Suggested short title:

THE EFFECTS OF INTRACAROTID METHOTREXATE ON THE RHESUS MONKEY. R. R. HANSEBOUT M. D.

THE EFFECTS OF INTRACAROTID METHOTREXATE ON THE RHESUS MONKEY

by

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A thesis submitted to the Faculty of
Graduate Studies and Research
in partial fulfilment of the requirements for the
degree of Master of Science.

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AUGUST, 1964.

PREFACE

Cancer in its diverse forms has plagued man through the ages. His inherent struggle for survival and natural curiosity have prompted man to try and devise methods to cope with this affliction, though even at present he is, in a sense, fighting the unknown.

In the time of Galen (150 A.D.) the role of surgery for cancer was already prominent (Heller, 1962). Modern surgery and irradiation therapy have vastly changed the palliative treatment and altered the course of the malignant process in man, but have seldom eradicated the disease. Though modern knowledge is vast and sophisticated compared to that of the ancients, the intimate secrets of cell division are still incompletely understood. It may be that forms of chemotherapy will prove to be man's most effective future agent in his effort to alter the course of neoplastic disease.

The medical treatment of cancer began in 1865, when Lissauer treated two cases of chronic myelocytic leukemia using arsenicals. During the last seventeen years a number of corrosive and antimetabolic compounds have been used in various ways, for varying types of malignancy, with limited success.

The resistance of some tumors of the brain, such as the glioblastoma multiforme to conventional means of therapy is well-known, and almost invariably the outcome is death in a few months. It is hopedthat chemotherapy may afford a means of active treatment, or at least palliation for patients with this

disease. However, chemotherapy using antimetabolites has not been extensively tried in the management of brain tumors, and its effects are for the most part unknown.

At the Montreal Neurological Institute where a number of brain tumor cases are seen, Dr. Charles Branch has become interested in the possibilities of chemotherapy, and having studied some of the effects of antimetabolites on cats, suggested this study, prior to the use of infusion chemotherapy for treatment of humans.

The first section of this thesis is an historical review of the development of the folic acid group of antimetabolites, and of their subsequent use as anti-cancer agents. The second section contains the results of continuous intracarotid infusion of one of these antimetabolites (Methotrexate) on the normal rhesus monkey.

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HISTORICAL BACKGROUND

A. Initial Studies

a. The folic acid group: The discovery of Methotrexate and some of the other antimetabolic agents that are currently being used for the treatment of neoplastic disease is a long and colorful epic, beginning about thirty years ago. The development of these "folic acid analogs" was intimately tied in with the elucidation of one of the B complex vitamins, folic acid.

Folic acid (pteroylglutamic acid) is found in many natural materials. Its structure is shown in Fig. 1.

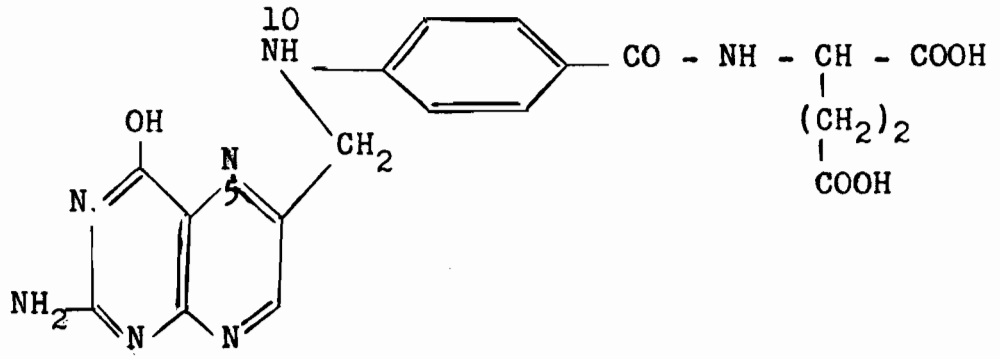

Fig. 1. Structure of folic acid
(pteroylglutamic acid)

Folic acid is a monoglutamate. In Nature there are a number of chemically related substances which differ structurally from this parent molecule in the number of glutamic acid residues

they contain in the side chains attached to nitrogen atoms 5 and 10, and in the degree of oxidation of the pteridine portion. These structurally related compounds are collectively referred to as "folic acid derivatives" or simply "folates."

In higher animals, ingested folic acid is reduced to dihydrofolic acid under the enzymatic influence of dihydrofolate reductase. The reduced form of nicotinamideadenine dinucleotide phosphate (NADP) acts as a hydrogen donor (Harper, 1963). The dihydrofolate form is the primary produce in the biogenesis of the folate derivatives (Friedkin, 1963). The dihydrofolate form is further reduced to the tetrahydrofolate form, again using reduced NADP under the influence of the enzyme dihydrofolate reductase (Harper, 1963). Some dihydrofolate is obtained nutritionally, but it is also regenerated from the tetrahydrofolates during thymidylate synthesis (Jukes, 1962). The dihydrofolate form is involved in a cycle of biochemical events, within which the tetrahydrofolates are formed (Jukes, 1962).

Under the influence of various enzyme systems, single carbon moieties are incorporated into the basic tetrahydrofolate structure to form the tetrahydrofolate coenzymes (Jukes, 1962). There are a number of these tetrahydrofolate forms which are metabolically active, but the most important ones are thought to be N^5-methyltetrahydrofolate, N^{10}-formyltetrahydrofolate, and $N^{5,10}$-methylene tetrahydrofolate, and $N^{5,10}$-methenyltetrahydrofolate (Herbert and Zalusky, 1962). There can be an interconversion of these tetrahydrofolate coenzymes from one form to another during metabolism (Herbert and Zalusky, 1962).

Single carbon moieties are carried by the tetrahydrofolate coenzymes into vital reactions leading to the synthesis of the purines and pyrimidines. The pyrimidines are essential in the formation of desoxyribonucleic acid (Huennekens et al,1958). Some of the biochemical reactions dependent upon the tetrahydrofolate coenzyme system are as follows (Boyland, 1962):

1) Conversion of glycine to serine;
2) Methylation of ethanolamine to choline;
3) Conversion of homocysteine to methionine;
4) Conversion of nicotinamide to N-methylnicotinamide.
5) Introduction of the amidine carbon into the histidine molecule.
6) Introduction of carbon atoms 2 and 8 in purine biosynthesis.
7) Conversion of some pyrimidine derivatives into thymine derivatives which are essential constituents of the nucleic acids.

The discovery of the importance of folic acid in biogenesis as outlined above was the result of many years of arduous investigation by numerous workers.

In the 1930's it had been observed that certain anemias in patients and in experimental animals could result from a deficiency in diet. Wills et al. in 1937 presented evidence that factors they had isolated from liver and yeast, when given parenterally, were curative for the nutritionally induced macrocytic anemias of rhesus monkeys. Stokstad and Manning (1938) described a factor U, occurring in yeast, having a growth

promoting effect on chicks. Hogan and Parrott (1940) stated that chicks given a diet deficient in a factor found in liver, developed anemias. They suggested that this factor was part of the B complex of vitamins and called it vitamin B_c. Absence of vitamin B_c decreased the hemoglobin, white blood count and thrombocyte count in chicks (Campbell et al. 1944). Vitamin B_c was soon isolated from liver (Pfiffner et al. 1943), and later from yeast (Pfiffner et al. 1945).

Snell and Peterson (1940) found a factor called the "norite eluate factor" which was required for the growth of Lactobacillus casei. This factor when isolated from liver and yeast (Stokstad, 1943) was designated the "liver L. casei factor" or the "fermentation L. casei factor," depending upon its respective source. It, too, was seen to be required for the growth and normal hemoglobin formation of the growing chick (Mills et al. 1942).

In 1941, Mitchell et al. obtained a product from spinach which they called "folic acid," and which they defined as "the material responsible for the growth stimulation of Streptococcus lactis R on a given medium." Wilson et al. (1942) found that monkeys who had developed leucopenia and later anemia on vitamin B deficient diets, responded by an increase in the white blood count to intramuscular injections of crude folic acid concentrate.

A compound active for the growth of both S. lactis R and L.casei was isolated from liver in 1944 by Hutchings et al. This compound which was the same as the liver L. casei factor and and termed "pteroylglutamic acid" (Angier et al. 1946) was

synthetized (Fig. 1), and also found to be active in promoting chick growth and hemoglobin maintenance (Angier et al. 1945). Pfiffner et al. (1947) isolated vitamin B_c from both liver and yeast and found it to be identical with the pteroylglutamic acid of Angier and his group.

Isolation of the pure fermentation L. casei factor (Hutchings et al. 1948), and its chemical degradation (Stokstad et al. 1948a; Hutchings et al. 1948b) soon followed. Concurrently the isolation of the liver L. casei factor (Stokstad et al. 1948b), elucidation of its structure (Mowat et al. 1948) and its synthesis (Waller et al. 1948) occurred.

It was realized that the compounds called factor U, vitamin B_c, the norite eluate factor, the fermentation L. casei, and the liver L. casei factor were either the same or closely related derivatives of the parent molecule pteroylglutamic acid (Jukes and Stokstad, 1948). Although they were all called "folic acid" they differed in the number of glutamic acid residues attached to the parent monoglutamate, pteroylglutamic acid (Boyland, 1962). The liver L. casei factor was simple pteroylglutamic acid (Waller et al. 1948).

In 1948 Sauberlich and Baumann described a growth factor occurring in liver slices which was essential for the growth of Leuconostoc citrovorum. They suggested that folic acid might be required for the synthesis of this substance they called the "citrovorum factor." It was later shown that under the influence of reducing agents such as ascorbic acid, citrovorum factor could be produced from synthetic pteroylglutamic acid and from compounds present in liver (Nichol and Welch, 1950b). This citrovorum factor

(N^5 -formyl-tetrahydropteroylglutamic acid) was synthetized by Brockman et al. in 1950.

In 1957, Futterman isolated the enzyme dihydrofolate reductase from chicken liver and found that it could reduce folic to the dihydrofolate and tetrahydrofolate forms. However, folic acid could also be reduced to dihydrofolate by other enzymes (Huennekens et al. 1958).

Jukes and Stokstad (1948) suggested the biological importance of folic acid in the synthesis of thymine and the purines in lactic acid bacteria. It was later suggested that in higher animals the tetrahydrofolates also acted as carbon carriers for thymine and purine production (Jukes, 1953). By 1958 it was known that folic acid served as a growth factor by controlling the metabolism of the one-carbon compounds, formate and formaldehyde, and most of the biosynthetic pathways involving the tetrahydrofolates in the synthesis of certain purines, pyrimidines and amino acid were known (Huennekens et al. 1958). The intricate cycles involving the tetrahydrofolate coenzymes were more extensively elaborated in 1963 by Friedkin, and by Brown and Reynolds.

Throughout the years, members of the folic acid group have been of great value in the treatment of certain megaloblastic anemias. Patients with some forms of chronic hemolytic anemias such as Thalassemia have also been found to be deficient in this vitamin (Jandl and Greenberg, 1959).

The clarification of the importance of folic acid in biogenesis set the stage for investigation of antagonism of the vitamin, since even in 1947 it was suggested that folic acid antagonism might modify blood dyscrasias marked by erythrocytosis

and leucocytosis (Franklin et al. 1947a).

The participation of the folic acid coenzymes in reactions required for the synthesis of purine and pyrimidine derivatives emphasized the fundamental role of this vitamin in growth and the reproduction of cells. It is not surprising therefore that interference with folic acid metabolism inhibits cell division in both normal and neoplastic tissues.

b. The folic acid antagonists: In living tissue, enzymes attract various molecules to their surface, allowing biochemical reactions to take place, with the formation of new compounds. Compounds called "antimetabolites" can displace the normal metabolites from the surface of the enzyme, stopping vital biochemical reactions. Characteristically, the antimetabolite, which is often structurally similar to the metabolite, is displaced from the surface of the enzyme when the concentration of the metabolite is increased, allowing the enzyme to resume its normal function (Jukes, 1962). This principle of competitive inhibition of a metabolite by a structurally similar antimetabolite was important in the development of some drugs currently used in the treatment of cancer.

The first great stimulus to planned approaches in chemotherapy came in 1940, when Woods found that p-aminobenzoic acid nullified the action of sulphanilamide, and suggested the mode of action of the latter. The idea of the competitive inhibition of a metabolite by an antimetabolite then came into vogue.

Laszlo and Leuchtenberger in 1943 found that the vitamin inositol given intravenously imhibited tumor growth in mice. Then

Leuchtenberger et al. (1944) showed that crude "folic acid concentrates" - glutamic acid derivates, given intravenously, inhibited the growth of Sarcoma 180 in mice. Next, they caused regressions of spontaneous breast cancers in mice with intravenous injections of an L. casei factor (Leuchtenberger et al. 1945). This factor was identical with the fermentation L. casei factor isolated in 1944 by Hutchings et al., which was subsequently shown to be pteroyltriglutamic acid (Hutchings et al. 1948). The liver L. casei factor which was shown to be pteroylglutamic acid (Waller et al. 1948) was ineffective when given in a similar fashion.

In 1946, Martin et al. produced methylfolic acid, and found that it inhibited the folic acid required for the growth of Streptococcus faecalis. Methylfolic acid when fed to rats caused weight loss, anemia and leucopenia, but these effects were prevented by the addition of high concentrations of pteroylglutamic acid to the diet (Franklin et al. 1947). These observations were confirmed in mice and chicks (Franklin et al, 1947), and in pigs (Welch et al. 1947). Hutchings et al. (1947) considered that these effects were due to the competitive inhibition of the metabolite, since large doses of folic acid could prevent this syndrome.

In the course of investigation for analogs of pteroylglutamic acid, 4-aminopteroylglutamic acid (Aminopterin) was made and was found to inhibit the growth of Streptococcus faecalis in spite of added folic acid (Seeger et al. 1947). Mice, when fed this substance, died in a few days despite the dietary addition of large amounts of folic acid (Franklin et al. 1948). This was

followed by the announcement of the synthesis of 10-methylpteroylglutamic acid (Consulich et al. 1948), and of 4-amino-N^{10}-methylpteroylglutamic acid (Amethopterin, Methotrexate) by Seeger et al. in 1949 (Fig. 2).

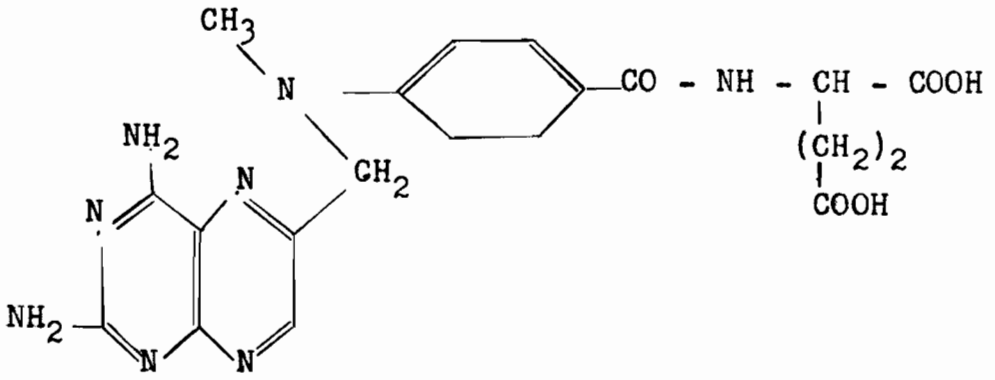

Fig. 2. Structure of 4-amino-N^{10}-methylpteroylglutamic acid (Methotrexate).

Other work showed that Rous sarcoma of chicks would not grow in folic acid deficient chicks; that Aminopterin inhibited the growth of Rous sarcoma in chicks; but the dose of the Aminopterin which prevented growth of the sarcoma also killed the chicks (Little et al. 1948). In 1949b, Burchenal found that Methotrexate doubled the survival time of mice with transplanted leukemia. This effect could be prevented somewhat by the administration of folic acid at the same time. The protective effects of Methotrexate could be almost entirely blocked by the prior administration of the citrovorum factor, which was several times as active in this respect as pteroylglutamic acid itself (Burchenal et al. 1950). It was assumed that Methotrexate was a true

antagonist of citrovorum factor rather than of pteroylglutamic acid (Burchenal and Babcock, 1951), and that citrovorum factor was the biologically active derivative of pteroylglutamic acid (Nichol and Welch, 1950).

Methotrexate when fed to mice also caused anemia, leucopenia and, in particular, granulocytopenia (Franklin et al. 1949). Other signs, such as alopecia, buccopharyngeal ulceration, and enteritis, which were not commonly seen in pure pteroylglutamic acid deficiency, called attention to the production of greater than one syndrome by pteroylglutamic antagonism in animals (Ibid).

In 1950a, Nichol and Welch suggested that Aminopterin prevented the conversion of pteroylglutamic acid to citrovorum factor, but also competed directly with citrovorum factor. Later it was said that the folic acid antagonists could be divided into two groups. The first group exemplified by methylpteroylglutamic acid was said to have the following characteristics (Jukes, 1953): a) They were competitively reversed by pteroylglutamic acid in animal experiments; b) These analogs caused no effect on Leuconostoc citrovorum in the presence of citrovorum factor; and these antagonists interfered with the formation of citrovorum factor from pteroylglutamic acid, but not with the utilization of the citrovorum factor. The second group of compounds was typified by Aminopterin and Methotrexate. In experiments with animals, the effect of these substances were not prevented by the administration of pteroylglutamic acid (Philips and Thiersch, 1949). The compounds in this group showed competitive reversal by citrovorum factor when studied with Leuconostoc citrovorum (Jukes, 1953). These

substances blocked the formation of the citrovorum factor from pteroylglutamic acid (Nichol and Welch, 1950a) and, in addition, were assumed to block the utilization of the citrovorum factor competitively (Jukes, 1953).

In 1957 a major step in the clarification of the site of action of the folic acid antagonists came when Futterman isolated the enzyme which reduced folic acid to dihydrofolate reductase from chicken liver. In this enzyme system, Aminopterin inhibited both the reduction of folic acid to dihydrofolic acid, and of dihydrofolic acid to tetrahydrofolic acid. This enzyme was dihydrofolate reductase (Condit, 1961).

In 1961 it was shown by Werkheiser that Aminopterin and Methotrexate bound themselves to dihydrofolate reductase in a stochiometric manner. Under certain conditions the drugs could be freed from the enzyme complex by dialysis with folic acid. The dihydrofolate reductase content of tissue, measured by the minimal amount of drug required for complete inhibition of the enzyme system, was the same as the amount of the drug bound to the tissues in a nondialyzable form. This showed that the 4-amino analogs of folic acid were actually bound to dihydrofolate reductase. That this was the site of interference in the folic acid cycle by Methotrexate was confirmed by Slavikova and Slavik (1961). They also found that due to the binding of Methotrexate to dihydrofolate reductase, the formation of the coenzymatically active tetrahydrofolates was prevented, and consequently single carbon transfer reactions in the synthesis of the purines and pyrimidines were prevented. The binding of these drugs to the enzyme was so

"tight" that Methotrexate could still be detected in animal tissue after 8 months (Werkheiser, 1961).

This quantitative type of binding implied that if the amount of the enzyme were to become increased beyond the point necessary to bind the antagonist, the excess enzyme would be able to carry out its normal function of reduction of the folates and dihydrofolates. The amount of enzyme to overcome this inhibition would have to be considerable, however, since the affinity of the enzyme for Methotrexate at pH 6 was found to be 100,000 times as great as for folic acid (Werkheiser, 1963).

The prime action of Methotrexate is the inhibition of the formation of the tetrahydrofolate coenzymes in metabolism secondary to its inhibition of the enzyme dihydrofolate reductase (Werkheiser, 1963). The inhibition of this enzyme is not as important to the formation of the dihydrofolates from folates, since some of the former are obtained from dietary sources, and folate can be reduced to dihydrofolate by other enzymes (vide supra). Dihydrofolate is also a by-product of some of the metabolic reactions involving the tatrahydrofolates. Although the citrovorum factor can inhibit the actions of Methotrexate (Condit, 1961), it is not an important tetrahydrofolate in the metabolism of higher animals. It is probably converted to the other coenzymatically active tetrahydrofolates by other enzymes (Herbert and Zalusky, 1962), and thus can reverse the actions of Methotrexate.

Certain strains of mouse leukemia cells (Burchenal et al. 1951) and human leukemic cells (Burchenal, 1956) have become

resistant to Methotrexate. Increased levels of dihydrofolate reductase were noted in these anti-folate resistant cells (Friedkin, 1963). This was due to the formation by the resistant cells of such high concentrations of dihydrofolate reductase, that the host would have been unable to tolerate systematically the larger doses of antimetabolite necessary to inhibit the resistant cells (Jukes, 1962).

Throughout the years, antimetabolites other than the folic acid antagonists have also been shown to have inhibitory effects on leukemic cells (Burchenal et al. 1949a). Such drugs as the purine antagonists, and the folic acid antagonists, have been shown to act synergistically due to their respective actions in different portions of biological cycles (Hochstein, 1957). Different types of antimetabolites have also been shown to potentiate each other therapeutically (Johnson et al. 1958).

Some preliminary work has already been done on the hydrogenated derivatives of Aminopterin and Methotrexate. It is thought that they inhibit the folic acid cycle at the tetrahydrofolate level competitively, rather than at the stage of the reduction of folate tetrahydrofolate (Kisliuk, 1960).

Since the folic acid analogs block the synthesis of desoxyribonucleic acid (O'Brien, 1962; Bertino, 1963), a synthesis essential to chromosome reduplication, there is mitotic arrest (Jacobson, 1959) and cell division does not proceed.

B. <u>The Era of Cancer Chemotherapy</u>

The work of Leuchtenberger et al. (1944) and of Lewisohn et al. (1946) regarding the inhibition of tumor cells in mice,

suggested clinical uses for folic acid antagonists as inhibitors of cell division. This idea was reinforced when it was found that Aminopterin could inhibit the growth of the chick embryo (Karnofsky et al. 1948) and the rat embryo (Thiersch and Philips, 1950), with the production of developmental anomalies.

Such reports, and their own studies on folic acid deficiency, prompted Farber and his associates to consider the use of folic acid antagonists in patients with malignant disease. The first report of this work (1947) indicated that 90 patients with various malignant processes, in whom established therapeutic procedures offered no hope, were given intravenous or intramuscular injections of pteroyltriglutamic acid (Teropterin) or pteroyldiglutamic acid (Diopterin). These injections were given over a period of a few days to a few weeks. Although arrest of the malignant processes were equivocal, no serious adverse effects were noted, and in some instances the patients became remarkably free of pain. It was soon observed that in children with acute leukemia receiving injections of Teropterin and Diopterin, the leukemic process in the marrow was apparently accelerated. This suggested to Farber and his associates (1948) that folic acid antagonists of a different type might be useful in the treatment of acute leukemia, but that further studies should be carried out.

When pteroylaspartic acid (Hutchings et al. 1947) was given intramuscularly to a four-year-old girl with acute myelogenous leukemia, it had no effect on the blood picture, but the bone marrow became hypoplastic (Farber et al. 1948). Injections of a more powerful antagonist, Aminopterin, were given to 16

moribund children with acute leukemia, 10 of whom showed temporary clinical, hematological and pathological remissions. Folic acid injections were given concomitantly to try and alleviate the stomatitis produced by this treatment (Farber et al. 1948). Later (1949) Farber reported that of 60 children treated for leukemia by Methotrexate and other antifolates, 50% had shown temporary clinical improvement, but none were cured. In another series (Sacks et al. 1950) 5 patients out of 14 with various types of leukemia treated by intramuscular injections of Aminopterin and Methotrexate were shown to have had temporary benefit.

Results of the early work with acutely leukemic children treated with Aminopterin were reported by Burchenal in 1956. Sixty-eight per cent of them had derived some clinical benefit, with an average remission in those responding of about 8 months. Methotrexate was less toxic than Aminopterin and thus had a wider therapeutic range. The drugs could be absorbed equally readily via the oral or intramuscular routes. The citrovorum factor sometimes re-exacerbated the leukemic process, and thus was to be given only if antifolate toxicity endangered the patient's life. Since the drugs were only palliative, the sequential use of different types of antimetabolites and steroids was recommended. Radiation therapy was considered to be the treatment of choice in the chronic leukemias (Burchenal, 1954).

It was also noted that in spite of administration of the folic acid antagonists, a child could still develop the central nervous system stigmata of the disease, although the blood picture

improved. However, Whiteside et al. (1958) induced remissions in such cases using intrathecal injections of Methotrexate, and disclosed a new avenue of administration of these drugs.

From observations on the patients they had treated with Aminopterin and Methotrexate, Schoenbach et al. (1950) reported that the side effects of these drugs on humans were as follows: buccopharyngeal ulceration, stomatitis, gastrointestinal cramps, diarrhea, leucopenia, thrombocytopenia, macrocytosis, anemia, drug rash, alopecia and hot flushes. The citrovorum factor in contrast to folic acid could reverse some of these established drug effects. This same group (1952) also emphasized the dangers of marked toxicity in patients with renal disease, since Methotrexate and Aminopterin were excreted via the renal route.

A further great impetus to the chemotherapy of malignant disease came in 1956 when Li et al. described clinical and radiological improvement in patients with choriocarcinoma and chorioadenoma following treatment with intravenous Methotrexate. There was unequivocal evidence of tumor regression with a drop in the urinary gonadotropin levels in eleven women with trophoblastic tumors treated with oral or intramuscular folic acid antagonists or 6-mercaptopurine. Five males with trophoblastic type tumors treated similarly showed no response. Other workers (Bagshawe and Brooks, 1959; Hertz et al. 1958) gave equally encouraging reports regarding the treatment of choriocarcinomas with Methotrexate.

Although the antifolates were of value in the treatment of the acute leukemias, such chemotherapy in patients with the more common forms of solid tumors gave only slight and variable

results (Schoenbach et al. 1952; Kofman, 1958). For example, in a series of 36 patients with carcinomas of the breast, treated with Methotrexate by conventional administration, only 10 showed temporary objective improvement (Wright et al. 1959). Attempts were thus made to increase the value of these agents by using combinations of antitumor drugs alone (Li et al. 1958), and with radiotherapy (Wright et al. 1959), or by different routes of administration (Whiteside et al. 1958).

In 1950, Klopp et al. tried fractionated intra-arterial injections of nitrogen mustard into the arterial supply of tumors and noted evidence of regression. This caused a more intense tissue reaction than with the intravenous injection of nitrogen mustard. However, this method of therapy was not specific, damaging the adjacent normal tissues as well as the tumor (Sullivan et al. 1953).

In order to try to prevent the systemic toxicity of the antitumor drugs with delivery of high concentrations of a drug to the tumor, Creech et al. in 1958 used extracorporeal perfusion techniques. The tumor-bearing part was isolated from the general circulation for periods of 30 to 60 minutes while high concentrations of a drug were perfused through it. By this method very toxic compounds could be used, and the drug-sensitive hemopoietic tissues and gastrointestinal organs were exposed to relatively small amounts of the drug which spilled over into the general circulation.

Woodhall in 1959 tried the effects on four brain tumors of localized perfusion via the carotid arteries and internal

jugular veins in combination with hypothermia. Various agents were used and the results were equivocal. The hypothermia was used in an effort to try to counteract cerebral edema. When it was thought that hyperthermic perfusion might accelerate the lethal actions of a perfused alkylating agent, Woodhall in 1960 perfused twenty patients in this way and got temporary regressions in only two head and neck tumors.

Some good results were obtained by the perfusion methods but the type of drug used had a marked acute cytocidal effect on all tissues, both normal and neoplastic. It was said that perfusion was of value in the palliation of advanced malignancy, where tumors were regionally confined and otherwise inoperable. The best results were obtained in melanomas and sarcomas of the extremities, where good isolation could be achieved (Clarkson and Lawrence, 1961). Perfusion methods with the antimetabolites did not result in substantial tumor regressions even at high concentrations (Sullivan, 1962), probably because a longer period of exposure was required for the oncolytic effects of the antimetabolites, compared with the alkylating agents (Clarkson and Lawrence, 1961).

Sullivan et al. in 1959 gave numerous types of antimetabolites intra-arterially to the tumor region in daily single doses, without enhancement of the local antitumor effects. It was then theorized that the continuous administration of the antimetabolite to the region of the tumor might enhance the oncolytic effects of the drugs. In studies it was found that the amount of citrovorum factor required to circumvent severe systemic toxic manifestations

of the drug, was increased tenfold when Methotrexate was given continually intra-arterially or intravenously rather than by single daily intra-arterial injections in the same dosage (Sullivan et al. 1959). In order to prevent the enhanced toxic effects of the drug when given in this fashion, the simultaneous intermittent administration of the metabolite might be of aid, for it was theorized that at the relatively high concentrations of the drug in the arterial supply of the tumor, the metabolite would not inhibit completely the oncolytic effects of the anti-metabolite (Sullivan et al. 1959).

Thus, in 1959 Sullivan et al. introduced a method of chemotherapy in which a pump continually infused an antimetabolite into the arterial supply of a tumor at pressure greater than that of the blood, via a catheter placed in a major artery of supply to the tumor. Intramuscular citrovorum factor was given intermittently during the infusion period. Carcinomas of the head and neck were sometimes infused in this manner for days by a catheter inserted into the external carotid artery via the superior thyroid artery. Some patients were given intravenous Methotrexate with intermittent intramuscular injections of citrovorum factor as a control series. Indeed, tumor regressions were sometimes noted in 3 or 4 days when the drug was given by continuous intra-arterial infusion, compared with regressions after a much longer time when the drug was given orally, intramuscularly, or even continually intravenously. Partial to complete tumor regressions were noted in 10 out of 18 patients treated in this new manner, so that further studies were prompted. The best results of treatment with this combination of drugs was in patients with previously untreated

epidermoid carcinomas of the head and neck, localized in the region of distribution of the external carotid arteries, or, in patients with untreated carcinomas of the cervix confined to the region of distribution of the hypogastric arteries (Sullivan et al. 1960).

Since the original reports of Sullivan et al. (1959), reports in the literature regarding the usage of continuous intra-arterial infusion therapy with Methotrexate are too numerous to cite. Two are noteworthy however. Westbury et al. (1962) reported on a series of 26 patients with previously irradiated head and neck neoplasms. Some responded to the therapy by partial tumor regression, while relief of pain was seen in all but one patient; only one patient was apparently free from disease after 3 months; three patients died of surgical complications, and one patient died of Methotrexate toxicity. Watkins and Sullivan (1964) reported on a series of 68 patients with head and neck tumors, treated by continuous intra-arterial Methotrexate infusion therapy. There were 42 cases of objective remission shown by reduction of the tumor to 50% of its initial size.

The status of the infusion and perfusion techniques was summarized in 1962 by Smith et al. who stated that perfusion could often be used with good success for tumors of the extremities, especially melanomas and sarcomas. Perfusion methods had not changed the survival time of 34 patients treated in this way for malignant brain tumors, and thus the degree of surgery involved was unwarranted. For tumors of the pelvis, axillae, brain, abdominal viscera and lungs, the intra-arterial methods were

better used.

In the opinion of Clarkson and Lawrence (1961), and Westbury et al. (1962), although infusion methods had increased the oncolytic effects of the antimetabolites, these methods were still experimental, and were not to be relied upon when the possibility of curative surgery or radiotherapy existed. Regarding tumors of the head and neck, the methods were better used with failures of surgery and radiotherapy (Westbury et al. 1962).

Watkins and Sullivan (1964) reviewed the results of the treatment by the continuous intra-arterial method of 136 patients with neoplasms of the head and neck, pelvis, brain, liver and chest wall. The patients were infused for periods up to 45 days, and the longest follow-up was 37 months. Seventeen patients had regressions for periods up to 37 months; 62 had partial regressions as evidenced by decrease in the size of the tumor mass by 50%; and 55 patients had one or more complications resulting from the treatment.

Several intresting observations have been made in the literature pertaining to the infusion method with Methotrexate. The best tumor response is obtained when a tumor is confined to the region of supply of the tumor. Clarkson and Lawrence (1961) noted that tumors which had an extension outside the distribution of the infused artery often regressed in the direct region infused, while portions of the tumor deriving their blood supply from other arteries showed only minor regression. They also noted that a patient's renal and general condition had to be satisfactory to cope with the toxicity resulting from the treatment.

Westbury et al. (1962) found that the minimal dosage of Methotrexate that could be given without causing local necrosis in previously irradiated cases, was less than in non-irradiated cases. They suggested that the infusion method without the simultaneous use of citrovorum factor should be further investigated. Espiner (1962) claimed that using Methotrexate in continuous intra-arterial infusion, better results were obtained when it was administered prior to irradiation therapy. On the other hand, Bagshaw (1963) claimed that the radiation effect could be potentiated by the concomitant administration of continuous intra-arterial Methotrexate.

The dosage schedules for the continuous intra-arterial infusion method using Methotrexate and intermittent intramuscular citrovorum factor were established by Duff et al. (1961). A dosage of 50 mg. / adult / 24 hrs. with 9 mg. citrovorum factor given intramuscularly every six hours during a 5 to 10 day course of therapy was considered to be adequate.

The main complications of this method were as follows (Duff et al. 1961): improper catheter placement, bleeding from the catheter insertion site, thrombosis of the artery, local infection, septicemia, leakage from the catheter, and premature displacement of the catheter. The erythema and the ulceration of the skin in the infusion region, regarded as an indication of a favorable response to therapy (Watkins and Sullivan, 1964), was distressing to Stovner et al. (1962) who prevented it by topical application of a solution composed of citrovorum factor and hyaluronidase.

Placement of the infusion catheter by angiography was abandoned by some observers, since the information obtained in this manner was misleading, showing only the major arteries of supply to the tumor, and not the capillaries. The pressure of the arteriographic injection filled vessels in a retrograde fashion, which would not ordinarily have filled by the slower therapeutic infusion. Instead, a slow injection of Fluorescein was made, and the area of infusion was shown by fluorescence under ultraviolet light. It was also thought that the use of small catheters which had a faster rate of flow probably prevented clot formation (Duff et al. 1961).

Of the considerable number of tumors of the head and neck reportedly treated by the continuous intra-arterial infusion method, only brief mention has been made of brain tumors. Up to 1964, Sullivan and his group considered that only 10 of the 16 neoplasms of the brain they had treated received an adequate course of therapy (Watkins and Sullivan, 1964). Seven out of 9 of these which were suitable for evaluation showed partial tumor regression. Talley et al. (1961) gave a short course of infusion chemotherapy with Methotrexate to 4 patients with primary brain tumors. One of these showed subjective and objective regression of tumor for about a month, while the others did not respond to the therapy. Using this same method, 10 patients with malignant astrocytomas, treated with Vinblastine, also showed little response (Mealey, 1962).

Primary tumors of the brain, such as the glioblastoma, do not readily metastasize, and remain localized for a long time.

They are thus theoretically ideal from the criteria which should make a tumor amenable to continuous intra-arterial infusion chemotherapy. However, there is evidence that the doubling time of these tumors may be a long time (Sullivan, 1964). Consequently, for antimetabolite infusion therapy to be effective, one would have to continue the infusion period long enough to catch all the cells when they sequentially reach the stage of active desoxyribonucleic acid production, prior to reduplication. Thus, several courses of treatment might be anticipated, or better, if possible, one long course of infusion. Sullivan (1964) has envisioned the treatment of these brain tumors with a low dose, such as 2 mg. of Methotrexate per adult, per 24 hours, for several months wihtout the use of citrovorum factor.

The development of the chronometric infusor (Watkins, 1964), a miniaturized, self-contained infusion assembly, might make long term therapy a reality. This tiny pump is designed to operate on an ambulatory basis, and it can be refilled on an outpatient basis. The unit is connected to an indwelling intra-arterial catheter.

With these realizations and advances in mind, the use of an infusion method of therapy for the glioblastoma multiforme which is resistant to treatment by both surgery and radiotherapy becomes an interesting problem for consideration.

EXPERIMENTAL OBSERVATIONS

A. Introduction

The utilization of the newer forms of chemotherapy in the treatment of malignant neoplasms, affecting various parts of the body, by clinicians in North America and abroad, prompted us to consider them for the therapy of inoperable malignant neoplasms of the brain.

After a consideration of the literature, it was evident that few preclinical trials of the total effects of the antimetabolites on animals had been recorded. Moreover, little was known of the effects of these drugs on the brain. Before embarking on the treatment of humans, it was considered wise to ensure that the drugs would not themselves harm the normal brain substance. Animal experimentation might also enable us to familiarize ourselves with a technique which could be used in humans.

In view of its equivocal results in therapy of brain neoplasms (vide supra), the necessity for the use of compounds harmful to normal tissues, and the requirement of elaborate equipment, the perfusion method was ruled out. The infusion method of therapy, though time-consuming, was more appealing since a simple operation only was required, which could be used even on moribund individuals, and relatively simple equipment could be used. It relied on the usage of compounds which theoretically, at least, should not harm the normal brain substance, since mitotic activity is rare in this tissue.

It was known that primary brain tumors contain higher amounts of desoxyribonucleic acid than normal brain tissue (Heller and Elliott, 1954) and, further, that the folic acid antagonists lower the desoxyribonucleic content of tissues by curtailing its production (Strength et al., 1954) together with cell division. Thus the folic acid antagonists seemed a logical choice. Of these, Methotrexate had already been extensively used in humans, and the dose schedule had been established.

With these goals in mind it was decided to use the rhesus monkey as the subject since it is a readily obtained primate. Moreover, neurological and behavioral changes can easily be observed in this animal, and it is large enough for intracarotid surgery.

Vastly supralethal doses of Methotrexate were to be given, without the simultaneous use of the metabolite, to test the hypothesis that the normal brain would not be harmed, but that the animals would die due to systemic toxic effects of the drug. Since the experiments were to be chronic ones, as many parameters of the effects of the drug as possible were to be studied.

B. Methods

Certain abbreviations are used in the text that follows:

cm. = centimeter

EEG = electroencephalogram

gm. = gram

hr. = hour

kg. = kilogram

M = molar
mg. = milligram
min. = minute
ml. = milliliter
$mm.^3$ = cubic millimeter
mo. = month
mug. = millimicrogram
sec. = second
ug. = microgram

(a) The operation. Fifteen adult rhesus monkeys aged 2 to 4 years, and weighing from 3.1 to 6.9 kg. were randomly selected. Thirteen of the animals received infusions of Methotrexate over a period of several days. Two of the animals served as controls, receiving only a buffered infusate. Eight of the experimental animals were infused with buffered solutions, while in five experimental animals the infusates were unbuffered. Drug dosages of Methotrexate were as follows: Four animals received 20 mg./kg./ 24 hrs. in unbuffered infusate, while two animals received this dosage in buffered form; at 10 mg./kg./ 24 hrs., one animal had an unbuffered infusate, while three had buffered infusates; in three animals receiving 1 mg./kg./ 24 hrs., all the infusates were buffered.

The organic buffer used to maintain the pH of the infusate in the physiological range was Talatrol (Tris [hydroxymethyl] aminomethane), commonly called "Tris" or "Tham". Thirty-six gm. of Tham were first titrated under sterile conditions with an amount of 2N hydrochloric acid required to bring each 1000 cc. of 5% glucose and water to a physiological range,

when the calculated dose of Methotrexate was added to the infusate. All were mixed under sterile conditions.

The animals were anesthetized, using intravenous Nembutal in doses varying from 22 mg./kg. to 70 mg./kg. injected into a sural vein. The hair of the face and neck was cropped, using an electric hair clipper, and the animals were placed prone in the Montreal Neurological Institute head-holder.

