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A Preliminary Study of the Effect of Methotrexate on the Labeling Index of the Tongue and Palate Epithelium in the Mouse

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A PRELIMINARY STUDY OF THE EFFECT OF METHOTREXATE ON THE LABELING INDEX OF THE TONGUE AND PALATE EPITHELIUM IN THE MOUSE

by

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CHAPTER I

INTRODUCTION

Through the ages man has tried to conquer disease with his scientific knowledge and ability. In the treatment of neoplastic diseases many chemotherapeutic agents have been introduced which have resulted in varying degrees of success. One such group of agents, the folic acid antagonists, has been utilized for the past twenty years in cancer chemotherapy. Today, methotrexate is the most widely used member of this group of agents. However, as is the case with most anti-neoplastic drugs, the concomitant side effects that occur with therapeutic doses have limited its optimum utilization.

The complaint of a sore, painful mouth with a hyperemic appearance is a well-known side effect from methotrexate therapy. Many hematologists use the oral mucosa as a general indicator of the therapeutic effectiveness of this drug. Once the stomatitis has appeared, the drug is discontinued, and it is assumed that the optimal drug effect has been attained. Of course, regular hematologic studies and analyses are performed in conjunction with the therapy.

Although the modern medical literature contains many case reports and experimental investigations concerning the activity of methotrexate, very little is to be found regarding the specific dynamic effect on the

oral epithelium. In order to study this effect, the autoradiographic technique has been employed in this study. This technique has previously been utilized to measure the turnover rate of various other tissues throughout the body, but has never been used on the oral epithelium for this purpose.

CHAPTER II

REVIEW OF THE LITERATURE

A. Methotrexate

Physical Properties

The chemical processes of life occur continuously in all living organisms with the aid of special proteins, the enzymes. These react with food substances and with small molecules arising from these substances, which are termed metabolites. Huennekens and Osborn (1959) stated that coenzymes, including certain vitamins as folic acid, are metabolites which are present in only very small amounts in living tissues, but are essential for normal and continued function of the tissues. Stokstad (1954) mentioned that deficiency states of folic acid were described in man, monkeys, chicks, rats, guinea pigs, insects and microorganisms long before the vitamin was isolated and characterized.

Willis, et al, (1931) in Bombay attempted to identify a factor in yeast autolysate and crude liver extract which prevented some forms of megaloblastic anemia. Pfiffner, et al, (1934, 1944) were the first to isolate folic acid, or more exactly, pteroylglutamic acid (PGA). (Fig. 1a)

Angier, Subbarow (1946) and their group at Lederle Laboratories found that a growth factor for Lactobacillus casei was similar, if not identical, to the factor above. They were able to determine its constitution and were the first to synthesize it.

Bessey (1953) stated that the vitamin folic acid has been shown to be essential in cells for their normal growth, maintenance, and functioning of their cellular processes. These reactions can be blocked by certain chemicals which displace metabolites from enzymic reactions, thereby interfering with biochemical processes.

Delmonte and Jukes (1962) noted that the folic acid antagonists owe their profound biological activities to their ability to compete with the vitamin folic acid. These metabolic antagonists, such as methotrexate and aminopterin, are very similar in molecular structure to folic acid, except for the presence of additional chemical group(s).

Seeger, et al (1947) were the first to synthesize certain derivatives of folic acid, namely aminoproterin and amethopterin. The brand name of amethopterin is methotrexate.

In aminopterin the folic acid hydroxyl group in the 4-position of the pteridine ring is replaced by an amino group, thereby giving this folic acid antagonist its potency. The chemical name for aminopterin is 4-aminopteroylglutamic acid. (Fig. 1b). Methotrexate has in addition a methyl group on the amino nitrogen between the pteroyl and benzoyl group and is named 4-amino-N^{10}-methylpteroylglutamic acid. (Fig. 1c)

Rall (1963) described methotrexate as a moderately large molecule with a molecular weight of 454.4. It is fairly soluble as the salt in aqueous solution, but is insoluble in lipoid solvents at body pH. The two carboxyl groups in the glutamic acid portion of the molecule render it a fairly strong acid. Furthermore, the amino group (NH_2) on the pteridine

ring can ionize to a limited extent as a weak base. In general, this description resembles that of penicillin. Therefore, he concluded that after methotrexate ingestion there would be a limited degree of oral absorption, a volume of distribution largely confined to the extracellular spaces, and a limited penetration into the cerebralspinal fluid and brain.

