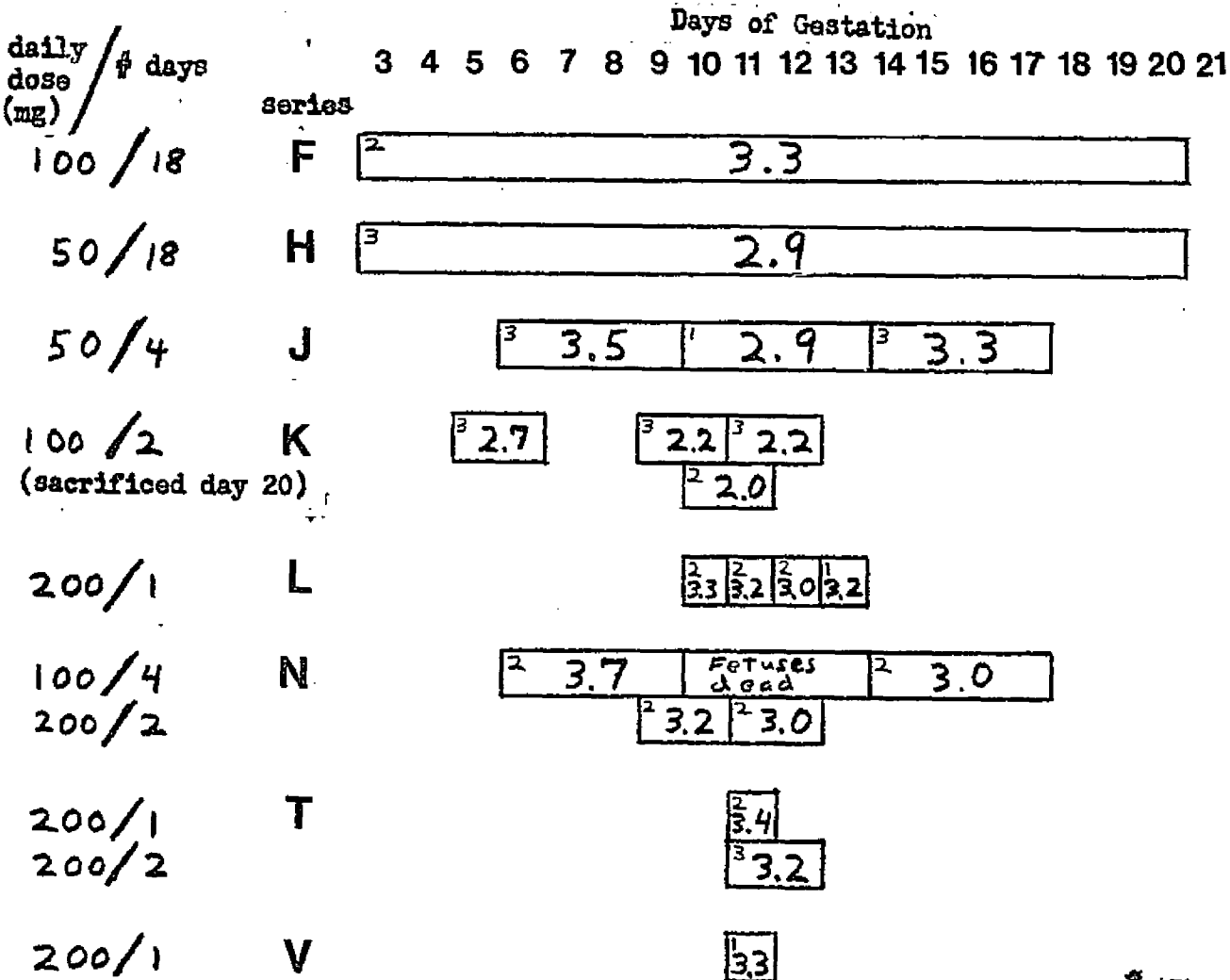


Fig. 10

Table V.

Dosage schedules of Win 18,446 administration for teratogenesis*



Controls		
	# dams	Av. Fetal Wt. (gm.)
	2	4.1
	2	3.7
	2	4.0
	3 (day 20)	2.2
	2	3.8
	2	4.2
	2	3.8
	—	—

Means: all live Win 18,446 treated fetuses (excluding series K), 33 mother rats, fetal weights $\bar{X} = 3.22 \pm .06$, controls (excluding ser. K), 12 mother rats, fetal weights $\bar{X} = 3.93 \pm 0.12$, $p < .005$.

* The small number in the upper left corner of each box is the number of mother rats in the group, the large number is the average weight of the live fetuses in grams.

1. Face:

The most obvious malformations were those of the head. These were seen only in dosage schedules that included any day between days 10 and 13. As seen in Fig. 11, severely malformed blunt snouts appeared in practically all fetuses whose mothers were given Win 18,446 on any schedule that included day 11. If day 12 was also included in the regimen, bilateral cleft lips were almost always seen. Regimens that did not include day 11 but did include either day 10 or days 12 or 13 often resulted in the less severe malformation, pointed snout. Deformations of the snout thus extended over a spectrum from the most severe, blunt snout with bilateral cleft lips if the drug was given on day 11 to pointed snout if the drug was given on adjacent days. The structure of the facial bones reflects these (Fig. 12). In addition, on close comparisons some of the fetuses from others that were given the drug on days 9 and 10, 13, and 14-17 exhibited slight alterations intermediate between normal and pointed, although they are recorded as normal.

2. Body:

Edema and small size were the main deformities seen in basic body form. The edema was most pronounced if the dosage schedule included day 11 or especially days 11 and 12. These fetuses, the same that had blunt snouts and bilateral cleft lips were especially small and grossly edematous. Skeletal structures were also effected with irregular or small ribs, pectoral girdle, sternebrae, pubic, and hind limb bones (Fig. 12B).