Having prepped the vertex of the head in succession with Phisohex, 70% alcohol, ether, and 2½% iodine, ½ inch longitudinal incisions were made down through pericranium in the mid-sagittal and bilateral frontal and parietal regions. Under sterile conditions, using a hand drill and a 0.078 inch bit, tiny holes were made through the cranium in the mid-vertex and symmetrically in both frontal and parietal regions. Sterile steel screws (No. $^{2}/_{56}$ x $^{3}/_{8}$") , previously insulated with three coats of Epoxylite (aside from the extreme tip and head) were screwed down flush, so that the tips rested in the epidural space. The wounds were sprinkled with Chloramphenicol powder, and were then closed snugly, using Michel clips (Fig. 3).

Next, leads plugged into a Gilson Medical Electronics Electroencephalograph machine were attached to each of the screws via alligator clip electrodes (Fig. 4). A bilateral, bipolar, fronto-parietal two-channel recording was then made for a baseline reference.

The animal was positioned in the left lateral decubitus position with the head inclined to the left to afford access to the right side of the neck (Fig. 5). Again, the neck region,

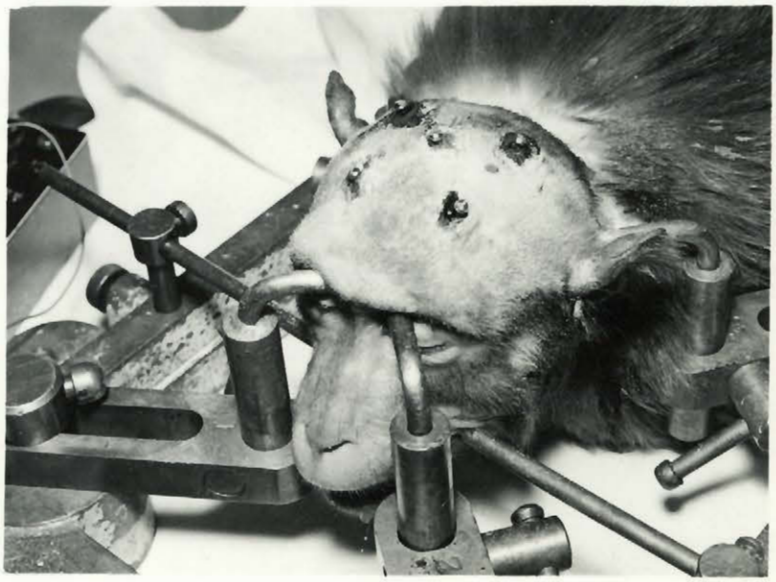

Fig.3. Animal under anesthesia in head holder. Screw electrodes are in place.

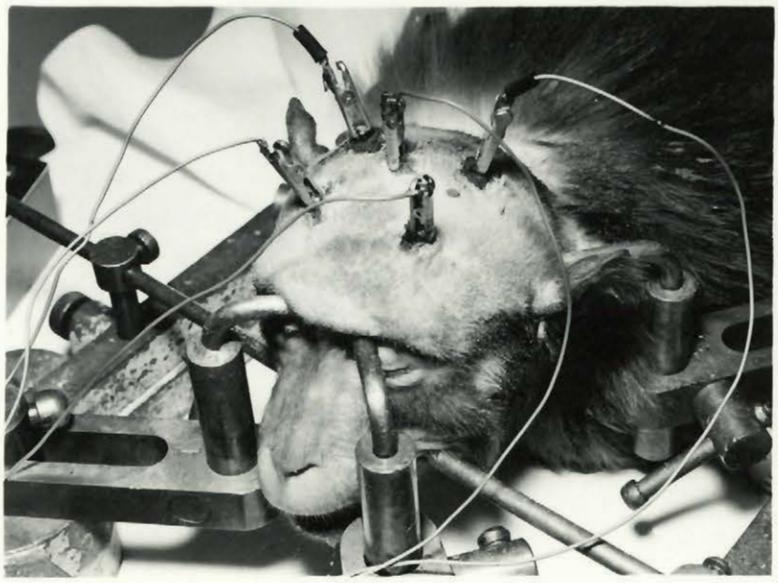

Fig. 4. Animal under anesthesia in head holder. Alligator-clip leads are attached to screw electrodes.

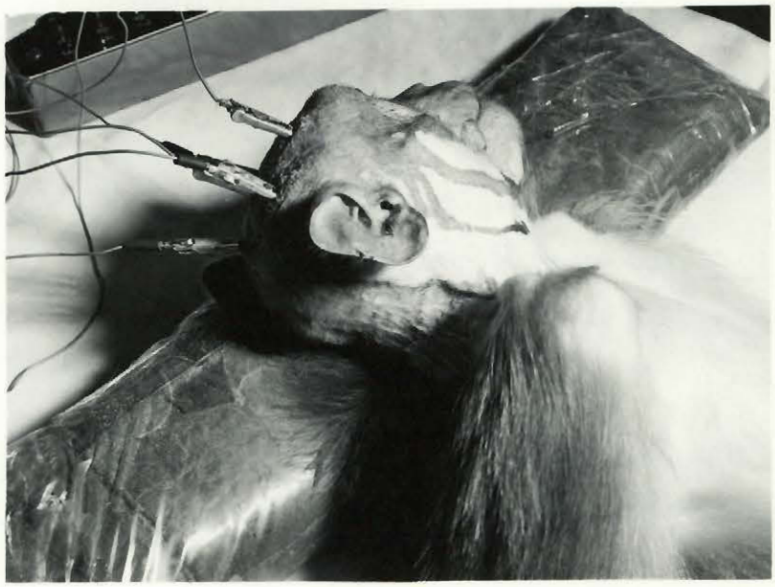

Fig. 5. Animal in left lateral position prior to surgery. Electroencephalographic recording is in progress.

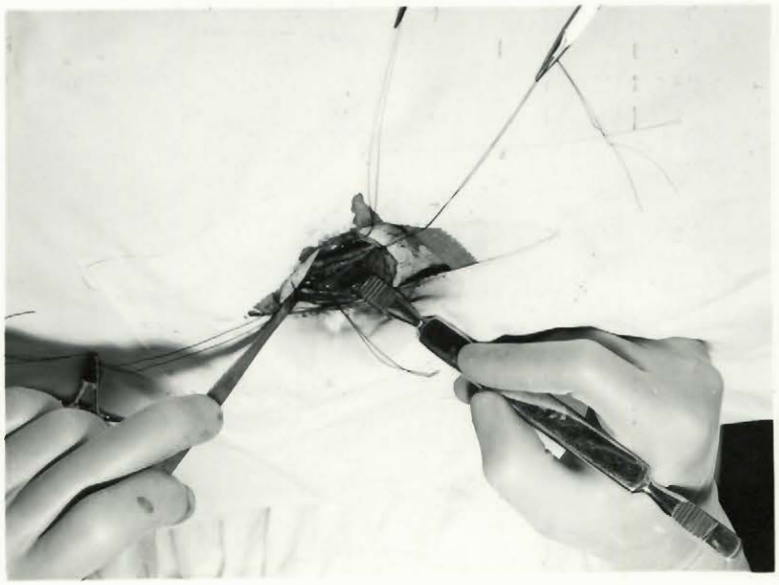

Fig. 6. The operative field, with structures of the anterior triangle of the neck exposed. (Refer to Fig. 5 for orientation).

face and shoulders were widely prepped in succession with Phisohex, 70% alcohol, ether and 2½% iodine. The animal was then draped in a suitable fashion.

At a level midway between the suprasternal notch and the ramus of the mandible, an incision was begun on the right side, 1 cm. lateral to the anterior midline of the neck, extended backwards parallel to the ramus of the mandible, and below the angle of the mandible it was curved upwards slightly. Using sharp dissection the platysma was exposed and then divided. The sternocleidomastoid muscle was next encountered, and retracted laterally, exposing the superficial structures of the anterior triangle of the neck. The carotid sheath was exposed, and using sharp dissection (Figs. 6 and 7), split longitudinally to expose its contents. The right hypoglossal nerve was sectioned to afford a better view of the bifurcation of the common carotid artery (Fig. 8). The proximal 1 cm. of the internal carotid artery and the external carotid artery and its branches were each meticulously dissected in turn.

Next a 2 cm. long vertical incision was made over the mid-portion of the right temporalis muscle. The incision was extended vertically downward from a level corresponding with the outer canthus of the right eye in a line bisecting the mid-portion of the right zygomatic arch. Using sharp dissection the right temporalis fascia was exposed. A Kelly curved clamp was then pushed from this incision subcutaneously to communicate with the larger neck dissection. A P50 polyethylene catheter, into one end of which a No. 22 needle had been inserted, was then

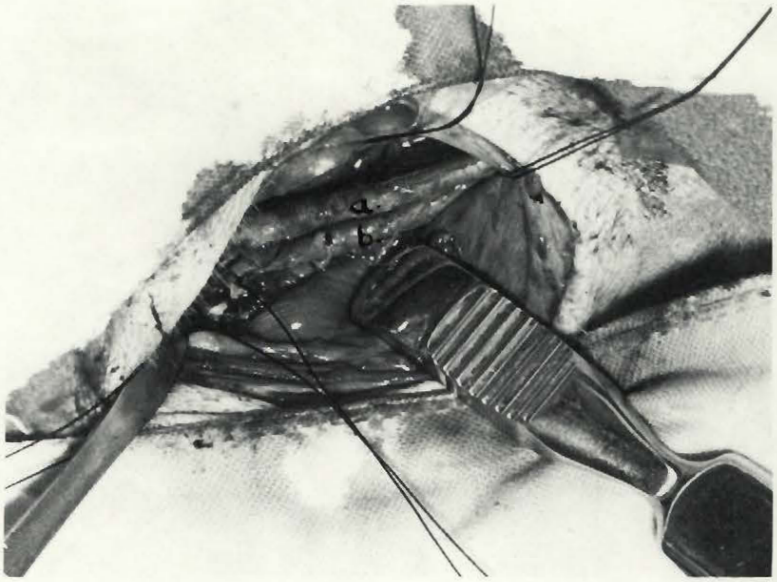

Fig. 7. Anterior triangle of the neck at higher magnification. The common carotid artery (a) is medial to the internal jugular vein (b).

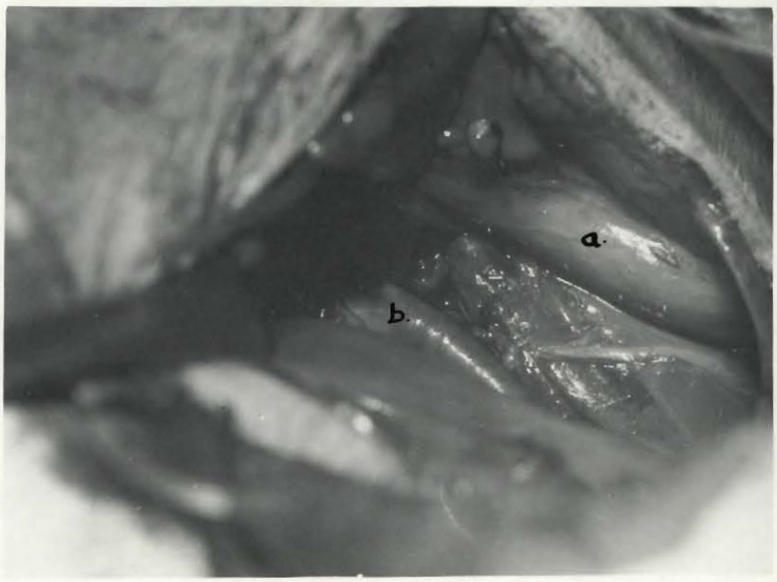

Fig. 8. Operative exposure, with esophagus (a) retracted medially, and carotid artery bifurcation (b) in view.

passed through the tract so created that one end protruded from the temporal incision while the other end came into the neck incision.

Five hundred ml. of 5% glucose and water were suspended from an intravenous pole and connected via an adapter to a 10 foot long piece of continuous plastic tubing, via a short piece of tough plastic tubing which had contact with the finger mechanism of a T11 Sigmamotor pump unit. The longer piece of hose was then connected via an adapter to the temporal part of the P50 polyethylene catheter. The machine was turned on, and the system of tubes which had been previously autoclaved, was allowed to fill with 5% glucose and water free of air bubbles.

All the branches of the right external carotid artery were tied off with 3-0 surgical silk. Next, the external carotid artery itself was tied off 2 cm. above the carotid bifurcation with a 3-0 double ligature. Under electroencephalographic control a bulldog clamp was placed across the common carotid artery 3 cm. below its bifurcation. The time of this manoeuvre was recorded. A similar bulldog clamp was then placed across the right internal carotid artery just above the carotid bifurcation. With this system of the carotids free from blood flow, a tiny transverse incision was made in the right external carotid artery ½ cm. above the bifurcation. The lower end of the P50 polyethylene tube was then threaded retrograde down the external carotid artery, past the bifurcation, and for about 1½ cm. below the carotid bifurcation into the right internal carotid artery. A double 3-0 silk ligature was snugly tied around the tubing and the external

carotid artery (Fig. 9). The two previously applied bulldog clamps were then released and the time noted. The Sigmamotor T11 pump was allowed to pump infusate slowly into the common carotid artery and, consequently, the internal carotid system.

After it was apparent that there was no bleeding in either wound, the divided portions of the platysma were approximated, using 3-0 interrupted through and through sutures. Next, the skin of both incisions was also approximated, using simple through and through 3-0 silk sutures (Fig. 10).

The P50 polyethylene tube was next divided from the larger plastic tubing by uncoupling the adapter, and a syringe, the plunger of which had been wetted with Heparin,was used to draw samples of blood for pH and routine hematological studies. A dry syringe was next used to draw blood from the carotid system for measurement of blood B12 and folic acid. While the animal was still asleep, a bone marrow smear was made from the right iliac crest, or the head of the right femur. A final EEG recording was made.

When moving all extremities, but still slightly drowsy, the monkey was placed in the sitting position in the animal chair, with the polyethylene catheter connected and the machine running. Another bottle containing a suitable dose of Methotrexate, calculated according to the weight of the animal, was then put in the place of the original bottle. The pH of the infusate was measured, since the infusate was either buffered or unbuffered.

(b) Postoperative studies.

1. Routine studies: It is well-known in this laboratory that obtaining blood samples by the intravenous route

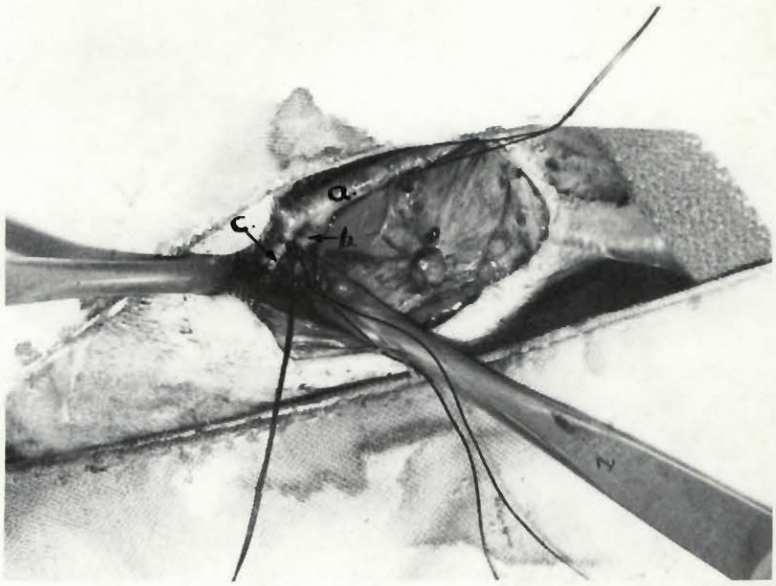

Fig. 9. Right common carotid artery (a), internal carotid artery (b), and catheter (c) tied into external carotid artery.

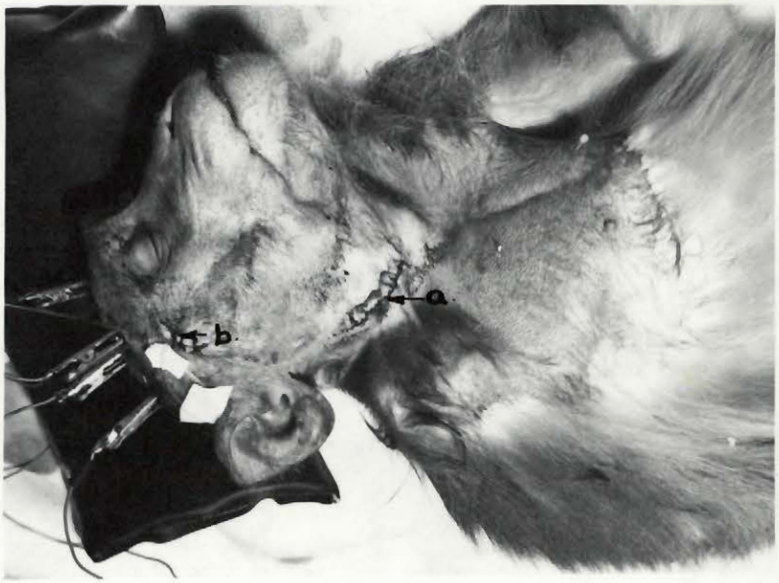

Fig. 10. Animal shortly postoperatively, with neck incision (a) and temporal incision (b) closed.

from monkeys is most difficult. This is due to the thinness of the veins and their collapse when suction is applied. Moreover, the animals are very hostile and would have to be sedated for each blood sample taken by the intravenous route. Since blood was readily available from the catheter implanted in the carotid artery without traumatizing the animals, it was obtained from here after the first animal. Blood was taken every other day for pH measurement on the last 10 animals, and for complete blood counts on all of the animals.

In the first 2 animals, small phonograph needles soldered onto the leads connected to the EEG machine served as electrodes. These proved impractical because even in the chair there was enough freedom of movement to allow the animals to shake them out. In the third animal needle electrodes, previously treated with Epoxylite except at the tips and the heads were positioned in the skin of the scalp in the previously mentioned positions. These, too, were impractical because of the muscle artifact in the recordings, and also because of the inconstant position of the tips of the electrodes. In the rest of the animals the alligator clips of the leads of the EEG machine were simply clamped on to the screws positioned in the animal's head during the operation. EEG's were thus taken every two days when possible, or more often if the status of the animal indicated its necessity (Fig. 11).

The animals were seen frequently, both by day and by night. Their behavior was closely observed and recorded, as well as oral intake and bowel and bladder output. Neurological examinations were done daily.

Fig. 11. Animal in special head-holding chair, with electroencephalographic recording in progress.

2. Autopsies: The infusion of Methotrexate was maintained until the death of the animal. Three of the animals (SVI, SXIV and SXV) were sacrificed by overdoses of intravenous Nembutal. Within minutes after death cardiac puncture yielded blood specimens for study of blood B12, folic acid and Methotrexate. The animals were then removed from the chair and weighed. A general autopsy was begun as soon after death as possible, usually within two hours.

The integument and mucosa of the animals was first observed. Next, a coronal ear to ear incision was made and the site of the electrode drill holes studied for symmetry. The calvarium was removed to reveal the brain. When the falx cerebri had been cut, the extreme frontal poles of the brain were removed, marked, and put in the refrigerator for later assays of Methotrexate content. Having next divided the tentorium cerebelli, the brain was severed from the spinal cord at the level of the medulla by sharp dissection. Another small slice from the frontal poles was then taken for studies of cerebral water content. The remainder of the brain was fixed in 10% formalin, after weighing.

A cruciate incision with its longer portion extending from the suprasternal notch to the suprapubic notch, and horizontal extensions along the intermammary line, exposed the thoracico-abdominal viscera. The great vessels, esophagus and trachea were ligated <u>en masse</u> in the mediastinum, using two ligatures spaced ½ cm. apart and then divided by incising between the ligatures. The posterior parts of the leaves of the diaphragm were divided. The thoracic viscera were removed <u>en bloc</u> and then the various

attachments of the abdominal viscera were divided in succession. Finally the sigmoid colon was doubly ligated and divided between the ligatures. The abdominal contents were also removed en bloc. The heart and lungs were examined in detail, as were the great vessels of the mediastinum, esophagus and trachea. Specimens were kept of the esophagus and lungs. The liver was sliced, and a specimen taken, and similarly for the kidneys. The gastro-intestinal tract was opened longitudinally from esophagus to sigmoid colon, and specimens kept from each of the major portions of gut. Lastly, several of the thoracic vertebrae were taken for a specimen of bone marrow. All specimens were fixed in 10% formalin.

The incision in the neck was opened and inspected for signs of infection and/or hematoma. The carotids were exposed and the position of the catheter in the right common carotid artery was determined. The right carotid system was checked for patency by tying off the common carotid artery below the site of the lower end of the catheter, and injecting water retrograde into the common carotid artery until it appeared in a jet above the intracranial portion of the right internal carotid artery. A similar procedure was followed for the left carotid system. The entire extracranial portion of the right carotid system was kept in 10% formalin, together with control specimens of the left carotid system. The remains of the animal were then cremated.

When the brain had been thoroughly fixed after several days, the brain stem was divided from the cerebral hemispheres. Sections of the brain stem and cerebellum were cut by hand.

Coronal sections of the cerebral hemispheres were made and measured for symmetry. All sections of the brain so made were photographed. One coronal section through the frontal lobes and another coronal section of the cerebral hemispheres at the level of the mid-thalamus, together with a horizontal section of the pons and cerebellum were submitted for histological processing. Slides prepared from each of the specimens were stained by the hematoxylin and eosin, phosphotungstic acid hematoxylin, Bodian, cresyl violet and Haidenhein's methods. A Gram's stain was done if indicated.

Similarly, specimens of the thoracic and abdominal viscera previously saved were processed histologically, and stained by the hematoxylin and eosin method. A specimen of bone marrow was decalcified and stained by the hematoxylin and eosin and Giemsa methods.

3. Studies on cerebral water content: The dry weights of specimens of cortex and white matter were determined by the method of Pappius and Gulati (1963). When the brain was removed, it was transferred <u>in toto</u> to a chamber which was closed except for two holes in the side to allow entry of the experimenter's hands. Air was allowed to bubble through a water filled beaker in the chamber to humidify the air, thereby preventing drying. In this milieu the anterior portions of the frontal poles were sliced off and marked.

The pia was pulled off the tissues of each of the sections so as not to allow the blood contained in the included vessels to interfere with the determinations. Using sharp

scissors, tiny slices of gray matter were removed from each of the specimens in duplicate. The specimens varied in weight from 50 to 100 mg. Similar slices were taken off the white matter of each of the specimens in duplicate. These were also carefully weighed.

Each of the specimens was put into pre-weighed glass beakers. The beakers were then maintained at 100° centigrade overnight. The beakers and contents were then weighed and, by subtraction, the water content of each of the specimens was easily determined.

4. Determination of brain and blood Methotrexate content: To make these determinations the folate assay method of Baker et al.(1959), and Herbert et al.(1961), as modified by Cooper and Lowenstein (1961) was used.

Lactobacillus casei were grown in the presence of a known quantity of folic acid and different concentrations of Methotrexate-containing material to be assayed. The quantity of folic acid per unit of material which overcame the growth inhibiting effect of the Methotrexate was determined and recorded as representing the quantity of Methotrexate within the sample.

The growth medium for the L. casei was BBL - folic acid assay - pteroylglutamic acid medium.

A standard curve was prepared by plotting the optical density produced by the growth of an inoculum of organisms added to the medium, versus the amount of pteroylglutamic acid required to produce this growth.

The marked brain samples from each side of the brain were

weighed. In some of the animals (SIII, SIV and SV) the brain samples were homogenized, and 5 ml. of 0.05 M phosphate buffer, containing 150 mg. % of ascorbic acid were added to the homogenized brain. For the other animals, 1 ml. of a solution containing 25 mg. of ascorbic acid was added to the homogenized brain sample.

The brain suspensions were then diluted 1 to 5 with water. This suspension, in turn, was diluted 1 to 10 with 0.05 M phosphate buffer at pH 6.1, containing 150 mg. of ascorbic acid. The brain buffer solutions were incubated at 37° centigrade for 90 minutes, and then autoclaved for 10 minutes at a pressure of 16 pounds per square inch. The coagulated proteins were centrifuged off and the clear supernatant saved. This supernatant was then diluted with water to concentrations of 1/10, 1/100, 1/1000 etc. One ml. of a solution containing 10 mug. of folic acid was added per tube. To this was added 1 ml. of the diluted supernatant, 3 ml. of water, and 5 ml. of the growth medium.

The tubes were autoclaved for 30 minutes at 118° centigrade at a pressure of 16 pounds per square inch, and then cooled to room temperature. A 6-hour culture of L. casei was diluted 1/10 and a drop added to each tube. The tubes were again incubated for 18 hours at 37° centigrade.

The growth density of the L. casei was then measured using a colorimeter at a wave length of 625 millimicrons. From the standard curve, previously plotted, the amount of folic acid required for this growth of the organism could be determined.

Since a known amount of folic acid had been added to each assay specimen, by subtraction, the amount of folic acid inhibited was obtained. Thus the Methotrexate concentration of the sample was expressed as "the amount of folic acid in ug. inhibited per gm. of brain."

The blood samples were processed in a similar fashion but the dilution factors were different. Each ml. of blood was diluted 1/5 with water in order to hemolyze the cells. This in turn was diluted 1/10 in the phosphate buffer as described above. The Methotrexate content was expressed as "ug. folic acid inhibited per ml. of whole blood."

C. Results

The animals are numbered chronologically in the order in which they were done, and designated SI (Simian one) SII (Simian two), and so forth. The operative day is day 0, whereas the postoperative days are referred to as day 1, day 2, etc., in order.

a. General behavior: During the first few days of the experiment the animals tended to be very hostile. Depending on the dosage of drug administered they became increasingly lethargic and apathetic after day 4 to day 6. When in this state they tolerated having their heads petted, and their mouths washed, although at times they still tried to bite when approached.

Anorexia was a major problem in the infused animals; those on pH controlled infusions ate for an average of 3.75 days, whereas animals in which the infusate pH was not controlled, ate

for an average of only 1.5 days. One control animal (SXV) receiving an infusate composed of 5% glucose and water buffered with Tham, ate four bananas per day for ten days.

Severe diarrhea began day 4 to day 6 depending on the drug dosage. The animals lost from 2 to 12% of their body weight during the experiments. However, the control animals also lost an average of 7% of their body weight during their infusion periods. The control animals were sacrificed at seven and ten days. The average survival times from the beginning of infusion until death in the drug-infused animals with high, intermediate and low doses were respectively 5.6, 6.3, and 7.3 days.

b. Neurological signs: All of the animals had slight tongue protrusion to the right as a consequence of cutting the right hypoglossal nerve during the operative procedure. Three animals had a right Horner's syndrome; four had varying degrees of right facial paresis, some of which cleared while others persisted; two animals had a combined right Horner's syndrome and facial paresis. All of the above complications were considered to be the result of surgical manipulation in the neck.

SI developed a left homonymous hemianopsia and a left hemiparesis five hours before death, but at autopsy was found to have some right cerebral infarcts. SII and SIII developed right cerebral seizures resistant to anticonvulsant medications. Both of these animals also had right cerebral infarcts, and SIII had meningitis. SIV and SX each had one short generalized

seizure with no visible permanent sequelae after the accidental entry of a small air bubble into the right carotid system. SVI had epilepsy partialis continuans on day 2, which was thought clinically to be due to meningitis since it cleared after Penicillin administration. SVII and SXI had no neurological sign of any kind.

Control animal SXIV had both a right Horner's syndrome and a right peripheral type facial paresis. On day 1 he developed a right sixth nerve paresis, and later a complete right ophthalmoplegia, though the pupil would still react to light. Control SXV had a slight right peripheral type facial paresis which cleared on day 1, and he was then neurologically sound to the end of the experiment.

c. Electroencephalographic studies: EEG's were taken preoperatively, during the operative procedure, and postoperatively.

In the preoperative tracings bilateral alternate slow and fast activity with spindles, characteristic of Nembutal anesthesia, were seen. In some of the animals (SII, SIV, SVI, SX, SXI, and SXII) there were occasional slow waves over the right hemisphere prior to surgery on the neck, but after electrode placement. In four of these animals the following was noted at autopsy; SII had a small left parietal intracerebral hematoma; SV had a 1 ml. epidural collection of blood over the right hemisphere; SX had a small 0.5 ml. film of epidural blood on the right side, and SXI had a 1 ml. collection of subdural blood over the right hemisphere. Thus, in animals SV, SX and SXI traumatic electrode

placement may have accounted for these slow waves. The reason for the slow waves seen on the right in the remaining animals prior to surgery is unexplained. These slow waves over the right hemisphere cleared postoperatively in SIV, SX and SXI.

Clamping of the right carotid system during the operative procedure accentuated or caused right cerebral slow waves in SVII, SVIII, SX and SV. This was particularly evident in SVIII whose EEG tracing was normal until the carotid arteries on the right went into spasm during the operation. This caused an immediate drop of electrical amplitude over the right cerebral hemisphere and the appearance of 2 to 3 per second slow waves. These slow waves in SVIII and SX disappeared postoperatively. In SVII and SV they remained throughout. However, SV had slow waves on the right prior to surgery.

Clinical seizures were seen in animals SII, SIII, SIV, and SX. SII had some epileptiform abnormalities over the right hemisphere before surgery. On day 2 an air bubble accidentally went into his right carotid system and he immediately had a generalized seizure which, clinically, was right cerebral. This was followed by numerous other seizures. Electroencephalographically, these began as spikes, sharp waves, and multiple spike and wave complexes over the right hemisphere, becoming an electroencephalographic seizure, with spread to the left hemisphere. Following this seizure, there was voltage suppression on the right and slow waves on the left. Similar tracings followed during repeated seizures.

SIII had a normal EEG on day 0. Later on day 0, while

unblocking his catheter assembly by injection of water from a syringe through the catheter, the animal suddenly had a generalized seizure. On day 1 the animal had numerous repetitive seizures which, clinically, came from the left hemisphere. On the EEG they started in the left hemisphere with slow waves, sharp waves and multiple spikes leading to an electrographic seizure on the left side, with gradual spread to the right hemisphere. There was marked postictal depression of electrical activity on the left and, later, slow waves appeared over both hemispheres. This animal was later found to have meningitis, and both right and left cerebral infarcts.

SIV had a small seizure on day 1 after accidental injection of an air bubble into the right carotid system. In a recording taken shortly afterwards no epileptiform abnormality was noted.

SVI had slow waves over the right hemisphere prior to neck surgery. Later he developed epilepsy partialis continuans which soon cleared after Penicillin. The seizures were clinically right cerebral although no epileptiform abnormality was seen in the EEG during the seizures.

SX also had right cerebral slow waves prior to surgery. An air bubble going into the right carotid system after obtaining a blood sample precipitated a generalized but unlateralized seizure. The EEG taken one hour after the seizure showed slow wave activity over the right cerebral hemisphere with decreased amplitude. The same day the EEG gradually reverted to normal, bilaterally.

Animals SI, SVII, SVIII, SIX, SXI, SXII and SXIII receiving variable doses of Methotrexate had neither seizures nor epileptiform abnormalities in the EEG. Animal SI who had right cerebral infarcts, had a normal tracing until day 5, when slow waves began to appear over the right hemisphere in the EEG recording.

Most of the animals developed bilateral slow waves near death, and these were usually accentuated over the right hemisphere.

Control animal SXIV had a completely normal EEG throughout. Control animal SXV had occasional slow waves over the right hemisphere prior to surgery. On days 5, 6, and 7, large air bubbles were purposefully injected into the right carotid system of SXV to study the effects. In each instance the animal would flush, gasp, and stare. There was an immediate left hemiparesis which cleared after a few minutes leaving no neurological sequelae. In the EEG tracing one or two seconds after air injection, there was a suppression of the amplitude of electrical activity over the right hemisphere, together with the appearance of slow waves. This cleared gradually to normal, bilaterally, in about an hour in each instance. On day 9 this animal again had a completely normal tracing.

d. <u>The gastrointestinal tract</u>: In the experimental animals the gastrointestinal tract was constantly severely damaged. Various parts of the system were affected in different degrees, the damage becoming greater the more distal the portion of the gut studied.

The gross changes were not too striking. In the esophagus of animals on high doses of drug there was a thinning of the wall. The gastrointestinal organs of the infused animals were the same yellow color as the infusate administered. This coloring was not seen in the control animals. Gross changes attributable to the drug were not seen in the stomach. In the duodenum of those animals on large and intermediate drug doses there was also mucosal thinning.

In the jejunum few gross changes were seen, apart from an apparent thinness of the mucosa. In three of the animals (SVII, SVIII and SXII) some dark mucosal hemorrhages and petechiae were noted, together with gross ulcerations of the mucosa (SVIII).

In the ileum, again the most common change was an apparent thinning of the wall. Five animals (SVII, SVIII, SXI, SXII and SXIII), some on large doses and some on small doses of the drug, had either injection of the mucosa, petechial mucosal hemorrhages, or generalized injection of the mucosa.

The most severe changes were seen in the colon. An apparent thinness of the wall was noted on palpation. Often some pink injection of the mucosa was seen. In SV the entire mucosa was grossly hemorrhagic, with bluish discoloration and areas of ulceration. In SVII the mucosa was completely denuded, with large hemorrhagic patches in the wall. There were mucosal petechial injections, and clots of blood in the colons of some of the other animals (SX, SXI, SXII and SXIII).

Histologically, the ravages of the drug in the

gastrointestinal system are much more striking. In the esophagus of those animals on high doses there was partial to complete denudation of the epithelium, with one or two layers of cells remaining in some areas (Fig. 13), and often with clumps of bacteria growing in the overlying necrotic debris. The remaining basal cells of the esophageal epithelium were bizarre in appearance, with huge, pale nuclei, nuclear pleomorphism, prominent nucleoli and eosinophilic cytoplasm. Histologically these cells appeared to be very active metabolically, though this was not proven.

In the stomach the changes were minimal, with no apparent loss of epithelial cells (Fig. 14). However, in seven of the animals on varying doses of the drug, there was pleomorphism of the basal epithelial cells (Fig. 15). Again, these cells had large hyperchromatic nuclei and prominent nucleoli. There were increased numbers of plasma cells and lymphocytes in the lamina propria in some animals. In some areas there was distention of the gastric glands.

In the jejunum, in many cases, there was striking denudation of the superficial mucosal epithelium (Fig. 17), with epithelial cells remaining only at the bases of the crypts of Lieberkühn. These cells were also bizarre, with large pleomorphic hyperchromatic nuclei containing one or more prominent nucleoli (Fig. 20), and eosinophilic cytoplasm. The lamina propria remained, but in many animals it was diffusely infiltrated with lymphocytes, plasma cells and, occasionally, eosinophiles. The eosinophiles were found in animals with parasitic infestations.

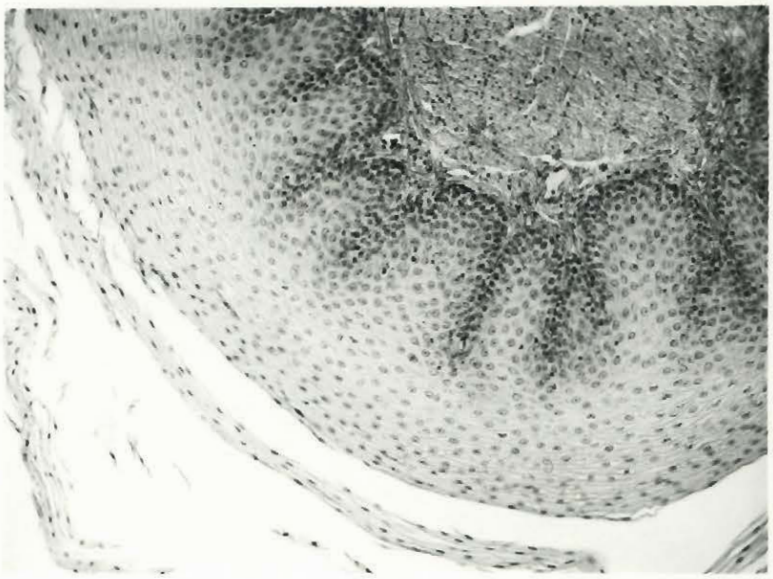

Fig. 12. Wall of normal esophagus, showing thick mucosa. Animal SXV. Hematoxylin and eosin stain, X110.

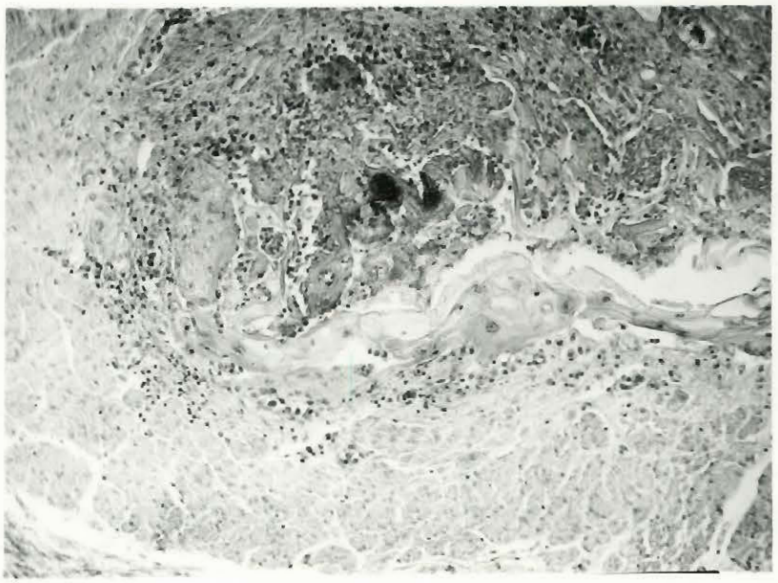

Fig. 13. Transverse section of esophageal wall of drug infused animal. Few epithelial cells remain (center) and bacterial colonies are present. Animal SVII. Hematoxylin and eosin stain, X110.

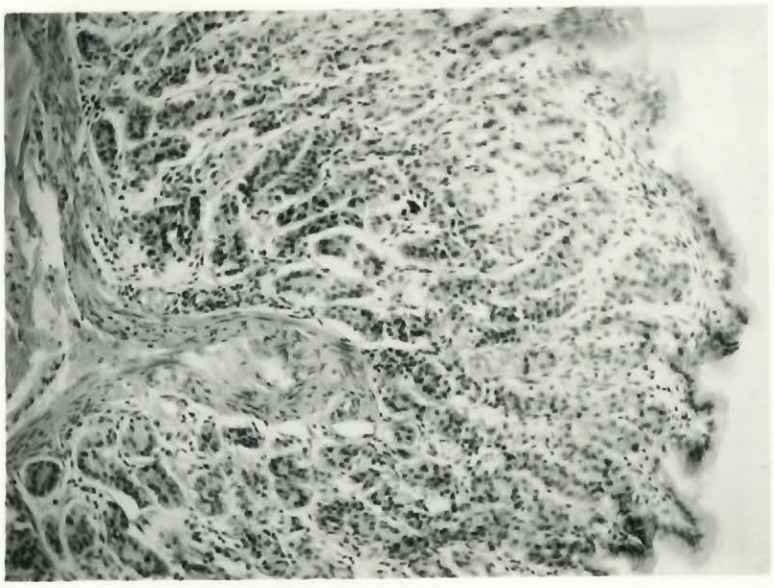

Fig. 14. Section of wall of stomach of drug-infused animal. No appreciable cell loss is noted. Animal SIV. Hematoxylin and eosin, X110.

