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Methotrexate As A Teratogenic Substance On The Central Nervous System Of CD-1 Laboratory Mice

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METHOTREXATE AS A TERATOGENIC SUBSTANCE
ON THE CENTRAL NERVOUS SYSTEM
OF CD-1 LABORATORY MICE

Submitted in Partial Fulfillment of the Requirements for
Graduation with Honors to the Department of Biology at
Carroll College, Helena, Montana

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ABSTRACT

This study evaluated the teratogenic potential of methotrexate on the central nervous system of CD-1 mice at low concentrations during early gestation. Pregnant mice were given 0.1 mg (2.5 mg/kg) of methotrexate by intramuscular injections on day 5 of gestation while pregnant control mice received an injection of distilled water. Fetuses were excised by cesarian sectioning on days 7 through 20 of gestation and sectioned for histological examination. Other mice from each group were allowed to go to term and the offspring were examined externally at 7 and 14 days of age. An increased mortality rate, a decrease in fetal size, and slight internal hydrocephaly were all observed in some of the mice which were prenatally exposed to the methotrexate.
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INTRODUCTION

Methotrexate is currently one of the most widely used compounds in the clinical treatment of cancer. As with all cancer chemotherapeutic agents, methotrexate has a mechanism of action which is most potent against rapidly proliferating cells such as malignant cells, but its action also affects normal cells to some extent. Because of its high level of possible toxicity and its widespread use, many studies have been done on methotrexate in an effort to determine its characteristic activities and effects.

Most of the previous teratogenicity research performed on methotrexate using laboratory animals has been directed towards determining its effects resulting from administration during the second trimester of gestation. This study, however, was meant to study the teratogenic effects of methotrexate on the central nervous system of mice when the drug is administered during the first trimester of gestation.

This manuscript explains the chemical composition of methotrexate as well as its pharmacokinetics, pharmacology, and toxicity. An overview of teratogenic studies of the central nervous system and of methotrexate-induced malformations is also given. The results obtained from this particular study are presented and they are discussed.
LITERATURE REVIEW

METHOTREXATE

DESCRIPTION

Methotrexate is an antimetabolite used in the treatment of certain neoplastic diseases such as gestational choriocarcinoma, choriocarcinoma, desmoids, hydatidiform moles (Baker, 1979), acute lymphocytic leukemia, meningeal leukemia, and lymphoblastic (stem-cell) leukemia in children (Boyd, 1981). Methotrexate is also used in the management of breast cancer, epidermoid cancers of the head and neck, Burkitt's tumor (the best results of remission occurring in stages I and II), advanced cases of mycosis fungoides, and in severe cases of psoriasis (Baker, 1979; Boyd, 1981; Morra, and Potts, 1980).

Methotrexate has a molecular formula of \( \text{C}_{20}\text{H}_{22}\text{N}_{8}\text{O}_{5} \), a molecular weight of 454.46, and its chemical name is \( \text{N-}((\text{2,4-diamino-6-pteridinyl})\text{ methyl})\text{-methylamino) benzoyl glutamic acid} \) (see Fig. 1) (Baker, 1979).

![Fig. 1. The chemical structure of methotrexate (from Windholz, Budavari, Blumetti, and Otterbein, 1983).](image)

PHARMACOKINETICS

ABSORPTION AND DISTRIBUTION

Methotrexate is available in several forms (Baker,
and can be administered by the oral, intramuscular, intravenous, intraarterial, or intrathecal route (Pratt, Ruddon, 1979). At low level doses, which are usually employed clinically, similar plasma levels are seen after oral or intravenous administration; however, at higher doses, intestinal absorption of methotrexate is less effective (Pratt, Ruddon, 1979). According to Pratt and Ruddon (1979), there is experimental evidence that low levels of methotrexate are transported through the intestinal wall via a transport system, but at high concentrations this system is saturated and the uptake then largely involves absorption by diffusion. Methotrexate administered orally is absorbed rapidly by most patients and reaches peak serum levels in one to two hours (Boyd, 1981). Peak serum levels are seen in about one-half this time after parenteral administration (Baker, 1979).

Methotrexate distribution seems to reflect both the cellular level of the enzyme dihydrofolate reductase and the ability of the cell to transport methotrexate (Pratt, Ruddon, 1979). Autoradiography of tritiated methotrexate carried out by Darzynkiewicz, Rogers, and Bernard (1966) revealed that methotrexate is preferentially localized in the kidney proximal tubules, intestinal epithelium, and nuclei of parenchymal liver cells of mice. Pratt and Ruddon (1979) claim that drug accumulation in these organs is correlated to their high levels of dihydrofolate reductase. To treat meningeal neoplastic disease the methotrexate must be given by the intrathecal route because these cells do not
have a very good ability to transport the drug, which can be seen by the very small amounts of methotrexate which pass into the cerebrospinal fluid after oral or parenteral administration (Pratt, Ruddon, 1979). About 50% of the absorbed methotrexate is reversibly bound to serum protein, but it easily exchanges with the body fluids and diffuses into the body tissue cells (Boyd, 1981; Baker, 1979). Methotrexate is apparently distributed to 67 to 90 per cent of the body (Pratt, Ruddon, 1979).

METABOLISM AND EXCRETION

Methotrexate is significantly oxidized to 7-hydroxy-methotrexate by hepatic aldehyde oxidase in some animals such as rabbits and guinea pigs, but in humans receiving conventional doses of methotrexate, 7-hydroxymethotrexate does not appear in their urine (Pratt, Ruddon, 1979). However, Pratt and Ruddon (1979) also mention research by S. A. Jacobs, R. G. Stoller, B. A. Chabner, and D. G. Johns which showed that significant amounts of 7-hydroxymethotrexate are excreted in humans after high-dose therapy. Since 7-hydroxymethotrexate is less soluble in water than methotrexate, it has been suggested that this 7-hydroxy compound is a factor in the nephrotoxicity in patients on high-dose therapy (Pratt, Ruddon, 1979).