Fig. 11. The pattern of facial malformations produced by Win 18,446. Any regimen that included day 11 produced facial alterations in all surviving fetuses, with blunt snouts predominating. (Blunt snout with bilateral cleft lip, an extreme malformation of the face, was obtained when treatment on day 11 was extended to include day 12.) Pointed snouts, a lesser malformation, was found most often when the drug was administered on days adjacent to (but not including) the critical day 11.

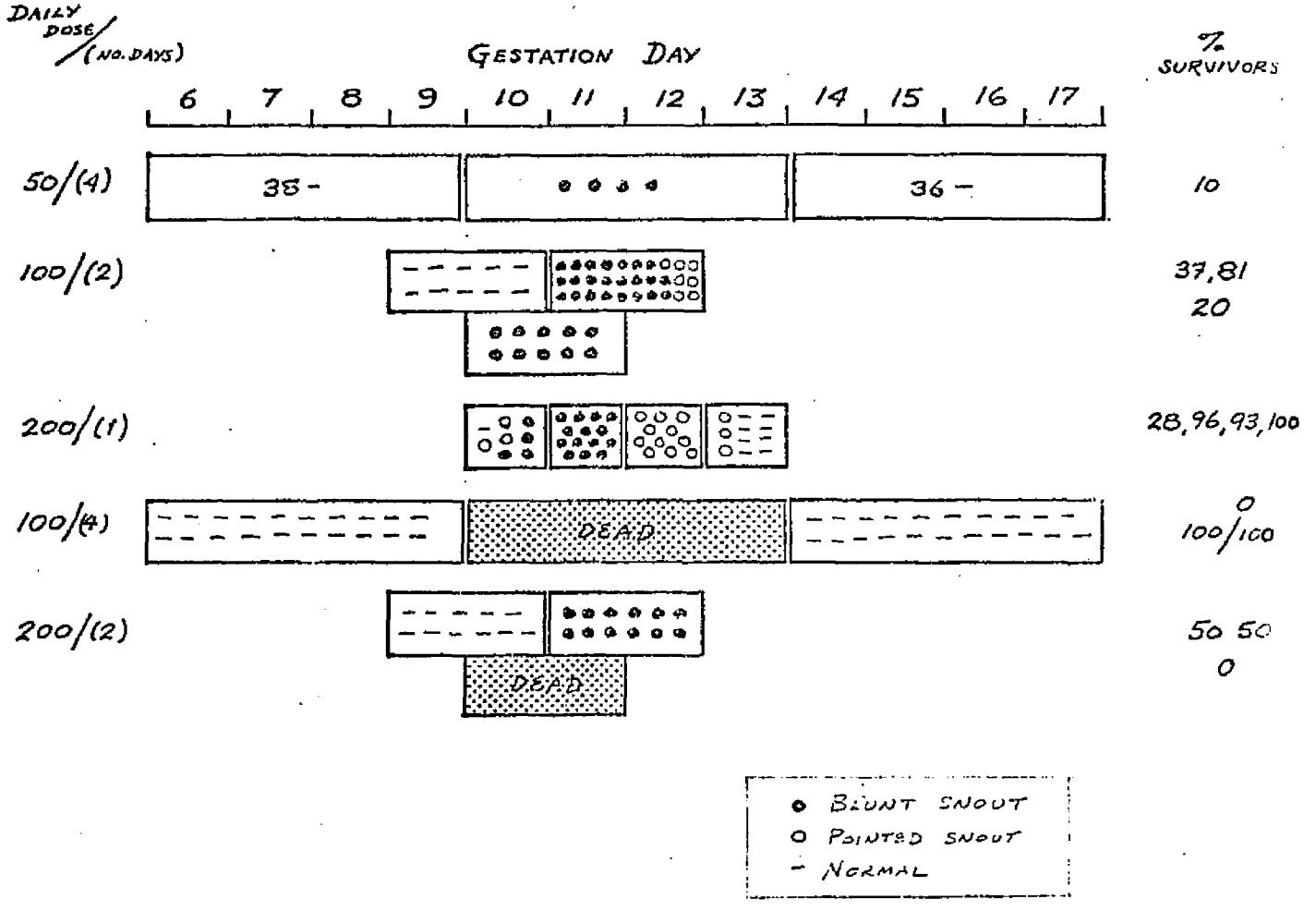

DAILY DOSE / (NO. DAYS)
GESTATION DAY
% SURVIVORS
6 7 8 9 10 11 12 13 14 15 16 17
50/(4)
38 -
36 -
10
100/(2)
37,81
20
200/(1)
28,96,93,100
100/(4)
DEAD
0
100/100
200/(2)
50 50
DEAD
0
BLUNT SNOUT
POINTED SNOUT
NORMAL

Fig. 11

Fig. 12. Cleared and bone-stained specimens of a normal (N), a pointed snout (P), and a blunt nosed fetus with bilateral cleft lip (B). In the latter, the development of the skull, especially the facial region, was most severely altered by the drug, but the ribs, sternum and scapulae were also strongly affected. The skeleton of the pointed snout fetus shows a similar spectrum of changes but to a lesser degree.