The molecular chemistry of the pteridines is complex and the separation of related compounds has been very difficult. Oliver (1961) was only able to attain an eighty-five per cent degree of purity of methotrexate.

Distribution and Fate of Methotrexate

In order to understand the action of methotrexate in the body, one must first strictly define its tissue distribution. Methotrexate has been observed to persist in human liver, kidney and spleen. These tissues have been attributed by Dietrich et al (1952) to normally have a high folic acid and citrovorum factor.

Timmis (1967) stated that when folic acid is reduced and formylated, and the product is treated with alkali, 5-formyltetrahydrofolic acid is produced. It is also known as folinic acid leucovorum or citrovorum factor and is used as the most effective antidote against methotrexate toxicity.

Cherache (1960) and Freeman (1958) both concluded that the duration and amount of drug persistence are independent of the dosage, route of administration and duration of drug therapy.

Condit (1957, 1960) with human subjects, and Rall (1959) with rats and dogs showed that better absorption and maintenance of drug blood levels

were obtained with repeated small doses rather than with single large doses. However, drug toxicity also increases when this method of intake is utilized.

Berlin(1963) suggested that some of the drug does penetrate cells since the folic reductase system is intracellular.

Fountain et al (1953) used bioautographs to show that high concentrations of methotrexate persist in the tissues of the normal mouse (20 grams) for a period of three weeks and, in one instance, as long as eight months in the liver. A decrease in kidney concentration of methotrexate was also noted within 24 hours. The mouse rapidly eliminated all the methotrexate in excess of its renal threshold. He assumed that the methotrexate which was retained for such long periods of time was incorporated up to a relatively constant concentration in enzymes ordinarily associated with folic acid and citrovorum factor and any excess was rapidly excreted.

More recently, Henderson and Oliverio (1965) studied several different groups of male mice with various dosages of tritiated methotrexate. In the first group, 15 mg/kg was given and they observed that after twenty-four hours, most of it had been excreted with the remainder being distributed throughout the body tissues. Only the liver, kidney, stomach and gonads consistently contained greater quantities of radioactivity than did the remaining organs of comparable size. The bulk of the drug was excreted in the urine, and about 15 per cent appeared in the feces, apparently derived from biliary excretion. The urinary excretion was over 50 per cent in four hours after injection and 60 - 80 per cent by eight hours. At the end of the twenty-four (24) hour period, almost all of the original dose was

recovered. This confirmed the earlier findings of Werkheiser (1963) who stated that methotrexate was excreted fairly rapidly through renal pathways also that some biliary excretion occurred. The second group was pretreated for five days with 0.5 mg/kg/day of unlabeled methotrexate. This did not significantly alter the pattern of excretion, although total recovery of the drug was slightly reduced. A third group was administered 0.5 mg/kg of tritiated methotrexate for six consecutive days. However, this too failed to change the total amount of retained radioactivity or the proportions in the fractions obtained. The relatively constant proportions in the counts in each fraction of liver tissue appeared to reflect the dynamic equilibrium between subcellular components rather than binding sites with either sequential or variable priority for the drug. The majority of the remaining labeled methotrexate was precipitated with the nucleus and stromal elements during the 600 times "g" centrifugation.

The distribution in the liver was similar to that reported by Werkheiser (1961) and is consistent with his proposal that methotrexate binds to the enzyme folic reductase which resides in the soluble fraction of liver cells.

Since the antagonists are essentially irreversibly bound to folic reductase, Werkheiser (1962) suggested that the drug remains in the cell until the cell's death and removal from the body; and that the half-times represent an estimate of the turnover of the two populations of cells in the liver, and of the turnover in the intestinal mucosal cells.

Henderson et al (1965) established that about fifty per cent of methotrexate in mouse plasma was reversibly bound to proteins. The majority of this binding was associated with the albumin fraction, but its stability has not been determined. However, they felt that the degree of this binding was relatively constant over a remarkably wide range of concentration.

Johns (1961) found a similar degree of protein binding with tritiated folic acid. Johns et al (1964) performed in vivo studies indirectly comparing the affinity of a variety of folic analogs for folic reductase, and this resulted in the following ranking as far as their ability to displace bound tritiated methotrexate: methotrexate dihydrofolic acid folic acid formyl tetrahydrofolic acid methyl tetrahydrofolic acid.