Single daily doses of methotrexate are excreted through the kidneys by a combination of glomerular filtration and active tubular transport in amounts 55 to 88 per cent or
more in 24 hours (Boyd, 1981; Pratt, Ruddon, 1979). Accumulation of methotrexate in the tissues may result from repeated daily doses which result in more sustained serum levels and some retention of the drug over each 24 hour period (Baker, 1979). The pharmacokinetics of methotrexate depends upon dihydrofolate reductase levels, cellular transport, enterohepatic circulation, and significant metabolism when high doses of the drug are given (Pratt, Ruddon, 1979).

PHARMACOLOGY

The principal mechanism of action of methotrexate is competitive inhibition of the folate reductase enzyme tetrahydrofolate reductase, which reduces dihydrofolate \( \text{FH}_2 \) to tetrahydrofolate \( \text{FH}_4 \) in the presence of NADPH (see Fig. 2) (Boyd, 1981). At pH 6 the drug is bound extremely tightly and there is virtually no dissociation of the enzyme-inhibitor complex (Pratt, Ruddon, 1979). However, as the pH is raised to physiological levels and higher, reversible competitive kinetics are observed. This strong binding of the drug by dihydrofolate reductase at low pH has been called "pseudo-irreversibility." The theory that binding may cause a dramatic conformational change in the enzyme is supported by the fact that the drug-bound reductase is stabilized to digestion of proteases (Pratt, Ruddon, 1979).

Tetrahydrofolate is normally converted to a variety of coenzymes that are necessary for one-carbon transfer reactions involved in the synthesis of thymidylate, purines,
methionine, and glycine (Pratt, Ruddon, 1979). Because of this, inhibition of dihydrofolate reductase by methotrexate can cause the inhibition of DNA, RNA, and protein synthesis. There is experimental evidence which suggests that the critical effect leading to death after exposure to methotrexate, in most cells, is the inhibition of thymidylate synthesis (Pratt, Ruddon, 1979). According to Pratt and Ruddon, there is a transfer of a one-carbon unit from $N^5,N^{10}$-methylene-tetrahydrofolate to deoxyuridine monophos-
Deoxyuridylic Acid (dUMP) under the direction of the enzyme thymidylate synthetase to synthesize thymidine monophosphate (TMP) (see Fig. 3). In most one-carbon transfer reactions, the one-carbon unit is simply transferred to the substrate and tetrahydrofolate is regenerated. However, in thymidylate synthesis the coenzyme $N^5,N^{10}$-methylene tetrahydrofolate is oxidized, producing dihydrofolate. The dihydrofolate must

Deoxyuridylic Acid (dUMP)  
\[
\begin{align*}
\text{HN} & \quad \text{C} \\
\text{C} & \quad \text{CH} \\
\text{O} & = \text{c} \\
\text{N} & \quad \text{N} \\
\text{Deoxyribose-P} & \quad \text{Deoxyribose-P}
\end{align*}
\]

Thymidylate Synthetase

\[
\begin{align*}
\text{HN} & \quad \text{C} & \quad \text{c-CH}_3 \\
\text{O} & = \text{c} \\
\text{N} & \quad \text{N} \\
\text{Deoxyribose-P} & \quad \text{Deoxyribose-P}
\end{align*}
\]

\[\rightarrow \text{DNA}\]

$N^5,N^{10}$-Methylenetetrahydrofolate

\[\text{FH}_2\]

Dihydrofolate Reductase

\[\text{FH}_2\quad \text{FH}_4\]

Methotrexate

Fig. 3: Synthesis of thymidine monophosphate from deoxyuridine monophosphate. In this reaction, a one-carbon fragment is transferred from the tetrahydrofolate cofactor to deoxyuridylic acid (dUMP) with the formation of thymidine monophosphate (TMP) and dihydroxyfolate ($\text{FH}_2$). Tetrahydrofolate ($\text{FH}_4$) must then be regenerated by reduction of $\text{FH}_2$, the reduction blocked by methotrexate. (from Pratt, Ruddon, 1979).
be reduced by dihydrofolate reductase to produce the tetrahydrofolate in order to keep the system running. Methotrexate may also be able to inhibit thymidylate synthetase directly by occupying the folate coenzyme site (Pratt, Ruddon, 1977). Even though the primary mechanism of methotrexate is the inhibition of the enzyme dihydrofolate reductase, the direct inhibition of thymidylate synthesis may be a contributing factor to the cytotoxic effect when the drug is used in high concentrations.

The methyl group of the amino acid methionine and the carbon atoms 2 and 8 of the purine structure are derived from the folate coenzymes, and tetrahydrofolate accepts a one-carbon fragment in the synthesis of glycine from serine (Stryer, 1981). The rate that cells enter the S phase of the cell cycle would be slowed by this RNA and protein synthesis (Pratt, Ruddon, 1979). Because of this slowed rate of entry of cells into the S phase and since methotrexate kills cells in the S phase, methotrexate is a "self-limiting" S phase specific drug.

L1210 leukemia cells have been studied extensively in regard to methotrexate uptake and it has been found that the drug is actively transported into the cell by a system that is also used by the two folate coenzymes leucovorin (N5-formyl-tetrahydrofolate) and 5-methyl-tetrahydrofolate, and also possibly weakly by folic acid (Pratt, Ruddon, 1979). Leucovorin and 5-methyl-tetrahydrofolate compete for methotrexate uptake and also stimulate the efflux of methotrexate out of the cell. Methotrexate is more effective against
actively proliferating cells, such as malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa, dermal epithelium, and cells of the urinary bladder (Boyd, 1981). Because cellular proliferation is greater in malignant cells than in most normal cells, methotrexate may impair the growth of the malignant cells without irreversible damage to normal cells (Baker, 1979).

TOXICITY

Toxic effects occur with both conventional methotrexate administration and high-dose therapy. With high-dose therapy close pharmacological monitoring of the therapy is required. Patients with 24-hour serum methotrexate levels higher than 10^{-5} M, 48-hour levels greater than 10^{-6} M, and 72-hour levels higher than 10^{-7} M are considered to be at high risk (Pratt, Ruddon, 1979). The levels of methotrexate in the blood should be assayed by the physician for patients receiving high-dose therapy. Since the majority of methotrexate is excreted via the kidneys, any condition that impairs renal excretion of the drug increases the risk of toxicity (Boyd, 1981). For this reason, the patient’s renal status should be carefully monitored. Also it is important that the urine receives both adequate hydration and alkalinization for proper drug clearance (Pratt, Ruddon, 1979).