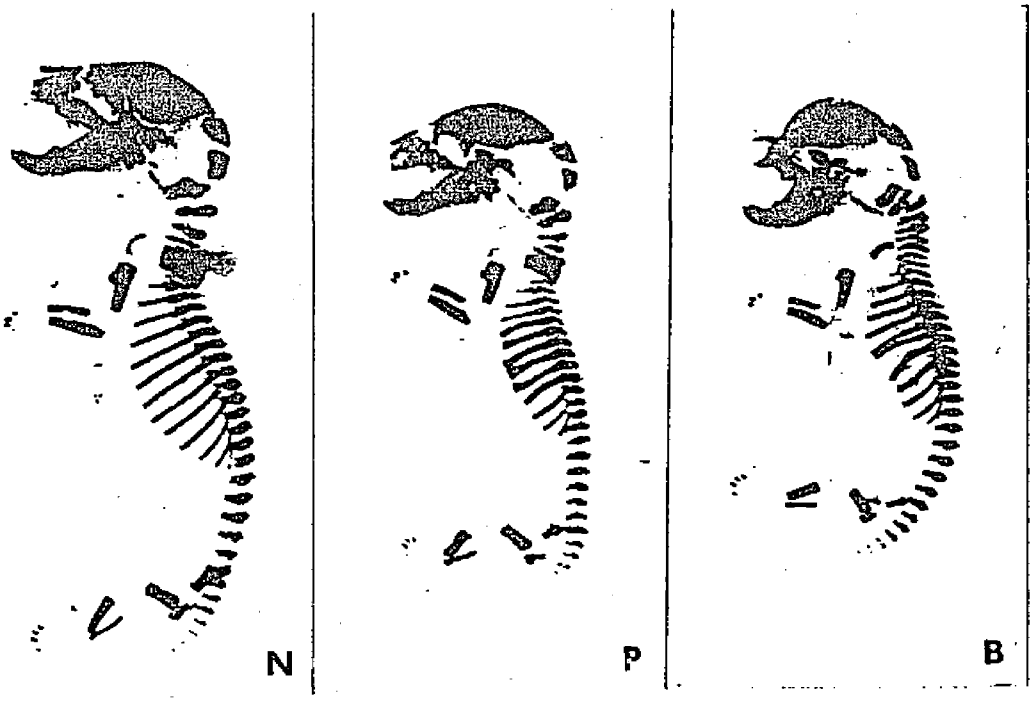


Fig. 12

For schedules that include days 10, 12 or 13 the body and skeletal malformations are reduced, while for schedules including days 9 or 14 the fetuses approach normal appearance, but are still smaller than controls (see below).

Regimens that included day 10 resulted in occasional forelimb abnormalities. There was some ectrodactyle (missing part or all of a digit) in the shorter regimens including day 10 and an increased incidence of ectrodactyly as well as hypoplasia of the forelimb in extended regimens (50 mg/day for days 3 to 20). In addition there was brachydactyly (short digits) in all the fetuses whose mothers were treated on days 14 to 17. This group also had different types of internal teratologies as compared to fetuses from rats treated on or near day 11 (see below).

3. Body Size:

The average weight for live fetuses from others which received Win 18,445 at any dosage schedule (see Table V) was 3.22 $\pm$ .12 gm, a statistically significant difference ($P < .005$). Average fetal weights for each dosage schedule group were arranged in rank order. No clear pattern of correlation of dosage schedule, daily dosage, or total dosage could be seen with weight of the live fetuses. Even the heaviest group of fetuses from Win 18,446 treated mothers (100 mg/day for days 6-9, average weight 3.66) were more than two times the standard error lower than the control average.

4. Internal Malformations

The internal malformations of the soft tissues were also most severe when Win 18,446 was given in dosage schedules that included

day 11. Many of these deformations occurred in high frequency and can be considered a characteristic syndrome. These malformations include persistent truncus arteriosus communis, absent or irregular thymus, fluid filled cavities in the neck, diaphragmatic hernias and chryptorchism. Other abnormalities also occurred but how often they occurred was difficult to establish by the examination procedures used (free hand razor blade slices). These include tracheoesophageal fistula, retinal coloboma (fissure of the retina) and interarterial septal defect.

If the drug administration schedule included days 10 or 12 but not day 11, the malformations were less severe. If the drug was given in days 14 to 17 a different pattern of abnormalities emerged. These included diaphragmatic hernias, cryptorchism and brachydactyly (short digits).

Septal Heart Defect. All fetuses from rats treated with Win 18,446 at any of the dosages tested, in regimens including day 11 had suppressed development of the septum dividing the truncus arteriosus into the aortic and pulmonary branches. As is usually the case with persistent truncus arteriosus interventricular septal defect was also found.

If the drug was given in a regimen that included days 10 or 12 but not day 11 then the variety of deformations seen were varying degrees of incomplete or faulty division of the truncus arteriosus. These included Fallot's tetrad (pulmonary stenosis, ventricular septal defect, detroposition of the aorta, right ventricular hyperplasia) or transposition of the great vessels and interventricular septal defect (see Fig. 13).

Fig. 13. The pattern of septal heart defects produced by Win 18,446. The division of the outflow tract and/or of the ventricle was affected in all of the fetuses examined when the drug was administered in any schedule that included days 10, 11, or 12. Day 11 was the most sensitive, and any treatment that included this day resulted, in all instances, in persistent truncus arteriosus (absence of septum). The adjoining days (10 and 12) are less sensitive, and administration of Win 18,446 on these days (but excluding day 11) resulted predominantly in imperfect septal division of the truncus.