Fischer (1962) and Wood et al (1961) have demonstrated a possible active transport mehcanism for methotrexate absorption.

In a recent experiment, Condit and Yoshino (1968) studied the displacement of 0.1 mg/kg of tritiated methotrexate by three compounds. They observed that after injection of tritiatiated methotrexate, the total radioactivity in the small intestine disappeared more rapidly than from the liver and kidney. Displacement of tritiated methotrexate from these three tissues increased following increasing doses of unlabeled methotrexate until almost all of the radioactivity in the organs was displaced by 10 mg/kg of unlabeled methotrexate. A dose of 25 mg/kg of folic acid displaced 25 per cent of tritiated methotrexate from the liver, but the same amount of citrovorum factor failed to displace any isotope.

Their chromatographic studies of the intestine revealed almost all the radiation tritiated methotrexate remained unchanged. The liver homogenate yielded a large peak of bound-form isotope, a relatively small peak of free tritiated methotrexate, and a very small peak of an unknown metabolite.

There is no evidence that methotrexate is metabolized in any mammals. Whatever amount is not bound by the folic reductase is excreted unchanged.

Mechanism of Action

Biochemical Alterations

Friedkin (1963) noted that the enzyme folic reductase, or as it is also known, dihydrofolic reductase, specifically catalyzed the reduction of folic acid to tetrahydrofolic acid and that in folic acid biosynthesis the dihydro form was the final product. Brown, Weisman and Molmar (1961) established that dihydrofolic acid was the initial compound required for the biosynthesis of tetrahydrofolic acid and its derivatives.

Tetrahydrofolic acid (FAH_4) is involved in the transfer of single carbon units in biochemical reactions involving the de novo synthesis of purines and pyrimidines, such as thymine, methionine, and serine, which eventually lead to the synthesis of nucleic acids.

Demonte and Jukes (1962) stated that mehtotrexate's usefulness is primarily due to the firm combination with the folic reductase system, thereby interfereing with these biosynthetic pathways among which is the synthesis of thymidylic acid which leads to DNA. (fig. 2). Methotrexate is not reduced by the folic reductase system and once it has combined with the enzyme, the antagonist takes no further part in the biochemistry of cells.

Mead (1963) felt that folic reductase was inhibited by a number of folic acid analogs. Through this inhibition, methotrexate and the other analogs could cause a depletion of various coenzyme forms, thereby reducing activity in the biosynthetic pathways which utilize them.

Mead also stated that the de novo biosynthesis of purines started with ribose-5-PO_4, and constructed the purine nucleus, step by step, to form the purine nucleotide inosinic acid. Two tetrahydrofolate enzymes are involved in this sequence, each contributing a one-carbon unit at the oxidation level of formate to the final purine nucleus. In this instance, methotrexate interfered with this biosynthetic pathway by inhibiting the folic reductase which supplies the two tetrahydrofolate enzymes.

Another key biochemical pathway known to be inhibited by folic acid antagonists is the biosynthesis of thymidylic acid which is essential for the synthesis of desoxyribonucleic acid (DNA). Here the enzyme thymidylate synthetase was found by Wahba and Friedkin (1961) to transfer a one-carbon unit from mehtylenetetrahydrofolate (from folic acid activation) to deoxyuridylic acid. The products of the reaction are thymidylic acid and dihydrofolic acid. (Fig. 3).

McDougall and Blakeley (1961) proposed that methotrexate, as is true of all folic acid antagonists, exerted an indirect inhibitory effect on this reaction by blocking dihydrofolate reductase. That is, since the folic acid cycle is dependent upon the regeneration of tetrahydrofolate from dihydrofolate, the inhibition of the reductase prevented further formation of methylenetetrahydrofolate and the overall effect was an inhibition of

thymidylate synthetase. (Fig. 3). This is in contrast to the direct inhibition of thymidylate synthetase by the fluorinated pyrimidines, which has been established by Hartmann and Heidelberger (1961).

A similar indirect inhibition by methotrexate occurs in the synthesis of methionine as reported by Larrabee (1961).