The most common adverse reactions observed in methotrexate use include ulcerative stomatitis, leukopenia, nausea, and gastrointestinal distress (Boyd, 1981). Boyd
also mentions malaise, fatigue, chills and fever, dizziness, and decreased resistance to infection as other reactions reported. The rate of occurrence and severity of the side effects is related to the dose of methotrexate received by the patient (Baker, 1979). Boyd (1981) and Baker (1979) report the following adverse reactions for the various systems:

**Skin:** Erythematous rashes, pruritus, urticaria, photosensitivity, depigmentation, alopecia, ecchymosis, telangiectasia, acne, and furunculosis. Concomitant exposure of ultraviolet radiation may aggravate lesions of psoriasis.

**Blood:** Bone marrow depression, leukopenia, thrombocytopenia, anemia, hypogammaglobulinemia, various stages of hemorrhaging, and septicemia.

**Alimentary:** Gingivitis, pharyngitis, stomatitis, vomiting, anorexia, diarrhea, hematemesis, melena, gastrointestinal ulceration and bleeding, enteritis, acute liver atrophy resulting from hepatic toxicity, necrosis, fatty metamorphosis, periportal fibrosis, or hepatic cirrhosis.

**Urogenital:** Renal failure, azotemia, cystitis, hematuria, defective spermatogenesis or oogenesis, transient oligospermia, menstrual dysfunction, infertility, abortion, fetal defects, and severe nephropathy.

**Pulmonary:** There have been reports of interstitial pneumonitis deaths and chronic interstitial obstructed pulmonary disease occasionally occurs.

**Central Nervous System:** Headaches, drowsiness, and blurred vision. Aphasia, hemiparesis, paresis, and convulsions have
also been reported following the use of methotrexate. There have been reports of leukoencephalopathy following intravenous administration in patients who have had craniospinal irradiation.

The CNS toxicity after intrathecal use of methotrexate can be classified as following (1) chemi arachnoiditis with recognizable symptoms such as headache, back pain, nuchal rigidity, and fever; (2) paresis which is usually transient and manifested by paraplegia associated with involvement with one or more spinal roots; (3) leukoencephalopathy recognizable by confusion, irritability, somnolence, ataxia, dementia, and occasionally major convulsions (Boyd, 1981; Baker, 1979).

Other adverse reactions that have been associated with the use of methotrexate include pneumonitis, metabolic changes, precipitating diabetes, osteoporatic effects, abnormal tissue cell changes, anaphylaxis, and sudden death (Boyd, 1981).

Since methotrexate has a high potential for toxicity, physicians should be familiar with the various characteristics of the drug and its established clinical use (Baker, 1979). Patients should be properly supervised so that symptoms or signs of possible toxic effects may be detected and evaluated as quickly as possible (Baker, 1979; Pratt, Ruddon, 1979).
TERATOLOGY

There has been a large amount of effort directed toward all aspects of teratology, including its causes and effects. The following pages are a brief sampling of some studies conducted on teratology of the central nervous system and on the teratogenic properties exhibited by the chemotherapeutic drug methotrexate.

TERATOLOGY OF THE CENTRAL NERVOUS SYSTEM

Research has revealed that many defects which occur in the central nervous system result from abnormal closure of the neural tube during embryonic development. A potent metabolic inhibitor called 6-aminonicotinamide (6-AN) was administered by David W. McCandless and William J. Scott (1981) on day 9 of gestation (a positive sperm smear was determined as day 0) in pregnant rats. These researchers then removed the embryos 12 and 24 hours later and found that energy metabolism of the neural tube changed with an increase in the metabolites ATP (29% increase) and PCr (37% increase). McCandless and Scott believe that these alterations may be related to decreased metabolic demands.

Another group of researchers investigated the effects of diazepam—a drug widely used by humans for treating anxiety and muscle spasms associated with neuromuscular and musculoskeletal diseases—on the development of explanted chick embryos (Nagele, Pietrolungo, Lee, and Roisen, 1981). They found that diazepam preferentially inhibited closure of
the neural tube at concentrations of 10-120 μg/ml. Nagele et al., found through electron microscopy that the affected neuroepithelial cells 1) had apical surfaces which were much smoother than usual and 2) that apical filament bundles—believed to provide motive forces for uplifting the neural folds—were not well organized and often lacked alternating dark and light areas along their length. These findings suggest that the neural tube closure defects are at least partially due to the impaired ability of the filament bundles to contract.

H. Lee and R. G. Nagele also conducted research on the importance of apical microfilaments in 1979 by using papaverine. These two investigators found that papaverine (50 μg/ml) inhibited the uplifting of neural folds in explanted chick embryos and that the affected neuroepithelial cells often lost their wedge-shaped and elongated appearance. It was also noticed that the luminal surfaces of the affected cells were smoother than usual due to a marked decrease in the number of cytoplasmic extensions. These changes in the topography of the cell surfaces were due, at least in part, to the impaired ability of the apical microfilaments to contract, resulting in their eventual relaxation (Lee, Nagele, 1979). Lee and Nagele found that subsequent treatment with ionophore A23187 would reverse the "relaxing" effect of papaverine. Since ionophore A23187 and papaverine are known to alter the normal distribution of intracellular calcium ions and since changes in cell-surface topography are correlated with the contractile activities of...
apical microfilaments, these researchers concluded that papaverine elicits neural-tube closure defects by lowering the levels of intracellular free calcium. This, in turn, causes the contracted apical filaments in the neuroepithelial cell which are responsible for the neural tube to close, to relax.