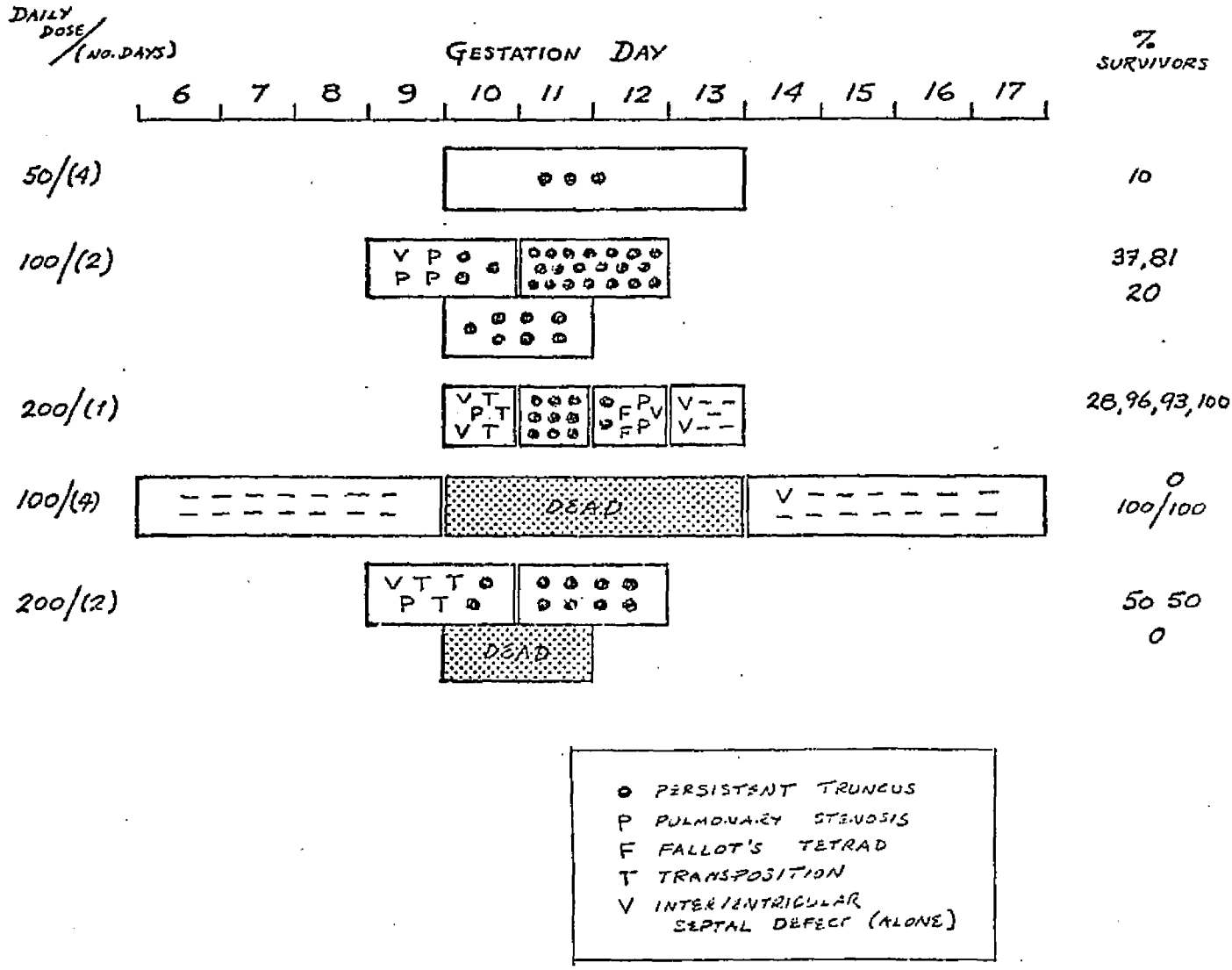

DAILY DOSE / (NO. DAYS)
GESTATION DAY
6 7 8 9 10 11 12 13 14 15 16 17
% SURVIVORS
50/(4)
10
100/(2)
37,81
20
200/(1)
28,96,93,100
100/(4)
DEAD
0
100/100
200/(2)
50 50
DEAD
0
P PERSISTENT TRUNCUS
P PULMONARY STENOSIS
F FALLOT'S TETRAD
T TRANSPOSITION
V INTERVENTRICULAR SEPTAL DEFECT (ALONE)

Fig. 13

Interatrial septal defects also occurred. These were in fetuses from rats on drug treatment regimens that included days 10 to 12 and especially if the drug was given on days 11 and 12. Other vascular abnormalities, were also seen with drug treatment around day 11.

5. Cervical:

Administration of Win 18,446 in regimens that included days 10 or 11 resulted in absent (day 11) or irregular (day 10) thymus.

Large cervical pockets and faulty separation of trachea and esophagus were also seen. The pockets were bilateral spaces in the anterior part of the neck below the inner ear, down to the sternum. These occurred in 89% of the fetuses from rats given 100 mg/day Win 18,446 or more in regimens that included day 11. Some fetuses from rats treated on days 10 or 12 also had these pockets. Faulty separation of trachea and esophagus was seen in at least 80% of the fetuses from rats treated on days 11 and 12, and a lower percentage of the fetuses from rats treated on day 11 but not 12. This faulty separation was usually seen as a tracheoesophageal fistula with atresia of the esophagus.

6. Diaphragmatic hernias

As seen in Fig. 14 most fetuses from rats treated with Win 18,446 from day 10 to 17 had some form of diaphragmatic hernia. The hernia was massive in fetuses from early treatment schedules (days 10 to 12) and smaller in the later schedules (days 14 to 17). Some of these latter had thin membraneous diaphragm with no musculature rather than a true herniation. The diaphragmatic hernias were predominantly

Fig. 14. The pattern of diaphragmatic hernias produced by Win 18,446. Sensitivity to the drug appears to begin on day 10 and continues to the 14-17 day experimental group whose time span embraces the closure of the pleuroperitoneal canals (gestation day 16 1/2). Those regimens that included days 10 and/or 11 (but not later times of development) produced hernias only of the left side. Right sided hernias, alone or bilaterally, occurred only when the drug was administered on day 12 or later.