Thus many of the effects of methotrexate can be explained by the inhibition of folic acid reduction, both with respect to its highly spcific tissue toxicity and to its pharmacologic effects.

Werkheiser (1961) illustrated the affinity of methotrexate for the enzyme folic reductase by the fact that nearly eighty per cent of its activity remained in the supernatant after sucrose fractionation of treated rat liver. He also showed that the affinity of the partially purified folic reductase for methotrexate was about 100,000 times greater than for folic acid at pH 6.0 and 20,000 times as great for dihydrofolic acid at pH 7.5. Therefore, the enzyme was quickly saturated by the inhibitor, leading to pseudoirreversibility and the exclusion of folic acid from the enzyme. This strength of association between the drug and the enzyme was so great that the drug acted as a titrating agent.

Werkheiser (1961) used the term "stoichiometric" to describe this inhibition, since the amount of methotrexate bound by the soluble supernatant proteins of the rat liver was claimed to be precisely the amount required to inhibit the folic reductase in the preparation. This allowed a titration of enzyme with drug. Removal of the drug from the protein by dialysis against folic acid resulted in precisely equivalent reactivation of the

enzyme. He concluded that folic reductase was the agent in rat liver which binded the 4-amino derivatives of folic acid, both *in vivo* and *in vitro*, and thus probably represented the mechanism of long term retention of these drugs in the animal.

Using this procedure he showed that about half of the methotrexate which was retained in mouse liver twenty-four (24) hours after a single injectinn was lost very slowly. He arrived at a half-time of over ninety days. This work confirmed the findings of Fountain (1953) and Cherache et al (1960). Werkheiser felt that this stoichiometric relationship indicated that methotrexate was a very potent inhibitor of folic reductase and that within twenty-four (24) hours no free drug was available to inhibit any other enzyme.

Timmis (1967) stated that subsequent work on the drug-enzyme relationship and their interaction indicated that the binding of the drug to protein was more than could be accounted for by the amount of folic reductase present. This suggested that binding to non-specific protein may be a prerequisite of the metabolism of folic acid and its antagonists.

Condit (1961) suggested that the activity of methotrexate was mediated by some additional mechanism. In 1965 he confirmed that the antifolics had important sites of action in addition to inhibiting folic reductase. He noted the blockage of folic reductase in mouse liver by aminopterin and methotrexate with *in vivo* doses of 0.1 mg/kg.

Werkheiser (1963) elaborated on the ever present reality of auxillary problems which are associated with methotrexate therapy and its success and failure. These included the drug's side effects on the host, chemical

modifications of the drug to make more effective agents, special excretory mechanisms, limitations of access of the drug to its site of action due to permeability or active transport restrictions, intracellular compartmentation of the drug, and rate of recovery from the drug's effect. These problems are recognized, but as yet unsolved, and he felt that they should be considered when evaluating the therapeutic efficacy and the success of methotrexate treatment.

In addition to the above, the inhibition of folic reductase by methotrexate has also been well documented by Osborn et al (1958), Peters (1958), and Zakrzewski (1960).

To understand the exact intracellular action of methotrexate, it is convenient to observe the course of events following a single dose of methotrexate as described by Werkheiser (1963). The drug enters the cell rather rapidly, perhaps in amounts in excess of that needed to abolish folic reductase activity. The resulting effect is a profound inhibition of thymine synthesis with a lesser but still very strong inhibition of purine synthesis.

Thiersch, et al (1949) found by microscopic observation that protein synthesis continued during a period of obvious methotrexate toxicity; yet the cells were inhibited by the drug. He observed increased cytoplasmic volume microscopically. Van Scott (1959) noted enlarged epithelial tonofibrils in the epithelialization of psoriatic patients treated with methotrexate. These observations led Werkheiser (1963) to deduce that there was a continuation of protein synthesis during a period of

obvious methotrexate toxicity. Therefore, he concluded that the presence of the drug in a cell causes an abrupt cessation of multiplication, but permits a moderate synthesis of proteins, including that of folic reductase. Hakala (1962) showed that this enzyme could be synthesized at a significant rate by the fact that the inhibitory level of methotrexate for resistant S-180 cells in culture was determined by the balance between the rate of drug entry and the rate of enzyme synthesis. Hence, if the supply of this critical enzyme has been replenished, the cells recover and resume mitosis.