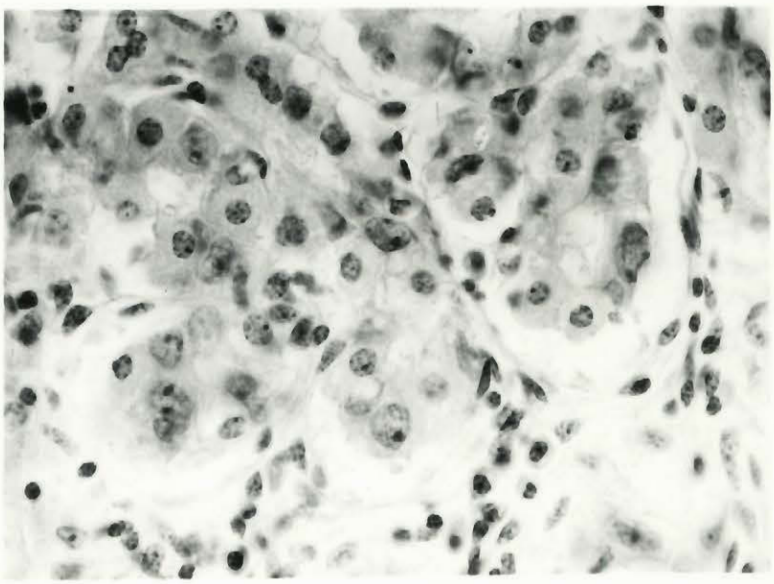

Fig.15. Basal epithelial cells of the gastric mucosa in same animal, showing pleomorphic nuclei, hyperchromatism, and prominent nucleoli. Animal SIV. Hematoxylin and eosin stain,X500.

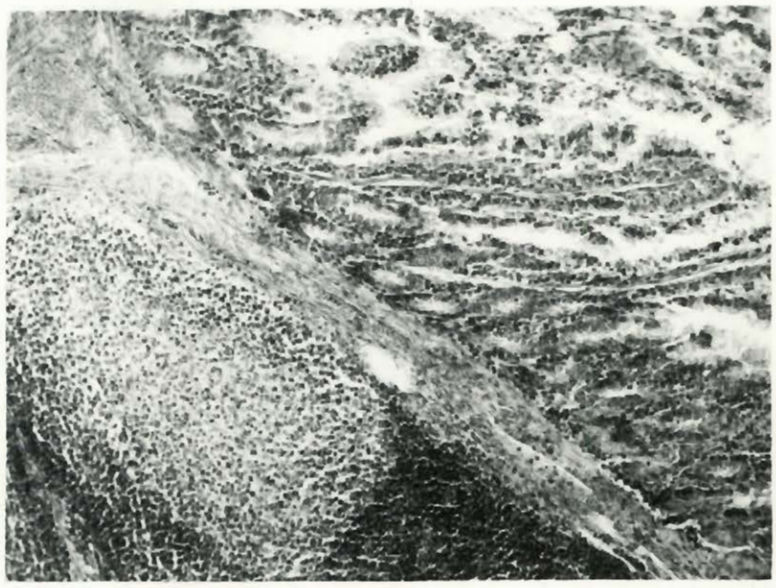

Fig.16. Section of normal jejunum, showing prominent Peyer's patch (left) and abundant epithelial cells (right). Animal SXIV. Hematoxylin and eosin stain,X110.

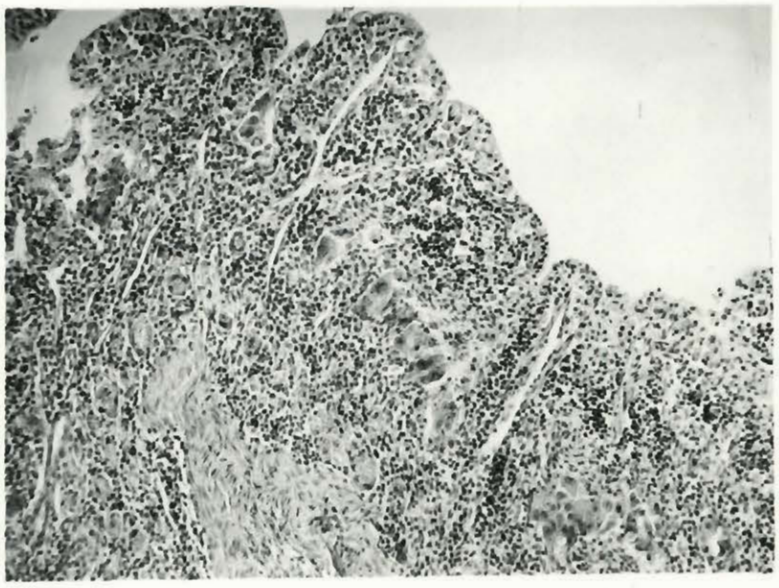

Fig.17. Section of jejunum of drug-infused animal showing adherent villi and absence of epithelial cells except at base of lamina propria. Note inflammatory cell infiltrate. Animal SVII. Hematoxylin and eosin stain,X 110.

The villi were flattened with adherence at the tips. No apparent difference was seen in the above changes regardless of the drug dosage. However, in the animals on the high and intermediate doses, there was striking ablation of the Peyer's patches, so that in many instances no germinal centers remained, small aggregations of mature lymphocytes replacing the lymphoid follicles. In animals on small doses of the drug, prominent germinal centers were still present in the lymphoid follicles.

In the ileum the changes were similar to those described for the jejunum (Figs. 19 and 21) but, in addition, in some areas there was proliferation and stratification of goblet cells. In SVII there was a pseudomembrane covering the remains of the epithelium; it was composed of necrotic tissues admixed with colonies of bacteria. There was marked underlying submucosal edema in this instance.

The most severe changes were seen in the colon. In many of the animals there was complete denudation of the mucosal epithelium (Fig. 23), while in other animals a few abnormal basal cells remained in the glands of Lieberkühn. These cells again had the bizarre qualities previously described (Fig. 24). In some of the animals (SV and SVII) there was complete hemorrhagic necrosis of the mucosa, including the lamina propria and portions of the muscularis mucosae in SV. Animals SIV, SV, SVII, SX and SXIII had pseudomembranes of necrotic debris, with colonies of bacteria overlying the lamina propria. In almost all instances there were lymphocytes, plasma cells, sometimes eosinophiles, and rarely polymorphonuclear leucocytes in the lamina propria.

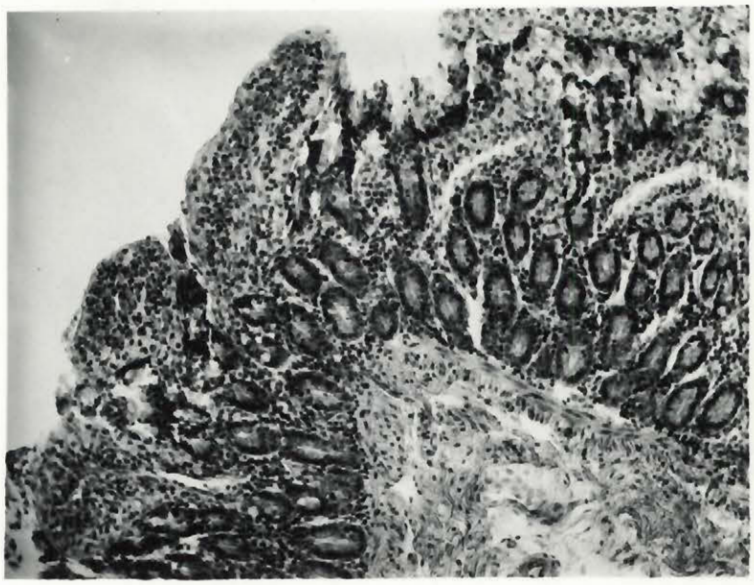

Fig.18. Section of normal ileum showing abundant epithelial cells. Animal SXV. Hematoxylin and eosin stain, X110.

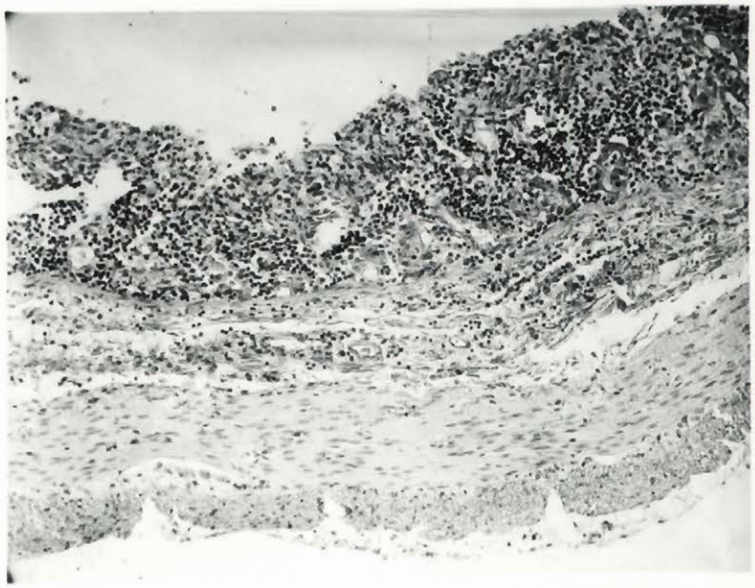

Fig.19. Section of ileum from drug-infused animal showing thin mucosa, with few remaining epithelial cells. Animal SI. Hematoxylin and eosin stain, X110.

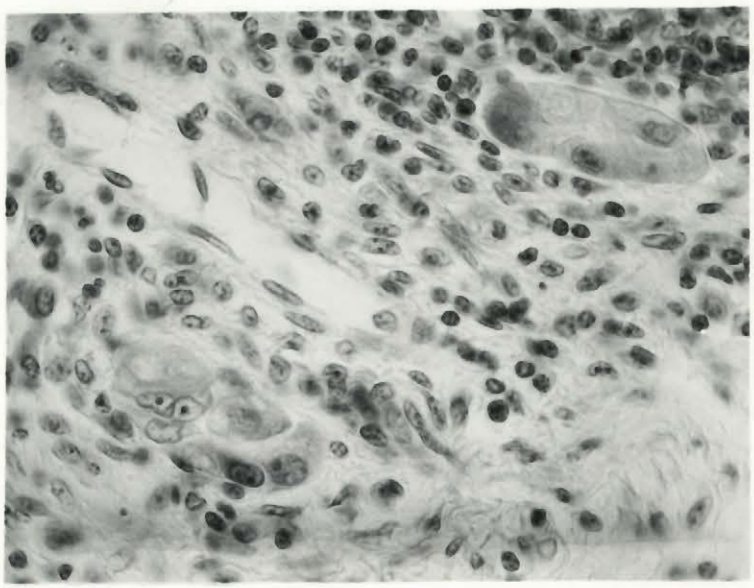

Fig.20. Bases of two remaining jejunal crypts of Lieberkühn in drug-infused animal. Surviving epithelial cells are bizarre, with hyperchromatism, pleomorphism of nuclei and prominent nucleoli. Animal SX. Hematoxylin and eosin stain, X 500

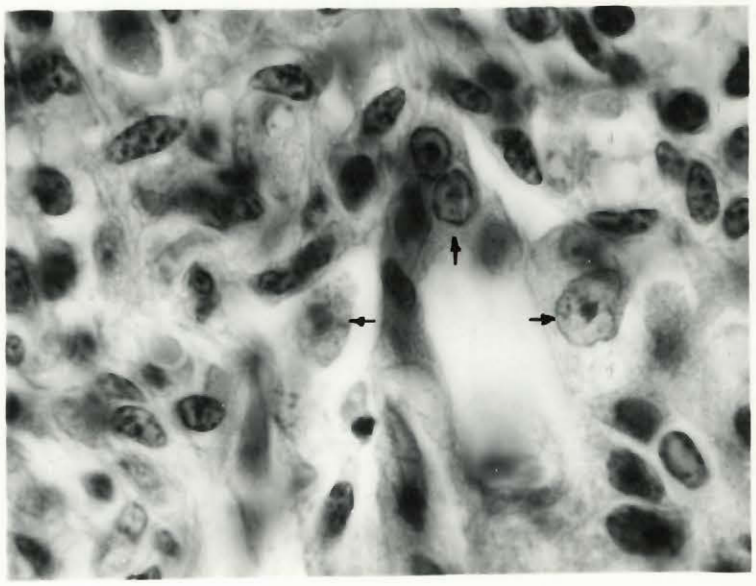

Fig.21. Altered remaining cells (arrows) in ileum of drug-infused animal. Animal SIV. Hematoxylin and eosin stain, X 1000

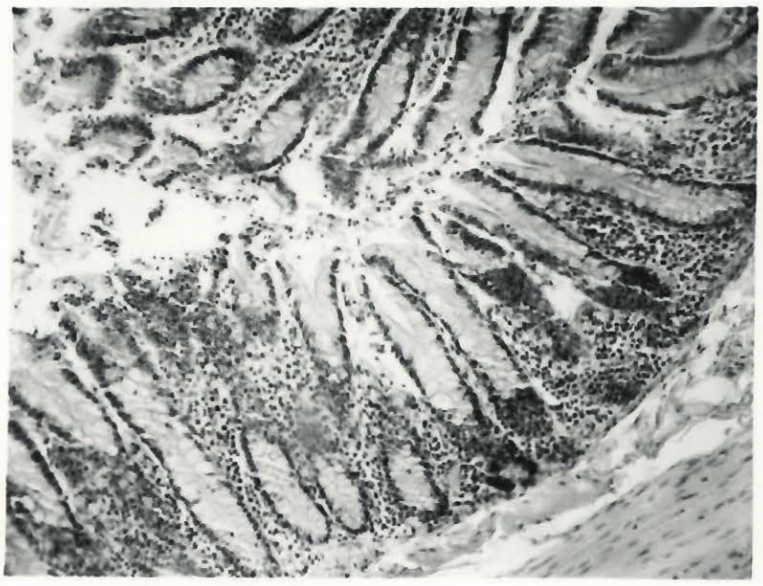

Fig.22. Wall of normal colon showing prominent epithelium. Animal SXV. Hematoxylin and eosin stain,X110.

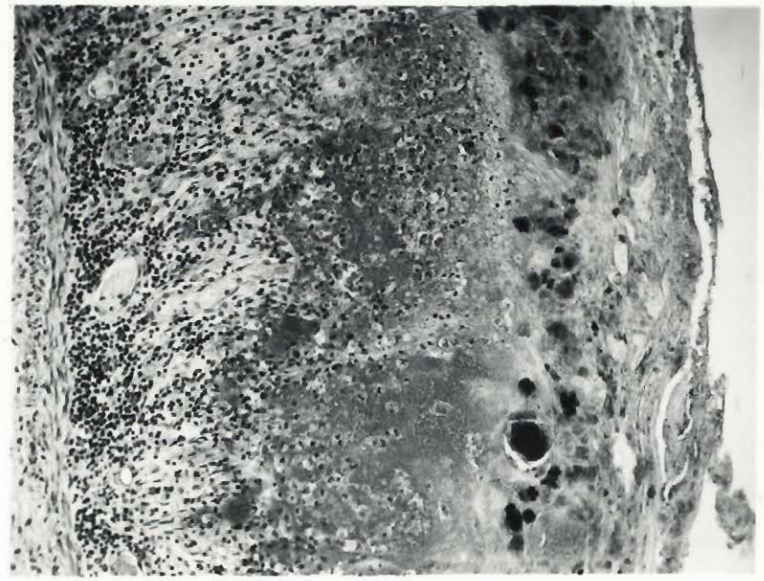

Fig.23. Wall of colon in drug-infused animal, showing hemorrhagic necrosis. Note superficial bacterial colonies and absence of epithelial cells. Animal SIV. Hematoxylin and eosin stain X,110.

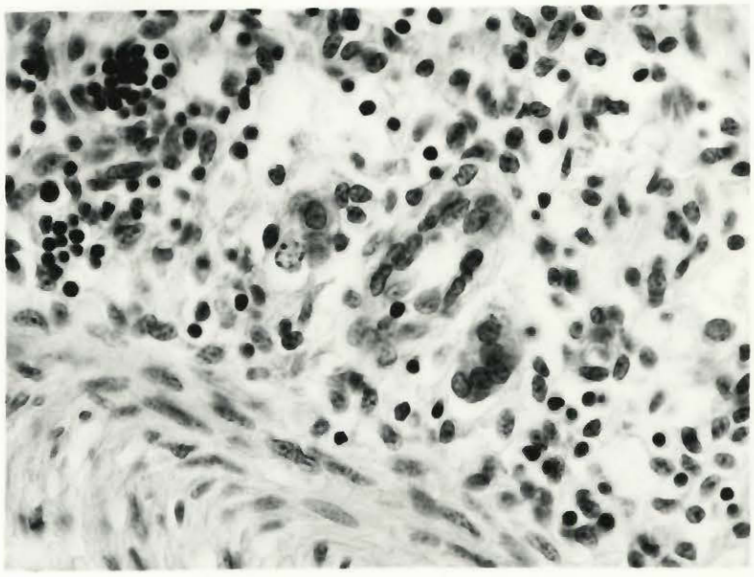

Fig.24. Section of colon from drug-infused animal. Three surviving groups of altered basal epithelial cells remain (center). Animal SIX. Hematoxylin and eosin stain X 500.

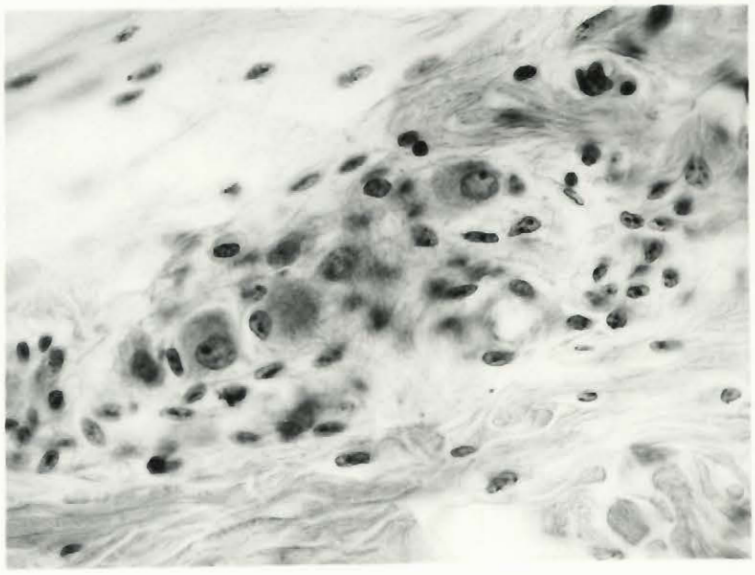

Fig.25. Aggregate of histologically normal neurones (center) in myenteric plexus of drug-infused animal. The nearby colonic mucosa was severely damaged (see Fig.23). Animal SIV. Hematoxylin and eosin stain, X 500.

There was sometimes submucosal edema underlying severely damaged regions of gut.

Throughout the gastrointestinal tract the damage was nearly always limited to the mucosa. The muscularis externa and serosa showed no effects attributable to the drug. The neurones of the myenteric plexus of the gut were normal, histologically, even in the most severely damaged, hemorrhagic, edematous portions of colon (Fig. 25).

The gastrointestinal tracts of the control animals (SXIV and SXV), and animal SVI, were histologically normal (Figs. 12, 16, 18, and 22).

e. Other organs: The kidneys of the animals were also studied, and minimal pathology of doubtful significance was seen here.

In three of the animals there was moderate passive congestion of the venous sinusoids of the liver. In one of the animals (SXIV) the changes were considered to be of long standing. In almost all of the animals there was mild fatty infiltration of the liver, compatible with the low dietary intake of the animals prior to death.

In animal SXII there was some cloudy swelling of the renal convoluted tubules. In animals SIII and SVII focal lymphocytic infiltration of the renal pelves was seen.

No ulcerations of the skin or oral mucosa were noted. It is of interest that, depending on the dosage of the drug, a zone of periorbital erythema appeared around the right eye during the infusion period, together with increased lacrimation of this eye.

f. The blood vessels: The peripheral blood vessels of all of the animals appeared grossly normal. There were some histological changes noted, however.

In SVII and SIX there was moderate sclerosis and perivascular fibrosis of the mesenteric blood vessels. In SXV there was marked sclerosis and fibrinoid necrosis of the vessels of the submucosa of the gut, together with lymphocytic and plasma cell infiltration. However, all three of these animals had marked nematode parasitic infestations of the mesentery.

When the catheter remained in the common carotid artery at death, in each instance it was found patent by injection of water through the catheter. In some instances considerable pressure had to be exerted on the syringe in testing for patency. In spite of this, there was gross recent ante-mortem thrombus in the common carotid arteries of SXIII and SXIV.

Histological sections of the carotid vessels of twelve animals were studied. SII and SIX had completely occluded internal carotid arteries. The thrombi, histologically, were very recent, composed of red cells and platelets. SX, SXI, SXII and SXIII had crescentric soft fibrin thrombi in the common carotid arteries around the site of placement of the catheter. Control animal SXIV had a solid recent ante-mortem thrombus of both the right internal and external carotid arteries (Fig. 26). There was also a soft fibrin thrombus around the end of the catheter in the common carotid artery of control animal SXV. SVI had a typical large atheromatous plaque of the left common carotid artery, just below the bifurcation.

Fig.26. Recent ante-mortem thrombus in right internal carotid artery of control animal. Animal SXIV. Hematoxylin and eosin stain, X 35.

In animals SX and SXIII there were gaps seen in the intima of the right common and internal carotid arteries. The fibroblasts of the media of the vessel wall peripheral to these gaps had large pale vesicular nuclei with prominent nucleoli.

The vessels of the brain, both major and minor, even in the animals who had brain infarcts, all appeared histologically normal. It is of interest that in the rhesus monkey there was seen to be a single pericallosal artery.

g. The lymphatic system: The lymphatic tissues and peripheral lymphocyte count of the drug-infused animals were altered. There was a decrease in size of the splenic Malpighian corpuscles, together with absence or decrease in the size of the germinal centers (Fig. 28). The white pulp was not prominent, and contained only mature lymphocytes, while the younger lymphocytes seen were very rare. There were large numbers of reticulum cells in the white and red pulp. These were hypertrophic, with large pleomorphic nuclei and prominent nucleoli (Fig. 31). In some of the animals on the high doses, there appeared to be a decrease in the number of reticulum cells. In the animals on low drug doses, ablation of the germinal centers and white pulp in general was not as striking as that seen in animals on the higher doses of Methotrexate.

The normal architecture of the lymph nodes in the drug infused animals was largely ablated (Fig. 29). The germinal centers in most instances were absent and, instead, decreased numbers of mature lymphocytes remained, together with the previously described hypertrophied reticulum cells (Fig. 32). In some lymph

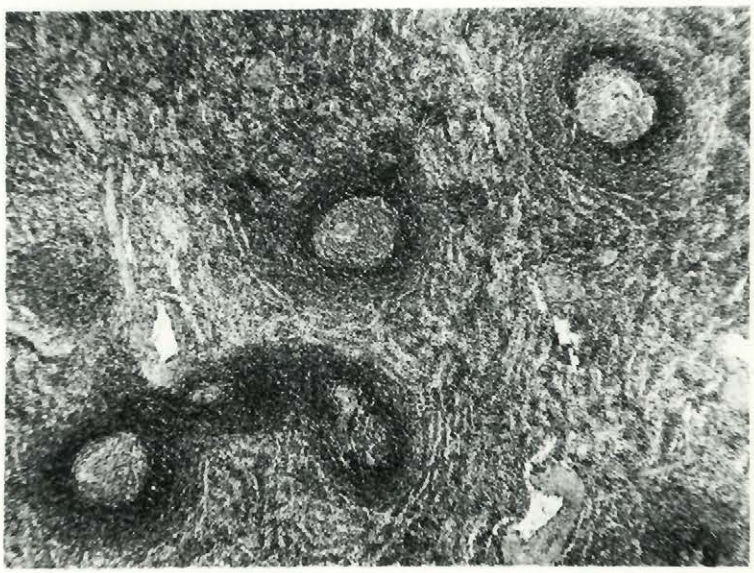

Fig.27. Section of normal spleen from control animal. Prominent germinal centers are seen in the Malpighian corpuscles. Animal SXV. Hematoxylin and eosin stain, X 35.

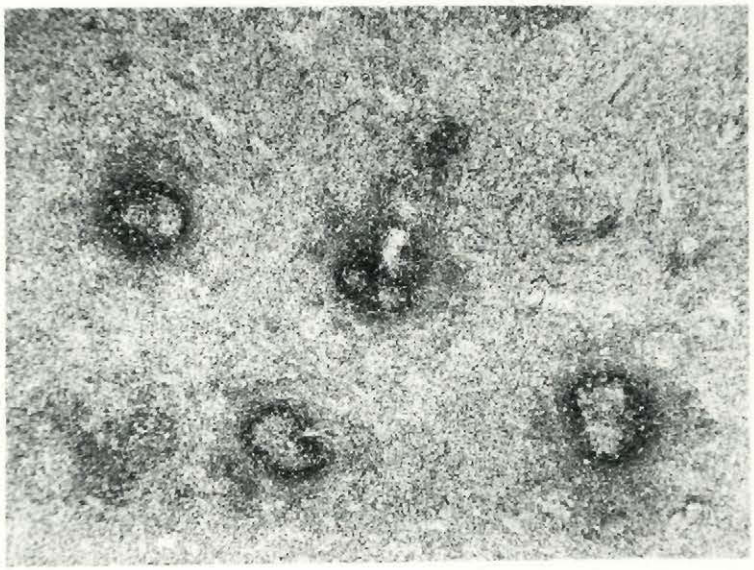

Fig.28. Section of spleen of drug-infused animal. Note the atrophy of the Malpighian corpuscles and decreased size of germinal centers. Animal SIV. Hematoxylin and eosin stain, X 35.

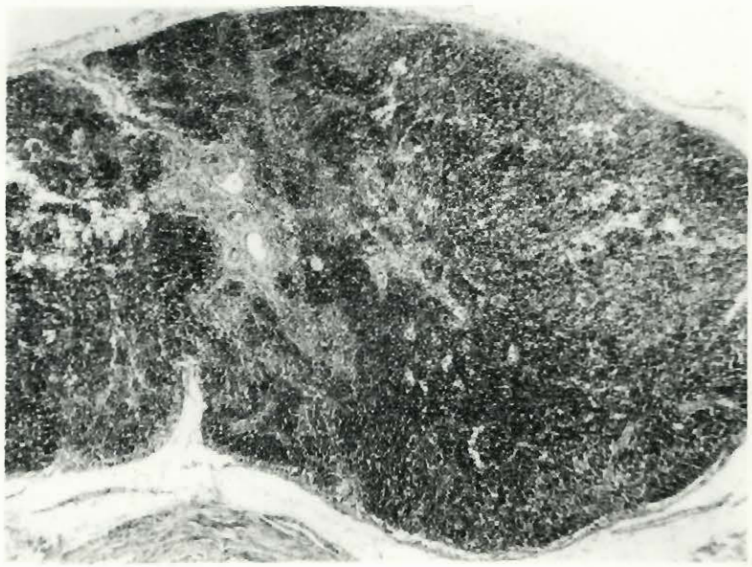

Fig.29. Mesenteric lymph node of drug-infused animal. There is hypoplasia of lymphoid tissue and absence of germinal centers. Animal SVIII. Hematoxylin and eosin stain, X 35.

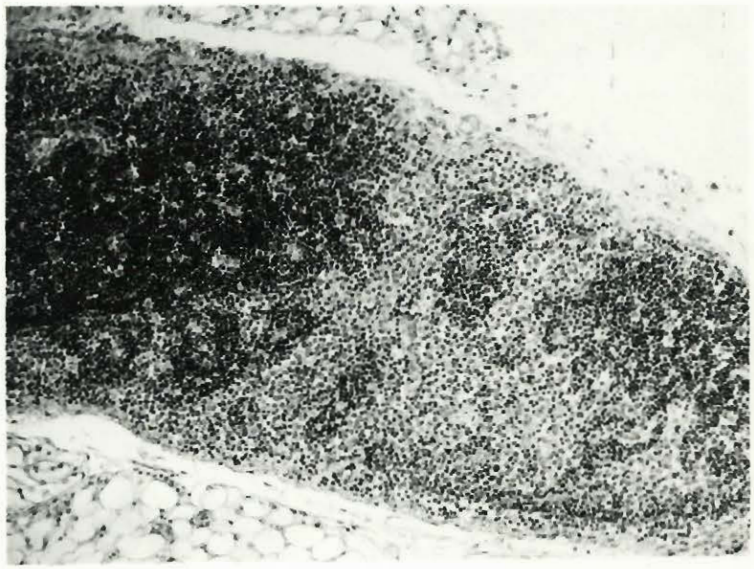

Fig.30. Abdominal lymph node with disrupted architecture and absent germinal centers, from drug-infused animal. Sheets of foamy histiocytes are seen centrally. Animal SVII. Hematoxylin and eosin stain, X 110.

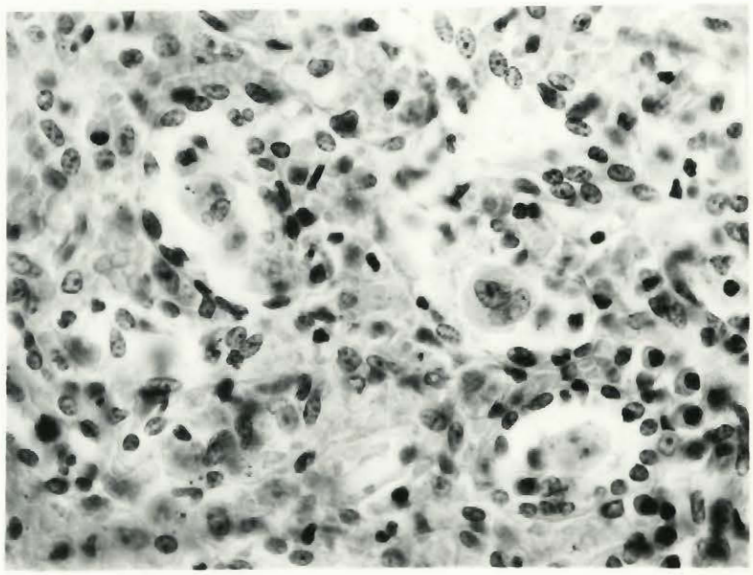

Fig.31. Red pulp of spleen in drug-infused animal showing large reticulum cells, some multinucleate. Animal SIV. Hematoxylin and eosin stain, X 500.

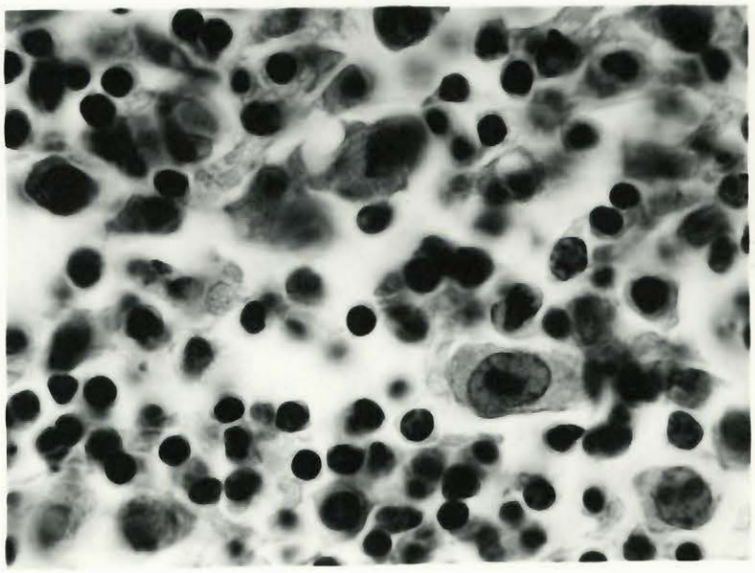

Fig.32. Hypertrophic reticulum cells in mesenteric lymph node of drug-infused animal. Only mature lymphocytes remain. Animal SVIII. Hematoxylin and eosin, X 1000.

nodes so affected, there were numerous foamy histiocytes in the sinuses (Fig. 30).

Atrophy of the lymphoid Peyer's patches of the gastro-intestinal tract were noted in the animals on large and intermediate drug doses. Again, the remaining lymphocytes were mature, while immature forms were very rare. The changes in the lymphoid tissues of the gut were milder in the animals on the low doses of the drug.

Nowhere was there any evidence of extramedullary myelopoiesis.

In the control animals (Fig. 27), and in animal SVI (which lived eight months after the test) the lymphatic systems were histologically normal.

The changes above were reflected in the peripheral blood by an absolute decrease in circulating lymphocytes, but a relative lymphocytosis, while in the control animals there were absolute increases of the circulating lymphocytes during the infusion period.

h. <u>The hematological system</u>: For reasons previously given (see Methods) blood samples were usually taken via the carotid catheter. In many of the animals, after a couple of days it was possible to inject substances into the carotid via the catheter, but blood did not reflux when the assembly was uncoupled. This may have been due to the formation of a one-way, valve-like endothelial flap (Sullivan, 1962a) around the catheter tip, or due to the formation of soft clot around the end of the catheter (See The blood vessels). Blood samples thus

were few, and were not necessarily taken on the same day for each group of animals. At death the last sample was obtained by cardiac puncture, when possible.

In the graphical representations of the blood counts, some of the points shown were single values (.), while others were averages of more than one animal (x). The various components of the blood count of the animals varied widely at the outset of the experiment. In order to graph the components, the preoperative values were taken as 100% for each animal, while the values on ensuing days were calculated as a percentage of this preoperative baseline value.

Inadequate blood samples were drawn from SXIV, which was not considered a good control, and was not represented in these graphs. Instead, animals SXV (a good control) and SVI (which received almost no drug and survived the experiment) were used as controls.

In all animals, including the controls, there was a drop in the hemoglobin values with time (Fig. 33). Little difference was seen between the controls and the animals receiving small drug doses. On day 5, which was the last day on which there were readings for all classes of animals, there appeared to be a more rapid drop of hemoglobin in the animals on high and intermediate drug doses than in the control and low dosage groups. The graphs became progressively longer with decreasing drug dosage, since the animals lived longer.

In studying the trends of the white blood count (Fig. 34), it was also seen that the graphs were short for animals on the

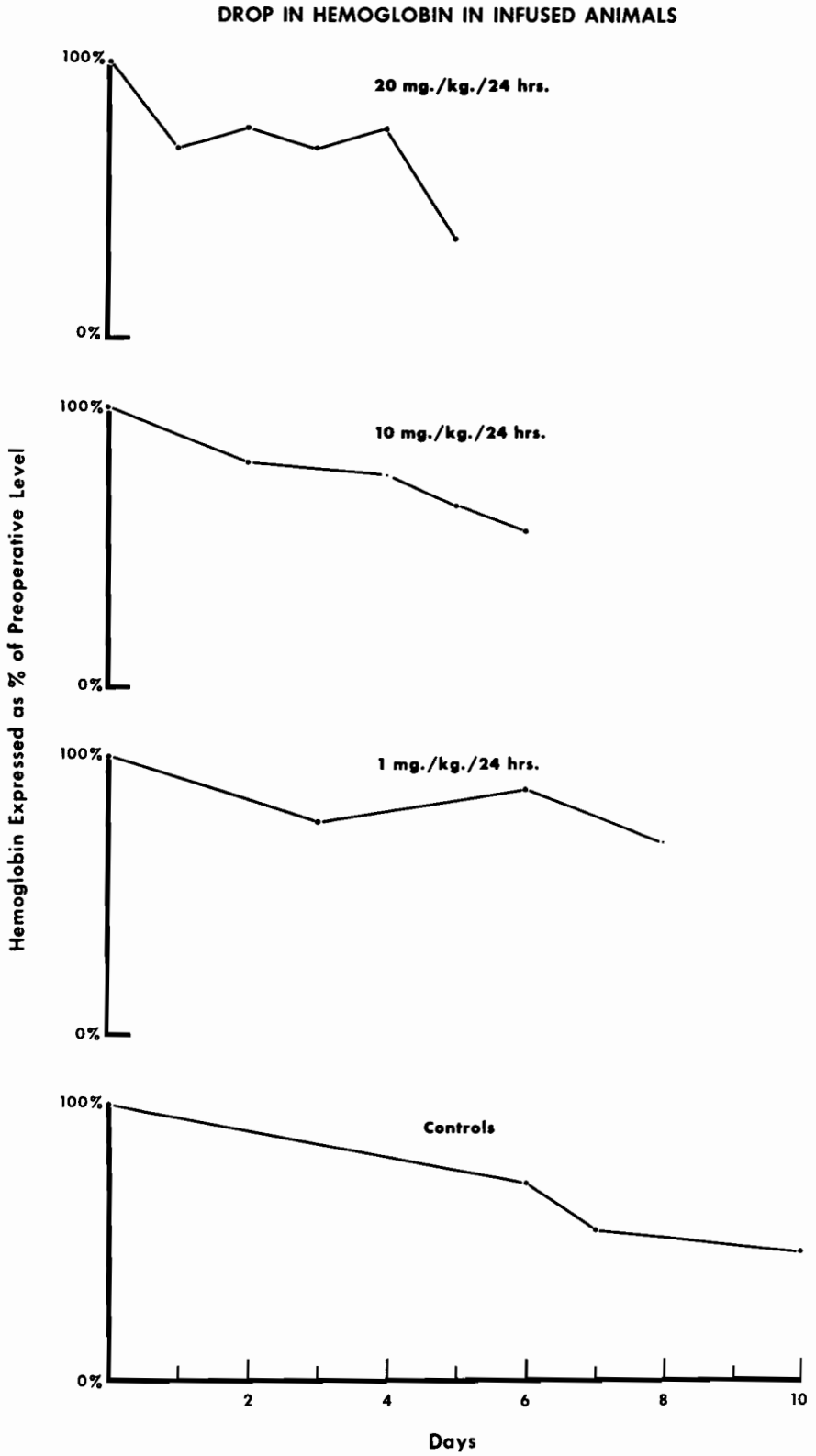
DROP IN HEMOGLOBIN IN INFUSED ANIMALS
100%
20 mg./kg./24 hrs.
0%
100%
10 mg./kg./24 hrs.
0%
Hemoglobin Expressed as % of Preoperative Level
100%
1 mg./kg./24 hrs.
0%
100%
Controls
0%
2
4
6
8
10
Days

Fig. 33.