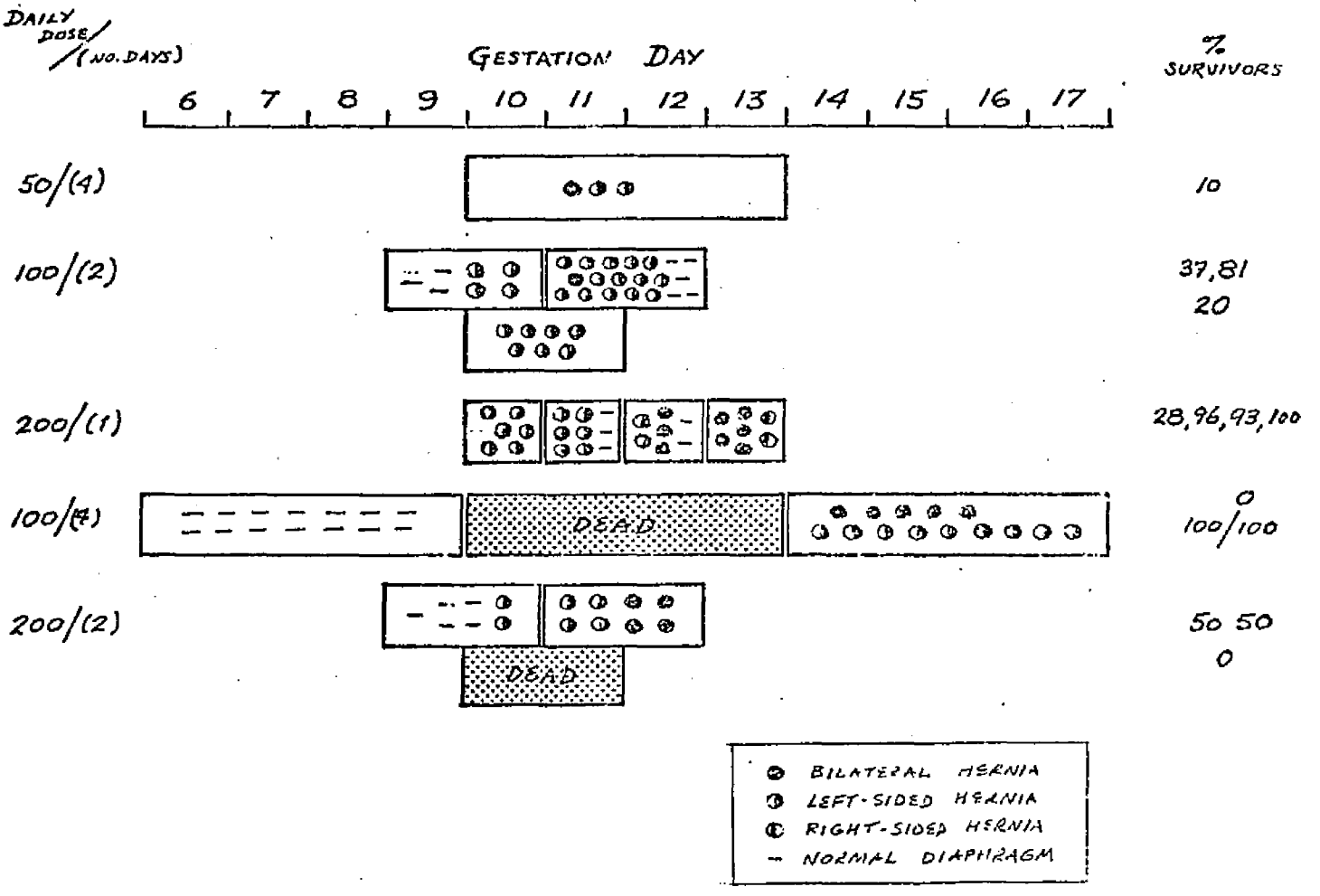

DAILY DOSE / (NO. DAYS)
GESTATION DAY
6 7 8 9 10 11 12 13 14 15 16 17
% SURVIVORS
50/(4)
10
100/(2)
37,81
20
200/(1)
28,96,93,100
100/(4)
DEAD
0
100/100
200/(2)
50 50
DEAD
0
BILATERAL HERNIA
LEFT-SIDED HERNIA
RIGHT-SIDED HERNIA
NORMAL DIAPHRAGM

Fig. 14

herniation. The diaphragmatic hernias were predominantly left sided. Right sided ones (or bilateral) tended to occur in regimens that included day 12 or 13. All fetuses in the group treated on days 14-17 had either the thin membraneous diaphragm or a true hernia.

7. Cryptochism

Like the diaphragmatic hernias, cryptorchism was seen in fetuses from rats treated with Win 18,446 as early as day 10 and as late as days 14-17. In the early period day 11 is again the most sensitive, with 92% of the male fetuses from schedules including day 11 having one or both testes incompletely descended. All male fetuses from rats treated on days 14-17 showed marked bilateral cryptorchism. Female fetuses showed a similar inhibition of descent of the gonads. In schedules with the drug given on day 11, one or both ovaries were high in 35% of the female fetuses. With treatment on days 14 to 17 all showed inhibited descent of the ovaries. Along with the gonadal defects were various defects of the kidneys and ureters.

8. Head:

Aside from the malformations of the snout and lips mentioned before, many fetuses from Win 18,446 treated rats had eye malformations. These included microphthalmia, retinal coloboma (part of the lens lacking) and other eye and eyelid malformations. Eye malformations occurred most frequently in regimens including day 11, but also in regimens including adjacent days (10 and 12). Changes in the shape of the skull of fetuses with bilateral cleft lips (Fig. 10, Fig. 12) resulted in compression of the brain especially of the front of the brain.

9. Controls:

As mentioned previously a total of 15 rats were given gum tragacanth alone at volumes and dosage schedules corresponding to various experimental groups. All 183 of the fetuses from these rats were externally normal. Of these 19 were preserved, 12 were examined internally and 7 were cleared and bone stained. No malformations were seen except for a dot like 14th rib in 2 of the bone-stained control fetuses. Other fetuses from rats given gum tragacanth, controls in other experiments, have likewise been examined and no malformations seen.

E. RELATED BIOCHEMICAL EXPERIMENTS

Male rats were partially hepatectomized and then treated with disulfiram (100 mg/day), Win 18,446 (100 mg/day) or gum tragacanth (1 ml/day) (controls) as described under Materials and Methods, sec. F. The livers from Win 18,446 treated animals showed much more fatty material than did the controls. The cytochrome c oxidase activity of the liver from Win 18,446 treated rats was one-third of that of the controls.

Mitochondrial protein synthesis, as measured by the incorporation of labelled leucine into mitochondrial protein, was considerably suppressed by as little as 1 microgram per ml of Win 18,446. Larger concentrations of disulfiram or of chloramphenicol were required to produce the same extent of inhibition.