Another approach in studying neural tube closure defects is seen in work done by A. G. Fantel et al. (1981) who gave intraperitoneal injections of cytochalasin D (CD) to pregnant rats on gestational days 7-11 and found doses of 400 \( \mu g/kg \) were only minimally and nonsignificantly teratogenic; only two exencephalic fetuses were discovered among 111 fetuses delivered. However, when these researchers exposed embryos to CD in vitro on day 10 of gestation, significant frequencies of neural-tube abnormalities were seen when the concentrations of CD used were at or above 3.1 ng/ml. Fantel and his colleagues then performed experiments in order to try to understand this discrepancy between the teratogenicity of CD in vivo and in vitro. These experiments were aimed at determining whether drug metabolism could inhibit the teratogenicity in vivo (Fantel, Greenaway, Shepard, Juchau, and Salleck, 1981). These men pretreated male rats with a mixture of polychlorinated biphenyls as cytochrome P-450 inducers and used the rats' livers to prepare microsome-rich fractions called S-9. This S-9 fraction, plus a source of NADPH, significantly inhibited the teratogenicity of CD. The teratogenicity of CD was restored
by omitting the NADPH, and was partially restored by adding
carbon monoxide.

These tests by Fantel and his colleagues (1981) led
them to the conclusion that the teratogenic effect of CD can
be inhibited by drug metabolism in vitro. They also con-
cluded that it is likely that this drug metabolism may
depend upon cytochrome P-450. These researchers further
speculated that CD may be inactivated in vivo by these same
mechanisms, which would explain the apparent discrepancy
between the teratogenicity of CD in vivo and in vitro.

The thymidine analog 5-bromodeoxyuridine (5BrdU) has
been shown by several researchers to strongly inhibit
neural-tube closure in explanted presomite-stage chick
embryos (Lee, Hikida, and Levin, 1976). Hsin-Yi Lee and his
fellow researchers (1976) conducted a study to investigate
the cytological effects of BrdU on the chick neural tube.
These researchers cultured explanted chick embryos for 24h on
thin albumen with 10 μg/ml BrdU, which inhibited the
closure of the neural tube in over 90% of the embryos. Lee
et al. found mitotic figures throughout the adversely
affected neuroepithelium, which suggested that interkinetic
nuclear migration had been inhibited. When these re-
searchers studied BrdU-treated neural-tube cells with elec-
tron microscopy, they found these cells showed fewer and
more amorphous cytoplasmic extensions, microfilaments, and
desmosomal tonofibrils than in neural-tube cells not exposed
to BrdU.

Several other agents were studied for their effects on
neural-tube closure by Allan R. Beaudoin and D. Dowell Fisher, whose results were published in 1981. Beaudoin and Fisher administered 2-amino-1,3,4-thiadiazole (thiadiazole), cadmium sulfate, 1,2-(sec-Butyl)-4,6-dinitrophenol (dinoeb), lead nitrate, polybrominated biphenyls (PBB), sodium arsenate, and trypan blue either 24 or 4 hours prior to recovery of day 10 embryos from pregnant Wistar-derived rats. The recovered embryos were then cultured in vitro using Waymouth's medium and fetal calf serum. Two-thirds of the embryos were recovered after 24 hours in culture and examined; the remaining one-third were examined after 42 hours in culture. All of the compounds tested inhibited the rate of neural tube closure in vitro.

M. J. Wiley (1980) administered single intraperitoneal injections of cytochalasin B (CB) in dimethylsulfoxide to pregnant Syrian hamsters on the eighth day of pregnancy at various dose levels. Wiley found that treatment with 7.0 mg/kg CB led to the failure of the cranial neural folds to approximate and close. As in the study carried out by Funtel et al. (1981) using CD, Wiley noted that the principal ultrastructural changes involved alterations in the topography of the apical membranes of the neuroectoderm cells. According to Wiley's results, this failure of the neural fold to close resulted in exencephaly and encephalocele in the mice that made it to term.

William S. Webster and Karin Messerle (1980) conducted a study to investigate neural tube defects induced by metal
Cadmium (Cd). Mice were injected with 4 mg/kg CdCl₂ on day 7, 8, 9, or 10 of gestation (the day vaginal plugs were found was referred to as day 1). These animals and control animals (injected with saline) were sacrificed at various times up to 48 hours after injection and the embryos were examined both grossly and histologically. Webster and Messerle reported that exencephaly occurred after Cd treatment on day 7 or 8. The exencephaly development was examined in day-8 embryos, revealing that eight hours after Cd injection, many cells of the closing neural plate contained dense-staining inclusions which were thought to be autophagic vacuoles. After 24 hours, this damage had almost disappeared, but the anterior neural folds were more open than in the controls even though the experimental neural folds were histologically normal. Forty-eight hours after the Cd injection it was apparent that this part of the neural tube was not going to close, resulting in exencephaly.

Another metal tested for its teratogenicity has been nickel. Because of the widespread use of its chemical compounds in various industries, nickel has begun to constitute a potentially serious hazard (Lu, Matsumoto, and Iijima, 1979). Chiung-Chen Lu and his coworkers (1979) administered nickel chloride to pregnant mice on the seventh to eleventh day of gestation which resulted in significant embryotoxic effects in terms of a decreased fetal rate, an increased resorption rate, a delay in skeletal ossification, and a high incidence of malformations including exencephaly.
rebral hernia. In this study the concentration of nickel chloride retained in embryonic tissues was 800 times higher in the exposed group compared to the control group and indicated that increased tissue levels of nickel chloride has a toxic influence on developing embryos.

Josef Warkany and Harold G. Petering (1972) have also directed research on metals as teratogenic substances. These researchers studied the teratogenicity of maternal zinc deficiency in rats with special attention given to malformations of the central nervous system and to tissue anomalies not recognizable by gross inspection of the fetuses. In their study, female Wistar rats were given a semipurified diet and drinking water containing 10 \( \mu g \) of zinc (as zinc acetate) and 2 or 4 \( \mu g \) of copper (as copper sulfate) per ml for four days prior to mating. After mating, when sperm had been found in the vagina (called the 1st day of pregnancy), the water was changed to water that contained no zinc but provided 2 or 4 \( \mu g \) of copper per ml.