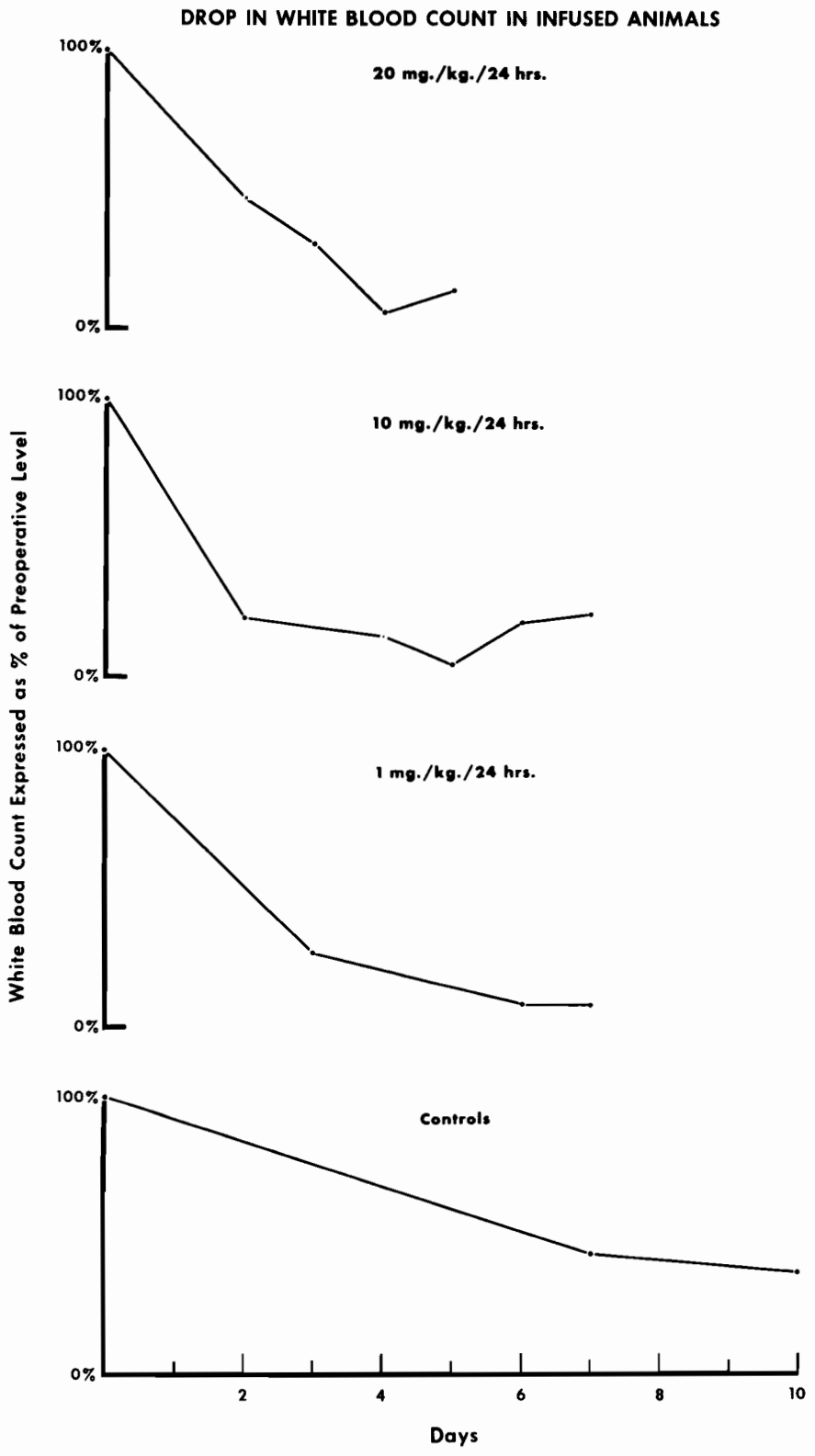
DROP IN WHITE BLOOD COUNT IN INFUSED ANIMALS
White Blood Count Expressed as % of Preoperative Level
100%
0%
20 mg./kg./24 hrs.
10 mg./kg./24 hrs.
1 mg./kg./24 hrs.
Controls
2
4
6
8
10
Days

Fig. 34.

high and intermediate drug-dose infusates, due to early death. In all of the experimental animals there was a steep drop of white blood cell counts during the first two to three days, after which the decline was slower. In the control animals there was a lesser decrease in the white blood count. Even on day 10 the white cell count in the control group did not reach the low level attained by the drug-infused animals on day 3.

The most striking effect of the drug on the peripheral blood was seen in connection with the platelets (Fig. 35). A constant drop in the platelet count occurred in all of the experimental animals, with little difference between the three drug dosages. This decline varied from about 35% to about 57% by day 4. In the control animals the peripheral platelet count rose to about 12% above the preoperative level during the same time interval.

In the drug-infused animals, there was a large absolute drop in peripheral leucocyte counts during the experiment. By day 3, a marked reduction of the circulating band polymorphonuclear leucocytes was noted, while by the end of the experiment nearly all the circulating polymorphonuclear leucocytes were hypersegmented. In the control animals during the same time interval, there was an increase in the number of circulating band polymorphonuclear leucocytes.

In the drug-infused animals, in many cases there was a relative lymphocytosis, with an absolute lymphocytopenia, often accompanied by a relative monocytosis. In the control animals, there was both a relative and an absolute lymphocytosis at the end

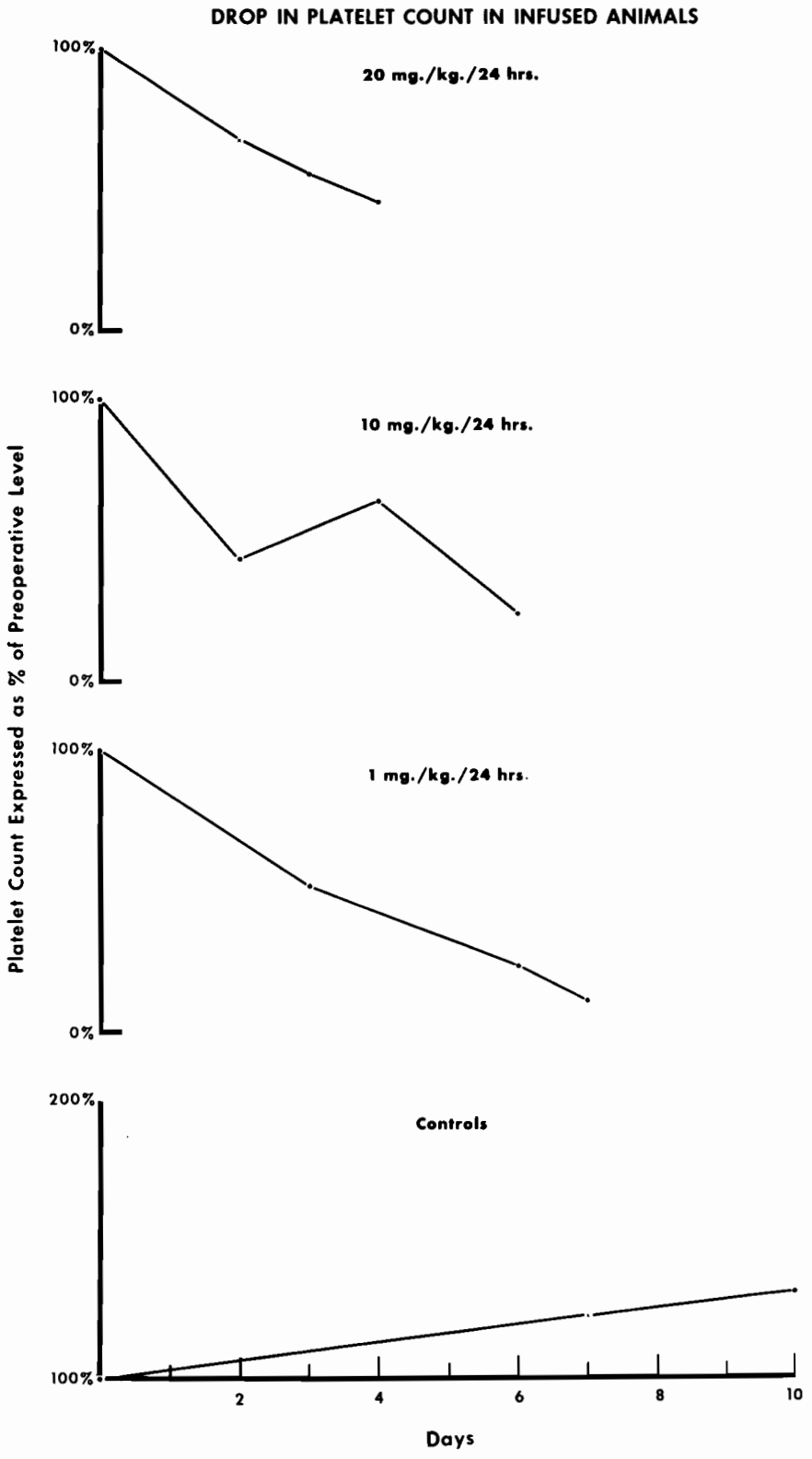
DROP IN PLATELET COUNT IN INFUSED ANIMALS
100%
20 mg./kg./24 hrs.
0%
100%
10 mg./kg./24 hrs.
0%
Platelet Count Expressed as % of Preoperative Level
100%
1 mg./kg./24 hrs.
0%
200%
Controls
100%
2
4
6
8
10
Days

Fig. 35.

of the experiment. Reticulocytes were absent in the peripheral blood of the drug-infused animals after day 4. In the control animals the reticulocyte count remained steady.

Throughout the experiments, the mean corpuscular hemoglobin concentration varied only slightly. In the drug-infused animals, when it changed it usually increased a point or two, whereas in the control animals it declined a point or two.

i. The bone marrow: Striking hypocellularity of the bone marrow was seen in the drug-infused animals (Fig. 36). This was most evident in the animals on the high drug doses, (SI, SII, SXIII, SVIII and SIX). In the marrow there were large numbers of remaining primitive reticulum cells. These were abnormally large, with prominent nuclei, and hyperchromatic nucleoli. Some were damaged, showing irregularity of the nuclei and eosinophilia of the cytoplasm. No remaining megakaryocytes were seen. In the myeloid series, a few immature cells were seen, rare mature cells, and no intermediate type cells. There thus appeared to be maturation arrest of myelopoiesis. In the erythroid series a few normoblasts were seen, together with mature erythrocytes. There were many examples of erythrophagocytosis, indicative of severe damage to the erythroid precursors. The overall picture was also one of maturation arrest.

In the high drug-dose group a few scattered plasma cells were seen in the marrow, and also clusters of lymphocytes, a usual reaction to hypoplasia of the marrow. There was an apparent tendency towards fatty infiltration of the marrow. In some areas there were clusters of amorphous, eosiniphilic, structureless

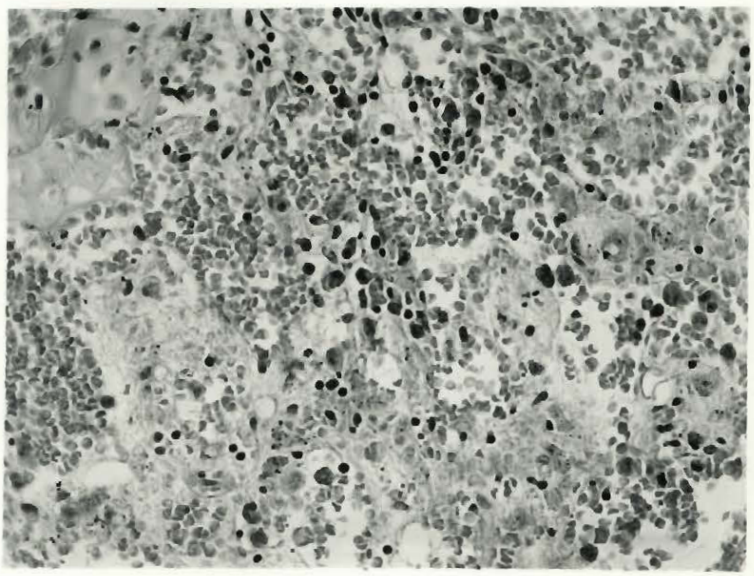

Fig.36. Hypoplastic bone marrow from drug infused animal. Animal SI. Hematoxylin and eosin stain, X 280.

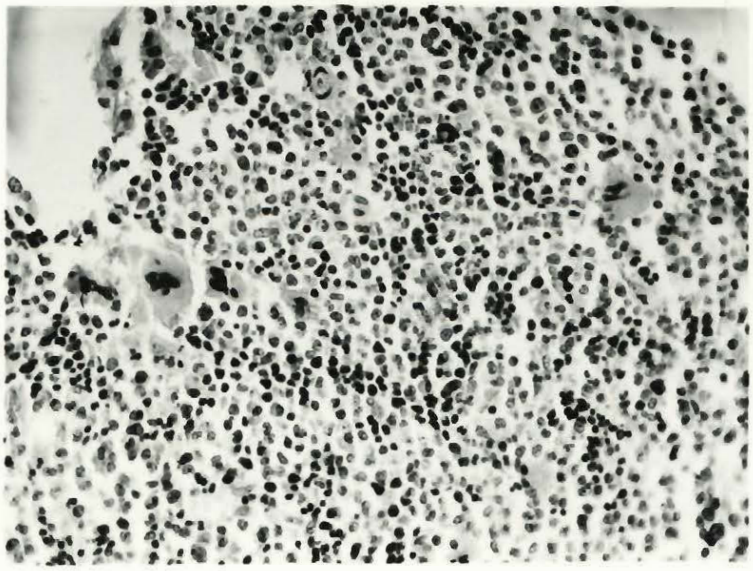

Fig. 37. Normal bone marrow from control animal. Animal SXV. Hematoxylin and eosin stain, X 280.

debris. There was some proliferation of the marrow connective tissue. In some areas there were normal appearing osteoclasts close by newly deposited bone. This last change in the bone may have been a chronic one.

In animals on the intermediate drug doses (SIV, SV and SVII) the changes were qualitatively the same as above regarding the damage seen in individual cells, but qualitatively a larger number of cells remained in any series. In comparing the myeloid series in this group with animals on high drug doses no apparent difference was seen. In the erythroid series, however, decreased numbers of immature nucleated red cells were seen, with numerous mature cells and rare intermediate cells. Thus, there may have been some maturation in the erythroid series. Megakaryocytes were rarely seen; these cells were abnormal, with enlarged, swollen nuclei and eosinophilic cytoplasm. Eosinophilic clumps of debris and the erythrophagocytosis were again noted, as were the scattered lymphocytes and plasma cells. There was less fatty infiltration of the marrow and less connective tissue proliferation here than in the animals on the high doses of the drug. No new bone formation was seen. Rare examples of mitotic activity were present.

In the animals on the low drug doses (SX, SXI and SXII), hypocellularity of the marrow involving all cell series was mild, the changes not being as severe as in the animals on the high drug doses. There was mild hyperemia of the marrow. Less primitive reticulum cells were seen than in the animals on the high drug doses, and the reticulum cells present were normal. Megakaryocytes were also decreased in number, but the remaining ones were histologically normal. There were decreased numbers of cells in the

myeloid series, but members of the complete series were seen, indicating some progression in maturation. Similarly, there was an apparent histological progression of maturation in the erythroid series. Neither the amorphous debris nor the erythrophagocytosis were seen here. Little plasma and lymphocyte cell infiltration of the marrow was evident here.

SVI, the animal which pulled out his catheter in the early stages of an experiment, and thus survived until he was sacrificed 8 months later, had a normal marrow. In the control animals (SXV and SXIV) there was apparent a slight hypercellularity of the myeloid series (Fig. 37), possibly as a consequence of the parasitic infestations seen in these animals. This was compatible with the initial increased white blood counts seen in these animals. Otherwise, the bone marrow of these animals was histologically normal. In the drug-infused animals the loss of megakaryocytes and members of the myeloid series in the marrow was rapidly reflected in the peripheral blood by a decrease in the numbers of thrombocytes and granulocytes respectively (see The hematological system); whereas, regarding the damage seen in the erythroid series of the marrow, the decrease in hemoglobin peripherally was much less striking.

j. Infections: In the drug-infused animals, disregarding the parasitic infestations, a high incidence of acute infections were noted in various organs.

Eight of the animals had chronic focal interstitial pneumonitis; however, lung parasites were identified in six of these animals, and this pneumonitis may have been a reaction to

the infestation.

SIII, which died on day 1 in status epilepticus was noted to have a meningitis at autopsy, and this was confirmed histologically. No microorganisms were seen in any of the special stains of the brain.

Five animals had fulminating bacterial colitides. Among the organisms seen after special staining of tissue sections were the following: Gram positive rods with spores, probably Clostridia; Gram positive cocci in chains, probably Streptococci; large Gram positive cocci, in groups and clusters, probably Staphylococci; Gram positive rods with spores, probably aerobic Subtilis; and, in one animal Gram positive yeasts were seen, probably Candida albicans. Though the organisms seen had the morphological and staining characteristics of the microorganisms named, the identification may not be positively correct since stool cultures and other special differentiating tests were not done on the animals during life.

In some of the animals which had parasitic nematode infestations, large mesenteric nodes were noted. Tuberculin tests were done on the animals and were all negative.

SVII (had a bacterial colitis) and SII had acute fulminating pneumonitides (Fig. 38), with clumps and colonies of microorganisms growing amidst the necrotic debris. These microorganisms had similar morphological and staining characteristics to those of Staphylococci. In addition, animal SVII had colonies of fungi growing in his denuded esophageal mucosa.

SV, included in the series of bacterial colitides above,

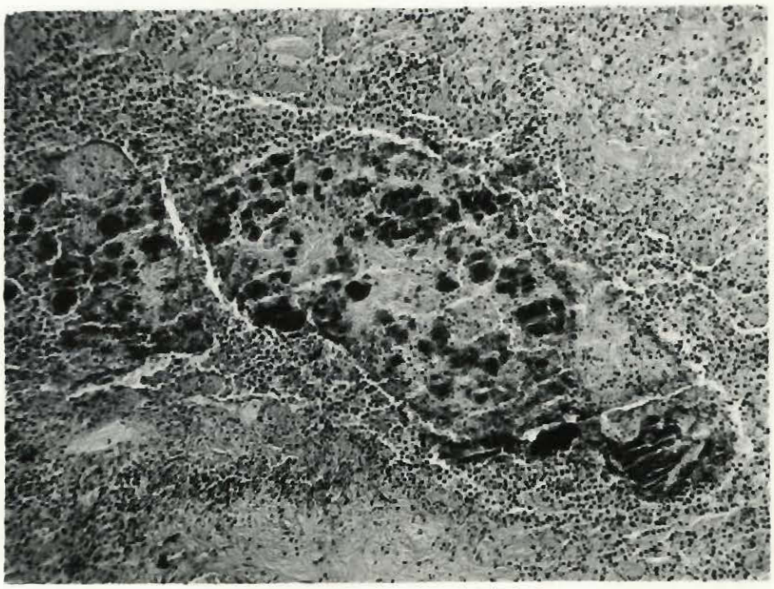

Fig. 38. Section of lung from drug-infused animal, showing pneumonitis. Colonies of bacteria are seen, together with an inflammatory cell infiltrate. Animal SII. Hematoxylin and eosin stain, X 110.

had small micro-abscesses in the frontal lobe cortex and white matter of the brain. No microorganism was identified in the brain sections.

Of the thirteen drug-infused animals, eight had some sort of acute superinfection, either bacterial or fungal. In the control animals no evidence of any acute infection was found.

k. Incidental findings: In the autopsies and detailed histological examinations of the animals, a considerable amount of incidental pathology was found, which was thought not to be related to the experimental procedure. Four of the animals (SVIII, SXI, SXIII and SXIV), were found to have parasitic arthropod infestations of the lungs; one animal had a nematode infestation of the mesentery, while three animals (SIX, SX, and SXV) had combined arthropod-lung and nematode-gut infestations. Three of these animals had increased peripheral eosinophile counts at the outset of the experiment.

Grossly, the lungs of the infested animals were studded with soft, circumscribed, yellow, well-demarcated lesions. The affected lobes were adherent to one another and to the parietal pleura. On section, the lesions were cavitated, containing cloudy fluid and one or more hard, formed organisms, barely visible to the naked eye. Under the dissecting microscope the organisms had from 6 to 8 tiny legs. At the rostral end there were tiny hairs.

Histologically, the parasites were tiny arthropods lying in cysts lined by flattened cuboidal epithelium (Fig. 39). In two instances the arthropods were seen in the terminal respiratory

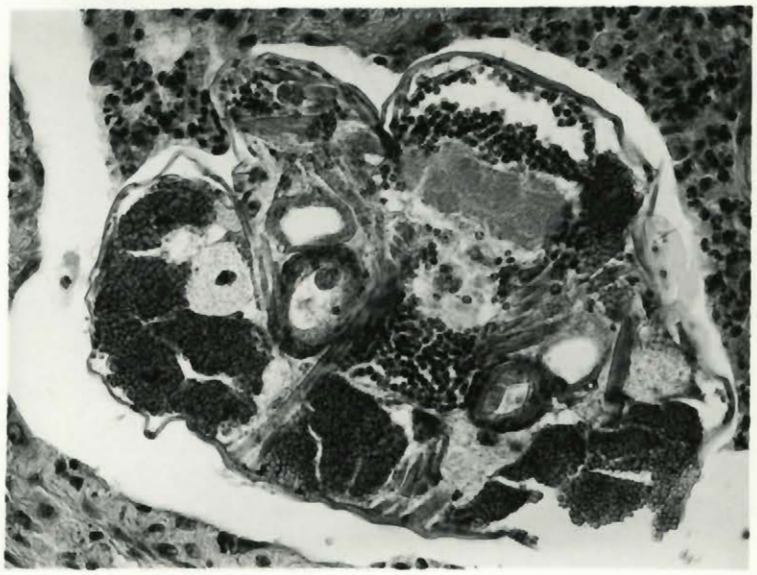

Fig.39. Section through an arthropod in lung. Animal SIX. Hematoxylin and eosin stain, X 280.

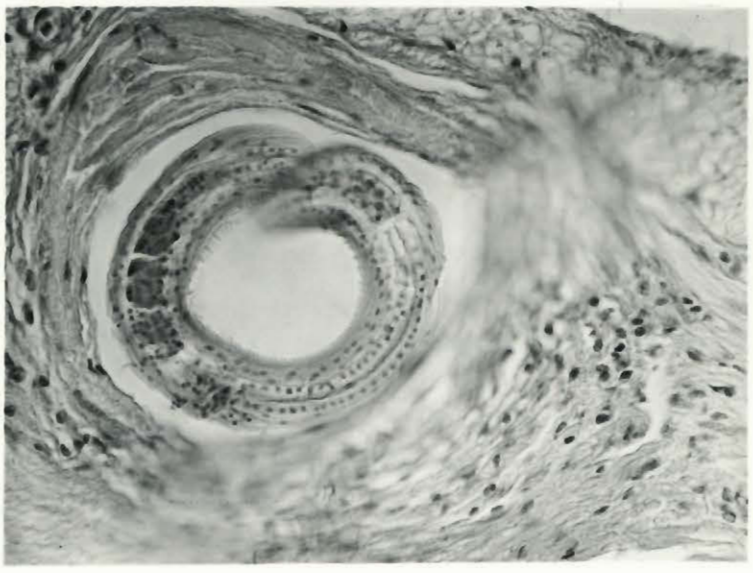

Fig.40. Longitudinal section of larval nematode seen in Muscularis of gut. Animal SXV. Hematoxylin and eosin stain, X 110.

bronchioles. The cysts, histologically, had the appearance of distended terminal bronchioles. They were surrounded by an area of fibrosis, foam cells, histiocytes, lymphocytes and eosinophiles. Nearby there were areas of chronic focal pneumonitis, probably a reaction to the parasites.

It was thought that these arthropods were of the genus Pneumonyssus (Meerovitch, 1964), an arthropod which is endemic in the lungs of Indian monkeys. Of the animals which came from India almost half had this infestation. These arthropods are thought to be spread via the respiratory route (Meerovitvh, 1964). The finding of some of the arthropods in bronchioles, and of others in what appeared to be terminal bronchioles, was consistent with this mode of spread. Since some of the arthropods had 6 legs, while others had 8, representing respectively adult and larval stages, the infestations were felt to be active ones.

There were numerous hard nodules in the mesentery and peritoneum of some of the animals. Histologically, in sections prepared from these nodules, round worms were seen. The organisms were surrounded by a zone of coagulation necrosis. This, in turn, was surrounded by a rim of polymorphonuclear leucocytes, lymphocytes, monocytes, plasma cells, histiocytes, and pigment-laden macrophages. Organizing granulation tissue surrounded some of the rims of inflammatory cells. The nearby mesenteric blood vessels had sclerotic walls and perivascular lymphocytic and plasma cell infiltrations. Some of the worms in the mesentery were adults, while some smaller worms in the muscularis of the gut (Fig. 40) were probably larval.

These nematodes could not be classified further from the sections studied: they, also, are endemic in the Indian monkey population, (Meerovitch, 1964).

A pyloric polyp, measuring 10 mm. x 4 mm. was found on the greater curvature at the level of the pylorus in SVII. This consisted of an inner core of lamina propria supporting cells of the usual gastric type. In the base of the polyp some of the cells formed aggregations which lay in the muscularis of the stomach (Fig. 41), a phenomenon suggesting malignancy. Since the animal had received Methotrexate, and consequently some of the cells in the base of the polyp had the previously described hyperchromatic and other nuclear changes, it is difficult to say histologically whether the cells were in fact malignant.

In SXI there was a white patch, 10 mm. in diameter, in the lower third of the stomach near the lesser curvature. The wall of the stomach was thin and hard in this region. Histologically, this was a superficial ulcer without any inflammatory exudate surrounding it. In the ulcer there was no remaining mucosa, while at the rim the mucosa was thin, with basophilism of the glands, suggesting healing (Fig. 42). The underlying submucosa in the ulcer region was thin and hyalinized.

In the spleen of SIX there was seen a red, well-circumscribed subcapsular mass 6 mm. in diameter. Histologically, this was a tumor consisting of well-formed aggregations of capillaries lined by plump endothelial cells (Fig. 43). It was a hemangioendothelioma or a hemangiopericytoma.

The gastric serosa and submucosa in SXV contained many vessels showing striking fibrinoid necrosis (Fig. 44). There was

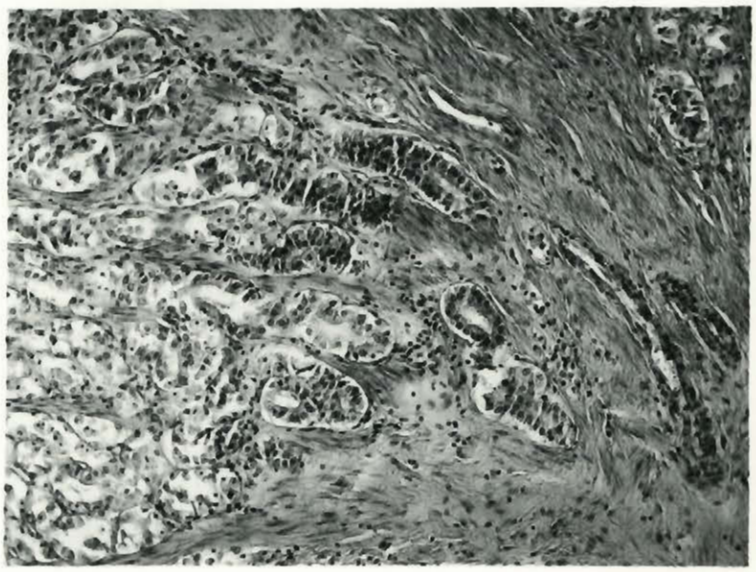

Fig.41. Nests of cells in the muscularis of the stomach, at the base of a gastric polyp. Animal SVII. Hematoxylin and eosin stain, X 110.

Fig.42. Edge of gastric ulcer showing basophilia of glands in healing portion. Animal SX. Hematoxylin and eosin stain, X 35.

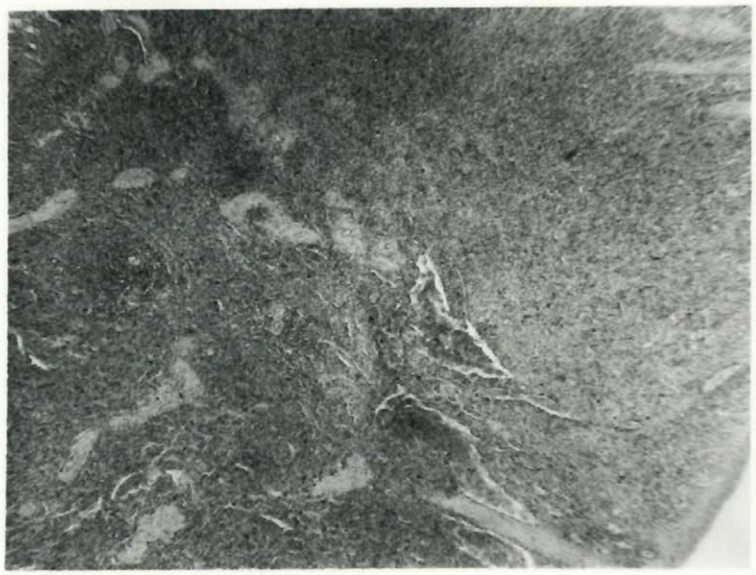

Fig.43. Spleen (left) adjoining splenic tumor (right). Animal SIX. Hematoxylin and eosin stain, X 35.

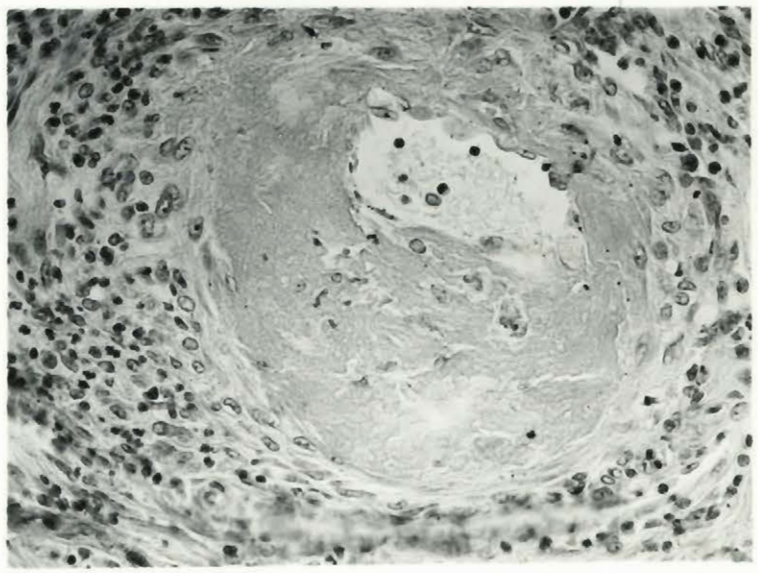

Fig.44. Fibrinoid necrosis of all of mesenteric artery. There is a periarterial infiltrate of inflammatory cells. Animal SXV. Hematoxylin and eosin stain, X 280.

a surrounding perivascular infiltrate consisting of polymorphonuclear leucocytes, eosinophiles and mononuclear cells. These changes were very similar to those seen in humans with polyarteritis nodosa, although the rest of the histological findings did not support this diagnosis. It is possible that these lesions were a consequence of the nearby parasitic infestation of the mesentery.

1. The brain: Four of the fifteen animals used in the experiments were found to have lesions of the brain.

Grossly, in the brain of SI the gyri were flattened and the sulci were shallow. Histologically, in the brain of SI there were numerous small, well-defined areas of destruction of the neuropil in the cortex of the right middle frontal gyrus (Fig. 45). One such tiny area was seen in the cortex of the right superior frontal gyrus. Small, confluent areas of fenestration of the cortex were seen in another portion of the right middle frontal gyrus (Fig. 46). In the central part of these lesions there was neuronal death, with eosiniphilia, and lack of detail of peripheral neurones. The included astrocytes were swollen.

In the subcortical white matter, beneath the most severely affected gyrus, some of the astrocytes were Alzyheimer like, with large, pale nuclei surrounded by an irregular nuclear membrane.

There was one tiny area of neuronal death in the right hippocampus. Other parts of the brain, including the brain stem and the cerebellum, and cerebral blood vessels of this animal were normal.

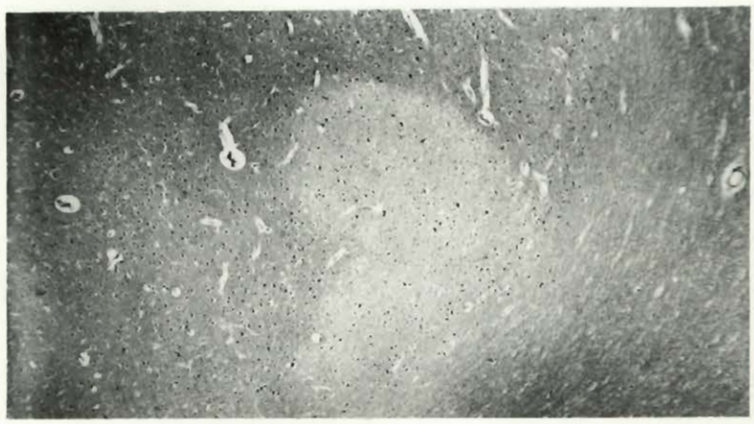

Fig.45. Well-defined area of brain destruction in cortex of right middle frontal gyrus. Animal SI. Bodian stain, X 35.

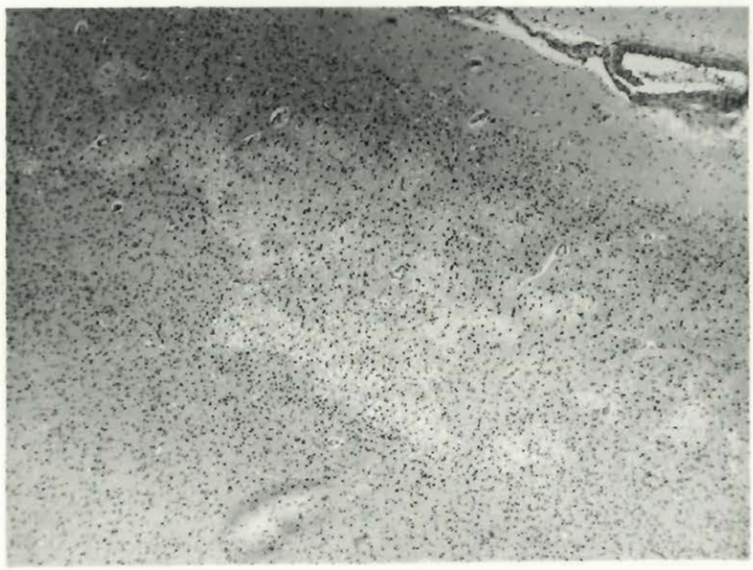

Fig.46. Confluent areas of fenestration and destruction of brain in the middle layers of the cortex of the right middle frontal gyrus. Animal SI. Hematoxylin and eosin stain, X 110.

In SII there was gross swelling of the right cerebral hemisphere (Fig. 47), with a 3 mm. shift of the midline structures to the left at the mid-thalamic level.

Histologically, there was increased space between the fibers and cells in the right hemisphere suggesting cerebral edema. On the right side of the brain, there were numerous intracerebral lesions, while none were seen in the left hemisphere.

One sharply demarcated lesion involved about one half of the right middle frontal gyrus (Fig. 48). It extended the depth of the cortex in one area, but ended abruptly at the subcortical white matter. In the hematoxylin and eosin stain the lesion was pale with necrosis of all tissues including neurones and supporting cells in its cerebral portion. The cortical margins were poorly defined. At the periphery of this area of brain destruction there were numerous hypertrophied astrocytes, some of which were binucleate (Fig. 49), while others were undergoing karyolysis. At the cortical periphery of this lesion many of the neurones showed eosinophilia, with lack of cellular detail.

In the sulcus between the right middle frontal and right inferior frontal gyri, a layer of laminar necrosis involved the 4th and 5th layers of cortex. A very recent, well-defined infarct was present in the right forceps minor, with destruction of all elements including the axons. There were other small, widely scattered areas of fenestration and destruction of the cortical neuropil in the region of supply of the right middle cerebral artery.

The left hemisphere, brain stem, cerebellum and cerebral

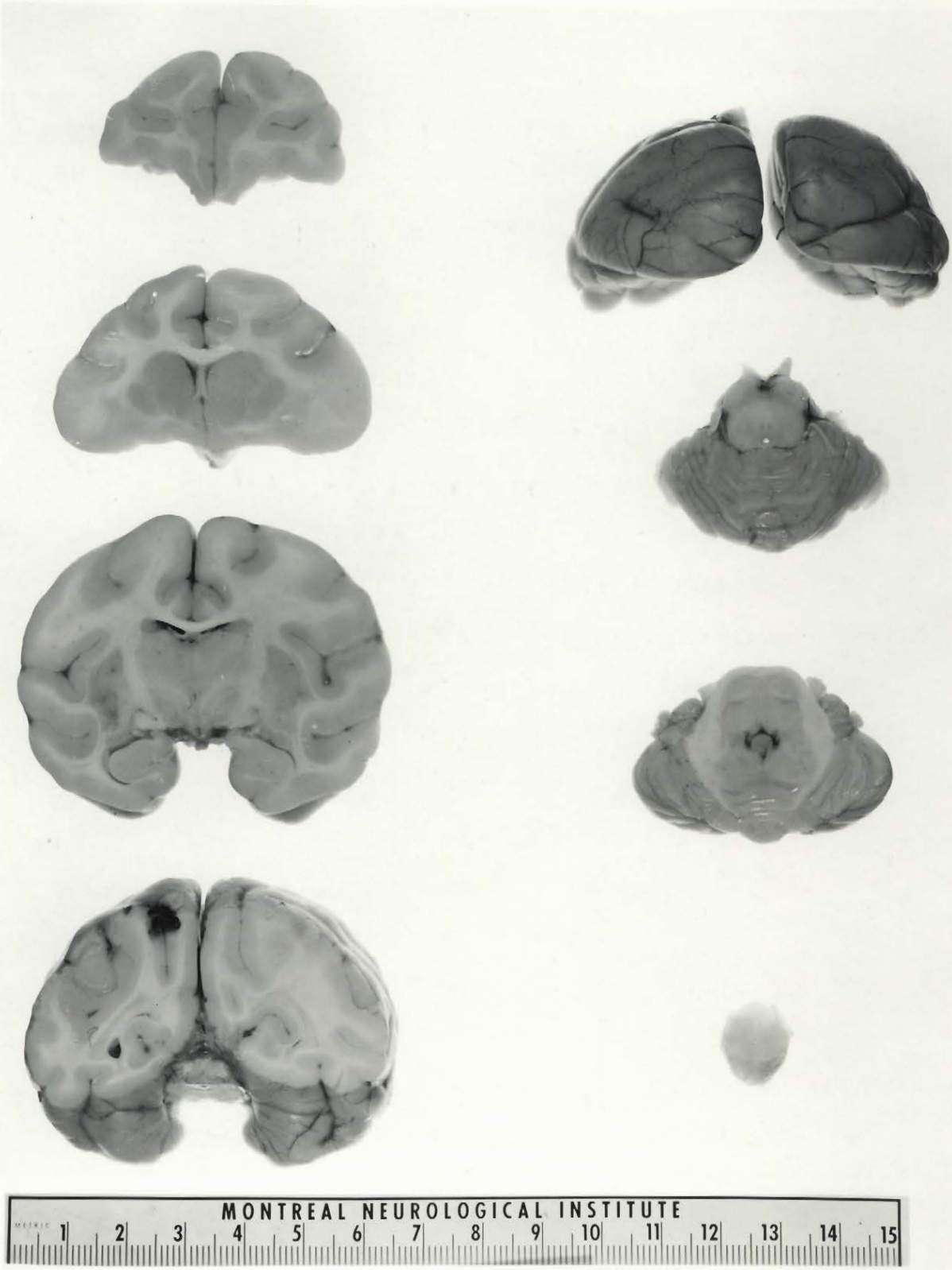

Fig.47. Sections of brain of SII. Note right cerebral swelling.