IV DISCUSSION

A. COPPER CHELATING AGENTS

The diet in the copper deficiency studies on rats (210,211,212) is milk treated with hydrogen sulfide to remove the last traces of copper. We thought simpler approach to the removal of copper from rats during pregnancy would be to feed the animals a copper chelating agent. Disulfiram given orally to rats at a dose of 100mg per day from day 3 of pregnancy causes fetal resorption. Just as in the case with the copper-deficient diet, implantation occurs for the disulfiram-tested rats and by day 13 most of the implants were resorbed.

Disulfiram itself is not, strictly speaking, a chelating agent but is reduced *in vivo* to form the diethyldithiocarbamate (310).

The carbamate is a well known chelating agent specific for copper with much less chelation for zinc (311). The water soluble sodium salt becomes soluble in non-aqueous solvents when complexed with copper. The disulphide, disulfiram, is lipid soluble. For rats, the copper-containing enzyme dopamine B-hydrolase is inhibited *in vivo* with a dosage of disulfiram of about 100mg per day (312). Disulfiram is called Antabuse because of the alcohol-intolerance it induces in humans. This effect is thought to be primarily due to the buildup of aldehydes brought about by the inhibition by disulfiram of aldehyde dehydrogenase (see 314). Inhibition of dopamine B-hydrolylase and acetadehyde-induced release of norepinephrine are also involved (315,316).

The similarity of our fetal resorption studies on rats with those produced by copper-deficient diets (211,212) strongly implies that disulfiram, as we have administered it, renders copper unavailable to

the developing implant. Neonatal liver has seven times the concentration of copper than does adult liver (217,218,also 209,Table 9). Maternal serum copper levels increase markedly during pregnancy and, indeed, a fall in maternal plasma levels of copper has been used as an indication of imminent fetal death (203,213,273,274). Copper could play a critical role in development in a variety of ways. It is known that copper deficiency can lead to elastin disorders resulting in internal hemorrhage in the chick embryo (330), and in the rat fetus (304). Copper is also known to be required for erythropoiesis (214,215) and bone development (215,304).

Our results with disulfiram differ from those with copper-deficient diets (211,212) in that fetal demise occurs with the chelating agent at various stages of development whereas with a copper-free diet it occurs more regularly. Thus, with the diet used by Howell and Hall (212) fetal death was found to occur at day 13 while with disulfiram fetal deaths occurred at various stages of development. This variability could have been caused by differences in food intake and, hence, copper ingestion. With less severe copper deficiencies Hall and Howell (211) found more variation in the timing and egree of fetal resorption. Copper may not be needed only at one critical period of embryonic development but rather increasing amounts may be required as development progresses. As we have shown, 50mg per day or less of disulfiram lead to smaller fetal weightsaat day 21 in agreement with the findings of others (331).

For fetal resorption in rats the dosages of disulfiram required cause toxic reactions in the mother. Toxic reactions included loss of weight, lethargy, hunching of the back, foul smelling discharge, and, with longer administration or higher doses, coma and death (231).

Others have noted the same symptoms (332). The lethargy and coma may be a result of the known inhibition of dopamine B-hydroxylase by disulfiram (312), blocking norepinephrine synthesis. Autopsies of the rats revealed hemorrhagic lungs and discoloration of the liver (231), also noted by others (332).

The toxic reactions seen in the mother rats could also have occurred in the fetuses, and the fetuses may have died from these toxic effects of disulfiram.

A striking characteristic of the treated animals was their almost total loss of appetite. This anorexia produced by copper chelating agents is attributed to the loss of taste and smell (see Sec. B1a).

The decrease in food intake of rats on disulfiram cannot of itself explain the fetal resorption effect as seen by the lack of resorption in the paired control rats (Table II). Deliberate starvation of mated rats to the point of maternal death does not affect early fetal development (333).

Although we did not measure serum or tissue copper levels of rats treated with disulfiram, the doses which we used are known to inhibit the copper-containing enzyme dopamine β-hydroxylase in vivo in rats (312). Disulfiram is metabolized to the copper chelating agent diethyldithiocarbamate (310). The diethyldithiocarbamate is known to affect copper metabolism, as it alters serum copper levels when given to dogs (334).

Penicillamine and Na_2EDTA are copper chelating agents yet we did not find them to induce fetal resorptions although they both caused a reduction in the weight of the 21 day old fetuses. Swenerton and Hurley (335) found that 3% Na_2EDTA added to an artificial feed caused fetal resorption and the effect could be reversed with addition of zinc salts.

Na_2EDTA also chelates calcium and it might be expected that the high level of calcium in the feed would render Na_2EDTa unable to chelate copper which is present in only trace amounts. The diet we use is lower in zinc (in parts per million, 58 for ours versus 96 for theirs) and lower in copper (18ppm versus 29ppm) but higher in calcium (1.2% versus 0.5%). It is possible that the additional zinc with which they reversed the effect ties up the Na_2EDTA so that more copper is available to the developing embryo. Penicillamine being a mercaptan binds copper but not calcium, and is used to increase copper excretion in Wilson's disease (224,226). It is possible that the dosages of penicillamine which we employed were not sufficient to induce fetal resorption. At higher doses penicillamine causes teratologies in animals and man (see 125). The difference of the effect on the fetus between disulfiram and the two other chelating agents may be due, in part, to the different solubility characteristics of the copper chelates. The complex of copper ion and diethyldithiocarbamate is lipid soluble whereas the copper complex of Na_2EDTA and of penicillamine are water soluble.

A different approach to the interruption of early pregnancy is to interfere with DNA synthesis. Thiersch and Phillips in 1950 (336) showed fetal resorption with injection of the folic acid antagonist, aminopterin. Although disulfiram is a less toxic drug, we applied it at levels far in excess of those noramlly used clinically for chronic alcoholism. Aminopterin is extremely teratological (336). Disulfiram in smaller amounts than we used as produced occasional teratologies when given to animals in combination with other agents (331,337). The evidence that it is teratological by itself at these levels is inconclusive (331,337).