In Warkany and Petering's preliminary experiment 30 females were used and 25 of these carried their young to term or near term. The rats with surviving young were sacrificed on the 20th or 21st day of pregnancy and the young were examined by inspection, clearing, or sectioning. After the examination, 70.5% (136 of the 193) of the young were found to be abnormal. Among the 136 abnormal young, only three had exencephaly, whereas 40 had external hydrocepha, and other anomalies were discovered after histo-
logical sectioning. The three exencephalies and most of the hydrocephalies diagnosed by inspection showed additional lesions by microscopic examination. These researchers found that some of the microscopic tissue malformations that were always present in grossly deformed species were sometimes found in species whose heads seemed normal upon external examination. The reason these researchers sectioned these animals with apparently normal heads was because they had an abnormal eye or palate or because of an obvious CNS malformation of a littermate. Another result of this experiment was spinal cord anomalies in fetuses that appeared short and had abnormal tails.

The administration of excess vitamin A during gestation of Sprague-Dawley rats by Curtis D. Eckhart and Lucille S. Hurley (1979) resulted in gross malformation of the fetuses. A proposed mechanism of vitamin A in producing malformations is a reduction in DNA synthesis and altered cellular differentiation; a similar mechanism has been suggested for the teratogenic effect of zinc deficiency (Eckhart, Hurley, 1979). These researchers tested high levels of vitamin A (3,000 or 30,000 IU/day) as well as simultaneous treatment of pregnant rats with excess vitamin A (3,000 or 30,000 IU/day) and low dietary zinc (0.4 or 9 ppm). Both treatments resulted in brain deformities. Exencephaly and hydrocephaly were the most common brain anomalies with a less frequent occurrence of anencephaly.

Developmental malformations are also produced by ribavirin, a synthetic nucleoside which shows promise as an
effective therapeutic agent in human and animal viral infections when it is administered to pregnant golden hamsters by oral, intraperitoneal, or intravenous routes (Ferm, Willhite, and Kilham, 1978). The most common defects found in the hamster were abnormalities of the limbs, eyes, and brains. The defects of the central nervous system, especially encephalocoeles and exencephaly, occurred when ribavirin was administered during the very early stages of embryogenesis (late on day 7 or early on day 8). Ferm et al. (1979) also found that doses approximately ten times greater were required to induce anomalies in CD-1 rat embryos and the malformations were generally restricted to the head region (exencephaly and encephalocoele). The results of these researchers provide evidence that in both rats and hamsters, oral administration of the drug is more teratogenic than administration by other routes. This suggests that the metabolism of ribavirin in the gastro-intestinal tract and/or liver may change it into its active form.

In 1981 the results of Calvin C. Willhite, Vergil H. Ferm, and Roger P. Smith were published on their study on teratogenic effects of aliphatic nitriles. These researchers gave intraperitoneal injections of acrylonitrile at 1.51-2.26 mmole/kg (80-120 mg/kg) or propionitrile at 0.54-1.51 mmole/kg (30-80 mg/kg) on the morning of day 8 of gestation in hamsters and found that exencephaly and encephalocoeles were among the resulting malformations. They then found that multiple intraperitoneal injections of
sodium thiosulfate at 4.03 mmole/kg (1 g/kg) protected both the dams and the embryos against toxicity. When the larger doses of the nitriles were given in the presence of sodium thiosulfate, the teratogenic results were observed, but the overt signs of maternal poisoning were absent. In the publication of their results, these researchers describe several studies that were done previously by other researchers which demonstrated that acrylonitrile and propionitrile are converted in vivo to toxicologically significant concentrations of cyanide and that sodium thiosulfate (an established cyanide antagonist) can provide protective actions against poisoning of either acrylonitrile or propionitrile. The observations, according to Willhite et al., suggest that the teratogenic effects of both acrylonitrile and propionitrile are related to the metabolic release of cyanide.

Two antitumor chemotherapeutic agents used in humans, vinblastine (VLB) and vincristine (VCR), when injected intravenously into pregnant golden hamsters on the eighth day of gestation, cause an increased fetal mortality rate (determined by the number of resorption sites) and a significant number of congenital malformations in the surviving fetuses (Ferm, 1963). Both VLB and VCR have similar chemical structures, yet they appear to produce definite differences in their effects on tumors and, as Ferm (1963) discovered, they produce different congenital malformations. When Ferm recovered the fetuses on the 14th day of gestation, he found that VLB produced spina bifida while VCR
induced mild exencephaly. These two compounds did, however, show similar malformations in the eye (microphthalmia and anophthalmia) and the skeletal development (rib fusion).

Researchers have also investigated environmental influences which may be teratogenic to the CNS during development. One example of this is the work done by Lawrence Kilham and Vergil H. Ferm on hyperthermia. These men induced hyperthermia by placing pregnant hamsters in a bacteriological incubator at 39, 40, or 41 degrees celcius for 1 to 1.25 hours. They found that exposure of the pregnant hamsters on gestation day 8 to 40 or 41 degrees for one hour caused an increased rate of resorption and a high frequency of exencephaly and encephalocele.

Charles Roux, Cecilia Horvath, and Rolande Dupuis (1979) demonstrated embryomortality and teratogenesis were provoked by inhibitors of cholesterol synthesis in Wistar rats. Their results supported prior studies in that the teratology which occurred was reflected by holoprosencephalies. They also obtained evidence that a hypercholesterolemia-provoked diet is completely effective for preventing holoprosencephaly.

Researchers Minoru Inouye and Ujihiro Murakami (1978) studied the teratogenic effect of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), which is an alkylating agent and is known as a potent mutagen in bacteria. Pregnant mice of a closed colony of JCL:CF#1 were injected on one of gestation days 7-12 (the day after mating was designated as day 0 of
gestation) with an intraperitoneal dose of 40, 60, or 80 mg/kg of MNNG and the fetuses were examined on day 18 of gestation. They found that the predominant malformations were of the brain which included hydrocephalus, hydromicrocephaly, and microcephaly.

Defects in the Long-Evans rat central nervous system during retarded brain development and congenital hydrocephalus were studied by J. C. Chamberlain (1970) following single maternal injections of the niacinamide antimetabolite 6-aminonicotinamide (6-N) late in development. Histological and histochemical analysis by Chamberlain indicated CNS hemorrhage and growth retardation of fetal brains 24 hours after treatment. There was also evidence of fluid movement into cerebral ventricles by secretion-like, circular, "blebs" along the ependymal lining. Chamberlain also found there was transitorily increased glycogen in hypoplastic choroid plexuses with a disruption in the cerebral layering of neuroblasts. He also found thinning of the cerebral cortex and an increased ventricular surface area. During the gestation period, macrophagic cells removed cellular debris and RBC's so that by term there was little evidence of early hemorrhage.