Fig.48. Extensive area of laminar necrosis of cortex, extending from normal cortex (top right) to subcortical white matter (bottom left). Animal SII. Hematoxylin and eosin stain, X 35.

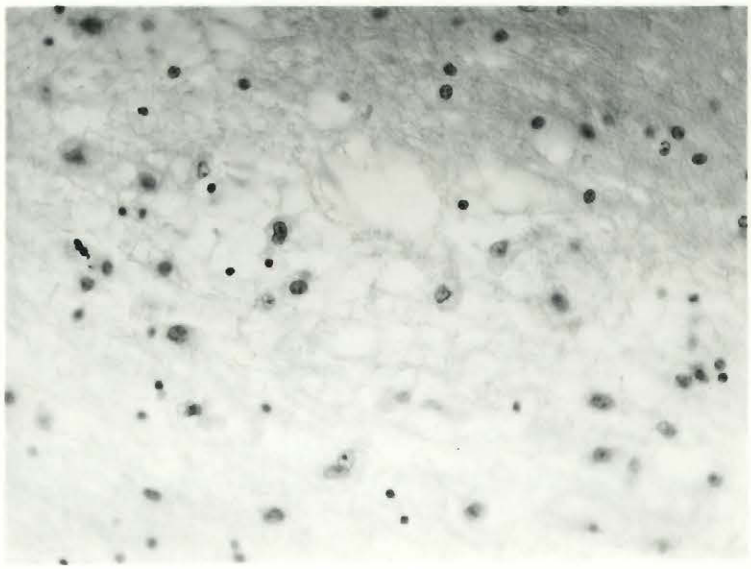

Fig.49. Hypertrophied astrocytes in periphery of area of laminar necrosis shown in Fig.48. There is an absence of polymorphonuclear leucocytes. Animal SII. Hematoxylin and eosin stain, X 280.

blood vessels were all normal.

At autopsy the right cerebral hemisphere of SIII was grossly swollen, mostly in the anterior portions, with a 2 mm. shift of the midline structures to the left at the level of the mid-thalamus (Fig. 50).

Histologically, there were numerous well-demarcated areas of pallor diffusely affecting the cortex of the right hemisphere in the regions supplied by the right internal carotid artery. Some of these lesions were discrete, while others were confluent. The white matter was involved to a lesser extent. These were recent areas of necrosis with loss of staining details in some neurones, and death in others. There was also swelling and destruction of some of the included axons. One large area extended the entire depth of the cortex in the sulcus between the right middle and right inferior frontal gyri, but ended abruptly at the subcortical white matter. There was another large area of confluent pallor, central necrosis, and cell death in the right centrum semiovale. A minute area of neuropil destruction in the left superior frontal gyrus was situated in the middle layers of the cortex.

Further back, the superior gyri of the right frontal and temporal lobes including both cortex and white matter, were a mosaic of tiny, irregular, but well-defined areas of necrosis. In these areas there was neuronal loss and astrocyte swelling, but a dearth of inflammatory cell and macrophage response.

There was extensive infiltration of polymorphonuclear leucocytes in the subarachnoid space, especially in the basal

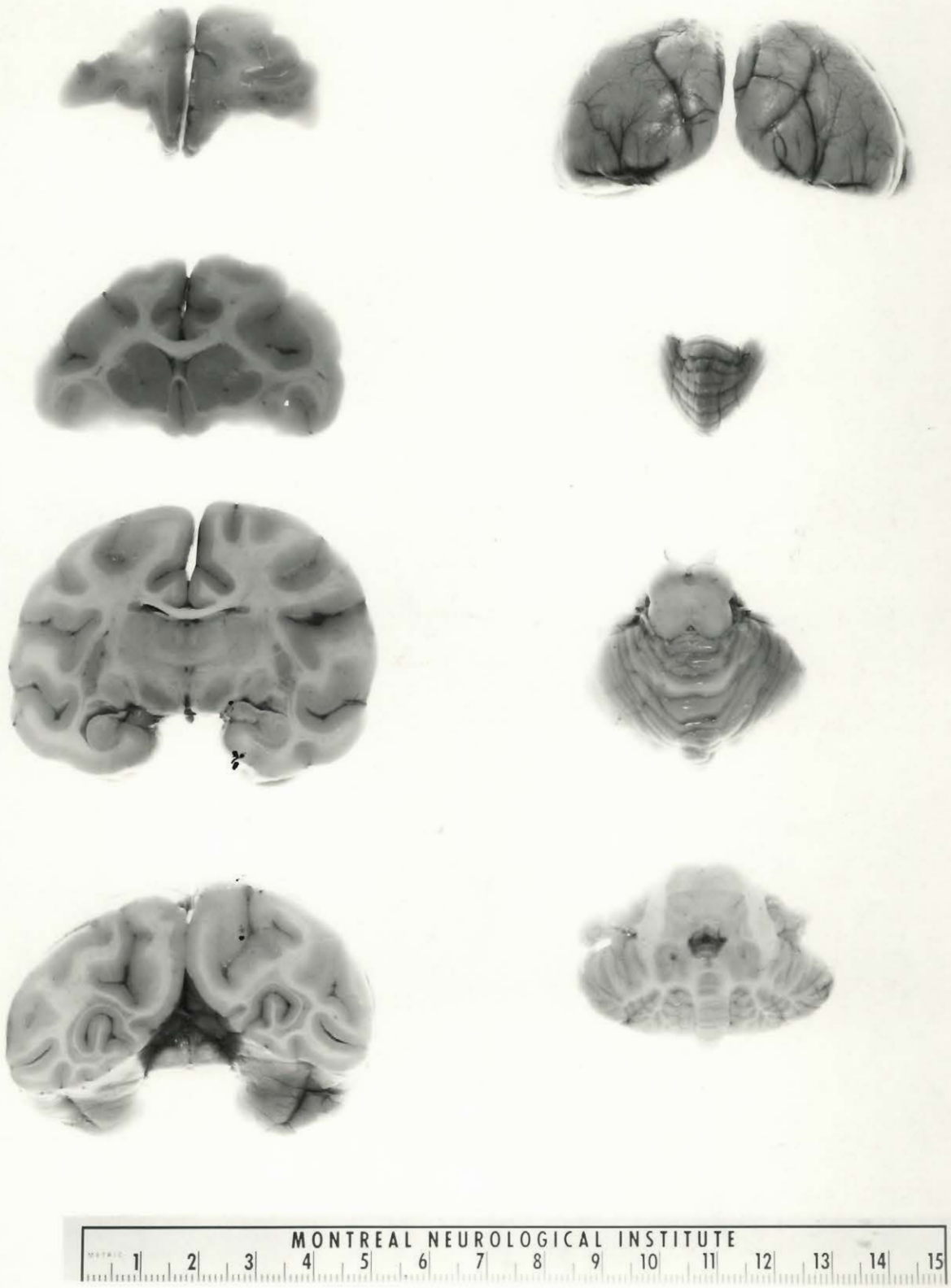

Fig.50. Sections of brain of SIII. Note right cerebral swelling.

cisterns, suggestive of acute meningitis. On the Gram stained sections no microorganisms were seen however. Nowhere was there any sign of vessel damage or of any vascular occlusion. The brain stem and cerebellum were normal, as were the highly anoxia-sensitive hippocampi of each hemisphere.

In SV, which received an intermediate dose of the drug buffered with Tham, the brain was slightly swollen in the right frontal region.

Histologically, there were two tiny, well-demarcated areas of necrosis in the cortex of the right middle frontal gyrus (Fig. 51). In addition, a similar tiny lesion was seen in the middle layers of cortex in the left superior frontal gyrus (Fig. 52), extending from cortical layers two to five. Two micro-abscesses were seen; one in the cortex of the right inferior frontal gyrus, and another more posteriorly in the white matter of the right inferior frontal gyrus (Fig. 53). Again, no micro-organisms were seen in the Gram stained sections.

Some of the animals had lesions as a consequence of the electrode placement. Ten of the animals had insignificant thin films of epidural blood under one or more of the drill hole sites. In three instances the dura was penetrated by the drill, causing a lesion. SII had a small right parietal cortical bruise. In SVII there was a hematoma in the white matter of the left middle frontal gyrus 1 mm. in diameter, and in SV there was a hematoma in the white matter of the right parietal lobe, 3 mm. in diameter.

Lesions attributable to the infusion were only seen in the animals described above. In the 10 other animals, including

Fig.51. Recent small area of cortical necrosis in the right middle frontal gyrus, showing destruction of neurones and axons. Animal SV. Bodian stain, X 110.

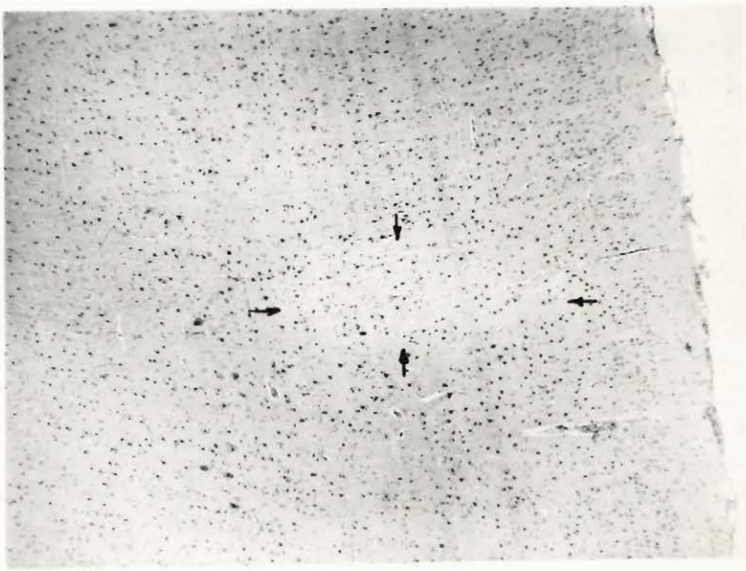

Fig.52. Small area of localized brain destruction in cortex of the left superior frontal gyrus. Animal SV. Hematoxylin and eosin stain, X 35.

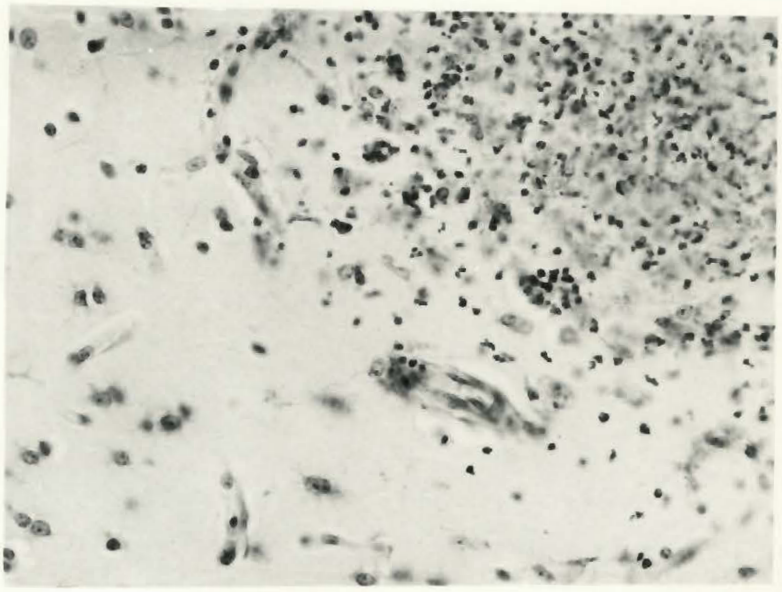

Fig.53. Micro-abscess in the cortex of the right inferior frontal gyrus. Animal SV. Cresyl violet stain, X 280.

the controls, the brains were all grossly normal, and in the 150 giant histological sections studied of the brains of these animals no lesion was found.

m. The cerebral water content: Unfortunately, the first five animals done were not included in this part of the study, hence samples from those brains having gross swelling were not included.

In order to establish normal values for the dry weights of cerebral tissue, it is necessary to obtain absolutely fresh samples from exsanguinated animals. No animals in this series were sacrificed specifically for this purpose. Figures for the normal dry weights of white and gray matter of the monkey brain have not been reported in the literature. However, these normal values are established for the rat, cat, and human, and they do not vary statistically when comparing one species with the other (Pappius, 1964). Consequently, the results in this study will be compared with the well established normal values for the cat. The dry weights for the cat are as follows (Pappius and Gulati, 1963): white matter 32 ± 1.3 mg. / 100 mg. fresh white matter ($n = 17$); gray matter 18.2 ± 0.8 mg. / 100 mg. fresh gray matter ($n = 17$).

A limited number of animals was done under each group of experimental conditions. Despite replicate values for each of the animals, it was felt that the number of observations was too small to be properly subjected to statistical analysis. Accordingly, no definite conclusions can be drawn, and only trends commented upon.

Two controls were available in this series; one animal (SXV) was infused without Methotrexate, while the other animal died of pneumonia without having been subjected to any experimentation. Despite the sampling of these controls respectively at 1 and 4 hours after death, the values obtained for the gray matter in both animals fell within the normal range for the cat, while the values for the white matter were slightly higher than the normal values in the cat (Figs. 54 and 55).

In this series of animals there was no apparent difference in the dry weights between the right and left hemispheres for either white or gray matter, (Figs. 54 and 55).

Considering the gray matter (Fig. 54) regardless of the dosage of Methotrexate infused, the cortical dry weights fell within the range of 14 to 18 mg. / 100 mg. / fresh brain. This was slightly below the normal values for the cat, but considerably below the values for the monkey control animals. Four of the drug-infused animals (SVI, SVII, SXI and SXIII) had cortical dry weights close to the normal range established for the cat. These animals were sampled respectively 1, 2, 1, and 4.5 hours after death. SVIII, SIX, and SXII had dry weights well below the accepted normal values for the cat, however, in these animals the brain samples were taken respectively at 14, 9, and 9 hours after death. SX had a relatively low cortical dry weight, and he was autopsied only two hours after death. The control animal (autopsied 4 hours after death) had a lower cortical dry weight than SXV (autopsied one hour after death).

Concerning the white matter (Fig. 55) the dry weights of

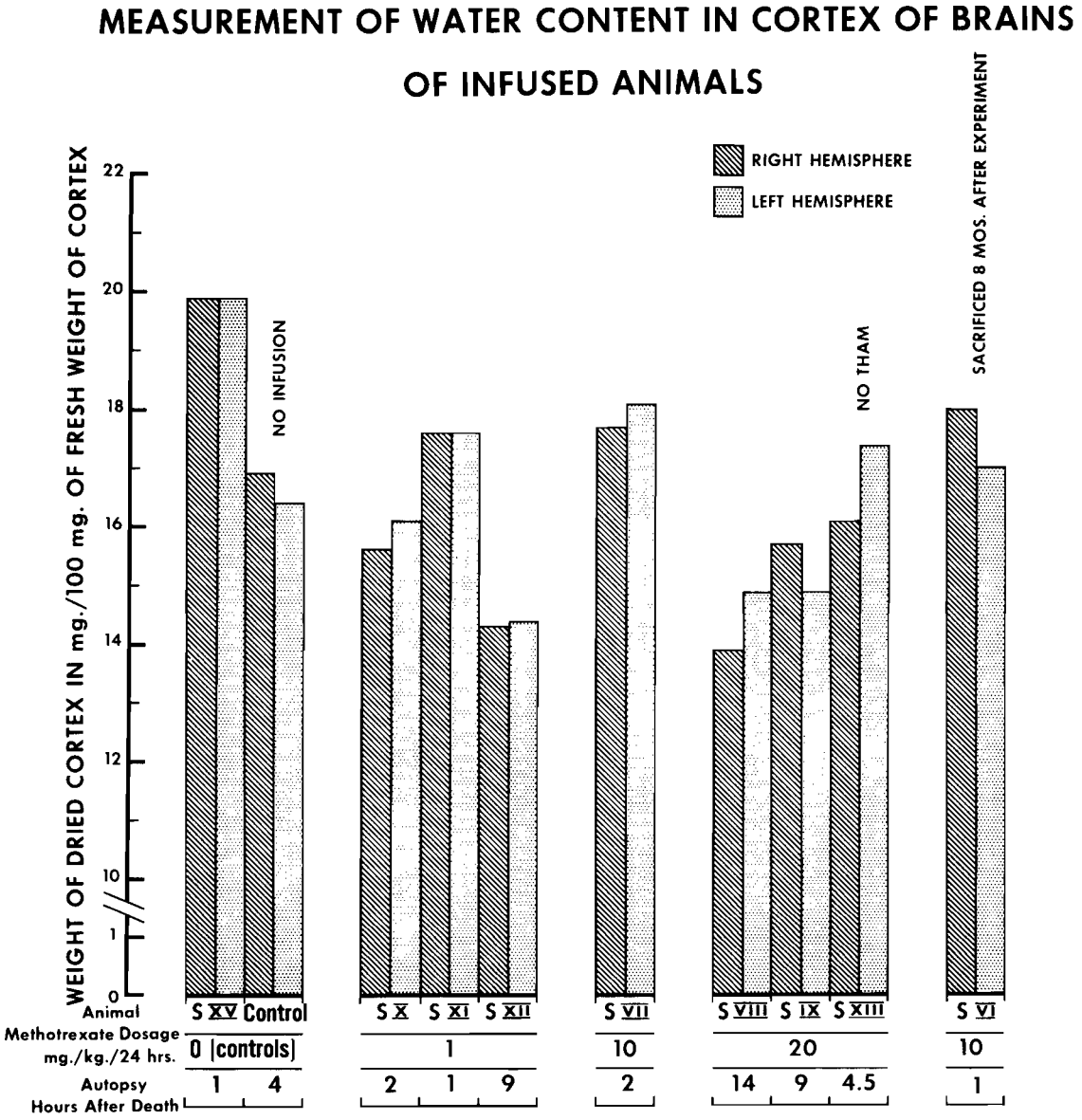
MEASUREMENT OF WATER CONTENT IN CORTEX OF BRAINS OF INFUSED ANIMALS
RIGHT HEMISPHERE
LEFT HEMISPHERE
WEIGHT OF DRIED CORTEX IN mg./100 mg. OF FRESH WEIGHT OF CORTEX
22
20
18
16
14
12
10
1
0
NO INFUSION
NO THAM
SACRIFICED 8 MOS. AFTER EXPERIMENT
Animal
S XV Control
S X
S XI
S XII
S VII
S VIII
S IX
S XIII
S VI
Methotrexate Dosage mg./kg./24 hrs.
0 (controls)
1
10
20
10
Autopsy Hours After Death
1
4
2
1
9
2
14
9
4.5
1

Fig. 54.

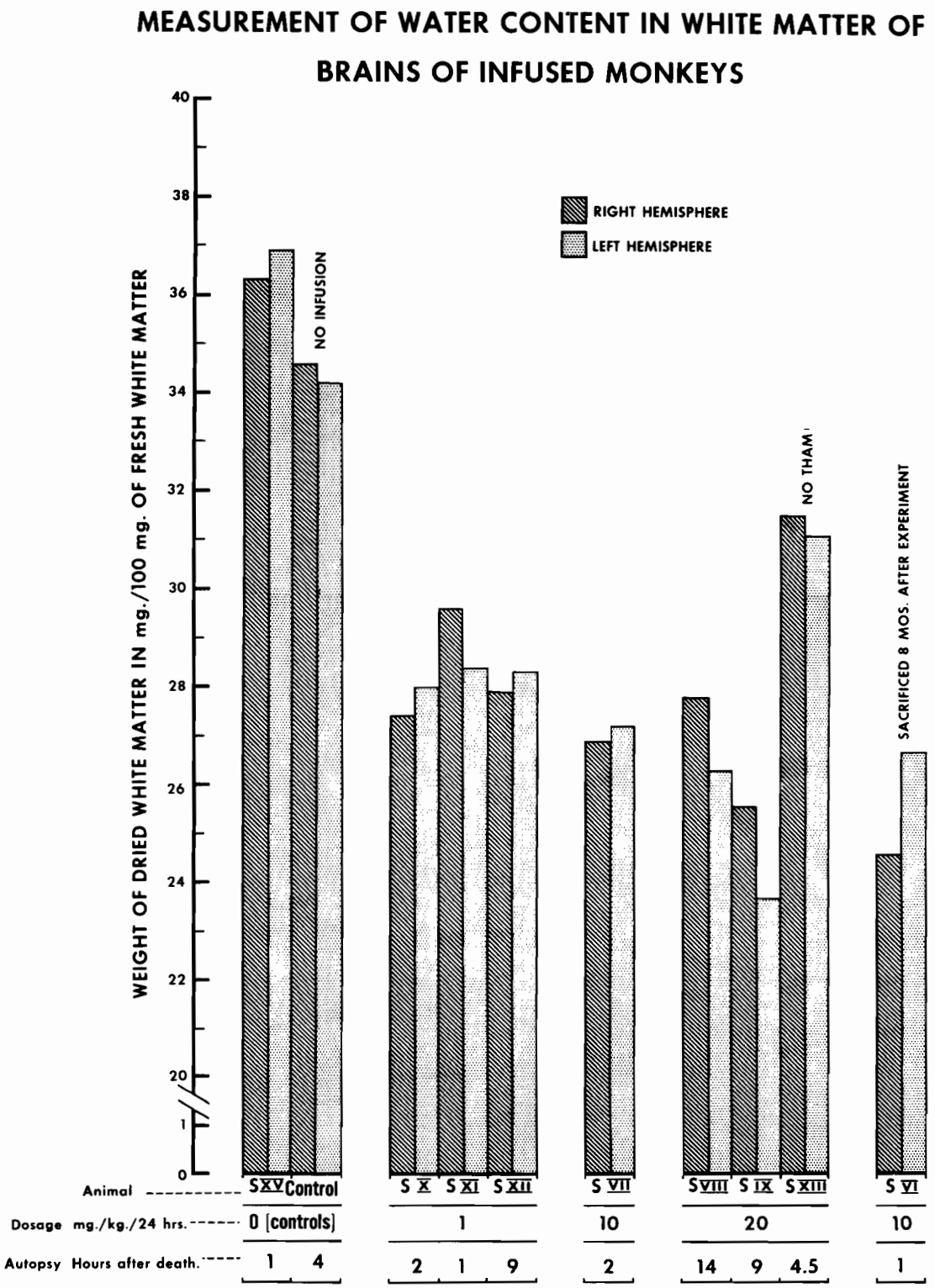
MEASUREMENT OF WATER CONTENT IN WHITE MATTER OF BRAINS OF INFUSED MONKEYS
WEIGHT OF DRIED WHITE MATTER IN mg./100 mg. OF FRESH WHITE MATTER
RIGHT HEMISPHERE
LEFT HEMISPHERE
NO INFUSION
NO THAM
SACRIFICED 8 MOS. AFTER EXPERIMENT
Animal
Dosage mg./kg./24 hrs.
Autopsy Hours after death.
S XV Control
S X
S XI
S XII
S VII
S VIII
S IX
S XIII
S VI
0 [controls]
1
10
20
10
1 4
2 1 9
2
14 9 4.5
1

Fig. 55.

all of the drug-infused animals fell within the range of about 24 to 29 mg. / 100 mg. of fresh white matter, which was well below the normal range established for the cat. This held true, regardless of the drug dosage. The dry weights of the white matter in the control animals were quite close to the established normal values for the cat.

Animals SVIII and SXII which were autopsied many hours after death, had values of the dry weights of white matter which were in the same range as the animals from which samples were taken soon after death.

All of the animals receiving the drug in Tham buffered infusates had dry weights of white matter below the normal values for the cat. SXIII, which did not have a Tham buffered infusate, and the control animals, one of which did have an infusion containing Tham, had dry weights of the white matter falling within the normal range for the cat. SVI, whose brain still contained Methotrexate at sacrifice 8 months after the experiment, had a dry weight of white matter below that of the normal cat white matter, and below that of the control animals.

n. Brain and blood content of Methotrexate: These determinations, as previously explained (see Methods) were made by an indirect bio-assay method.

Animals on the high dose infusates of Methotrexate will be considered first. Animals SI, SII, SIII and SXIII all received unbuffered infusates. Animals SI and SII had exceedingly high uptakes of brain Methotrexate (Fig. 56). Both were infused for longer than 5 days. At autopsy both were found to have right

METHOTREXATE LEVELS IN BRAIN SAMPLES FROM INFUSED MONKEYS

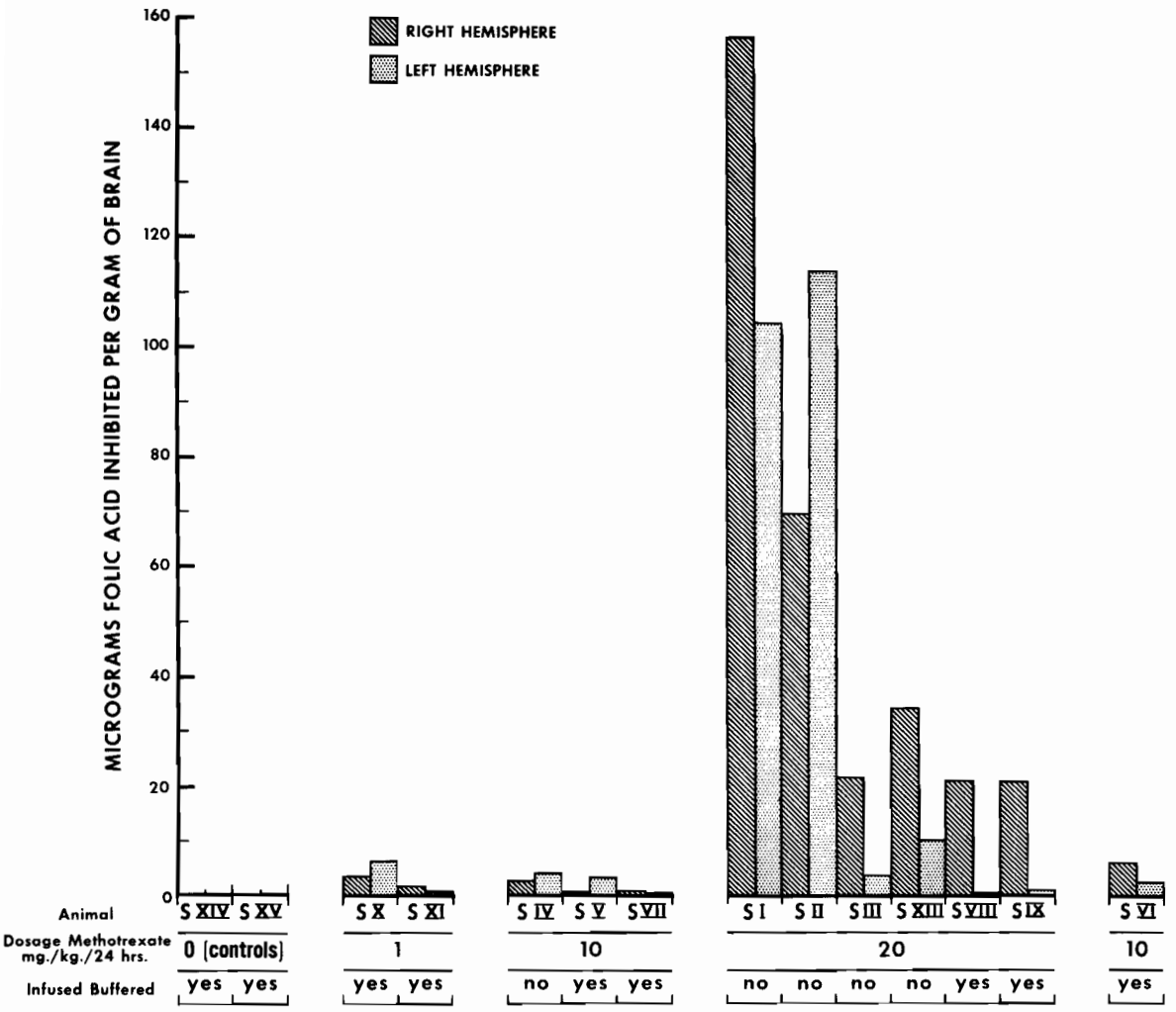

Fig. 56.

cerebral infarcts. Animal SII had a higher Methotrexate content on the left side of the brain, but also had a right internal carotid thrombosis, suggesting that the infusate had perhaps flowed retrograde down the right common carotid, and thence into the left side of the brain in high concentration via the left common carotid artery. SXIII was infused for 6.6 days and also had a high Methotrexate uptake, but this was relatively low as compared with SI and SII. This animal, however, had no cerebral infarcts. Animal SIII was infused for less than one day, under the same conditions as the above three animals, and had an uptake as high as SXIII infused for a much longer period of time. However, SIII also had cerebral infarcts.

Animals SVIII and SIX, both on high doses of Methotrexate buffered with Tham, had fairly high uptakes on the right side, but lower uptakes on the left side (Fig. 56). Animal SVIII received infusion for only 2.5 days, since he pulled out his catheter. SIX was infused for 4.7 days. Neither of these animals had cerebral infarcts.

In animals SIV, SV, and SX, on intermediate doses of Methotrexate, and animals SX and SXI on low doses of the drug, there was little apparent difference in brain content of Methotrexate, regardless of the dose, or whether or not the infusate was buffered. In animals SIV, SV, and SX there was a higher uptake on the left side of the brain than on the right, but in all three of these animals difficulties were encountered in the standardization of the brain suspension solutions, and these differences may fall within the error of the bio-assay procedure. SX had a

right common carotid thrombosis at the catheter site.

SVI was sacrificed 8 months after a short infusion period on a buffered intermediate dosage of Methotrexate, and still had an appreciable uptake of brain Methotrexate at that time. There was no Methotrexate in the brains of the control animals.

Regarding blood Methotrexate concentration (Fig. 57), SI and SIX on high doses of Methotrexate had relatively high concentrations of blood Methotrexate. Animals SIV and SVII, on intermediate doses of the drug, had intermediate concentrations of the drug in the blood. Animal SVII had two blood samples drawn; one on day 4, and another on day 7. The latter sample had the higher blood Methotrexate concentration. Animals SX and SXI, on low doses of the drug, had the lowest blood Methotrexate levels.

Controls SXIV, SXV and SVI had no blood Methotrexate activity, but instead had appreciable folic acid levels (Fig. 57); It was not possible to compare the blood uptakes in regard to whether the infusates were buffered or not, since the number of observations were too few.

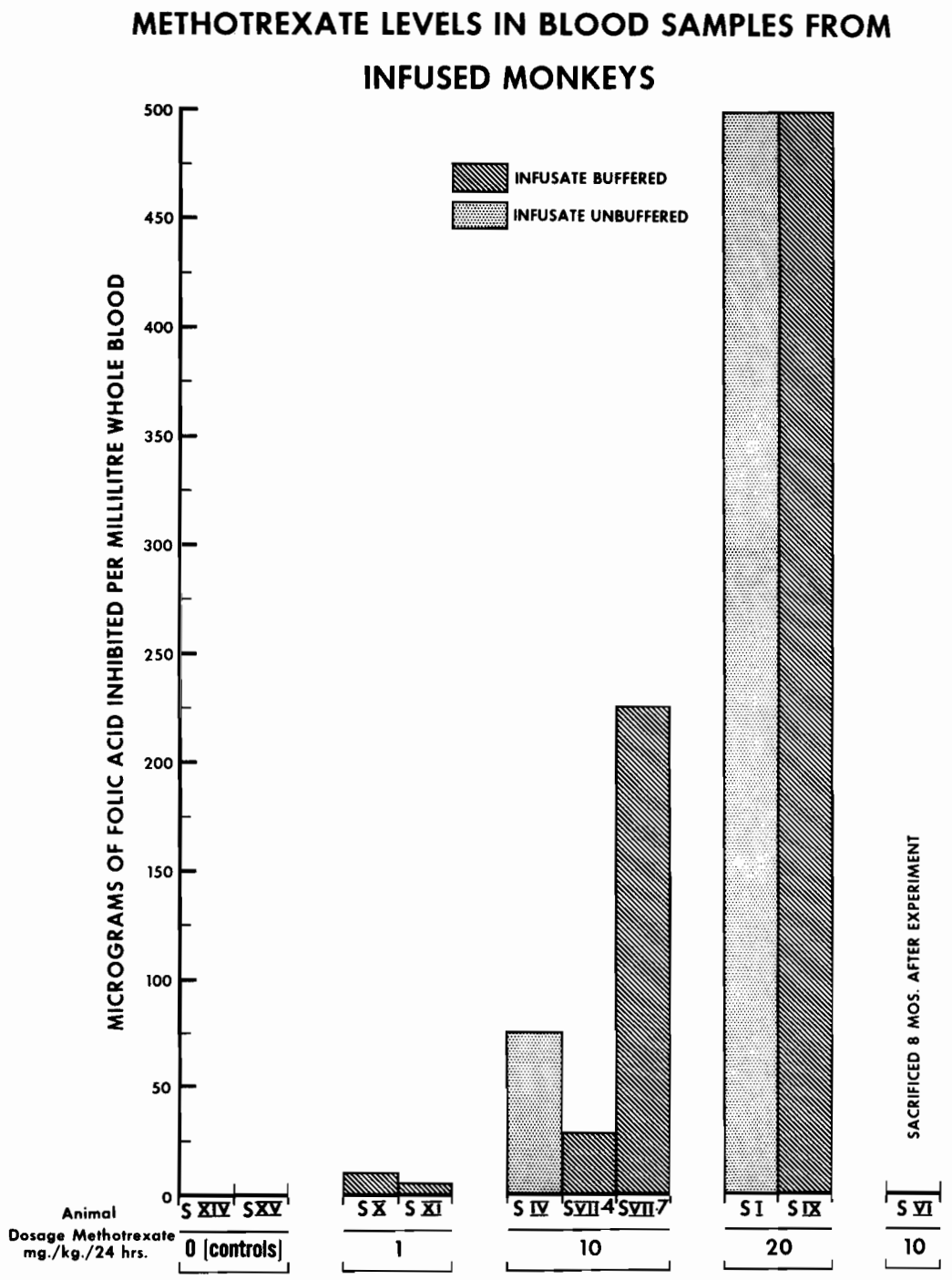
METHOTREXATE LEVELS IN BLOOD SAMPLES FROM INFUSED MONKEYS
INFUSATE BUFFERED
INFUSATE UNBUFFERED
MICROGRAMS OF FOLIC ACID INHIBITED PER MILLILITRE WHOLE BLOOD
500
450
400
350
300
250
200
150
100
50
0
SACRIFICED 8 MOS. AFTER EXPERIMENT
Animal
Dosage Methotrexate mg./kg./24 hrs.
S XIV
S XV
S X
S XI
S IV
S VII-4
S VII-7
S I
S IX
S VI
0 (controls)
1
10
20
10

Fig. 57.

D. Discussion

This study confirms the well established in vivo effects of Methotrexate on the bone marrow and gastrointestinal epithelium.

In all of the drug-infused animals except SIII, there was loss of gut epithelial cells, with a few of the basal epithelial cells remaining. These remaining cells were larger than normal, with pleomorphic hyperchromatic nuclei and prominent nucleoli. These changes were not seen in SIII which died on day 1 having received a high dose of the drug for less than one day. In SIX, which died after 4.7 days of infusion, the epithelial cell loss and the abnormalities of the remaining cells were well established. Thus the loss of epithelium probably occurred between days 1 and 4 in these animals. This would be within the estimated time of regeneration of the gut epithelium (Leblond and Messier, 1958), and is in agreement with the observation that the effects of Methotrexate are due to the prevention of multiplication of cells, and not due to direct toxic action of the drug itself.

Generally similar effects to those described above were observed in the gastrointestinal systems of rats after oral administration of 4-amino-pteroylglutamic acid (Philips and Thiersch, 1949); and in mice after oral administration of Aminopterin (Jacobson, 1954). In 1962, Trier noted a reduction in the mitotic activity of the gut epithelium of humans within 3 hours after 2-5 mg. of Methotrexate given intravenously. Trier also noted that despite marked reduction in mitotic activity, gross changes were few in the gut after the administration of

Methotrexate. This coincides with the scant gross changes seen in the proximal gut of these animals, despite almost complete denudation of the epithelium.

Jacobson (1954) claimed that the tetrahydrofolates were required for division of the chromosome halves during the metaphase of mitosis, where in the presence of folic acid antagonism cell division was arrested. He further stated that, even in the presence of the folic acid antagonists, a cell could enter into mitosis, be arrested in the metaphase and, without dividing, reconstruct a resting nucleus which was larger than the original cell. Trier did not notice the abnormally large cells in his study of the short-term effects of Methotrexate on the gut epithelium, even when there was complete mitotic arrest, nor did he see apparent arrest at the metaphase stage. Since there was evidence that desoxyribonucleic acid (DNA) synthesis occurred in the premitotic period, Trier suggested that the actual interference with mitotic inhibition was in this earlier DNA-synthetic phase. Electron microscopy of these human gut epithelial cells revealed dilatation of the cisternae of the endoplasmic reticulum and mitochondrial fragmentation. These changes were reversible to normal within 96 hours after discontinuation of Methotrexate administration.

In the present series, the abnormally large cells were frequently seen and mitotic activity was not seen in the remaining basal epithelial cells. Further, pyknotic nuclear remnants in enlarged cells having eosinophilic cytoplasm, as described by Trier, were frequently seen. Regardless as to whether the

interference with the cell division occurs in the premitotic or metaphase stages, an explanation for these large cells has been offered by O'Brien (1963). According to him, there is a greater reduction of production of desoxyribonucleic acid than of ribonucleic acid (RNA) in folic acid deficiency due to folate antagonism, and thus the processes of cellular division are inhibited relative to the processes of growth of the cell. Even if cell division is prohibited, cytoplasmic protein can thus increase even in the face of folate antagonism.

It is known that Methotrexate can be stored in the gut (Werkheiser, 1963). The finding of a yellow coloration of the gut and mesentery similar to the color of the infusate, compared with the normal coloration of these organs in the control animals, may confirm this. Since this yellow coloration was present in all of the drug-infused animals, regardless of the drug dosage, there is a suggestion that the gut mucosa of all of the infused animals was saturated with Methotrexate. This coincides with the observation that there was little apparent histological difference seen in the gut mucosa regardless of the dose of Methotrexate administered. Philips and Thiersch (1949) found that the course of lethal intoxication was not altered using doses ten times the medial lethal dose ($L.D._{50}$) in folate antagonism.

Various parts of the gut were affected to different degrees for reasons which are unclear. This may be due to a differential in the rate of regeneration of the epithelium of these different members, but this is not known. Respecting decreasing severity of epithelial cell loss, the order was as follows: colon, small intestine, esophagus, and stomach.

Even in the most severely damaged colon the neurones of the myenteric plexus were histologically normal. The tissues of the adventitia, muscularis and submucosa of the gut were unaffected aside from some secondary changes such as edema and inflammatory cell infiltration.

The effects of the drug on the bone marrow resulted in marked suppression of mitotic activity and, in high doses, generalized hypoplasia of the marrow. The cells dependent on mitotic activity were reduced in number, while the primitive precursors remained. This confirmed the antimitotic effects of the drug on the bone marrow.