In summary, the similarity of our results with those found by others for rats fed on a copper-free diet indicates that disulfiram renders copper unavailable to the developing embryo and causes its death and resorption.

B. WIN 18,446: EMBRYOCIDAL ACTION

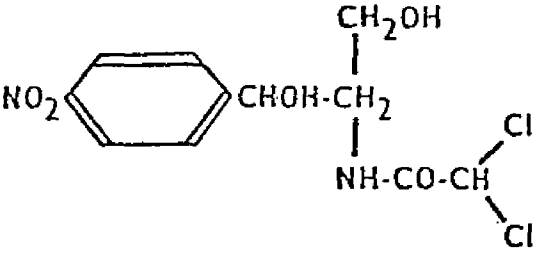
In our further search for oral abortifacients we tried the experimental drug N,N'-bis(dichloracetyl)-1,8-octamethylenediamine (Win 18,446 Sterling-Winthrop). This bis(dichloracetyl) diamine has many pharmacological properties similar to disulfiram. Both compounds cause alcohol intolerance presumably by their known inhibition of aldehyde dehydrogenase (318). Disulfiram also has some striking similarities to Win 18,446 in regard to inhibition of mitochondrial oxidation and electron transport (318). Here both compounds inhibit oxidation of pyridine nucleotide-linked respiration while oxidation of succinate is not affected. Disulfiram at near-toxic dosages is also an embryocidal drug and acts at about the same time period in gestation as does Win 18,446 (231). All of these facts suggest that the mechanism of embryocidal activity of Win 18,446 and disulfiram are similar.

Our results demonstrate that for rats Win 18,446 is a highly efficient embryocidal agent. It can be given orally and, even at levels higher than the minimal effective dose, there is no toxicity to the mother. Although the drug is most effective when administered at days 10 through 11, a longer schedule that includes these two days is not harmful to the mother.

Win 18,446 has very low toxicity: for mice the LD_{50} is greater than 16,000mg per kilogram (338). For human male subjects dosages of 500mg per day have been given continuously for 23 weeks (339). The effects, aside from the documented suppression of spermatogenesis and alcohol intolerance, are minor complaints of "gas." To suppress spermatogenesis in rats required a dosage of 125mg per kilogram daily as compared with 7mg per kilogram for human subjects, i.e., a ratio of 18:1.

In adult rats, mice, rabbits, and dogs, Win 18,446 has been shown not to affect any tissue other than the testes (340). With the high dosages we use, there is the occasional side effect of xerophthalmia. Bone marrow, pituitary, adrenals, and ovaries are unaffected and the LH and FSH are unaltered (340). Inhibition of spermatogenesis is not reversed by addition of various vitamins, amino acids, or hormones (340). Vitamin A was not tested. These facts demonstrate that Win 18,446 is not a classical antimetabolite.

What then, is the mechanism of embyrocidal action of Win 18,446? This drug is one of a series of bis-dichloroacetylamine compounds developed for their ambicidal action in analogy with chloramphenicol, which contains a single dichloroacetylamine group (341) (compare Sec. IIA).



CHLORAMPHENICOL

Of these bis compounds, Win 18,446 has the lowest amebicidal activity (341). These compounds (especially Win 18,446) in this series which have the least amebicidal actvity have the greatest inhibition of several mitochondrial enzymes (342). In addition, these same compounds, and particularly Win 18,446, are most effective in inhibiting spermatogenesis (318). The fact that chloramphenicol is known to inhibit mitochondrial reproduction (322) leads us to a hypothesis of the mode of action of Win 18,446 both on spermatogenesis and on embryonic development. Since this dichloroacetylamine drug is the most powerful

inhibitor of the mitochondrial enzymes, and acts in a manner similar to chloraamphenicol (318), one might expect that it would also be a potent suppressor of mitochondrial reproduction. Spermatogenesis and embryogenesis are characterized by period of intense cell replication followed by differentiation. These periods require not only considerable energy dervied from mitochondrial metabolism, but, in addition, require the replication of mitochondria themselves.

Win 18,446 acts on the rat embryo within the known critical period when antimetabolites have their teratogenic and embryocidal effect (343,344). These are roughly days 8 to 14, corresponding in humans patients to the period from the beginning of the third week to the middle of the sixth week after conception (345). Our studies show that Win 18,446, in order to be effective, need only be administered to the rats on days 10 and 11, corresponding in humans beings to approximately days 21 to 26 after conception. Of the standard antineoplastic agents, only a few have been demonstrated to effect complete resorption of embryos in rats (344). Some are impractical to apply orally and most have serious side effects (346).

C. WIN 18,446: TERATOGENIC ACTION

When Win 18,446 is administered to rats at the appropriate dosage and time of gestation it produces the resorption of 100% of the embryos with no toxic effect to the mother. The optimal regimen for embryocidal action is 200mg daily at days 10 and 11 of pregnancy. When the total dose is less than optimal for fetal resorption and when applied during the period of organogenesis, teratologies appear (329). Day 11 is the most sensitive time. The teratologies are of high incidence, often occurring in 100% of the offspring of mothers with a certain regimen.

The characteristic teratologies which occur with Win 18,446 administration to the mother give us additional clues as to how Win 18,446 exerts its embryocidal and teratological effects. This section of the discussion will focus on the time of action of the drug and what is happening in the particular affected tissues of the differentiating embryo at this time. A more detailed discussion of the time and effect of Win 18,446 in teratogenesis is presented by Taleporos et al.(329).

Most of the malformations described involve mesodermal structures, the two exceptions being the retinal coloboma and the thymus irregularities.