Ethylenethiourea (ETU) is often found in ingested foods as a result of degradation of ethylenemedi thiocarbamate fungicides (Khera and Iverson, 1980). The interaction in vivo of ETU with sodium nitrite, a food preservative, may form N-nitrosoethylenethiourea (NO-ETU). To evaluate the pre- and postnatal effects of NO-ETU in Wistar rats, K. S.
Khera and F. Iverson administered the following single oral doses: 120, 160, 200, and 240 mg/kg on day 13 of pregnancy in the prenatal study; 60 and 120 mg/kg on day 15 of pregnancy in the postnatal study. The results these investigators obtained for the prenatal study were that 200 and 240 mg/kg doses were maternally lethal, and the 120 and 160 mg/kg doses produced high incidences of malformed fetuses with hydrocephalus, exencephaly, hypoplastic cerebellum, hydronephrosis, and other anomalies. In the postnatal study they found that 60 and 120 mg/kg doses were associated with hydrocephalus and microphthalmia.

Ethynitrosourea (ENU) is a neurotropic oncogen which produces central and peripheral tumors in rats (Drukery, Ivankovic, and Freussman, 1966). Michael J. Pfaffenroth, Gopal D. Das, and James P. McAllister (1974) intravenously injected Wistar rats with ENU between days 14 and 21 of gestation with day 1 of gestation being the morning in which vaginal smears contained sperm. The offspring were killed 6, 3, 6, 10, 15, 21, and 75 days after birth and their brains were examined macroscopically. These examinations revealed that ENU produced various degrees of micrencephaly which was reflected predominantly in the reduced dimensions of the cerebrum and cerebellum. The degrees of micrencephaly were first evident to the researchers in 15-day-old animals and persisted throughout adulthood.

Cerebellar malformations have also resulted from prenatal exposure to X-irradiation. Minoru Inouye (1979)
investigated this in an attempt to analyze and quantify the cerebellar malformations systematically. Inouye exposed pregnant WKA/Kok rats to 100 R and 200 R X-irradiation on one of the gestation days 16-21. He then sacrificed the offspring at 60 days of age and examined their cerebellum. He found that the cerebellum of animals exposed to 200 R was slightly reduced in weight but not in width and that reduction in the dorsoventral length of the cerebellum was more evident when exposure to X-irradiation was early in gestation. Inouye also noted that the anteroposterior length of the hemispheres increased following exposure to X-ray on days 16 through 19 and that the length of the vermis and paravermis decreased following treatment on days 17 through 21. Histologically, this study revealed that ectopic Purkinje cells in granule cell layer and white matter appeared following X-irradiation on days 20 and 21, but they were not found following earlier treatment. Inouye found that the cerebellum of animals exposed to 100 R had only a mild reduction in size.

W. Schmahl (1979) also conducted experiments using X-irradiation. He combined the X-irradiation with 5-azacytidine (AzaCr) and determined that the sequence in which these treatments were applied was important. Schmahl found that a single treatment of pregnant NMRI mice on day 12 post conception (the morning a vaginal plug was found was considered day 1 post conception) with AzaCr followed by a single irradiation dose of 200 R two hours later is exclusively neurotoxic to the fetus as shown by a severe hypo-
plasia of the parieto-occipital regions of the telencephalon. However, Schmahl found that applying these two hazards in the reverse manner, in other words, irradiation followed by Azacr, resulted in no general hypoplastic effect in the forebrain and only caused a depletion of cells in the marginal cortex. Schmahl believes that this indicates a significantly diminished Azacr sensitivity of fetal cortical cells subsequent to X-irradiation.

**METHOTREXATE-INDUCED MALFORMATION**

E. P. Schmid (1984) cultivated KFM-WSA rat embryos during their organ formation phase (days 9.5 through 11.5 post coitum with a positive vaginal sperm smear designated as day 0 post coitum) in the presence of an Aroclor 1254 pretreated liver microsomal preparation (S9-mix). Schmid added various concentrations of methotrexate at the beginning of the culture period and 43 hours later he observed malformations in the embryos treated with methotrexate concentrations as low as 0.05 μg/ml. The methotrexate selectively affected the rhombencephalic and telencephalic brain regions. Schmid also noticed malformations in the caudal trunk, the heart and forelimb regions, and in the vesicular structures. The higher concentrations of methotrexate in this study (0.10 μg/ml, 0.15 μg/ml, and 0.20 μg/ml) caused concentration-dependent increases in the types and incidences of malformations and also inhibited the overall growth and differentiation of the embryos.

Another study was concerned with the distribution and
embryotoxicity of methotrexate in pregnant rats and rhesus monkeys (Wilson, Scott, Ritter, and Fradkin, 1979). Dosages of methotrexate of roughly comparable embryotoxicity were determined by Wilson et al. to be 0.30 mg/kg on gestation day 10 in Royaihart (a Webster-derived strain) rats and 3.0 mg/kg/d on days 29-32 of gestation in rhesus monkeys--these times represent similar periods of development. This regimen was moderately embryolethal, slightly teratogenic, and caused some intrauterine growth retardation in rats; it was mildly embryolethal, not teratogenic, and caused only transitory growth retardation in monkeys.

In this experiment the methotrexate was largely cleared from the maternal plasma of both species within eight hours. The rate of fall, however, was faster in the monkey in spite of a higher administered dose and initial concentration. Both species also showed fractions of total drug concentration remaining unbound in plasma to be approximately 30 to 40% during the first four hours of treatment, but the embryo concentrations were strikingly different during the first eight hours (108 to 209 ng/g in the monkeys; 3.4 to 7.7 ng/g in the rats). These researchers estimated that a slow rate of fall in embryo concentrations in both species was inversely proportional to the rate of growth of the embryos, which was supported by the fact that the absolute amount per embryo changed little in 24 hours.

Wilson et al. concluded from their study that the degree and type of embryotoxicity was not closely correlated with the level or duration of concentrations in the embryos--a
small maternal dosage in the rats produced moderate embryo-toxicity and very low embryo concentrations; a large maternal dosage in the monkeys produced slight embryo-toxicity and high embryo concentrations.