The megakaryocytes seemed extremely sensitive to the drug, since they were rarely seen in the drug-infused animals. The granulocytic series of the marrow was early and severely affected. In the low-dose infusates there was decrease in maturation, while in high-dose infusates there was complete maturation arrest. Although marrow counts were not done in this series, the decreased maturation of cells did not appear to be as severe in the erythroid series as in the myeloid series for animals on the low and intermediate doses of the drug. In animals on high drug doses there was maturation arrest in both erythropoiesis and granulopoiesis. This is in accord with the observations by Franklin et al. (1947a) of the effects of folic acid antagonism on the bone marrow of rats, and later (1949) of Methotrexate in rats. Similar observations have been made of the effects of Methotrexate on human bone marrow (Farber et al. 1948; Li et al. 1958).

In the present series the bone marrow suppression was reflected peripherally by granulocytopenia, thrombocytopenia and, later, anemia. Similar observations have been described in rats receiving folic acid antagonists (Franklin et al, 1949; Philips and Thiersch, 1949) and in humans treated with Methotrexate (Farber et al. 1948; Li at el. 1958). Although the diminution of erythropoiesis paraleled the decrease in myelopoiesis with high drug doses, the drop in the peripheral hemoglobin was less striking than the peripheral decrease of white blood cells. This may be because the erythrocyte lives for about thirty times as long as the granulocyte (Keele and Neil, 1961), accordingly the destruction of the precursors of both in the marrow would take longer to be reflected peripherally in the erythrocyte series.

Although a few mitotic figures were seen in the bone marrow, this does not mean that the cells would actually divide, since they may have been arrested in the metaphase (Jacobson, 1954). The explanation for the abnormally large reticulum cells seen in the marrow, and for the increased mean corpuscular hemoglobin concentration of the erythrocytes, could also be due to the relatively greater decrease in DNA production than RNA, thus inhibiting division of cells but allowing continuing growth of the cytoplasm.

Structural changes in the lymphoid tissues were seen in animals on high doses of the drug. The white pulp of the spleen became less conspicuous than in the normal animals, together with atrophy of the germinal centers. Similar changes were seen in

the lymph nodes, in which the structure was altered; and in the Peyer's patches of the gut, which became atrophic. These changes were less severe in the animals on lower doses of Methotrexate. Histologically, in drug infused animals, few immature lymphocytes were seen in the lymphoid tissues and those remaining were mainly mature forms; hence the production of lymphocytes was curtailed. In the peripheral blood, these changes were reflected by an absolute decrease in lymphocytes, with a relative lymphocytosis. Since both the granulocytes and lymphocytes live less than a couple of days (Keele and Neil, 1961) this suggests that the lymphatic tissues are less sensitive to Methotrexate than the myeloid tissues. The effects on the lymphatic tissues, and the lesser sensitivity of the lymphatic tissues to folic acid antagonists than the myeloid series, was described by Franklin et al. (1947), and Philips and Thiersch in 1949.

The reason for the absolute lymphocytosis seen in the control animals is not clear; it may be related to the administration of Tham, however, since this may increase the white blood count when given chronically (Nash, 1969).

Effects of the drug on the integument of the animals were minimal. The buccopharyngeal ulceration reported by Schoenbach et al. (1952), and Li et al. (1958), when Methotrexate was given orally or intramuscularly or into the external carotid artery (Sullivan et al. 1959) were not noted in these animals. On about day 3 to day 5, however, a band of periorbital erythema was noted around the right eye, presumably a drug effect on the skin via the right ophthalmic artery. This coincided with the

pattern of fluorescence seen by Watkins and Sullivan (1964) after Fluorescein injection into the right internal carotid artery. This confirmed that the drug was in fact being delivered to the brain, and heralded more severe signs of systemic toxicity. It was also noted recently on the fourth day of infusion in a human being treated by intra-carotid Methotrexate for a brain tumor in our hospital (Branch, 1964).

The drug infused animals became anorexic in a few days, and then quite lethargic. These effects were probably due to a number of factors. Folic acid deficiency causes anorexia per se (Beckman, 1958), and so does the administration of Methotrexate (Li et al. 1958). Damage to the gastrointestinal tract, with denudation of the epithelium and bleeding into the gut would aggravate this anorexia. The lethargy is probably due to a combination of anemia, fluid and electrolyte loss via the gut, and lack of food absorption, due to anorexia and damage to the gut epithelium. This combination of factors, plus severe agranulocytosis with superadded infection may have contributed to the animals' early death.

This brings us to a consideration of the lethal dose of Methotrexate for the rhesus monkey. The acute medial lethal dose ($L.D._{50}$) is variable for different species. For example, in rats it is 15 mg. / kg. / 24 hrs., while in mice it is 89 mg. / kg. / 24 hrs. (Werkheiser, 1963). When Methotrexate is given continuously intravenously the toxic effects are markedly increased, and when given continually intra-arterially, the toxic drug effects are increased tenfold (Sullivan et al. 1959). Despite the protective effect of concomitant intramuscular citrovorum factor, the

continuous administration of 50 mg. Methotrexate / adult human / 24 hrs. can produce dangerous toxicity in a few days (Sullivan et al. 1959). Without citrovorum factor, if the drug is given continually intra-arterially, the human may die due to toxic effects from a dosage of 10 m. / kg. / 24 hrs. in a few days (Westbury, 1962). Assuming that the weight of the average adult is about 70 kg., this may put the lethal dose in humans at less than 0.14 mg. / kg./24 hrs. With these considerations in mind it was predicted at the outset that the lowest dose chosen for this group of animals (1 mg. / kg. / 24 hrs.) would be a supra-lethal dose, far above the chronic $L.D._{50}$ for the rhesus monkey.

This prediction proved correct and all the drug infused animals (except SIII) died obviously due to drug toxicity, even at the lowest dose chosen. It follows that the chronic $L.D._{50}$ for the rhesus monkey during continual intra-arterial infusion with Methotrexate is less than 1 mg. / kg. / 24 hrs. Even this smallest dose used is larger than any therapeutic dose one would consider on a weight basis for the human, while the intermediate and high doses used in this study are vastly supralethal.

Wilson et al. in 1942 commented on the abortive leucocyte response to infection seen in monkeys on vitamin B deficient diets. Philips and Thiersch in 1949 noted numerous infections in rats receiving Aminopterin. Infections have also been noted in humans receiving Methotrexate therapy for malignant disease (Schoenbach et al. 1952; Duff et al. 1961; and Westbury et al. 1962). In our series, acute fulminating infections, either bacterial or fungal, were seen in eight out of the thirteen drug infused animals.

The agranulocytosis in the animals undoubtedly predisposed to this high infection rate; moreover, the lymphatic system and consequently antibody production were curtailed. Further, Methotrexate itself is a very powerful antibody inhibitor per se (Brandriss, 1963). These effects could account for the disproportionately high rate of infections in the animals.

There was, as previously described (see Incidental findings) a high rate of parasitic infestation in the animals as well. Since these animals appeared healthy on examination at the onset of the experiments, and since the types of infestation seen are endemic in the natural habitat of the animals, it was felt that this added parameter did not affect the experimental results.

A number of pathological changes were noted in the blood vessels of the animals. The sclerotic changes and fibrinoid necrosis seen in the mesenteric vessels of SVII, SIX, and SXV, all occurred in regions of marked parasitic infestation. These changes may have represented an irritative of hypersensitivity reaction of the vessels to nearby parasites.

Of more importance, we must concern ourselves with the frequency of thrombotic changes observed in the right carotid system. Six of the twelve animals in which the carotid system was studied, had some sort of thrombotic complication of either the right common carotid, the right internal carotid artery, or both. This occurred in both the drug infused and control animals. Thrombotic complications have also been noted in humans subsequent to catheter placement in an artery during infusion chemotherapy (Sullivan et al. 1953; Clarkson and Lawrence, 1961; Branch, 1964).

Thrombotic complications were noted regardless of the drug dosage, and whether the infusate was buffered or not. The common feature was that all animals in which thrombosis occurred had a catheter in their right common carotid artery. The catheter was 1/4 to 1/3 the diameter of the vessel which contained it. Many of the thrombi appeared, histologically, to have started around the catheter in the common carotid artery. In the histological sections the thrombus was peeled away from the wall of the artery, accounting for the patency of the carotid system found in each instance on injection of water via the catheter.

These thrombi were so recent and soft that they were recognized grossly in only two of the animals. All of this may be a good argument for the addition of an anticoagulant to the infusate as advocated by Watkins and Sullivan (1964), and the usage of siliconized catheters.

This brings us to the most important aspect of this study, that is, the effects of the infusion method and Methotrexate on the central nervous system.

When brain swelling was noted in some of the first animals infused, it was thought that cerebral water content studies might prove useful. Definitive conclusions cannot be reached, however, due to the lack of a properly established normal value for the dry weight of rhesus monkey white matter and cortex, and because the sampling was not done immediately after death.

In all of the animals there was no apparent difference in the dry weight between the right and left hemispheres for either cortex or white matter. This suggested that changes in the cerebral water content were unrelated to the site of

Methotrexate infusion and the dosage of drug administered. This cannot be definitely proven since in the rhesus monkey the pericallosal artery is single (Campbell and Foster, 1944), and thus the left frontal lobe may have received almost as much Methotrexate as the right frontal lobe.

There appears, however, to be a relation between the cortical water content and the time of sampling after death. Edstrom and Essex (1955) showed that even after decapitation of an animal there was an increase of 4% of the original brain weight in the first thirty minutes, while after this, absorption of fluid continued more slowly for several hours. Furthermore, they stated that no conclusion regarding increased water content of the brain in the living state could be drawn from post-mortem findings unless fluid absorption was taken into account. The present study confirms this observation, since the cortical dry weights of animals SVIII, SIX and SXII (autopsied 14, 9, and 9 hours after death) were below the accepted normal values for the cat, while the remaining animals (autopsied from one to 4 hours after death) had cortical dry weights falling within the accepted range. The same trend was not seen in the white matter, suggesting that this post-mortem uptake of water may have been mainly cortical.

The drug infused animals receiving Tham all had dry weights of the white matter considerably below the control animals, one of which received Tham, and SXIII which received Methotrexate without Tham. It is thus possible that the simultaneous administration of Methotrexate and Tham resulted in a decreased dry weight of the white matter.

For both the white and gray matter in the drug infused animals there was no apparent correlation between the water content and the dosage of the drug administered. However, if there truly is edema of the brain due to this combination of drugs, this study suggests that it is equal in both sides of the brain, and mainly in the white matter.

Accumulation of edema fluid in the white matter has been observed in association with a variety of lesions resulting in damage to the cerebral blood vessels (Pappius and Gulati, 1963). Triethyltin poisoning can also result in edema of the white matter(Katzman et al. 1963). Whether Methotrexate which has been buffered with Tham results in fluid uptake by the white matter, due to a metabolic effect such as in triethyltin poisoning, or whether this apparent uptake of fluid by the white matter is due to submicroscopic damage to the vessels cannot be established from this study.

Regarding the Methotrexate content of the brain and the blood, there is good correlation between the observed results and the results expected clinically, despite the fact that the values were obtained indirectly using a bio-assay method. This method has not been described in the literature.

Methotrexate is stored in high concentration in the liver and kidney (Bertino, 1963) and to a lesser extent in the gut and bone marrow as well (Werkheiser, 1963). Reports that it may be stored in the brain have not been seen in the literature. According to Werkheiser (1961), Methotrexate is bound by some mouse tissues for as long as eight months or more. In animal SVI

which received an intermediate dose of Methotrexate for less than one day, then pulled out his catheter, and survived the experiment, there were still appreciable amounts of Methotrexate in the brain. This monkey was sacrificed 8 months after the experimental procedure. There was no detectable Methotrexate in the blood, but instead there was an appreciable level of folic acid. This level of folic acid was only half as high as the blood folic acid levels in the control animals. SVI still had a marked leucopenia at the time of sacrifice. This may be of some significance, since Bertino (1963) claims that Methotrexate may remain in some human tissues for months and thus may continue to inactivate dihydrofolate reductase for weeks.

Blood samples for Methotrexate assay were usually taken within a few minutes after death by cardiac puncture. Animal SIX had samples taken 5 hours after death. All of the drug infused animals had Methotrexate activity in the blood, except SVI. In fact the control animals and SVI had folic acid activity in the blood. There appeared to be a direct relationship between the amount of Methotrexate in the blood and the drug concentration of the infusate. Since a blood sample taken on day 7 showed a higher Methotrexate content than a sample taken on day 4, in SVII, there may be a relationship between the length of time of infusion and the blood concentration. There is support for these findings in the literature (Bertino, 1963; Sullivan, 1962).

It is not possible to draw any conclusions regarding a difference in blood levels for animals whether on Tham or not since the number of observations are few. One would not expect Tham to modify the blood Methotrexate levels if the Methotrexate

is carried in the serum, since the infused drug is simply poured into the blood, whether buffered or not. In this instance the blood would act as a vehicle. The drug level might be altered secondarily if pH changes were to induce faster renal excretion of the drug. Methotrexate may, however, be carried by the formed elements of the blood (Bertino, 1963; Cooper, 1964) and, if this is so, buffering may change the cell Methotrexate content. In this study the blood was hemolyzed and the Methotrexate content of the whole blood was studied.

The animals on high doses of Methotrexate apparently had a higher brain content of Methotrexate than animals on the lower doses. All of the animals on the high drug doses had a higher right-sided brain Methotrexate content than on the left side, except SII. This animal's right internal carotid thrombosis may have resulted in a higher concentration of Methotrexate flowing to the left side of the brain. The comparison of Methotrexate contents in the two cerebral hemispheres of the same animal may lack validity, since, as previously mentioned, the pericallosal artery in the rhesus monkey is single. Thus, blood from the right carotid system may have carried some Methotrexate into the part of the left frontal lobe from which samples were taken.

According to Bertino (1963) most tissues can only bind a limited amount of Methotrexate during a constant length of time regardless of the dosage of the drug. There is, however, a statistically significant correlation between the dose of Methotrexate infused in this series and the brain content of Methotrexate when one considers both sides of the brain in the animals.

(r = 0.46; 0.05 is greater than p is greater than 0.02). This could be a result of the sustained high blood levels resulting from the continuous administration of the drug.

Animals SI and SII (both infused for 5 days) had greater brain Methotrexate uptakes than SIII (infused for less than one day). All were done under the same conditions, and all had right cerebral infarcts. Similarly SX (infused for 8.2 days) had a higher uptake than SXI (infused for 7.3 days). However, animals SVIII (infused for 2.5 days) and SIX (infused for 4.7 days) infused under the same conditions, had similar uptakes. There may thus be a correlation between the length of time of infusion and brain uptake. This is certainly true for other tissues (Sullivan, 1962), although since tissues can only pick up a given amount of Methotrexate in a given time, regardless of the dose (Bertino, 1963), this might be hard to prove if the brain cell production of dihydrofolate reductase were slow.

There is an apparent correlation of uptake and brain pathology. SI and SII (with brain infarcts) had much higher brain uptakes than SXIII (no infarcts) done under the same conditions. SIII (with infarcts) had a brain uptake in the same range as SXIII, although the respective infusion periods were 0.8 and 6.6 days. On the other hand, SV with a few small infarcts, but on an intermediate drug dose, had a relatively small brain uptake. There is thus a suggestion that brain infarcts (and disruption of the blood-brain barrier) may allow greater uptake of Methotrexate. This suggestion is guarded, since if it were assumed that the highest uptake of Methotrexate were in the region of the infarcts,

and knowing that it is bound to dihydrofolate reductase (an enzyme found in living cells) it may have to be bound in another way to account for this high uptake. The other factor that comes to mind is that around the region of an infarct some of the cells are viable. If the blood-brain barrier were compromised, Methotrexate might be able to reach these viable cells by diffusion or some other process with greater facility for binding with dihydrofolate reductase. This high uptake cannot be accounted for by increased blood in the infarcted brain tissues, since the infarcts were pale ones.

The other factor that must be considered is the effect either of Tham, or of pH itself on the Methotrexate uptake. Despite the powerful blood-buffer system, it is possible that the pH of blood in Tham-buffered infusates may have been different from those without Tham. Tham itself may have some effect upon the brain uptake of Methotrexate. In considering the animals on the high doses, there appeared to be a greater uptake of brain Methotrexate for unbuffered infusates than for buffered infusates. Thus, SI, SII, and SXIII (unbuffered) apparently had higher uptakes than animals SVIII and SIX (buffered).

Without buffering the infusates, the pH's were as follows: For a dose of 20 mg. / kg. / 24 hrs. - pH 7.7;

" " 10 mg. / kg. / q4 hrs. - pH 6.4;

" " 1 mg. / kg. / 24 hrs. - pH 6.1;

Tham buffered infusates ranged from pH 7.1 to 7.4.

Bertino (1963) found that leucocytic dihydrofolate reductase had a pH optimum of 8.3, with a second small optimum at

pH 5.5. However, the maximum inhibition of the enzyme system by a fixed amount of Methotrexate occurred at a pH of 5.9. Furthermore, dialysis of the Methotrexate-enzyme complex at pH 7.5 with 0.15 M KCl caused almost complete dissociation of the enzyme inhibitor complex, whereas dialysis against Tham buffer at pH 7.5 or against citrate buffer at pH 5.9, with or without 0.15 M KCl did not result in appreciable dissociation of the enzyme inhibitor complex. Thus it appeared that the tightness of the enzyme - Methotrexate binding depended not only on the pH, but on salt concentration as well. In fact, nine times as much inhibition of the enzyme by Methotrexate occurred at pH 5.9 than at pH 7.6. This implied that if the enzyme were functioning in a cell at the pH of 5.9, a much smaller amount of Methotrexate would be required to inhibit a fixed amount of the enzyme than at a pH of 7.6. Since the binding was less tight at the higher pH this implied that less drug might remain fixed to the tissues at a higher pH, and higher intracellular concentrations of the Methotrexate might be difficult to attain unless the drug were given continuously (Bertino, 1963).

In these monkeys it was not possible to measure the <u>in vivo</u> pH of the cerebral tissues, and enzyme-Methotrexate dissociation was not studied as such. Moreover, the pH of the infusate did not necessarily reflect the pH of the inside of the cells in the brain, unless of course the cells were damaged.

In this series the highest brain Methotrexate contents were recorded in the high dose animals, where the pH of the infusate was the highest (7.7). If the enzyme-Methotrexate complex in our animals followed the rules established by Bertino (1963) one would

have expected to find the greatest uptakes in the animals on controlled pH's, and SIV who had a low infusion pH to have relatively high uptakes, since these animals were infused at pH's nearer the optimal binding pH of the Methotrexate by dihydrofolate reductase. This is not true, however, (Fig. 56) and several reasons come to mind why it is not so:

1) It has been shown that the brain uptake of Methotrexate in this series is related to the dose of Methotrexate infused. The drug here was given continuously, and the effects of the constant bombardment of the enzyme at a pH of 7.7, may, with time, in the high dose infusates have resulted in higher Methotrexate uptakes than in animals on a lower dose infused at a more optimal pH from the standpoint of enzyme-Methotrexate binding.

2) The infusate when buffered contained Tham plus NaCl and KCl in physiological concentrations. Bertino (1963) commented upon the inability of 0.15 M KCl to dissociate the enzyme-inhibitor complex at pH 7.5 when the system was buffered with Tham, whereas at this same pH the 0.15 M KCl readily dissociated the complex. In the animals with Tham buffers, the infusion pH's ranged from 7.1 to 7.5. It is thus possible that Tham, being very stable, remained in the blood of the brain samples in high enough concentration to inhibit the dissociation of the Methotrexate to be assayed from any dihydrofolate reductase remaining in the samples. This would tend to make the values of the brain Methotrexate assays low in animals on Tham-buffered infusates.

3) The pH of the blood does not necessarily reflect the pH of the cells inside the brain. The pH's of the infusates in

the animals with the high brain uptakes of Methotrexate were in the range of 7.7. This is alkaline and unphysiological. In such a milieu, to remain in the physiological range of pH, the cells of the brain would tend to rid themselves of bicarbonate ion, secondarily lowering the pH. This may have resulted in bringing the pH's of the brain cells in the high dose unbuffered infusates near 5.9, resulting in firm binding of the enzyme-Methotrexate complex, with a high brain uptake. In an animal on an unbuffered, intermediate dose infusate, such as SIV, this phenomenon would not be expected to occur, and perhaps since the pH of this infusate was near 6.4, the cells of the brain would tend to be in a more alkaline environment, which would decrease enzyme-Methotrexate binding. Also, in the animals having infusates buffered within the physiological range, this lowering of the pH of the brain cells would not be expected to occur. Thus, if this were true, one would expect to find Methotrexate uptakes in the brain of the animals on intermediate doses to be similar, whether the infusate was buffered or not. Such values are, in fact, similar (Fig. 56).

4) Another possible explanation is that all of the complex variables above have no bearing here, and that the high uptakes in SI and SII relate to a simpler physical breakdown of the blood brain barrier.

5) Our bio-assays were carried out at a pH of 6.1, where binding is almost maximal. It could be that in the animals SI and SII, the Methotrexate was pooled in infarcted areas where no dihydrofolate reductase remained. Thus all of the Methotrexate here would be available for assay. In the animals done without

infarcts, any remaining dihydrofolate reductase in the assay sample would firmly bind Methotrexate (Werkheiser, 1961). This may have given a false low reading of Methotrexate in the assays on uninfarcted brain samples.

6) Perhaps the Methotrexate readings in the brain samples come from the blood contained in the brain, and the brain tissue actually contains no Methotrexate. In order to rule this out, let us assume that the weights of a ml. of either brain or blood are about the same. Wolfe (1964) says that the weight of the blood in one gram of brain comprises about 2% to 5% of the total. Take as an example SI. In one gram of brain there would be about 50 mg. of blood (i.e., 5% of 1 gram). Each ml. of blood in SI has about 500 ug. of folic acid inhibition by Methotrexate. Thus 50 mg. of blood has $\frac{50}{1000}$ X 500 equals about 25 ug. of folic acid inhibition. However, one gram of the brain of SI had 156 ug. of folic acid inhibition on the right side, and 104 ug. of folic acid inhibition on the left side. Thus in one gram of this animal's brain, there is more folic acid inhibition than could be accounted for by that of the Methotrexate in the contained blood.

By similar calculations the brain Methotrexate contents found in SII, SVI, SX, SXI, SXIV, and SXV are not accounted for by the Methotrexate in the blood. Since blood samples are unavailable for the animals SIII, SV, SVIII, and SXIII this calculation cannot be made, and the values are uncertain. In SIV, the values for the blood contained in the brain, and the brain values themselves, are almost equal. Thus, out of the 12 test animals for which there are brain Methotrexate values, it can only be stated

with certainty in six, that the readings cannot be accounted for by Methotrexate in the contained blood.

Probably some of the other animals for which this calculation cannot be made have valid readings of the brain Methotrexate content as well, for the following reasons:

1) SVIII died 3.5 days after the infusion was stopped. Freeman-Narrod (1962) stated that even after large doses of Methotrexate, given parenterally, the blood levels were almost nil after a few hours. It is therefore unlikely that there would have been any blood Methotrexate left in the brain samples to give a false reading in this animal.

2) Animals SI and SII which had the highest uptakes of brain Methotrexate had the lowest amounts of blood in the brain since the infarcts were pale, and not hemorrhagic. This suggests that the brain uptake values in these animals are valid.

In considering only the values of brain uptakes which cannot be accounted for by the contained blood, the following observations are probably still valid:

1) That the content of Methotrexate in the brain and blood apparently varies with the dose of the drug infused.

2) The content of Methotrexate in the blood, and possibly in the brain, varies with the length of the period of infusion.

3) Brain pathology may account for an increased uptake of brain Methotrexate.

4) Methotrexate can be retained in the brain for a long period of time. It must be very stable <u>in vivo</u>, since several of the brain and blood assays were repeated several months after they were first done, having been kept at 20° centigrade. There

was little difference in the values when they were repeated.

All of the animals had a slight tongue protrusion to the right, as a consequence of cutting the right hypoglossal nerve during the surgical procedure. Some animals had a right Horner's syndrome and/or a right peripheral facial paresis . These signs diminished with time, and since they were all on the operative side, it follows that they were probably the result of the operative technique. The right Horner's syndrome could have come from the dissection of the carotids. The right peripheral facial paresis was probably produced during the passage of the catheter from the upper to the lower incision through the region of the right parotid gland.

There were, however, neurological signs which cannot so readily be blamed on the operation. Four of the animals (SI, SII, SIII, and SV) had areas of brain destruction having the histological appearance of recent infarcts. It would be tempting to say that these lesions were due to technical problems in the infusion, since they occurred in the first five animals done. It remains then to try and elucidate some of these technical problems.

The lesions were seen in the region of distribution of the right internal carotid artery in each instance. Rarely, they occurred on the medial aspect of the left hemisphere as well (SIII and SV). This is still in the region of supply of the right carotid system, since in the rhesus monkey the pericallosal artery is single and receives blood from each carotid. This artery supplies a variable portion of the mesial frontal lobe, plus some of the medial dorsolateral frontal surface on each side of the brain (Campbell and Foster, 1944).

Some of the parameters to be considered in the production of these infarcts are as follows: Methotrexate, Tham, air bubbles, emboli, carotid clamping, and drugs put into the arterial system.

When SIII went into shock, Polybrene and Ephedrine were each injected into the animal's right internal carotid artery. This animal died with meningitis, and had the most severe infarcts seen in the entire series of animals. One of these drugs neutralizes the effects of Heparin and enhances clotting of blood. The other drug is a vasoconstrictor: these drugs alone may explain the pathology seen in SIII, but this is not proven.

It would be difficult to implicate carotid clamping in the production of these infarcts. In some animals the EEG gave evidence of brain ischemia after carotid clamping, but in a few minutes the tracing returned to normal. Some of the animals with no brain pathology had the carotids clamped for a much longer period of time than the animals which had infarcts. If the damage were due to the carotid clamping alone, one would not have expected infarcts to be on the left side, since the carotid artery from the left side, which in all of these animals was patent, should have given adequate collateral circulation.

Some of the animals had seizures. From our experience in this hospital with a large number of epilepsy patients, the production of these infarcts as a consequence of the seizures is unthinkable.

Could the brain lesions have been due to the Methotrexate itself? This also is unlikely since, for example, in SI the brain levels of the Methotrexate in both frontal poles was higher than those seen in other animals who had infarcts. It follows that if

the Methotrexate alone were responsible for the infarcts, there should have been infarcts in SI's left hemisphere, while in fact there were none. It is moreover unlikely that Methotrexate produced the seizures per se since many of the test animals had neither seizures nor any epileptiform abnormality, even on the high doses.

Could Tham alone have been responsible for the infarcts? This is also unlikely since the control animals which were on the same dosage of Tham as the test animals had no infarcts. Besides, infarcts occurred only in one animal on Tham, while the most severe infarcts were seen in three of the animals not on Tham. It has been noted that Tham, in large doses, may sometimes predispose to seizures (Nash, 1964) but, again, the most severe seizures occurred in the animals that were not on Tham.

Furthermore, it is not likely that the air bubbles seen entering the carotid system were alone responsible for the infarcts. In one control animal (SXV) numerous air bubbles were injected into the right carotid system, causing transient neurological signs (see Appendix), but without the production of infarcts. These air bubbles were much larger than those seen in any of the test animals.

In view of the great frequency of thrombotic complications in the carotid system on the side of the infusion, one wonders about the possibility of embolic phenomena causing the infarcts. No emboli were seen in any of the 225 giant sections studied of the brains of these animals.

Although the severe brain pathology occurred in animals

on the unbuffered infusates with a pH of 7.7, SV, in which the pH was controlled to within physiological limits, also had a few small infarcts.

It becomes apparent that there were probably multiple interacting factors responsible for the brain infarcts. The factors most common to all of the animals with the severe brain pathology were a high drug concentration, air bubbles, and the pH of the infusate. Air bubbles were not specifically noted in animal SI, but not as much significance was placed on them at first as in the later animals, and this animal may have had some. One wonders if the triad of Methotrexate in high concentration, inadequate pH control of the infusate, and air bubbles, may have been in part or totally responsible for the lesions.

A highly speculative suggestion is offered, that in the presence of high concentrations of the drug, in the absence of adequate control of the pH of the infusate, the brain might in some way be damaged or sensitized. A tiny, noxious stimulus such as an air bubble may, in such a state, precipitate a seizure without infarction. If the sensitization or damage is greater, this noxious stimulus, such as an air bubble or small embolus, may lead to infarction with or without generalized seizures. In support of this the following is offered:

1) Three out of the first five animals without controlled pH had cerebral infarcts. Only one of the animals on the controlled pH of the infusate had infarcts, and these were small in degree when compared with the animals in the first group.

2) Of the four animals which had seizures, three were not

on controlled pH while one was. Two of the animals which were not on the controlled pH had status epilepticus.

3) Of the four animals who had seizures, three had cerebral infarcts.

4) In three of the animals who were previously seizure free, there was a definite cause and effect relationship between an air bubble seen entering the right carotid system, and the appearance of the first seizure. Furthermore, two of the animals had only one seizure each shortly after the entry into their carotids of one air bubble each.

5) Animal SXIII done later in the series on a high dose of Methotrexate had neither pH control nor air bubbles, nor did he have seizures or infarcts.

6) Three of the animals while undergoing surgery on day 0 got small air bubbles into the right carotid system before the drug was started, and these animals had neither infarcts nor seizures.

7) Air bubbles many times greater than those affecting the test animals were deliberately injected into the right carotid system of control SXV, which had a pH controlled infusate, but these caused neither infarcts nor seizures.

From this study no definitive conclusions regarding the causation of the lesions in the brain can be reached. Once the probable importance of the role of the air bubbles was realized, special precautions were taken with the assembly to prevent these, and no further trouble was seen in the remaining ten animals when on high, intermediate, or low doses of Methotrexate. Even when the

high doses were later repeated in two of the animals, with and without pH control, no brain pathology was seen. Of interest, Sullivan et al. (1959), and Balla et al. (1962) have reported the deaths, due to air embolization, of humans on Methotrexate infused via one internal carotid artery.

The neurological signs seen in the animals with the brain pathology (see Neurological signs) were probably due to the infarcts. The complete right ophthalmoplegia seen in animal SXIV may have come from thrombosis of the whole right carotid system including the ophthalmic artery.

Tumors of the brain probably interfere with the "blood-brain barrier." In this regard it is not known whether buffering the infusate with Tham should be recommended or not, due to the possible greater uptake of diseased areas of the brain when it is not used, although there may be complications if the pH is not controlled. It is probable that if air bubbles could be technically prevented, it would be safe not to buffer the infusate with Tham, especially in the relatively low doses of Methotrexate used in the human.

Thus the original hypothesis proposed may not be entirely correct when considering the effects of gigantic doses of Methotrexate on the brain. However, in the animals on the low doses, which are still many times higher than those that would be considered in human therapy, there was no gross evidence of histological damage to the central nervous system.

It would appear that the dangers inherent in using this method of therapy are a greater risk to the central nervous system

than the drug itself. There is a steady decrease in the amount of pathology to the central nervous system seen in those animals with decreasing drug dosage until, in the low dose infusates, no brain pathology is seen, and the animals die due to the systemic hematological and gastrointestinal effects. It is thought that if used in the recommended therapeutic dosage, in the absence of certain technical factors, such as air bubbles, Methotrexate could ethically be given via the continuous infusion method for the therapy of inoperable neoplasms of the human brain.

Regarding the usage of this drug, some further studies might be considered:

1) Further experiments should be carried out regarding the simultaneous administration of the drug with intermittent folinic acid, to study the modification the latter drug might have on Methotrexate given by the infusion method.

2) Control specimens for establishing the normal dry weights of specimens of monkey brain might validify the results of the cerebral water study.

3) When a method of assay is perfected, using dihydrofolate reductase (Bertino, 1963), a repetition of the brain assays may clarify some of the results of the brain Methotrexate study.

4) Studies with anticoagulants should be undertaken to try to circumvent the tendency for thrombus formation in the infusion method.

5) If further studies are undertaken in the manner

suggested above, assays of the drug in the tissues, the blood, amd the urine should be done as well.

6) Further studies might be devised regarding the inter-relationships of the drug, pH, and air bubbles.

From the result of such studies a few recommendations can be made:

1) Besides the well established rules for following the blood counts in the human on this therapy, it would be wise to do stool cultures on these patients routinely, and perhaps even consider the use of routine bactericidal antibiotics.

2) The signs of Methotrexate toxicity have been described (see Historical aspects). An early sign of toxicity is the zone of erythema around the right eye. This sign usually heralds more severe toxic manifestations.

3) In order to prevent air bubbles, several bottles of infusate might be hooked up in tandem to prevent any chance of the system emptying. If the infusion system has to be uncoupled, a water-filled syringe should be kept on hand to fill the uncoupled ends of the assembly before rejoining them to ensure that no air bubbles are included in the system.

Summary and Conclusions

In order to study the total effects of the antimetabolic agent Methotrexate on primates, rhesus monkeys were infused continuously via the right carotid system with massive but variable supralethal doses of this drug.

1) The experimental animals died after a period of infusion inversely proportional to the dosage of the drug used.

2) It is confirmed that this drug affects primarily the tissues of the body which are dependent on mitotic activity for maintenance, such as the bone marrow, the gastrointestinal epithelium and the lymphatic tissues. The gastrointestinal system is very sensitive to the effects of this drug, with damages increasing the more distal the portion of gut studied. Little difference in gastrointestinal epithelial damages are noted for ten and twentyfold dosage increases of Methotrexate. Suppression of the bone marrow and lymphatic tissues results in hypoplasia, its degree varying with the dosage of the drug infused. Granulocytopoiesis was most severely suppressed, next erythropoiesis and, finally, lymphocytopoiesis. This was reflected in the peripheral blood by a marked decrease in the number of circulating formed elements.

3) In the drug infused animals toxic signs were observed in the following order - anorexia, periorbital erythema on the side of the infusion, lethargy, apathy, and finally diarrhea, followed by death.

4) The chronic $L.D._{50}$ for the rhesus monkey via the continuous intra-arterial infusion method using Methotrexate is less than 1 mg. /kg. / 24 hrs.

5) This method of administration of the drug resulted in the following complications: thrombotic changes in the vessels containing the infusion catheter, systemic and local bacterial and/or fungal superinfections, air bubbles introduced into the blood stream possibly causing neurological signs, and accidental pulling out of the catheter by the animals.

6) This study confirms the uptake of water by the cerebral tissues after death. It is suggested that this uptake is mainly cortical. The combination of an organic buffer, Tham, and Methotrexate may increase the water content of the cerebral white matter.

7) A bio-assay method for the determination of Methotrexate content of tissues is described. The Methotrexate content of the blood appears to vary with the dose of the drug and the length of time of infusion. The brain can take up Methotrexate and hold it for several months. The brain content of Methotrexate varies with the infusion dosage and possibly with the length of time of infusion. It is suggested that even after some months this stored Methotrexate may continue to suppress the peripheral white blood count. The brain content of Methotrexate is apparently dependent also upon pH. Pathological lesions of the brain, such as infarcts, may by unknown mechanisms increase the brain Methotrexate content.

8) Destructive lesions of the brain were seen in 4 of the 13 test animals. This study suggests that the lesions may

have been due to a number of variables, for example, a combination of high Methotrexate concentration, lack of control of the infusion pH, and noxious stimuli such as air bubbles.

9) Further studies are suggested regarding the action of the drug, the prevention of thrombotic infusion complications, and the interrelationship of the parameters causing the brain lesions.

10) Recommendations are made for the prevention of air bubbles in the infusion assembly. The routine use of bactericidal antibiotics may be indicated when this drug is used therapeutically.

11) Provided that some of the infusion complications can be prevented, it is felt that the use of Methotrexate administered in therapeutic dosage by the prescribed method, and with the above precautions, should not harm the normal brain.

It is hoped that the intra-arterial infusion method using antimetabolites may add something useful to the armamentarium of the physician for the treatment of some of the inoperable tumors of the brain which are only temporarily arrested by irradiation therapy.

F. Appendix: Case histories of the animals

The responses of the animals to the experimental procedure varied greatly. In studying the various interrelationships of the parameters, it was essential to consider chronologically the events of the experimental procedure. For this reason and for the sake of completeness it was considered necessary to include the histories of the animals, despite their length, for unfortunately they do not lend well to tabulation.

A history of each of the animals follows, together with the pertinent autopsy findings, and a brief account of the histological observations. In the preparation of this appendix it was considered useful to present the animals in order of decreasing concentrations of the Methotrexate infusions, ending with the controls.

Since the animals lost appreciable amounts of weight during the experimental procedure, the final drug and fluid values were calculated on the basis of the average weight of the animal, this average being calculated from the animal's weight at the beginning of the experiment, and the weight after death.

The animals are presented in the following order: SI, SII, SIII, SXIII, SVIII, SIX, SIV, SV, SVII, SX, SXI, SXII, SVI, SXIV and SXV.

Animal SI.

This 2½-year-old male weighing 4.65 kg. was catheterized on November 13, 1962, as previously described, and an unbuffered infusate calculated to give 20 mg. Methotrexate / 24 hrs. was begun at 3.05 p.m. Baseline, operative and postoperative EEG's were normal. Postoperatively the animal took food and water well orally. He would only sip water until day 2, and thereafter did not eat or drink. He remained alert and very hostile until day 4, when he became quite restless. Urinary output was good throughout the experiment. By day 5 he was lethargic, moved slowly, but tolerated petting his head. On day 5 he defecated large amounts of loose brown stool which were positive for occult blood.

Four hours before death he kept his eyes deviated to the right and had a horizontal nystagmus on right lateral gaze. Later, there were deconjugate eye movements and a left homonymous hemianopsia. Two hours before death a mild left hemiparesis, which gradually progressed to a complete left hemiparesis, was noted. A few minutes before death there was a ptosis of the right eye with a complete ophthalmoplegia, and anopsia. Just before death the EEG of the right side showed low voltage with loss of normal background activity. He died at 6.30 p.m. on day 5, the total length of the infusion having been 5.12 days, during which he received 44 ml. fluid / kg. / 24 hrs., and 18.6 mg. Methotrexate / kg. / 24 hrs.

At autopsy, one hour after death, the animal weighed 4.25 kg. The hair came out easily, and the integument and mucosa were pale. The skull and dura were normal. Aside from

pallor no gross abnormality of the brain or its vessels was seen. On section of the brain there was likewise no gross abnormality.

On examination of the thoracico-abdominal viscera, there was some passive venous congestion of the organs. The gastro-intestinal viscera and mesentery were yellow, the color of the infusate. The mucosa of the gut felt rather thin. No other abnormality was noted. The bone marrow was pale and dry. The catheter was in place in the right common carotid artery, and both carotid systems were found to be patent by inspection and by injection.

On histological examination of the brain, in the cortex of the right frontal lobe, there were small areas of focal destruction of the neuropil (Fig. 46) together with smudginess, eosinophilia and loss of detail of the neurones. The astrocytes in these areas were swollen. There were several of these abnormal cortical areas, mainly in the right middle frontal gyrus (Fig. 45), although one tiny similar area was seen in the left superior frontal gyrus. In the subcortical white matter below the most severely affected gyrus, there were patches of Alzyheimer-like glia. There was one tiny area of neuronal death in the right hippocampus. The brain stem and cerebellum were normal.

The other changes seen were confined to the gastrointestinal tract. The gastric mucosa was well preserved, but at the bases of the gastric glands, some of the epithelial cells were swollen with large hyperchromatic nuclei and prominent nucleoli.