The peak of sensitivity to the drug in practically all tissues which showed malformations, was day 11, with sensitivity increasing to this peak on day 10, and receding from it on day 12. This period, in the rat is during early embryogenesis, before many of the affected parts are even present. For example, the actual formation of the snout is not until the end of day 12 (347). The precursors of the snout region, the neural crest cells, are however undergoing differentiation at this time (348,349). These cells are migrating to form the embryonic facial features (348,349,see 329).

Other malformations reflect this same pattern. The septal heart malformations peak with treatment at day 11. At this time no septal growth is seen, but the precursory tissues are being set up. The endocardium, at about this stage, begins to proliferate mesenchymal tissue, which infiltrates the cardiac jelly (350,see 329).

Mesenchyme proliferation is thought to play a role in most of the other malformations seen (329). The closing of the diaphragm is a clear-cut example of this. From these and other observations, it was concluded that Win 18,446 inhibits the proliferation and/or spread of

embryonic mesenchyme. It is postulated by Taleporos et al., 1976 (329), that the immediate action of the drug is to inhibit the production of the embryonic ground substance, namely the mucopolysaccharides which provide the necessary framework for the proliferation and movement of the mesenchyme cells. It was further noted (329) that the teratologies produced by Win 18,446 have certain similarities to those produced by vitamin A deficiency (351). Vitamin A is required for the synthesis of mucopolysaccharides (352) and Win 18,446 probably inhibits the synthesis of the mucopolysaccharide ground substance (see Taleporos et al., 1976) (329). The only obvious adverse effect of prolonged administration of Win 18,446 to the mother rat is xerophthalmia (328), the first obvious sign of vitamin A deficiency (353).

Organogenesis is characterized by periods of intense cell replication followed by differentiation. These periods require not only considerable energy derived from mitochondrial metabolism but, in addition, require the replication of mitochondria themselves. We have tested three drugs, namely disulfiram, Win 18,446 and chloramphenicol for their effects on rat liver mitochondria. Chloramphenicol, which like Win 18,446 is a dichloroacetylamine, is usually considered the most powerful inhibitor of mitochondrial protein synthesis (354, for reviews see 327). It also inhibits mitochondrial reproduction (322). We found that isolated mitochondria are affected by Win 18,446 more than by chloramphenicol. To inhibit tritiated leucine incorporation into mitochondrial protein by two-thirds requires 150 micromolar chloramphenicol but only $\overset{25}{\wedge}$micromolar Win 18,446. We further found that Win 18,446 and disulfiram are inhibitors of cytochrome oxidase production in the mitochrondria of regenerating rat liver. Male rats

which had been partially hepatectomized were fed the drugs for three days and sacrificed. Aside from the enormous increase in liver fat in the treated rats the cytochrome oxidase content was markedly depressed. These drugs may be acting directly to withhold copper from cytochrome oxidase syntehesis. Copper is considered an essential constituent of cytochrome oxidase and undergoes valence changes in its action in the last step of electron transfer to molecular oxygen (for reviews see 355,356). One of the first indicators of copper deficiency is a decrease in cytochrome oxidase (357,358). In extreme cases of copper deficiency, the oxidative capacity of mitochondria is lost due to a decrease in cytochrome oxidase (359).

The indications that Win 18,446 is a copper chelating agent <u>in vivo</u> are indirect; we have not measured serum or tissue copper levels in Win 18,446 treated rats. That Win 18,446 may be acting as a chelating agent is inferred from five lines of evidence.

First, the parent compound of Win 18,446, namely 1,8 octamethylenediamine is a copper chelating agent as are many other diamines (311). The metabolism of the structurally-related drug chloramphenicol is partially known (360) and hydrolysis to give the amide is seen. Reduction in a subsequent step could produce the amine. Similar metabolism of Win 18,446 could produce the diamine. Even Win 18,446 in its original form shows an interaction with copper, at least in an indirect manner. We found that the cuprous-biquinoline complex in chloroform shows enhanced color if Win 18,446 is added. This implies that Win 18,446 might interact with copper in membranes.

The second line of evidence is that both Win 18,446 and disulfiram have similar inhibitory actions on mitochondrial oxidation and electron transport (318). Third, both drugs cause an alcohol intolerance (318).

Fourth, we have found that both drugs have similar effects on cytochrome oxidase production, and fifth, both drugs are embryocidal at about the same time of gestation.

The indications that Win 18,446 interferes with vitamin A metabolism or usage are also indirect; we have not measured vitamin A levels in Win 18,446 treated rats. Win 18,446 treatment produces three effects which resemble symptoms seen with a vitamin A deficiency. First, Win 18,446 produces male sterility (339,340). Vitamin A deficiency can lead to male sterility in animals (353,361). Second, the one side effect of prolonged Win 18,446 treatment which we found was xerophthalmia (329), the classical symptom of vitamin A deficiency (353,361). Thirdly, the teratologies which we found with Win 18,446 administration (330) are very similar to the teratologies seen in fetuses born to vitamin A deficient rats (351).

These two hypotheses - that Win 18,446 is acting as a copper chelating agent, and that Win 18,446 somehow interferes with vitamin A metabolism or utalization, could be related. Moore (362) has described a variety of stiuations in which copper metabolism and handling of vitamin A seem to be interrelated. With experimental copper poisoning in sheep, there is a drastic reduction in plasma vitamin A. For humans during pregnancy when ceruloplasmin levels are high, plasma levels of vitamin A are low (362). Under more normal conditions, there is a positive correlation of serum copper and serum vitamin A levels (362). Although the copper-vitamin A interaction is not clear, it seems that there is an interrelationship (362).

One possible interrelationship, which deserves further study, is whether copper is involved in the conversion of β-carotene to vitamin A.

This oxiditative cleavage, which occurs in the intestinal mucosa, is known to be inhibited by chelating agents such as dipyridyl and o-phenanthroline (363). Thus, Win 18,446 could well be acting as a copper chelating agent and inhibiting the conversion of β-carotene to vitamin A.

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