R. L. Dixon and his associates demonstrated that salicylates can displace methotrexate from proteins, thereby increasing the free methotrexate concentration (Woo, McClain, and Roar, 1978). Woo et al. (1978) gave methotrexate or aspirin, alone or in combination, on gestation day 9 or 12 of pregnant Charles River CD rats to study the augmentation of methotrexate-induced embryo toxicity by aspirin. They found that pretreatment with aspirin (200 mg/kg) significantly enhanced the embryolethality of methotrexate given at doses of 0.2 mg/kg on day 9 and 1.5 mg/kg on day 12. Studies with tritiated methotrexate in pregnant rats demonstrated that aspirin delayed the renal excretion of methotrexate and increased the methotrexate concentrations in maternal plasma and embryos. It is believed by Woo and his associates that these effects are responsible for the observed potentiation of embryolethality.

K. S. Khera (1976) administered methotrexate orally in gelatin capsules in single daily doses (0.5 mg/kg) on days 11 through 14, 14 through 17, or 17 through 20 of gestation of adult female cats (shorthaired European and Persian breeds). Methotrexate produced maternal toxicity and when given on days 11-14 and 14-17 produced high frequencies of malformations of the skeletal and visceral systems as well
as umbilical hernias.

Many of the studies conducted on methotrexate are conducted on humans who had been treated with methotrexate or who were exposed to methotrexate prenatally. H. R. Powell and H. Ekhert (1971) described fetal abnormalities in a newborn child whose mother took methotrexate during the first two months of pregnancy before it was realized that she was pregnant. The mother was taking 5 mg/day of methotrexate for moderately severe psoriasis which was diagnosed seven years previously.

According to Powell and Ekhert, at birth the baby (female) had a grossly abnormal appearance, with the major abnormalities affecting the skull. The head was oxycephalic (due to fusion of the coronal sutures, which were represented by a ridge of bone rather than the normal palpable sutures). The eyes were widely separated and the bridge of the nose was wide and depressed. The infant received corrective surgery and since then she has continued to do well up to the age of four months, the time Powell and Ekhert made their report.

Another infant born after the unsuccessful abortifacient use of methotrexate resulted in multiple congenital anomalies (Milunsky, Graef, and Gayner, 1968). According to Milunsky et al., the anomalies included absence of the frontal bone, synostosis of the lamboid and coronal sutures, multiple anomalous ribs, unusual facies, and the absence of digits on the left foot with only one digit on the right foot. The mental and motor development of this infant was reported to
be normal, but growth was markedly retarded.
MATERIALS AND METHODS

ANIMALS

Fifty female and 18 male CD-1 (a Swiss-Webster strain) mice were purchased from the Montana State University Animal Resources Center, Bozeman, Montana. All the mice were born within a three-day span (October 7, 1984-October 9, 1984). The animals were caged at 70-72 degrees with a 12 h day/12 h night cycle in groups of three with the males and females separated until they reached maturity for breeding. All the mice received Purina Lab Chow and water.

The animals were randomly divided into cages for mating with three females and one male per cage when the mice were approximately seven weeks of age. The females were checked every morning for the presence of vaginal plugs and the day on which a vaginal plug was found was designated as day 1 of gestation.

DRUG

Mexate® methotrexate sodium for injection, (Bristol laboratories, Syracuse, New York) was obtained from the Great Falls Deaconess Hospital, Great Falls, Montana. The recommended dosage of Mexate® for clinical use is 2.5 mg/kg.* The Mexate® was prepared immediately prior to each injection by diluting 2.0 mg of the chemical with 5.0 ml of distilled water. Each injection was 0.25 ml of this solution, which provided a dosage concentration of 0.1 mg of

* Mexate® package insert (Bristol Laboratories, 1982)
TERATOGENICITY STUDY

Thirty of the impregnated female mice were divided into two groups (control and experimental) with each group consisting of 15 mice. The mice in the experimental group received a 0.25 ml (0.4 mg/ml) intramuscular injection of Mexate® solution in the right gluteal muscles at 2:00 p.m. on day 5 of gestation. The mice in the control group each received a 0.25 ml injection of distilled water on gestation day 5 in a similar manner.

One pregnant female from each group was sacrificed by decapitation on each day of the gestation period, beginning on day 7 and continuing through the final day of gestation (day 20). The sacrifices were made from 12:00-2:00 p.m. each day and three fetuses were removed from each mouse by cesarian section. The fetuses removed were taken from the same areas of the uterus in all mice (see Fig. 4). The number of fetuses and resorption sites were recorded for each animal. The excised fetuses were fixed in formalin and embedded in paraffin for sectioning with a microtome. The number of resorption sites was used to determine the mortality rate 1) of each gestation day and 2) of each group. The remaining females in the experimental and control groups were allowed to go through term. The offspring of these mice were then externally examined when they were 7 and 14 days old for the presence of abnormal head shapes.

The embedded fetuses were sagitally sectioned 4 μm
thick and mounted on microscope slides using Elmer's glue as a section adhesive. The slides were stained using hematoxylin-eosin stain and coverslipped. A dissecting microscope and light microscope were used to examine the

![Diagram of the uterus of a pregnant female mouse](image)

**Fig. 4:** Diagram of the uterus of a pregnant female mouse. The numbers 1, 2, and 3 represent the areas from which the fetuses were excised for this study. (adapted from Rowett, 1957).

slides for abnormalities in the central nervous system. The slides prepared from fetuses taken from the experimental group were compared to those prepared from the control group for each day of gestation represented.
RESULTS

MORTALITY RATE

The first and most noticeable adverse effect the Mexate had on the developing mice was the increased resorption rate (see Table 1 and Table 2). There were only four resorption sites observed in the control group, producing an average mortality rate of 2.3%. However, in the experimental group 35 resorption sites were noted, which is an average mortality rate of 21.0%. There was no difference between the fetal rates of the two groups: the average control fetal's rate being 13.2 and the average experimental fetal's rate being 12.7.

Table 1. The number of resorption sites, fetuses, and the mortality rate of the control group for each day of gestation.