In the small intestine there was almost complete ablation of the mucosa, with largely only the lamina propria remaining (Fig. 19). The remaining villi were flattened and adherent. The remaining epithelial cells tended to be at the bases of the glands, and these cells were large, swollen, with large hyperchromatic nuclei and prominent nucleoli. In the lamina propria there was a chronic inflammatory cell infiltrate composed of eosinophile leucocytes and plasma cells.

In the spleen, the Malpighian corpuscles showed prominent germinal centers, and the reticuloendothelial cells lining the sinusoids and cords of Billroth had pleomorphism of nuclei, with prominent nucleoli and eosinophilia of cytoplasm.

In the bone marrow there was marked hypocellularity, (Fig. 36). There were large numbers of primitive reticulum cells; these were large, with prominent hyperchromatic nuclei. Some were damaged and showed eosinophilia of the cytoplasm. Only a few members of the myeloid series were seen, mainly immature cells with rare remaining mature cells. In the erythroid series, few normoblasts were seen, but mature members were prevalent. There were many examples of erythrophagocytosis. No megakaryocytes were seen. There were clusters of lymphocytes in the marrow. In a few areas there was early proliferation of connective tissue.

Animal SII.

This 2½-year-old rhesus monkey, weighing 5.15 kg. was anesthetized and catheterized on November 27, 1962. The unbuffered infusate (pH 7.8) designed to deliver 20 mg.

Methotrexate / kg. / 24 hrs. in 40 ml. of 5% glucose and water /kg. / 24 hrs. was started at 1.30 p.m. The preoperative EEG showed some slow waves on the right side only. The carotids were clamped for four minutes. After this, there was decreased amplitude of the EEG on the right side, with slow waves and occasional epileptiform-like sharp waves, together with slow waves on the left side. The animal rallied well after surgery, and was awake when put into the chair at 5 p.m. However, fifteen minutes after being upright, he developed large hematomas of both incisions and went into shock, presumably from receiving too much Heparin during the operation. After receiving intra-carotid Polybrene (40 mg.) to neutralize the Heparin, the oozing stopped, but he remained in shock. Shortly thereafter he received 45 mg. of Ephedrine, also by the intracarotid route. His right pupil became dilated and fixed five minutes after the Ephedrine, and there was a left convergent strabismus, together with shallow breathing, rapid pulse, and coma. He remained stuporous to 9 p.m., and then awakened and began taking sips of water. A right Horner's syndrome remained, and a left convergent strabismus. The EEG at 10 p.m. showed low amplitude on the right side, with some residual slow waves. The animal was kept in the recumbent position all that night.

On day 1 the animal was alert, but frightened. He barked, bit and scratched when approached. He moved all extremities, but the right Horner's syndrome remained. On day 2 a very small air bubble went into the right carotid system, and seconds after the animal suddenly turned his head to the right and

developed clonic-tonic movements of the left side of the body, with apnea. The whole episode lasted about 20 seconds. The animal was given sodium Luminal, 15 mg. intraperitoneally, but had a further similar episode at 6.00 p.m. on the same day. He was noted to have a severe cough later on in the day. After the seizures, he was alert after a postictal period of about one minute, but refused to eat or drink following day 2.

On day 3 the animal was noted to have cerebral seizures every 3 to 5 minutes. These were mainly right cerebral, since the head and eyes went to the left, followed by left facial twitching, lifting of the left arm into the air, and then clonic tonic contractions of the left arm and leg. Voiding and excessive salivation were observed during the episodes. There was postictal stupor, and a residual left hemiparesis between seizures. Several of these seizures were recorded electrographically, and they clearly started in the right hemisphere, with spread to the left hemisphere. There was persistence of the electrographic abnormality on the right side, with bilateral slow waves and low voltage bilaterally between attacks. Frequent sharp waves were seen over the right hemisphere. Despite a total of 135 mg. of sodium Luminal given either intraperitoneally or intravenously during the day, and 140 mg. of Dilantin given intraperitoneally, the seizures persisted. Finally, 75 mg. of intravenous Nembutal resulted in a deep sleep, and a seizure-free period of one hour.

On day 4, the animal again persisted in having cerebral seizures, which were generalized, and clearly right cerebral both clinically and electrographically. These continued every

5 minutes despite heavy doses of antiseizure medications. He was conscious between attacks but the right Horner's syndrome persisted, with a profound hemiparesis, hypalgesia, and homonymous hemianopsia, all on the left side.

On day 5 he continued in the same fashion as on day 4 to have seizures, along with low voltage and sharp waves over the right cerebral hemisphere. The animal in the last four days had refused all food and drink, but continued to void regularly. He did not have diarrhea. He died at 5.45 p.m. on day 5 after a total infusion period of 5.1 days, having received 20.1 mg./ kg. Methotrexate / 24 hrs., in 42 ml. of 5% glucose and water /kg. 24 hrs.

During the experiment the animal's hemoglobin dropped from 11.6 gm.% to 4.2 gm.%, and the white count from 16,900 to 2,400 cells / mm^3. There was also a decrease in the number of platelets, polymorphonuclear leucocytes and reticulocytes, leaving the animal with a relative lymphocytosis on the 5th day of 47%.

At autopsy, 1½ hours after death of the animal, he weighed 5.00 kg. The integument and oral mucosa were very pale. The electrode drill placements in the skull were symmetrically placed. The right cerebral hemisphere was tense and swollen, with flattened sulci (Fig. 47). The cerebral blood vessels were grossly normal. The left frontal and right parietal electrodes had penetrated the skull and left tiny holes in the pia. On section of the hemispheres after fixation, there was marked right hemisphere swelling, especially of the right frontal lobe, with

a 3 mm. shift of the midline structures to the left at the level of the mid-thalamus. A small 2 x 3 mm. intracerebral hematoma was noted in the deep cortex and white matter of the left superior parietal lobule.

On examination of the thoracico-abdominal viscera, the middle lobe of the right lung was dark, firm, and sank in the fixative. The stomach was distended with yellow fluid. The mucosa of the gastrointestinal tract felt rather thin. The rest of the viscera were grossly normal. The bone marrow was pale and dry. The catheter was well in place in the right common carotid artery. Both internal carotids were patent by both inspection and injection.

On examination of the histological sections there was increased space between the cells and fibers of the right hemisphere suggesting cerebral edema. On the right side there were rather extensive areas of well-demarcated necrosis. One such area involving half of the right middle frontal gyrus, ended at the subcortical white matter, but was less abruptly defined at its cortical margins (Fig. 48). The central tissue of this area was necrotic, but in the margins there were numerous hypertrophied astrocytes, some binucleate, while others were undergoing degeneration (Fig. 49). In the central area the neurones had disintegrated, while at the periphery some remaining were smudgy and eosinophilic. In the depths of the sulcus between the right middle frontal and right inferior frontal gyri there was also an area of laminar necrosis. A very recent, well defined area of necrosis was seen in the white matter of the

right forceps minor. There were other scattered small areas of necrosis and fenestration of tissues in the fourth and fifth layers of cortex in the right frontal lobe. It is of interest that the polymorphonuclear and macrophage infiltrate that one would expect to see in these areas of necrosis was here strikingly absent. The left hemisphere, brain stem and cerebellum were normal, as were the blood vessels throughout.

On examination of the thoracico-abdominal viscera, the changes in the gastrointestinal, hemopoietic, and lymphatic systems were similar to those described for animal SI. In addition, there was a fulminating bacterial pneumonitis in the upper lobe of the right lung, with a fibrinopurulent pleuritis.

A section of the right internal carotid artery showed occlusion, with recent ante-mortem thrombus. One part of the thrombus had peeled from the wall of the vessel, presumably due to testing for patency by water injection at autopsy.

Animal SIII.

This 3-year-old male, weighing 6.9 kg., was catheterized on January 18, 1963. The carotids were clamped for eleven minutes. This resulted in decreased amplitude in the EEG on the right, with low voltage slow waves. This partially cleared in about an hour, leaving a few random sharp waves on the right, suggesting that clamping of the carotids may have insulted the brain in some way.

The animal was awake by 3.00 p.m. and taking sips of water. At 3.30 p.m.while blood samples were being taken, the catheter blocked and, in unblocking it, a tiny air bubble entered the right

carotid system. The animal immediately uttered a little cry, extended both arms, and fell asleep for a few minutes. He was then well, and taking sips of water until 9.15 p.m. of day 0, when suddenly his head turned to the right, his right arm extended, his eyes blinked, and he voided. Then there were clonic-tonic contractions of the right arm and right leg. Finally, after 40 seconds all activity stopped and the monkey fell into a deep postictical sleep from which he could not be aroused for several minutes.

On day 1 the animal was having generalized seizures, similar to that described above, every 3 to 5 minutes, lasting 35 to 50 seconds. He was comatose between seizures. He received 15 mg. of sodium Luminal and 25 mg. of Dilantin intraperitoneally. An hour later the attacks came only every 15 minutes. Electroencephalographically, the attacks came from the left hemisphere, where there were seen to be multiple spike and wave complexes with spread to the right hemisphere. Postictally there was bilateral low voltage for a while, with persistence on the left side. Despite medications, the animal died at 11.45 a.m. on day 1, after an infusion time of only 0.8 days, having received 17.8 mg. Methotrexate / kg. 24 hrs. in 49 ml. of 5% glucose and water / kg. / 24 hrs. The experimental time was too short to present adequate data for hematological studies.

At autopsy, 1½ hours after death, the animal weighed 6.8 kg. The integument and oral mucosa were pale, but intact. The drill holes for the electrodes were symmetrically placed, and the dura was not penetrated. The brain was tense, with flattening of the

gyri and narrowing of the sulci over the right hemisphere. On section of the brain after fixation, there was a 2 mm. shift of the midline structures to the left at the mid-thalamic level (Fig. 50). The ventricles were slit-like, with the right lateral ventricle almost occluded. The swelling was mainly in the regions of the distribution of the right internal carotid artery. The brain stem and cerebellum were normal.

The thoracic viscera were normal, grossly. The stomach contained some yellow fluid but was grossly normal, as was the rest of the gastrointestinal tract. The marrow was red and greasy. The rest of the abdominal viscera were all grossly normal. The catheter was only ¼" into the right external carotid artery. The carotids were patent by inspection and injection.

On examination of the histological sections prepared from the brain, it was noted that there were numerous discrete and confluent, rather well-demarcated areas of very recent necrosis indiscriminately involving cortex and white matter. One large area involving the entire width of cortex between the right superior and middle frontal gyri ran from the surface to the subcortical white matter. There was also a similar large area in the center of the right frontal centrum semiovale. Further back, the superior gyri of the right frontal and temporal lobes, including cortex and white matter, were a mosaic of tiny irregular but well-defined areas of necrosis, with loss of neurones, and swelling of astrocytes. There was a dearth of inflammatory cells and macrophages in these lesions. There was also one small area of cortical necrosis in the left superior frontal gyrus.

There was an extensive infiltrate of polymorphonuclear leucocytes in the subarachnoid space. especially in the basal cisterns, suggestive of meningitis, though Gram stains showed no microorganisms. Nowhere was there any sign of vessel damage or occlusion. The areas of necrosis were in the area of distribution of the branches of the right internal carotid artery. The brain stem and cerebellum were normal, as were the hippocampi in each hemisphere. The middle lobe of the right lung showed increased cellularity with polymorphonuclear and mononuclear leucocytes in the alveolar septa, together with a partial atelectasis of the alveoli. The changes were those of an interstitial pneumonitis. No other significant pathology was seen in any of the other thoracico-abdominal viscera.

Animal SXIII.

This 4-year-old-male weighing 5.1 kg. was catheterized on October 24, 1963. The infusion, designed to give 20 mg. Methotrexate / kg. / 24 hrs., was begun at 1.45 p.m. The carotids were clamped for 14 minutes. All EEG tracings were normal. His postoperative condition was satisfactory, and he was sitting in his chair, eating, one hour after operation.

The animal remained alert but hostile throughout the experiment until day 5, when he became increasingly lethargic and apathetic. Bowel movements of hard, formed stool were noted on each day to day 5, when he developed severe diarrhea. Voiding was regular and adequate throughout. He ate well, but in decreasing amounts to day 5, after which he refused all food and drink. Neurological signs were not noted until day 5, when

he developed a mild right facial weakness, and in addition, on day 6, some deconjugate eye movements. He died at 6.00 a.m. on day 7, after a total infusion period of 6.6 days, during which he received 13.5 mg. Methotrexate / kg. / 24 hrs. / in 28.1 ml. of 5% glucose and water / kg. / 24 hrs. During the experiment the blood pH went from 7.38 on day 0, to 7.47 on day 5. The hemoglobin went from 11.1 gm. % on day 0, to 8.4 gm. % on day 4, while the white blood count went from 34,500 to 2,200 cells / $mm.^3$ in the same period of time. There was a neutrophile leucopenia on day 4, with a relative lymphocytosis.

At autopsy, 4.5 hours after death, there was a pressure abrasion under the chin. The incision was clean. The animal weighed 4.3 kg. The oral mucosa and skin were pale, and the hair came out easily.

The drill holes for the electrodes on the skull were not symmetrical, those on the right side each being 1 cm. anterior to those on the left side. There were small 0.5 ml. epidural collections of blood under both drill holes on the left, and under the right frontal one. The brain and its major vessels were grossly normal, both freshly, and on section after fixation.

The entire left lung and apex of the right lung had many sharply circumscribed yellow, soft nodules on its external surface and in the fissures. On section these nodules were cystic, containing white glairy fluid. The stomach was grossly normal, but in the rest of the gastrointestinal tract the mucosa was thin, with small submucosal and mucosal bluish, hemorrhagic exudates, particularly in the colon. The catheter was in place

in the right common carotid artery, but this vessel was occluded with ante-mortem thrombus for 2 cm., the upper portion of the occlusion extending to within ½ cm. of the bifurcation of the carotids. The right internal carotid was patent by injection via the catheter, and also by inspection. The bone marrow was pale and dry.

On examination of histological sections of the brain, no abnormality was found.

In the lungs there was focal chronic pneumonitis. Cysts were seen containing tiny, legged parasites, surrounded by an exudate of chronic inflammatory cells and histiocytes, with strands of fibrin running through the exudate. The cysts were probably remnants of bronchioles, since in some areas they were lined by a layer of cuboidal epithelium, which had some of the characteristics of an attenuated respiratory epithelium.

In the gastrointestinal tract, changes similar to those described for SI were seen. In the colon there was a necrotic superficial pseudomembrane with colonies and clusters of Gram positive cocci infiltrating it. The hematological system was also as described for animal SI.

In the mid-portion of the right common carotid artery there was a recent ante-mortem thrombus occluding the vessel. Near the bifurcation, this vessel was partially occluded, with a crescent of patency at the catheter placement site.

Animal SVIII.

This 2-year-old male, weighing 4.7 lbs, was catheterized on April 25, 1963. The infusate, buffered to a pH of 7.25, and

designed to give 20 mg. of Methotrexate / kg. / 24 hrs., in 40 ml. / kg. / 24 hrs., of 5% glucose and water, was begun at 1.45 p.m. The carotids were clamped for 3 minutes, but during this time the right common carotid artery went into spasm. Immediately the previously normal EEG showed slow waves on the right side, with decreased amplitude. This abnormality gradually improved in the next 24 hours, when the EEG was considered normal and remained normal to day 5. The postoperative course was satisfactory aside from development of a neck hematoma amounting to 20 ml. which had to be aspirated. He was sitting in the chair, sipping water, 1½ hours postoperatively.

The animal remained alert and very hostile, trying to bite when approached, until day 3, after which he became progressively more lethargic and apathetic. On day 1 there was a mild right peripheral facial weakness, which then cleared, and he was neurologically intact afterwards until death. He ate well until day 3, after which he refused all food and took only occasional sips of water. He voided regularly throughout, and passed well-formed stool to day 5, when he began to have foul-smelling liquid diarrhea. On day 3 at 8.00 a.m. the monkey was found with his catheter bitten in two. There had been no evidence of bleeding. Since he had received three days of infusion the procedure was not begun again. He was found dead at 3.00 a.m. on day 6. Assuming he may have pulled out his catheter just after he was last seen at 11.00 p.m. on day 2, he received a minimum of 23.5 mg. of Methotrexate / kg. / 24 hrs. in 46.5 ml. of 5% glucose and water /kg. / 24 hrs. The total length of the infusion lasted a minimum of 2.4 days.

At autopsy, 14 days after death, the animal weighed 3.95 kg. There was a small abrasion under the chin. The oral mucosa and skin were pale .

The drill holes in the skull were symmetrically placed, penetrating the dura. There were small insignificant epidural collections in each instance. The left posterior parietal drill hole had penetrated the dura and made a small hole in the left posterior parietal area of the brain. No other gross abnormality of the brain or its major vessels was noted. On section of the brain after fixation there was a shift of the midline structures 1.75 cm. to the left at the level of the head of the caudate nucleus.

There were again many circumscribed soft, yellow areas studding the external surfaces of the lungs, especially at the apices. On section, these were cysts, containing a soft, grumous material. There were numerous hard mediastinal lymph nodes. The stomach contained yellow fluid, but the mucosa was grossly normal. The mucosa of the rest of the gastrointestinal tract was thin, and in the jejunum and ileum there were some small bluish mucosal hemorrhagic areas. The bone marrow was pale and dry. There was no catheter in the right carotid system. Both carotid systems were patent to inspection and injection.

On examination of the histological sections of brain, no abnormality of the vasculature of brain parenchyma was seen.

In the lungs, the previously described cysts containing parasites were again seen, surrounded by areas of cellular exudate, fibrosis, and hemosiderin-laden macrophages. There

were areas of focal atelectasis, emphysema, and interstitial fibrosis. In one area, a parasite was seen in a bronchus.

The basal cells of the gastric mucosa showed changes similar in nature to those described for the other animals. In the small and large intestine there was marked ablation of the mucosal epithelium, the remaining cells showing changes similar to those previously described in the other animals. The splenic Malpighian corpuscles were devoid of germinal centers, containing only mature lymphocytes, and large reticulum cells with hyperchromatic nuclei and prominent nucleoli. The lymph node architecture was ablated (Fig. 29), there being complete disappearance of germinal centers, and hypertrophy of the reticulum cells (Fig. 32). The bone marrow was hypoplastic.

Animal SIX.

This 1½-year-old male, weighing 3.15 kg., was catheterized on May 1, 1963. The carotids were clamped for 10 minutes. There were some right-sided slow waves in the EEG on day 1, which later cleared. The animal awoke shortly postoperatively and was placed in the chair, awake, one hour after surgery. The infusate buffered to a pH of 7.5, and designed to deliver 20 mg. / kg. of Methotrexate / 24 hrs. in 5% glucose and water, was started at 2.00 p.m.

The animal remained alert but hostile to day 3, when he became progressively more lethargic, apathetic and drowsy. A right Horner's syndrome which appeared postoperatively and lasted the length of the experiment was the only neurological sign seen. He refused food after day 2, but continued to take small

sips of water to day 3, when he vomited twice. He voided and defecated regularly throughout, and on day 3 passed large amounts of soft, black stool. The hemoglobin dropped from 10.6 gm. % on day 0, to 8.1 gm. % on day 2, while the white blood count went from 40,000 to 22,600 cells / $mm.^3$ in the same time period. The animal died at 6.00 a.m. on day 4, having been infused for 4.7 days with 15.8 mg. / kg. of Methotrexate / 24 hrs., in 25.5 ml. 5% glucose and water / kg. / 24 hrs.

At autopsy, 5 hours after death, the animal weighed only 2.75 kg. The skin and oral mucosa were pale. There were small pressure abrasions on the inferior aspect of the chin.

The drill holes for the electrodes were symmetrically placed, the left frontal screw having penetrated the dura and made a small nick in the surface of the left superior frontal gyrus. Grossly the brain and its major vessels were normal. On section the only abnormality noted was a 1 mm. in diameter hematoma in the white matter underlying the left superior frontal gyrus.

The left lung was adherent to the chest wall at the apex. There were numerous small firm nodules on the external aspect of the upper lobe of the left lung. In addition there were many firm mediastinal nodes. The peritoneum was studded with firm grey nodules. The stomach was grossly normal . The intestinal mucosa was thin upon palpation. There were numerous hard nodules in the mesenteric wall of the intestine. The bone marrow was pale and dry. The catheter was in place in the right common carotid artery, and the carotid systems were patent to

injection and palpation.

Aside from the hematoma previously mentioned, no histological abnormality of the brain was seen. In the lungs there were areas of chronic pneumonitis, atelectasis, and emphysema. The previously described lung parasites were again seen (Fig. 39).

Histological changes in the gastrointestinal tract were similar to those described for animal SI (Fig. 24). In addition, there were nematodal parasites in the mesentery, surrounded by zones of coagulation necrosis, and a chronic inflammatory cell infiltrate. The nearby blood vessels were sclerotic. In the spleen there was absence of germinal centers, and only mature lymphocytes were seen. In one pole of the spleen there was a tumor composed of aggregates of capillaries, lined by plump endothelial cells, having the structure of a capillary hemangioma (Fig. 43). There was moderate hypoplasia of the bone marrow.

The right internal carotid artery had loose ante-mortem thrombus partially occluding the lumen. The wall of the vessel was intact, but there was a fibrinopurulent exudate in the adventitia, with necrosis of the walls of some of the included small blood vessels.

Animal SIV.

This 2½-year-old female, weighing 4.7 kg. was catheterized on January 24, 1963. The EEG preoperatively showed some slow waves over the right hemisphere. These were accentuated after carotid clamping, when some were also noted over the left hemisphere. A few hours postoperatively the EEG became normal and

remained normal throughout the experiment. An infusate, unbuffered, and at a pH varying from 5.6 to 6.3, calculated to give 10 mg. Methotrexate / kg. / 24 hrs. in glucose and water was started at 1.45 p.m. The animal awakened soon after the operation was finished, and was sitting in her chair, awake, 1 hour postoperatively.

She refused to eat after day 0, and would not drink. The animal voided regularly throughout, but passed soft stool only on day 4. At first she was alert and hostile, but became increasingly more tired and lethargic from day 2 until death. A right peripheral facial weakness, due to surgery, was noted on day 1 and remained throughout. On day 1, after uncoupling the catheter assembly, a small air bubble entered the right carotid artery. The observer was gone 15 seconds and, on returning, the animal was apneic, with her head turned to the right. She had voided, and was unresponsive for 10 minutes. After sleeping for a few minutes she awoke with no new neurological deficit. A few hours prior to death her left arm was weak and she did not move it in response to pinprick. During the experiment the hemoglobin went from 13 gm.% on day 0, to 8.5 gm.% on day 5, while the white blood count during a comparable time period went from 21,000 to 700 cells $mm.^3$ The platelet and reticulocyte counts also dropped markedly. She died at 4.30 p.m. on day 5, after an infusion time of 5.1 days, during which she received 10.9 mg. Methotrexate / kg. / 24 hrs., in 44.6 ml. of 5% glucose and water / kg. / 24 hrs.

At autopsy, 3 hours after death, the animal weighed 4.3 kg.

The skin and oral mucosa were pale. The neck incision was clean.

The electrode drill holes were symmetrically placed, and did not penetrate the dura. The brain and its major vessels were normal. On section after fixation the midline structures were shifted 1 mm. to the left.

The lingula of the left lung was collapsed, red, and would not float, but no emboli were seen. The gastrointestinal mucosa was normal in the stomach, but felt thin elsewhere. The rest of the thoracico-abdominal contents were normal. The bone marrow was pale and dry. The catheter was well in place in the right common carotid artery, and the internal carotid arteries were patent by injection and dissection.

Histological sections of the brain and brain stem were completely normal.

In the lungs there were areas of interstitial fibrosis and atelectasis. The changes seen in the gastrointestinal tract consisted of loss of mucosal epithelial cells, and bizarre changes in the remaining cells, as previously described (Figs. 14, 15 and 21). In the colon there was almost complete ablation of the mucosa, with only a few abnormal epithelial cells remaining. There was a pseudomembrane composed of necrotic tissue and bacterial colonies superficially (Fig. 23). These colonies were composed of Gram positive rods with spores, small Gram positive cocci in chains, and large Gram positive cocci in groups and clusters. These, histologically, were consistent with Clostridia, Streptococci, and either Staphylococci or Micrococci respectively.

In the spleen, the Malpighian corpuscles were atrophic, with small germinal centers (Fig. 28). Only mature lymphocytes were seen (Fig.31). The bone marrow was hypoplastic. Sections of the right internal carotid artery were normal.

Animal SV.

This 3-year-old female, weighing 4.2 kg. was catheterized March 6, 1963. The carotids were clamped for 5 minutes. This animal had slow waves on the EEG over the right hemisphere after electrode placement. These were accentuated after carotid clamping and then decreased in frequency on day 0. They were occasionally seen over the right hemisphere throughout the experiment. An infusate buffered to a pH of from 7.4 to 7.6, calculated to give 10 mg. Methotrexate / kg. / 24 hrs. was begun at 2.20 p.m. The animal was awake in the chair 1 hour and 45 minutes after closure.

The animal remained aloof, alert and hostile to day 5, when she became increasingly lethargic and apathetic. Aside from a right Horner's syndrome which she was noted to have post-operatively, and which remained until death, no neurological sign was noted. The animal voided regularly throughout, but had bowel movements consisting of copious amounts of soft, brown stool on days 4 and 6. She ate small amounts of banana to day 5, but refused drink after day 1. During the experiment her hemoglobin went from 8.2 gm.% on day 0, to 4.6 gm.% on day 6, and the white blood count went from 8,000 to 1,600 cells/mm.^{3} in the comparable time period. There was an absolute neutropenia with a relative lymphocytosis at the end of the experiment.

She died between 3 and 7 a.m. on day 7, after an infusion period of 6.6 days, having received 9.1 mg. Methotrexate / kg. / 24 hrs. in 36.1 ml. 5% glucose and water / kg. / 24 hrs.

At autopsy, about 5 hours after death, the animal weighed only 3.7 kg. The skin and oral mucosa were very pale, and the hair tended to come out easily. The electrode drill holes were symmetrically placed, and the right posterior parietal drill had penetrated the dura and nicked the brain at the junction of the right parietal and occipital lobes, 1 cm. from the midline. Aside from a film of blood over the right dorsolateral surface due to this trauma, no other abnormality of the brain or its vessels was noted. On section of the brain after fixation there was a 1 mm. shift of the midline structures of the brain to the left, at the level of the head of the caudate nucleus. There was a 3 mm. in diameter hematoma in the white matter of the right occipitoparietal junction, at the site of the drill hole entry.

The right lung was adherent posteriorly to the chest wall. The gastric mucosa was grossly normal, but the rest of the gastro-intestinal mucosa was thin, with small hemorrhagic areas in the colon. The other thoracico-abdominal organs were normal. The bone marrow was pale and dry. The catheter was well in place in the right common carotid artery, and both internal carotid arteries were patent to injection and dissection.

On examination of the sections of the brain, two tiny areas of recent, well-demarcated necrosis were seen in the cortex of the right middle frontal gyrus (Fig. 51). In addition,

a tiny, recent area of necrosis was seen in the cortex on the medial aspect of the left superior frontal gyrus (Fig. 52) extending from layers 2 to 5. Two micro-abscesses were seen, one in the cortex of the right inferior frontal gyrus (Fig. 53), and another more posteriorly in the white matter under the right inferior frontal gyrus. No organisms were seen in the Gram stained sections.

In the upper gastrointestinal tract, the loss of mucosal epithelial cells and the altered histological characteristics of remaining cells were as previously described. In the colon, however, there was complete ablation of the mucosal epithelium and hemorrhagic necrosis of the entire mucosa in some regions. In some places even the muscularis mucosae was necrotic. The submucosa was congested and edematous, There was a superficial pseudomembrane composed of necrotic materials admixed with colonies of bacteria. Some of these microorganisms were Gram positive spore-forming rods, probably Subtilis. Others were Gram positive cocci which were small and not identifiable on the stain. The spleen showed small Malpighian corpuscles with absent germinal centers. There was marked hypoplasia of the bone marrow. The right internal carotid artery was patent.

Animal SVII.

This 3-year-old male, weighing 5.0 kg. was catheterized on April 4, 1963. The infusate, buffered to range in pH from 7.3 to 7.5, and calculated to give 10 mg. Methotrexate / kg. / 24 hrs., in 5% glucose and water, was begun at 2.00 p.m. The carotids were clamped 7 min. 15 secs. A few small air bubbles

were seen to enter the right carotid system while the animal was asleep. The EEG postoperatively showed somewhat less rapid background activity over the right hemisphere with some slow waves. This state persisted to the end of the experiment. The animal was awake and placed in the chair 2 hours after completion of surgery.

The animal was very restless during the experiment, constantly trying to get out of the chair. He tore open his incision on day 0, and it had to be resutured. He became more and more lethargic on day 4, but remained hostile to the end, trying to bite when approached. He ate small amounts of apple and banana to day 5, and after that refused food or drink. He voided regularly throughout, but on day 4 he began to pass soft, dark stool which by day 6 became liquid and tarry. On day 5 the animal developed a severe intractable cough. There was no neurological sign until a few hours before death, when the right pupil started to dilate. During the experiment the hemoglobin went from 10.4 gm.% on day 0 to 8.7 gm.% on day 7, while the white blood count dropped from 26,000 to 5,900 cells $mm.^3$ in the comparable time period. The platelet count also dropped markedly. The blood pH remained fairly stable at from 7.36 to 7.39 during the experiment. He died at 6.15 p.m. on day 7, having received infusion for 7.17 days and 8.3 mg. Methotrexate / kg. / 24 hrs. in 33.5 ml. 5% glucose and water / kg. / 24 hrs.

At autopsy, 2 hours after death, the animal weighed 4.5 kg. The skin and oral mucosa were pale. There was a stomatitis at both corners of the mouth. The electrode drill holes were

symmetrically placed and the electrodes had penetrated the dura in the left frontal and bilateral parietal areas. In the comparable areas of the brain there were tiny nicks, with a thin film of subarachnoid blood. On section of the brain after fixation there was a small 1 mm. in diameter hematoma in the white matter under the left middle frontal gyrus. The midline structures were shifted 1 mm. to the left in the frontal region at the level of the head of the caudate nucleus. No other gross abnormality was seen in the brain or its vessels.

The left lung was adherent to the chest wall at the apex. There was a 1 cm. long pyloric polyp in the stomach. In the small intestine the mucosa was thin and hemorrhagic with ulceration in some places. The colon also had ulcerations of the mucosa with extensive underlying hemorrhagic areas. The other thoracico-abdominal organs were normal. The catheter was well in place in the right common carotid artery, and the internal carotid arteries were patent to both inspection and injection.

Histological sections of the brain and brain stem were normal.

There was a fulminating bacterial pneumonia of the lower lobe of the left lung, with clumps of Gram positive cocci throughout, consistent histologically with Staphylococci. There was marked denudation of the esophageal mucosal epithelium (Fig. 13), with clusters of large Gram positive cocci, and large Gram positive yeasts, resembling Candida albicans, scattered in the pseudomembrane of necrotic tissue covering the remaining epithelium. The denudation of mucosal epithelium and histological

abnormalities of the remaining cells as previously described (Fig. 17). Atypicality of the cells in the base of the gastric polyp, some of which were found in the muscularis, suggested that the base had undergone adenocarcinomatous changes (Fig. 41). In view of the drug, however, no mitoses were seen. In the colonic mucosa there were large areas of hemorrhagic necrosis, with an overlying necrotic pseudomembrane containing colonies of Gram positive cocci in clusters, and colonies of yeasts suggesting, respectively, Staphylococci and Candida albicans. A nematode infestation of the mesentery had produced areas of caseation necrosis surrounded by histiocytes, lymphocytes and plasma cells. The nearby blood vessels were sclerotic, with perivascular chronic inflammatpry cell infiltrates. Hypoplasia of structures in both the lymphatic and hemopoietic systems were as previously described (Fig. 30).

Animal SX.

This 2-year-old male, weighing 3.9 kg. was catheterized on May 23, 1963. The carotids were clamped for 6 minutes. Slow waves in the EEG, which were noted after electrode placement, were accentuated after carotid clamping. The EEG became normal later in day 0. An infusion buffered to a pH of 7.0 to 7.3, designed to give 1 mg. Methotrexate / kg. / 24 hrs., was started at 1.00 p.m.

The animal did very well postoperatively, and was sitting in the chair 2 hours later. There was a right Horner's syndrome which persisted throughout the experiment, but no other neurological sign. The animal was very hostile throughout, constantly

trying to bite and scratch the experimenter. He ate well until day 3, after which he would no longer eat or drink. Urinary output and bowel movements were regular, but on day 6 he developed a diarrhea, with profuse amounts of foul, green stool. On day 3 he was found to have severed the catheter some time during the night. There was a pool of about 15 ml. of clotted blood around the animal. The catheter assembly still contained liquid blood. This, together with his good urinary output, implied that the accident had occurred within only a few hours and possibly only a few minutes before it was noted. While the animal was struggling with the experimenter, the catheter was unblocked and reconnected. A tiny air bubble went into the right carotid system, whereupon he immediately dropped both arms and legs, and his pupils dilated. There were then clonic and tonic contractions of the arms and legs for 20 seconds, followed by 2 minutes of semisopor, and a weak left handgrip for a few minutes. The EEG taken minutes afterwards showed low voltage with some slow waves over the right hemisphere. The EEG was normal again on the next day and remained normal throughout. He became more lethargic in the last three days and died at 6.00 p.m. on day 8, having been infused for 8.21 days with 1.5 mg. Methotrexate / kg. / 24 hrs. in 40.5 cc. 5% glucose and water / 24 hrs. During the experiment the hemoglobin had dropped from 10.1 gm.% on day 0, to 4 gm.% on day 8.

The autopsy was done 2 hours after death, and the animal weighed 3.3 kg. The hair came out easily, and there was pallor of the skin and oral mucosa. The skull electrode drill holes

were symmetrically placed, and there were small 1 ml. epidural collections of blood under the mid-sagittal and right parietal drill holes. No other abnormality of the brain or its major vessels was noted at autopsy or on sectioning after fixation.

The left lung was adherent to the diaphragm, and contained the small cystic lesions previously described. In the stomach, which was filled with green fluid, there was a white patch, 1 cm. in diameter, in the lower third of the stomach near the lesser curvature. The gastrointestinal mucosa of the intestine was thin and there were flecks of blood under the mucosa of the cecum and colon. The bone marrow was pale and dry. The carotid catheter was in place in the right common carotid artery. The carotid arteries were patent to inspection and injection. The rest of the thoracico-abdominal orgams were normal.

The histological sections of the brain were normal. In the lungs, cysts lined by cuboidal epithelium and surrounded by chronic inflammatory exudates were seen. Some of these cystic lesions contained formed parasites. In other areas of the lungs, there were areas of focal chronic pneumonitis. The white area seen in the stomach was, histologically, a healing ulcer (Fig. 42). In the duodenum there was incomplete denudation of the epithelial cells. The loss of mucosal epithelium, and the histological changes of the remaining cells were as previously described (Fig. 20). On the surface of the remaining colonic mucosa there were numerous colonies of bacteria. Atrophy of the splenic germinal centers, and hypoplasia of the bone marrow were as previously described, but only moderately severe.

In the common carotid artery on the right side there was soft ante-mortem thrombus, with a hole in one side for the catheter. In the right internal carotid artery there was a gap in the elastica on one side. The nuclei of underlying cells in the media had large vesicular nuclei, with prominent nucleoli; histologically they had the appearance of very active cells.

Animal SXI.

This 2-year-old male, weighing 3.75 kg. was catheterized on June 5, 1963. The carotids were clamped for 4 minutes. After electrode placement, slow waves were seen in the EEG over the right hemisphere. These were accentuated after carotid clamping. The EEG was normal on days 1 to 5, after which slow waves appeared over both hemispheres and remained until death. The infusate, buffered to a pH between 7.2 and 7.35, and designed to give 1 mg. Methotrexate / kg. / 24 hrs. was started on June 5, 1963, at 2.00 p.m. He remained semicomatose for 6.5 hours after closure and had to be kept in the recumbent position during this time. This was due to an overdose of anesthetic. the animal recovered and remained alert throughout, except for the last three hours before death. He was very friendly and allowed the experimenter to pet him during the last 4 days of the test. He ate well to day 5, and then refused all food. Through the experiment the right pupil had been slightly larger than the left. In the last four hours before death there was a slight ptosis of the right eyelid. The catheter assembly leaked a bit in this experiment, and in the last two days the urinary output became scanty. On day 5 the animal developed a profuse, foul,

watery diarrhea. He died at 9.20 p.m. on day 7, having received the infusate 7.29 days, and an average dose of Methotrexate of 1.2 mg. / kg. / 24 hrs. in 41 ml. of 5% glucose and water / kg. / 24 hrs.

During the experiment the blood pH varied from 7.47 to 7.48, and the hemoglobin went from 11.8 gm.% on day 0 to 10.4 gm. % on day 6, while the white blood count dropped from 20,400 to 1,700 cells $mm.^3$ in the comparable time interval, along with a drop in platelets and reticulocytes. At the end there was a neutropenia, with a relative lymphocytosis.

At autopsy, 1 hour after death, the animal weighed 3.55 kg. The hair on the back came out easily, and the oral mucosa and skin were pale. The drill holes for the electrodes were symmetrically placed, but there were small 0.5 ml. epidural collections of blood under both right drill holes. The right frontal drill had penetrated the dura and created a small nick in the right middle frontal gyrus, with a 1 ml. overlying subdural hematoma. No gross abnormality of the brain or its major vessels was seen in the fresh specimen, or on the coronal sections made after fixation.

There was a small calcified nodule joining the middle and lower lobes of the right lung. The stomach was distended with 400 cc. of green fluid. In the small intestine an injected submucosal patch 8 cm. long was noted 20 cm. above the cecum. In the ascending colon there were also two injected patches, 2 cm. in diameter. The bone marrow was pale and dry. An aberrant branch of the right carotid system, coming off the

right common carotid artery below the bifurcation had not been tied off. Both internal carotid arteries were patent by water and inspection.

On examination of the histological sections of the brain no abnormality was seen.

In the lungs, numerous cysts due to the previously described parasites were seen. There were also small areas of atelectasis, and focal chronic inflammatory changes. In the small and large intestines there was partial denudation of the mucosal epithelium, the remaining cells showing changes as previously described. The germinal centers of the Peyer's patches were more prominent in this animal, however, than in previous animals on high doses of the drug.

In the spleen the only abnormality seen was that of chronic hyalinization of the central parts of the germinal centers.

The bone marrow showed mild hypoplasia, without full maturation arrest. In the right common carotid artery there was a recent ante-mortem crescentic fibrin thrombus, with a round space where the catheter had been. Occasional multinucleated phagocytes were seen in the adventitia of the vessel.

Animal SXII.

This 1¾-year-old male, weighing 3.21 kg. was catheterized on June 19, 1963. The carotids were clamped for 3.5 minutes. The EEG's showed a few slow waves over the right hemisphere after electrode placement. On day 1 the EEG was normal. By day 4, slow waves returned over the right hemisphere, and remained to the end. The infusate buffered to a pH between 7.0 and 7.1

was started at 1.25 p.m. It was calculated to give 1 mg. of Methotrexate / kg. / 24 hrs.

The animal was placed in the chair at 3.00 p.m., while still asleep. During this transfer the apparatus had to be uncoupled for a moment, and two tiny air bubbles were seen to go into the right carotid artery uneventfully. The animal remained semicomatose and his breathing became stertorous, so intermittent tracheal suctioning was begun and he was placed on his back in the chair. By 10.00 p.m. of day 0 he had not voided and there was beginning gastric dilatation. Carbachol 0.1 ml. was given intraperitoneally, and thereafter the animal voided. He was left sleeping in the prone position for the remainder of the night. The following morning the animal was alert, but friendly, and remained so until the end. The only neurological signs appeared postoperatively and consisted of right pupillary enlargement, and a slight right peripheral facial paralysis. Both of these signs remained to the end of the experiment. The animal ate well to day 5, and then refused food, vomited twice, and began to have tarry, dark, fluid stools. His voiding became scant by day 5, since some of the infusate was lost due to development of a small leak in the apparatus. He became more lethargic after day 5, and weak on day 6. He died at 3.00 a.m. on day 7, having been infused for 6.5 days, receiving 1.4 mg. Methotrexate / kg. / 24 hrs., in 46 ml. of 5% glucose and water / kg. / 24 hrs.

At autopsy, 9 hours after death, the animal weighed 2.5 kg. The hair did not come out easily, but the mucosa of

the mouth and the skin were pale. The electrode drill holes were well placed and symmetrical. There were 0.5 ml. collections of epidural blood under the parietal drill holes. No abnormality of the brain or its major vessels was noted on examination in the fresh state, or on examination of the sections after fixation.

The lower lobe of the right lung was boggy and wet, while the middle lobe of the right lung was collapsed. A few small petechiae were seen in the mucosa of the intestine. The bone marrow was pale and dry. The catheter was in place in the right common carotid artery, and both internal carotid arteries were patent to inspection and injection.

Histological examination of multiple sections of the brain and brain stem showed no abnormality.

Sections from the right lung were atelectatic, with focal areas of emphysema. There was some ablation of the mucosa of both the large and small intestines, with changes in the remaining cells similar to those previously described.

In the spleen, no significant change was seen, but in a nearby lymph node there were no remaining germinal centers, and there were numerous histiocytes in the sinusoids.

The common carotid artery contained a recent ante-mortem crescentic fibrin thrombus, with a space in which the catheter had been housed. The right internal carotid artery was patent, but there were numbers of foreign body giant cells in the adventitia of the vessel, due to remnants of surgical silk. The bone marrow showed mild hypoplastic changes.

Animal SV1.

This 3-year-old male, weighing 5.4 kg. was catheterized on March 27, 1963. The carotids were clamped for 7 minutes. A few slow waves were noted in the EEG after electrode placement. These cleared by day 1. The animal was put in the chair 2 hours postoperatively. He quickly became very alert and was hostile and frightened; he would bark, bite and scratch when approached. The infusion, buffered at a pH of 7.1 and calculated to give 10 mg. Methotrexate / kg. / 24 hrs., was begun at 2.30 p.m.

Postoperatively he had a right Horner's syndrome which persisted during his stay in the chair. On the morning of day 1 he was found with the catheter pulled out, so that the infusion had lasted a minimum of 0.36 days and a maximum of 0.75 days.

He ate well, voided regularly and had regular bowel movements to day 3. On that day he was found to have frequent staring spells, a spastic right arm, and a continuous tremor of the right hand with flexion of the right wrist. The tremor was intermittent. There was a left hand weakness. Decreased amplitude in the EEG and some slow wave activity were observed over the right hemisphere. After one of the attacks of staring, with head turned to the left and tremor of the left arm, the EEG showed flattening of the voltage on the right side. Epilepsy partialis continuans, probably due to meningitis, was diagnosed, and the animal was given intramuscular antibiotics and anti-epileptic drugs. In about 2 hours he looked better and the seizures were less frequent. The medications were repeated at 11.00 p.m. of day 3. The next day the animal looked lethargic,

but no more seizures were seen. The animal would not eat after day 3, nor would he drink. He remained in the chair to day 6, when he was put back in the cage in a somewhat weakened condition. Two hours after having been returned to the cage he began to eat and drink voraciously. During the experiment the hemoglobin had gone from 12.9 gm.% to 12.1 gm.%, while the white blood count had gone from 12,800 to 10,300 cells mm.^{3}

He remained in the cage for the next 8 months, during which the Horner's syndrome cleared. The keeper said that the animal acted strangely after the experiment, since he would often make grimaces which were considered strange, even for a monkey.

On November 14, 1963, the animal was sacrificed with an overdose of intravenous Nembutal. The hemoglobin was 12.4 gm.% and the white blood count only 1,900 cells mm.^{3}

At autopsy, one hour after death, the animal weighed 5.1 kg. The neck wound was well healed, and the mucosa and skin healthy and pink.

On examination of the skull there were no longer any drill holes noted. The brain and its major vessels were all normal in the fresh specimen, and no abnormality was seen in the coronal sections after fixation.

There was a small cyst in the lingula of the left lung. The mesentery was white, not yellow as in the recently drug infused animals. The gastrointestinal tract was full of food and feces. The bone marrow was dark and greasy. The branches of the right external carotid artery were tied off and, grossly, there

was no abnormality of the internal carotid arteries.

Histological sections of the brain and brain stem were normal. In the esophagus the epithelium was a bit thinner than in the control animals.

In the spleen there was abnormal prominence of the Malpighian corpuscles, which had markedly enlarged germinal centers containing many young lymphocytes. In the left common carotid artery, just below the bifurcation, a large plaque of subintimal atheroma was seen.

Animal SXIV.

This 3-year-old male, weighing 4.85 kg. was catheterized on November 15, 1963. The carotids were clamped for 16 minutes. Despite this, no EEG abnormality was seen throughout. The infusion of 5% glucose and water, buffered to a pH between 7.1 and 7.3 was started at 1.00 p.m.

The animal was put into the chair 1½ hours after surgery. There were then no neurological signs. Later that evening he managed to bite the catheter and it became occluded. It was unblocked uneventfully, shortened and reconnected. The animal remained lethargic on day 0. On day 1 in the morning he developed a right 6th nerve palsy, which persisted. Later that day there was a right facial paresis. On day 2 there was a complete right ophthalmoplegia, besides a postoperative right Horner's syndrome and a right peripheral facial paresis. The pupils, however, were bilaterally reactive to light and accomodation. There was also suggestion of a right 5th nerve paresis, since the right corneal reflex was diminished, and the right

temporalis muscle contraction was not as strong as that on the left. Vision was adequate in both eyes.

The animal remained very alert, irritable and hostile throughout the experiment, eating small amounts throughout. When the catheter assembly was uncoupled, blood came back up to day 4, after which it did not. The neck began to swell, and the urinary output decreased, suggesting that the catheter had come out. On days 5 and 6 the animal was bleeding from the gums easily. Numerous large air bubbles were deliberately injected into the right carotid system on days 5 and 6, without any ill effects on the animal. Finally, on day 7 at 3.30 p.m. the animal was sacrificed by an overdose of intravenous Nembutal. Through the experiment the hemoglobin went from 13.3 gm.% on day 0, to 8.1 gm.% on day 7, and the white blood count went from 18,390 to 2,500 cells $mm.^3$ There was an increase in platelets in this time, and a relative lymphocytosis.

At autopsy, 1 hour after death, the animal weighed 4.3 kg. The right skull burr holes were ½ cm. closer to the midline than those on the left side. There were very small epidural films of blood under the left parietal and mid-sagittal drill holes. No abnormality of the brain or its major vessels was seen in the fresh specimen, or in the coronal sections of the brain after fixation.

The deep paravertebral part of the neck incision contained food particles, and there was a hole demonstrable in the upper part of the right side of the esophagus, caused, presumably, during the operation. The middle lobe of the right lung had

numerous small, yellow, circumscribed lesions, as previously described in other animals. The rest of the thoracico-abdominal viscera were normal. The right common and internal carotid arteries were hard to palpation and felt thrombosed. On injection, water squirted out of the external carotid artery at the carotid bifurcation and, at a tiny pull, the catheter was out. The branches of the right external carotid were all tied off. The left carotid system was grossly normal.

On examination of the histological sections of the brain and brain stem no abnormality was noted.

In the lungs some of the parasitic cysts previously described were seen. In the gastrointestinal system there were occasional perivascular infiltrates of mononuclear cells and eosinophiles with a very high number of eosinophile leucocytes in the mucosa. No other gastrointestinal abnormality was seen. The right common carotid artery was occluded by early ante-mortem thrombus, and there was a periadventitial inflammatory cell infiltration. There was an extensive infiltration of inflammatory cells in the media of the artery. In the right internal carotid artery there was also extensive acute inflammatory cell infiltration of the vessel wall (Fig. 26). Early ante-mortem thrombus occluded the lumen. The bone marrow was normal.

Due to the severity of the postoperative complications shown in this animal, and in view of the uncertainty as to whether the animal in fact received infusate, another animal (SXV) was to be done as a more definite control.

Animal SXV.

This 3½-year-old female, weighing 5.3 kg. was catheterized on November 29, 1963. The carotids were clamped for 5 minutes. The EEG's were normal on day 0. The infusate buffered to a pH varying from 7.3 to 7.6 and consisting only of 5% glucose and water, was started at 2.00 p.m.

The animal recovered well from the operation, and was placed in the chair by 4.00 p.m. on the operative day. A slight right facial weakness, and a minimal right pupillary dilatation were noted on the first three postoperative days, after which they disappeared. The animal remained alert, irritable and hostile throughout the experiment, eating an average of three bananas per day. There were bowel movements with hard, formed stool every day, and the animal voided regularly.

On days 3, 5, and 7, air bubbles, perhaps 10 times as large as those inadvertently injected into the other animals were deliberately injected into the right carotid system under EEG control. Five injections were made in all. Once the monkey gasped, her face turned red, and both arms and legs extended for about 5 seconds. There was a slowing of the EEG activity and decreased voltage over the right hemisphere; this gradually returning to normal in 10 minutes after such an injection. The animal had a mild paresis of the right arm, lasting 14 minutes, after which the strength in this arm was considered normal. On another occasion the monkey stared for a couple of seconds, gripped her throat with her right hand, extended the left arm and leg, and was left with a left hemiparesis which cleared to normal after 10 minutes. Disappearance of the slow waves in the EEG

and return of the voltage to normal were observed after a few minutes. On day 6 the effects were very similar, but the amount of air injected was about twice as much, and the hemiparesis took about 20 minutes to clear. Slow waves seen over the right hemisphere returned to normal in a few minutes. After the last injections on day 7, with similar episodes, the EEG returned to normal and remained so to the end. On day 9 the catheter assembly began to leak and the animal's urinary output dropped. The blood in the catheter refluxed and clotted. The animal was sacrificed by an overdose of Nembutal given intravenously at 11.00 a.m. on day 10.

During the experiment the hemoglobin dropped from 12.8 gm.% to 8.3 gm.% from day 0 to day 10, while in the same time interval the white blood count went from 32,300 to 11,700 cells mm.^{3} Reticulocyte and platelet counts increased however. There was an increase of circulating lymphocytes throughout the experiment.

At autopsy, 1 hour after death, the animal weighed only 5 kg. The incision was healed, but there was a small pressure abrasion under the chin. The oral mucosa and skin were pink. The electrode drill holes were symmetrically placed in the skull, and there were small biparietal epidural films of blood. No abnormality of the brain or its major vessels was noted, grossly, on examination of the fresh specimen, and on examination of the coronal sections of the brain and brain stem after fixation.

The left lung was adherent to the chest wall, and scattered in the lungs on both sides there were numerous examples of the previously described parasitic cysts. No abnormality was seen

in the gross examination of the thoracico-abdominal contents. The bone marrow was red and greasy. The carotid arteries were patent to inspection and injection.

No abnormality was seen on examination of histological sections of the brain and brain stem. In the lungs, cysts as previously described were seen, but there were also areas of focal interstitial fibrosis, and areas of chronic inflammatory cell infiltration, presumably a reaction to the parasitic infestation.

The sections of spleen were normal (Fig. 27). In the submucosa and mucosa of the stomach, many of the small blood vessels had striking perivascular infiltrates of polymorphonuclears, mononuclear cells, and eosinophiles. In many instances there were striking examples of fibrinoid necrosis of the tiny blood vessels (Fig. 44). Histologically, these changes appeared subacute. In one part of the small intestine a longitudinal section of a larval nematode was seen in the muscularis (Fig. 40). There were many eosinophiles in the mucosa throughout the gastrointestinal tract. No other abnormality of the gastrointestinal tract was noted (Figs. 12, 18, and 22).

In the right internal carotid artery there was some adventitial cellular reaction. In the right common carotid artery there was a crescentic recent ante-mortem thrombus, with a defect where the catheter had previously been. The bone marrow was normal (Fig. 37).

BIBLIOGRAPHY

Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H., Semb, J. et al. Synthesis of a compound identical with the L. casei factor isolated from liver. Science, 102: 227-228, 1945.

Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H., Semb, J. et al. The structure and synthesis of the liver L. casei factor. Science, 103: 667-669, 1946.

Bagshawe, K.D. and Brooks, W.D.W. Subacute pulmonary hypertension due to chorionepithelioma. Lancet, 1: 653-658, 1959.

Bagshaw, M.A. Approaches for combined radiation and chemotherapy. Laval Med., 34: 124-133, 1963.

Baker, H., Herbert, V., Frank, O., Pasher, I., Hutner, S.H. et al. A microbiologic method for detecting folic acid deficiency in man. Clin. Chem., 5: 275-280, 1959.

Balla, G., Mallams, J.T., Hutton, S., Aronoff, B.L. and Byrd, L. The treatment of head and neck malignancies by continuous intra-arterial infusion of Methotrexate. Amer. J. Surg., 104: 699-704, 1962.

Beckman, H. Drugs that effect improvement in anemias (Hematinics), In Drugs, their nature, action and use. Philadelphia, Saunders, 1959. p. 119.

Bertino, J.R. The mechanism of action of the folate antagonists in man. Cancer Res., 23: 1286-1306, 1963.

Boyland, E. The biochemistry of Methotrexate and its analogues. In R. Porter and E. Wiltshaw (Editors). Methotrexate in the treatment of cancer. Bristol, Wright, 1962. p. 12-16.

Branch, C.B. Personal communication.

Brandriss, M.W. Methotrexate: Suppression of experimental allergic encephalomyelitis. Science, 140: 186-187, 1963.

Brockman, J.A., Jr., Roth, B., Broquist, H.P., Hultquist, M.E., Smith, J.M., Jr., et al. Synthesis and isolation of a crystalline substance with the properties of a new B vitamin.
J. Amer. Chem. Soc., 72: 4325-4326, 1950.

Brown, G.M. and Reynolds, J.J. Biogenesis of the water-soluble vitamins.
Ann. Rev. Biochem., 32: 419-462, 1963.

Burchenal, J.H. The treatment of leukemia.
Bull. N.Y. Acad. Med., 30: 429-447, 1954.

Burchenal, J.H. Current status of clinical chemotherapy.
Curr. Res. Cancer Chemother., Rep. No. 4., 1956. p. 3-30.

Burchenal, J.H. and Babcock, G.M. Prevention of toxicity of massive doses of Aminopterin by citrovorum factor.
Proc. Soc. Exp. Biol. Med., 76: 382-384, 1951.

Burchenal, J.H., Babcock, G.M., Armstrong, R.A. and Robinson, E. Mechanism of resistance in leukemia.
Acta Un. Int. Cancr. 7: 436-442, 1951.

Burchenal, J.H., Babcock, G.M., Broquist, H.P. and Jukes, T.H. Prevention of chemotherapeutic effects of 4-amino-N^{10}-methyl-pteroylglutamic acid on mouse leukemia by citrovorum factor.
Proc. Soc. Exp. Biol. Med., 74: 735-737, 1950.

Burchenal, J.H., Bendick, A., Brown, G.B., Elion, G.B., Hitchings, G.H. et al. Preliminary studies on the effect of 2, 6 - diaminopurine on transplanted mouse leukemia.
Cancer, 2: 119-120, 1949a.

Burchenal, J.H., Johnson, S.F., Burchenal, J.R., Kushida, M.N., Robinson, E. and Stock, C.C. Chemotherapy of leukemia. IV. Effect of folic acid derivatives on transplanted mouse leukemia.
Proc. Soc. Exp. Biol. Med., 71: 381-387, 1949b.

Campbell, C.J., Brown, R.A. and Emmett, A.D. Influence of crystalline vitamin B_c on hematopoiesis in the chick.
J. Biol. Chem., 152: 483-484, 1944.

Campbell, J.B. and Foster, F.N. The anterior cerebral artery in the macaque monkey (macaca mulatta).
J. Nerv. Ment. Dis., 99: 229-242, 1944.

Clarkson, B. and Lawrence, W. Jr. Perfusion and infusion techniques in cancer chemotherapy.
Med. Clin. N. Amer., 45: 689-710, 1961.

Condit, P.T. On the site of action of Amethopterin.
Science, 134: 1421, 1961.

Cooper, B.A. Personal communication.

Cooper, B.A. and Lowenstein, L. Evaluation of assessment of folic acid deficiency by serum folic acid activity measured with L. casei.
Canad. Med. Ass. J., 85: 987-988, 1961.

Cosulich, D.B. and Smith, J.M., Jr. Analogs of pteroylglutamic acid. I. N^{10}-alkylpteroic acid and derivatives.
J. Amer. Chem. Soc. 70: 1922-1926, 1948.

Creech, O., Jr., Krementz, E.T., Ryan, R.F. and Winblad, J.N. Chemotherapy of Cancer: Regional perfusion utilizing an extracorporeal circuit.
Ann. Surg., 148: 616-632, 1958.

Duff, J.K., Sullivan, R.D., Miller, E., Ulm, A.H., Clarkson, B.D. and Clifford, P. Antimetabolite - metabolite cancer therapy using continuous intra-arterial Methotrexate with intermittent intramuscular citrovorum factor: a method of therapy.
Cancer, 14: 744-752, 1961.

Edstrom, R.F.S. and Essex, H.E. Swelling of damaged brain tissue.
Neurology (Minneap.) 5: 490-493, 1955.

Espiner, H.J. Intra-arterial Methotrexate therapy in inoperable cancer, with special reference to cases not previously irradiated. In R. Porter and E. Wiltshaw (Editors). Methotrexate in the treatment of cancer. Bristol, Wright, 1962. p. 59-68.

Farber, S. Some observations of the effect of folic acid antagonists on acute leukemia and other forms of incurable cancer.
Blood, 4: 160-167, 1949.

Farber, S., Cutler, E.C., Hawkins, J.W., Harrison, J.H., Pierce, E.C. and Lenz, G.G. The action of pteroylglutamic conjugates on man.
Science, 106: 619-621, 1947.

Farber, S., Diamond, L.K., Mercer, R.D., Sylvester, R.F. and Wolff, J.A. Temporary remissions in acute luekemia in children produced by folic acid antagonist 4-amino-pteroylglutamic acid (Aminopterin).
New Eng. J.Med. 238: 787-793, 1948.

Franklin, A.L., Belt, M., Stokstad, E.L.R. and Jukes, T.H. Biological studies with 4-amino-10-methylpteroylglutamic acid.
J. Biol. Chem., 177: 621-629, 1949.

Franklin, A.L., Stokstad, E.L.R., Belt, M. and Jukes, T. Biochemical experiments with a synthetic preparation having an action antagonistic to that of pteroylglutamic acid. J. Biol. Chem., 168: 427-435, 1947a.

Franklin, A.L., Stokstad, E.L.R. and Jukes, T.H. Acceleration of pteroylglutamic acid deficiency in mice and chicks by a chemical antagonist. Proc. Soc. Exp. Biol. Med., 65: 368-370, 1947b.

Franklin, A.L., Stokstad, E.L.R. and Jukes, T.H. Observations on the effect of 4-amino-pteroylglutamic acid on mice. Proc. Soc. Exp. Biol. Med., 67: 398-400, 1948.

Freeman-Narrod, M. The pharmacology of Methotrexate. In R. Porter and E. Wiltshaw (Editors). Methotrexate in the treatment of cancer. Bristol, Wright, 1962. p. 12-16.

Friedkin, M. Enzymatic aspects of folic acid. Ann. Rev. Biochem., 32: 185-214, 1963.

Futterman, S. Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. J. Biol. Chem., 228: 1031-1038, 1957.

Harper, H.A. In his Review of Physiological chemistry. Los Angeles, Lange Medical Publications, 1963, p. 85-89.

Heller, J.R. Cancer chemotherapy, history and present status. Bull. N.Y. Acad. Med., 38: 348-359, 1962.

Heller, I. H., and Elliott, K.A.C. Desoxyribonucleic acid content and cell density in brain and human brain tumors. Canad. J. Biochem. Physiol., 32: 584-592, 1954.

Herbert, V., Fisher, R. and Koontz, B.J. The assay and nature of folic acid activity in human serum. J. Clin. Invest., 40: 81-91, 1961.

Herbert, V. and Zalusky, R. Interrelationships of vitamin B_{12} and folic acid metabolism: folic acid clearance studies. J. Clin. Invest., 41: 1263-1276, 1962.

Hertz, R., Bergenstal, D.M., Lipsett, M.B., Price, E.B. and Hilbish, T.F. Chemotherapy of choriocarcinoma and related trophoblastic tumors in women. J.A.M.A., 168: 845-854, 1958.

Hochstein, P. Synergistic effects of 6-mercaptopurine and Methotrexate on anaerobic glycolysis by subcellular fractions of S 91 mouse melanoma. Proc. Amer. Ass. Cancer Res., 2: 214, 1957.

Hogan, A.G. and Parrott, E.M. Anemia in chicks caused by a vitamin deficiency.
J. Biol. Chem., 132: 507-517, 1940.

Huennekens, F.M., Osborn, M.J. and Whiteley, H.R. Folic acid coenzymes. Metabolic reactions involving "active formate" and active formaldehyde are surveyed.
Science, 128: 120-124, 1958.

Hutchings, B.L., Mowat, J.H., Oleson, J.J., Stokstad, E.L.R., Boothe, J.H. et al. Pteroylaspartic acid, an antagonist for pteroylglutamic acid.
J. Biol. Chem., 170: 323-328, 1947.

Hutchings, B.L., Stokstad, E.L.R., Bohonos, N., Sloane, N.H. and Subbarow, Y. The isolation of the fermentation Lactobacillus casei factor.
J. Amer. Chem. Soc., 70: 1-3, 1948a.

Hutchings, B.L., Stokstad, E.L.R., Gohonos, N. and Slobodkin, N.H. Isolation of a new Lactobacillus casei factor.
Science, 99: 371, 1944.

Hutchings, B.L., Stokstad, E.L.R., Mowat, J.H., Boothe, J.H., Waller, C.W. et al. Degradation of the fermentation L. casei factor.
J. Amer. Chem. Soc., 70: 10-13, 1948b.

Jacobson, W. The mode of action of folic acid antagonists on cells.
J. Physiol. (London) 123: 603-617, 1954.

Jandl, J.H. and Greenberg, M.S. Bone marrow failure due to relative nutritional deficiency in Cooley's hemolytic anemia. Painful "erythropoietic crises" in response to folic acid.
New Eng. J. Med., 260: 461-468, 1959.

Johnson, W., Corte, G. and Jasmin, R. Effect of 4-amino folic acid antagonists on biological acetylations.
Proc. Soc. Exp. Biol. Med., 99: 677-680, 1958.

Jukes, T.H. Folic acid and vitamin B_{12} in the physiology of vertebrates.
Fed. Proc., 12: 633-638, 1953.

Jukes, T.H. Introduction. In R. Porter and E. Wiltshaw (Editors). Methotrexate in the treatment of cancer. Bristol, Wright, 1962, p. 1-10.

Jukes, T.H. and Stokstad, E.L.R. Pteroylglutamic acid and related compounds.
Physiol. Rev., 28: 51-101, 1948.

Karnofsky, D.A., Patterson, P.A. and Ridgway, L.P. Effect of folic acid, "4-amino" folic acids and related compounds on growth of chick embryo.
Proc. Soc. Exp. Biol. Med., 67: 447-452, 1948.

Katzman, R., Aleu, F. and Wilson, C. Further observations on triethyltin edema.
Arch. Neurol (Chicago) 9: 178-187, 1963.

Keele, C.A. and Neil, E. (Editors). Samson Wright's applied physiology. 10th Ed. London, Oxford University Press, 1961.

Kisliuk, R.L. Reduced folic acid analogues as antometabolites.
Nature (London) 188: 584-585, 1960.

Klopp, C.T., Alford, T.C., Bateman, J., Berry, G.N. and Winship, T. Fractionated intra-arterial cancer chemotherapy with methyl bis amine hydrochloride; a preliminary report.
Ann. Surg., 132: 811-832, 1950.

Kofman, S. Cancer of head and neck.
Ann. Intern. Med., 48: 731-746, 1958.

Laszlo, D. and Leuchtenberger, C. Inositol, a tumor growth inhibitor.
Science, 97: 515, 1943.

Leblond, C.P. and Messier, B. Renewal of chief cells and goblet cells in the small intestine as shown by radioautography after injection of thymidine-H^3 into mice.
Anat. Rec., 132: 247-259, 1958.

Leuchtenberger, C., Lewisohn, R., Laszlo, D. and Leuchtenberger, R. "Folic acid", a tumor growth inhibitor.
Proc. Soc. Exp. Biol. Med., 55: 204-205, 1944.

Leuchtenberger, R., Leuchtenberger, C., Laszlo, D. and Lewisohn, R. The influence of "folic acid" on spontaneous breast cancer in mice.
Science, 101: 46, 1945.

Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., and Keresztesy, J.C. The influence of liver L. casei factor on spontaneous breast cancer in mice.
Science, 104: 436-437, 1946.

Li, M.C., Hertz, R. and Bergenstal, D.M. Therapy of choriocarcinoma and related trophoblastic tumors with folic acid and purine Antagonists.
New Eng. J. Med., 259: 66-74, 1958.

Li, M.C., Hertz, R. and Spencer, D.B. Effect of Methotrexate therapy upon choriocarcinoma and chorioadenoma.
Proc. Soc. Exp. Biol. Med., 93: 361-366, 1956.

Lissauer, --. Zwei Fälle von Leucaemie.
Berl. Klin. Wschr., 2: 403, 1865.

Little, P.A., Sampath, A. and Subbarow, Y. The use of antagonists of pteroylglutamic acid in controlling Rous chicken sarcoma.
J. Lab. Clin. Med., 33: 1144-1149, 1948.

Martin, G.J., Tolman, L. and Moss, J. d (-) Methylfolic acid displacing agent for folic acid.
Arch. Biochem., 12: 318-319, 1947.

Mealey, J. Jr., Treatment of malignant astrocytomas by intra-arterial infusion of Vinblastine.
Cancer Chemother. Rep., 20: 121-126, 1962.

Meerovitch, E. Personal communication.

Mills, R.C., Briggs, G.M., Jr., Elvehjem, C.A. and Hart, E.B. Lactobacillus casei factor in the nutrition of the chick.
Proc. Soc. Exp. Biol. Med., 49: 186-189, 1942.

Mitchell, H.K., Snell, E.E. and Williams, R.J. The concentration of "folic acid." J. Amer. Chem. Soc., 63: 2284, 1941.

Mowat, J.H., Boothe, J.H., Hutchings, B.L., Stokstad, E.L.R., Waller, C.W. et al. The structure of liver L. casei factor
J. Amer. Chem. Soc., 70: 14-18, 1948.

Nash, P. 1963. Personal communication. (Data from Abbott Laboratories Ltd.)

Nichol, C.A. and Welch, A.D. On the mechanism of action of Aminopterin.
Proc. Soc. Exp. Biol. Med., 74: 403-411, 1950a.

Nichol, C.A. and Welch, A.D. Synthesis of citrovorum factor from folic acid by liver slices; augmentation by folic acid.
Proc. Soc. Exp. Biol. Med., 74: 52-55, 1950b.

O'Brien, J.S. The role of the folate coenzymes in cellular division. A review.
Cancer Res., 22: 267-281, 1962.

Pappius, H.M. Personal communication.

Pappius, H.M. and Gulati, D.R. Water and electrolyte content of cerebral tissues in experimentally induced edema.
Acta Neuropath. (Berlin) 2: 451+460, 1963.

Pfiffner, J.J., Binkley, S.B., Bloom, E.S., Brown, R.A., Bird, O.D. et al. Isolation of antianemia factor (vitamin B_c) in crystalline form from liver.
Science, 97: 404-405, 1943.

Pfiffner, J.J., Binkley, S.B., Bloom, E.S. and O'Dell, B.L. Isolation and characterization of vitamin B_c from liver and yeast. Occurrence of acid labile chick antianemia factor in liver.
J. Amer. Chem. Soc., 69: 1476-1487, 1947.

Pfiffner, J.J., Calkins, D.G., O'Dell, B.L., Bloom, E.S., Brown, R.A. et al. Isolation of an antianemia factor (vitamin B_c conjugate) in crystalline form from yeast.
Science, 102: 228-230, 1945.

Philips, F.S. and Thiersch, J.B. Studies of the actions of 4-amino-pteroylglutamic acid in rats and mice.
J. Pharmacol. Exp. Ther., 95: 303-311, 1949.

Sacks, M.S., Bradford, G.T. and Schoenbach, E.B. The response of acute leukemia to the administration of the folic acid antagonists, Aminopterin and Amethopterin: report of 14 cases.
Ann. Intern. Med., 32: 80-115, 1950.

Sauberlich, H.E. and Baumann, C.A. A factor required for the growth of Leuconostoc citrovorum.
J. Biol. Chem., 176: 165-173, 1948.

Schoenbach, E.B., Colsky, J. and Greenspan, E.M. Observations on the effects of the folic acid antagonists, Aminopterin and Amethopterin in patients with advanced neoplasms.
Cancer, 5: 1201-1220, 1952.

Schoenbach, E.B., Greenspan, E.M. and Colsky, J. Reversal of Aminopterin and Amethopterin toxicity by citrovorum factor.
J.A.M.A., 144: 1558-1560, 1950.

Seeger, D.R., Cosulich, D.G., Smith, J.M. Jr., and Hultquist, M.E. Analogs of pteroylglutamic acid. III. 4-amino derivatives.
J. Amer. Chem. Soc., 71: 1753-1759, 1949.

Seeger, D., Smith, J.M., Jr., and Hultquist, M.E. Antagonist for pteroylglutamic acid.
J. Amer. Chem. Soc., 69: 2567, 1947.

Slavikova, V. and Slavik, K. On the mechanism of action of some 4-amino analogues of folic acid.
Experentia, 17: 113-115, 1961.

Smith, D.F., Klopp, C.T. and Alford, T.C. Present status of isolation perfusion and intra-arterial infusion techniques.
Postgrad. Med., 32: 135-145, 1962.

Snell, E.E. and Peterson, W.H. Growth factors for bacteria. Additional factors required by certain lactic acid bacteria.
J. Bact., 39: 273-285, 1940.

Stokstad, E.L.R. Some properties of a growth factor for Lactobacillus casei.
J. Biol. Chem., 149: 573-574, 1943.

Stokstad, E.L.R., Hutchings, B.L., Mowat, J.H., Boothe, J.H., Waller, C.W. et al. The degradation of the fermentation Lactobacillus casei factor.
I.J.Amer.Chem.Soc., 70: 5-9, 1948a.

Stokstad, E.L.R., Hutchings, B.L. and Subbarow, Y. The isolation of the Lactobacillus casei factor from liver.
J. Amer. Chem. Soc., 70: 3-5, 1948b.

Stokstad, E.L.R. and Manning, P.D.V. Evidence of a new growth factor required by chicks.
J. Biol. Chem., 125: 687-696, 1938.

Stovner, J., Engseth, A. and Brennhovd, I. Cancer chemotherapy by intra-arterial Methotrexate infusion. Protection of mucous membranes by topically applied citrovorum factor with hyaluronidase.
Cancer Chemother. Rep., Rep. No. 21, 1962. p. 147-148.

Strength, D.R., Mondy, N.I. and Daniel, L.J. The effect of Aminopterin on diphosphopyridine nucleotide.
Arch. Biochem., 48: 141-148, 1954.

Sullivan, R.D. Continuous arterial infusion cancer chemotherapy.
Surg. Clin. N. Amer., 42: 366-388, 1962a.

Sullivan, R.D. The dose, duration, and route of administration studies of Methotrexate in clinical cancer chemotherapy.
In R. Porter and E. Wiltshaw (Editors). Methotrexate in the treatment of cancer. Bristol, Wright, 1962b. p. 50-55.

Sullivan, R.D. Personal communication.

Sullivan, R.D., Jones, R., Schnabel, T.G. and Shorey, J.M. The treatment of human cancer with intra-arterial nitrogen mustard. (methylbis 2 - chloroethyl amine hydrochloride) utilizing a simplified catheter technique.
Cancer, 6: 121-134, 1953.

Sullivan, R.D., Miller, E. and Sikes, M.P. Antimetabolite-metabolite combination cancer chemotherapy. Effects of intra-arterial Methotrexate - intramuscular citrovorum factor therapy in human cancer.
Cancer, 12: 1248-1262, 1959.

Sullivan, R.D., Wood, A.M., Clifford, P., Duff, J.K., Trussell, R. et al. Continuous intra-arterial Methotrexate with simultaneous intermittent intramuscular citrovorum factor therapy in carcinoma of the cervix.
Cancer Chemother. Rep., Rep. No. 8, 1960. p. 1-6.

Talley, R.W., Vaitkevicius, V.K., Brennan, M.J. and Kelly, J.E. Intra-arterial chemotherapy of malignant diseases. Henry Ford Hospital Med. Bull., 9: 1-10, 1961.

Thiersch, J.B. and Philips, F.S. Effect of 4-amino-pteroylglutamic acid (Aminopterin) on early pregnancy. Proc. Soc. Exp. Biol. Med., 74: 204-208, 1950.

Thiersch, J.B. and Philips, F.S. Studies of the actions of 4-amino-pteroylglutamic acid in rats and mice. J. Pharmacol. Exp. Ther., 95: 303-311, 1949.

Trier, J.S. Morphologic alterations induced by Methotrexate in the mucosa of human proximal intestine. I. Serial observations by light microscopy. Gastroenterology, 42: 295-305, 1962.

Trier, J.S. Morphologic changes in human small intestinal mucosa induced by Methotrexate. Proc. Amer. Ass. Cancer Res., 3: 273-, 1961.

Waller, C.W., Hutchings, B.L., Mowat, J.H., Stokstad, E.L.R., Boothe, J.H. et al. Synthesis of pteroylglutamic acid (liver L. casei factor) and pteroic acid. I. J. Amer. Chem. Soc., 70: 19-22, 1948.

Watkins, E. Jr. Chronometric infusor - an apparatus for protracted ambulatory infusion therapy. New Eng. J. Med., 259: 850-851, 1963.

Watkins, E. Jr. and Sullivan, R.D. Cancer chemotherapy by prolonged arterial infusion. Surg. Gynec. Obstet., 118: 3-19, 1964.

Welch, A.D., Heinle, R.W., Sharpe, G., George, W.L. and Epstein, M. Chemical antagonism of pteroylglutamic acid in a pig. Hematopoietic effect of extrinsic and intrinsic factors. Proc. Soc. Exp. Biol. Med., 65: 354-368, 1947.

Werkheiser, W.C. The biochemical, cellular, and pharmacological action and effects of the folic acid antagonists. Cancer Res., 23: 1277-1285, 1963.

Werkheiser, W.C. Specific binding of 4-amino folic acid analogues by folic acid reductase. J. Biol. Chem., 236: 888-893, 1961.

Westbury, G. Experiences with intra-arterial Methotrexate therapy in head and neck cancer previously treated by irradiation. In R. Porter and E. Wiltshaw (Editors). Methotrexate in the treatment of cancer. Bristol, Wright, 1962. p. 56-58.

Westbury, G., Newton, K.A., Humble, J.G., Ford, H.T., Pegg, D.E. and White, W.F. Recurrent cancer of head and neck. Treatment with continuous intra-arterial Methotrexate and intermittent intramuscular citrovorum factor. Brit. Med. J., 1: 1238-1242, 1962.

Whiteside, J.A., Philips, F.S., Dargeon, H.W. and Burchenal, J.H. Intrathecal Amthopterin in neurological manifestations of leukemia. Arch. Int. Med. (Chicago) 101: 279-285, 1958.

Wills, L., Clutterbuck, P.W. and Evans, B.D.F. A new factor in the production of certain macrocytic anemias. Lancet, 232: 311-314, 1937.

Wilson, H.E., Doan, C.A., Saslaw, S. and Schwab, J.L. Reactions of monkeys to experimental respiratory infections. V. hematologic observations in nutritional deficiency status. Proc. Soc. Exp. Biol. Med., 50: 341-343, 1942.

Wolfe, L.S. Personal communication.

Woodhall, B., Hall, K., Mahaley, S., Jr. and Jackson, J. Chemotherapy of brain cancer: experimental and clinical studies in localized hypothermic cerebral perfusion, Ann. Surg., 150: 640-652, 1959.

Woodhall, B., Pickerell, K.L., Georgiade, N.G., Mahaley, M.S. and Dukes, H.T. Effect of hyperthermia upon cancer chemotherapy; application to external cancers of head and face structures. Ann. Surg., 151: 750-759, 1960.

Woods, D.D. The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide. J. Bact., 21: 74-90, 1940.

Wright, J.C., Cobb, J.P., Golomb, F.M., Gumport, S.L., Lyall, D. and Safadi, D. Chemotherapy of disseminated carcinoma of the breast. Ann. Surg., 150: 221-240, 1959.

ACKNOWLEDGEMENTS

I thank Dr. Theodore Rasmussen for the privilege of being able to carry out this project at the Montreal Neurological Institute.

I am indebted to Dr. Herbert Jasper for permitting me the use of the facilities of the Laboratory of Neurophysiology, and for his helpful suggestions.

To Dr. Charles Branch I owe a personal debt of gratitude for suggesting this project and for the many hours of help and useful criticism he rendered throughout the experiments.

Dr. Bernard Cooper's advice and aid in the Methotrexate assays are deeply appreciated. Mrs. Heli Girey of the Vitamin Research Laboratory did the Methotrexate bio-assays.

Without the cooperation of Miss Mary Roach and her staff this project would not have been possible. Mr. Charles Hodge and Mr. Mike Smith deserve full credit for the quality of the photography, while Mrs. Linda Nyeste prepared many of the graphs.

Dr. Hanna Pappius aided in the interpretation of the cerebral water study. Mrs. Hannah Szylinger carried out the dry weight determinations on the brain samples.

Doctors Gordon Mathieson, Eugene Meerovitch, Witold Pirozynski and Jaime Zamuco verified the pathological findings. It was especially kind of Dr. Mathieson to allow me to complete some of this project while working in the Laboratory of Neuropathology.

Animal SXIII sitting in his chair on the third day of the experiment. The electroencephalographic recording apparatus and the infusion pump are in the background.

Some six hundred histological sections were prepared in the Laboratory of Neuropathology, and I thank Miss Barbara Nuttall, Mis Robin Richmond, and Mr. Alan Mitchell for this mighty task.

Mr. Lewis Henderson suggested methods for recording the EEG data and helped with its interpretation.

Miss Ann Thomas helped during the typing of the first draft.

Abbott Laboratories Limited supplied the Talatrol (Tham) buffer, while the Lederle Division of Cyanamid of Canada donated the Methotrexate used in this study.