<table>
<thead>
<tr>
<th>GESTATION DAY</th>
<th>NUMBER OF RESORPTION SITES</th>
<th>NUMBER OF FETUSES</th>
<th>MORTALITY RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0</td>
<td>12</td>
<td>0.0</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>14</td>
<td>0.0</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>12</td>
<td>7.7</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>13</td>
<td>0.0</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>12</td>
<td>7.7</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>14</td>
<td>0.0</td>
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<tr>
<td>14</td>
<td>0</td>
<td>14</td>
<td>0.0</td>
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<tr>
<td>13</td>
<td>2</td>
<td>10</td>
<td>16.7</td>
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<tr>
<td>12</td>
<td>0</td>
<td>14</td>
<td>0.0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>13</td>
<td>0.0</td>
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<tr>
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<td>0</td>
<td>13</td>
<td>0.0</td>
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<tr>
<td>7</td>
<td>0</td>
<td>12</td>
<td>0.0</td>
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<tr>
<td>6</td>
<td>0</td>
<td>14</td>
<td>0.0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>00</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table 2. The number of resorption sites, fetuses, and the mortality rate of the experimental group for each day of gestation.

<table>
<thead>
<tr>
<th>Gestation Day</th>
<th>Number of Resorption Sites</th>
<th>Number of Fetuses</th>
<th>Mortality Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
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<td>11</td>
<td>15.4</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>6</td>
<td>57.1</td>
</tr>
<tr>
<td>18</td>
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<td>12</td>
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</tr>
<tr>
<td>17</td>
<td>5</td>
<td>7</td>
<td>41.7</td>
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<td>3</td>
<td>9</td>
<td>25.0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>10</td>
<td>23.1</td>
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<tr>
<td>13</td>
<td>1</td>
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<td>7.7</td>
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<tr>
<td>7</td>
<td>1</td>
<td>12</td>
<td>7.7</td>
</tr>
</tbody>
</table>

**SIZE**

The size of the fetuses of the experimental group was smaller than those of the experimental group. Size variation was slight for many of the fetuses, especially those excised early during the gestation period. However, some of the experimental fetuses that were closer to term were about 25% smaller (both in length and height) than those of the control group. The decrease in the size was associated with a retarded development rate of the fetal mice, which was noticeable in several of the experimental specimens by comparing the central nervous systems of these to the control specimens.

**HISTOLOGICAL RESULTS**

External examination revealed no anomalies in the central nervous system; however, upon sectioning, slight
internal hydrocephaly was seen in some of the experimental animals. The internal hydrocephaly was characterized by enlarged ventricular spaces due to a thinning of the ventral walls of the ventricles. The thinning of the first and second ventricles was slight enough that the walls still enclosed the ventricular system completely so there was no sign of any hydranencephaly. The area of the third ventricle showed the greatest extent of expansion, but in all cases, as with the first and second ventricular walls, the walls were always confluent and no apparent gaps in the neural tissue occurred.

Rosettes were occasionally observed in the brain tissue in the experimental group but did not appear in the control group. The rosettes consisted of cells arranged radially around a lumen and would stain darker than other cells with hematoxylin-eosin stain. No anomalies were observed in the spinal cord of any of the animals.
DISCUSSION

The present study demonstrates that methotrexate, when injected maternally early in gestation, does have a direct effect on the developing offspring. The reduced size of the experimental fetuses is in all probability due to an inhibition of DNA, RNA, and protein synthesis in the normally rapid proliferating cells during embryonic development. This inhibition would slow the proliferation rate and thus interfere with the fetuses' normal development, resulting in a "stunted" growth.

The normal development of the tissues is required for appropriate differentiation of the various specialized systems, such as the tissue of the central nervous system. As noted earlier, the hydrocephaly produced in this research was only mild and restricted to internal hydrocephaly. This abnormality was slight enough that no noticeable physical or mental retardation was observed in the offspring allowed to go to term and mature. Their behavior, as well as their morphological appearance, was indistinguishable from the offspring of the control group. These results suggest that single, low doses of methotrexate administered early in gestation have no greatly significant teratogenic effects. The key aspects in this research were that only low doses of methotrexate were administered and that they were administered during early gestation. It is my belief that changing either one or both of these points would produce greater detrimental effects on the offspring.
Increasing the levels of methotrexate could be achieved by larger doses—which is presently being used experimentally in the clinical use of methotrexate—or by multiple doses. Both of those methods would result in increased plasma levels of the drug maternally which would allow for a greater concentration to cross the placental barrier into the fetal circulation and, in turn, into the fetus. The increased fetal concentration would intensify the inhibition of the DNA, RNA, and protein synthesis, thus producing more dramatic teratogenic effects, such as more severe hydrocephaly, encephalocranium, and spina bifida, to mention a few. The increased levels would also be likely to result in cytotoxic effects on the fetal cells by the proposed mechanism of thymidylate synthesis.

Teratogenicity to the central nervous system by chemicals has been shown to be greatest during the second trimester of pregnancy such as days 9 through 12 in the mouse. For this reason, administration of methotrexate during this "critical time" would naturally result in more profound teratogenic results. Multiple doses prior to this time phase—allowing increased levels of the drug to be maintained either partially or completely during this period—would also have a good probability of dramatic abnormalities in the central nervous system.

Even with the relatively slight teratogenic results obtained in this study, there is evidence that females receiving methotrexate therapy should be informed on the dangers of methotrexate associated with pregnancy and women
should receive medical advice about contraception during the use of methotrexate. The results of this study may be useful in helping to determine what steps should be taken if a pregnancy does occur during methotrexate therapy. If pregnancy is determined early, during the first trimester, and if the doses of administrated methotrexate are low, evidence from this research would suggest a good chance of the child's being born with only minimal, if any, anomalies of the central nervous system as long as the treatments can be discontinued for the remaining period of pregnancy. These "minimal anomalies" would be likely to produce no noticeable head malformations or mental retardation.

This study, therefore, provides evidence that methotrexate does have a potential to be a teratogenic substance, but low single doses, administered during early gestation, produces no highly significant abnormalities of the central nervous system.
LITERATURE CITED