The free drug in the cell now has two alternate pathways open to it. Fischer (1962) found that part of it will pass out of the cell at a relatively slow rate, while part of it will combine with newly formed enzyme. When enough free enzyme is present, normal division processes would resume provided that the activity of folic reductase had not been abolished for twenty-four hours or more. Such cells would suffer irreversible damage.

Werkheiser (1962; et al 1963) found that a particular dose of the drug,which was high enough to inactivate all the enzyme that could be found during the critical period, was so closely related with the toxicity and the tumor sensitivity. He also determined that the dose necessary to reduce the free enzyme level of the intestinal mucosa to negligible levels was almost precisely the LD_{50}.

Fisher (1962) and Werkheiser (1962) both proposed that the primary parameter for defining the sensitivity of a tissue to methotrexate would

be the rate of entry of the drug into that tissue. Therefore, they felt that other phenomena besides the level of the enzyme in the tissue were responsible for the effect of this drug on different tissues.

As long as free drug exists, activity of folic reductase would be abolished. During this time no tetrahydrofolate could be formed from dihydrofolate. For non-dividing tissues such as liver and kidney, this would be of little consequence, since these tissues have large stores of tetrahydrofolate compounds. However, rapidly dividing tissues, for example, intestinal mucosa, bone marrow, oral mucosa, and tumors, contain much smaller amounts of tetrahydrofolate compounds (Nichol, 1953), but continue to oxidize them to dihydrofolate in the process of thymidylate synthesis. Therefore, Werkheiser (1963) explained that these tissues could become depleted of their stores of reduced folate cofactors quite readily, despite the normal circulating blood level of these compounds, which apparently is too low. If this situation were maintained, they would undergo "thymineless death" as Cohen (1956) has described.

Both Werkheiser (1961) and Williams (1955) established that the synthesis of thymidylate appeared to be more sensitive to the action of methotrexate than were the other reactions requiring tetrahydrofolate.

Since Werkheiser et al (1963) showed that the rate of methotrexate entering cells differs from one tissue to another, a dose adequate to provide free intracellular methotrexate for a considerable period in one tissue, may not suffice even to saturate all of the folic reductase in another tissue.

Burchenal et al (1961) investigated the effect of the administration of the final amino acid end products of the folic reductase system reactions on the toxicity of methotrexate therapy. They noted that Skipper was unable to prevent the inhibiting anti-leukemic effects of methotrexate by the administration of thymidine (TDR), adenine, histidine, methionine and serine either alone or in combination when they were given on a three times weekly dosage schedule. Burchenal used various dosages of methotrexate in order to find out which of the metabolic pathways interrupted by the drug was responsible for the toxicity. He found that methotrexate on a 0.2 mg/kg per dose five times daily for ten days was lethal to seventeen of twenty (20) mice. Using this drug dosage schedule, 20 mg/kg per dose of thymidine gave no protective effect. The same was true of serine, methionine, and histidine at 10 mg/kg. However, adenine at 10 mg/kg gave complete protection against methotrexate at 0.2 mg/kg., but not against 0.4 mg/kg of methotrexate. The addition of thymidine alone at 20 mg/kg or of thymidine plus the three amino acids gave no additional protection. They also stated that methotrexate in the mouse and man produced toxicity at significantly smaller dosages when given by frequent or continuous infusion.

Grant (1958, 1960) using the Rana pipiens embryo as the experimental system, revealed the effect of antifolic compounds on both DNA and RNA. The regions showing the highest mitotic activity, as in mammalian tissues, were most easily inhibited by the antagonists. This effect was completely prevented by folinic acid in a non-competitive manner. Thy-

midine gave protection at a later stage of development, but had no protective effect in the egg stage. He felt that the drug concentration in the cells was not enough to interfere with the de novo synthesis of RNA, but that the thymidine biosynthesis was sufficiently inhibited to prevent DNA synthesis and accounted for the tissue-specific nature of the observed effects.

Delmonte and Jukes (1962) stated that during methotrexate therapy, abnormal chromosomes and mitotic figures were seen which probably reflected the drug's interference with intracellular nucleic acid synthesis and provided morphologic evidence of direct damage to the cells of the host. However, they also stated that there was no morphologic evidence of direct damage to the cell dissolution.

Histologic changes

This section is devoted to the review of microscopic tissue alterations that have been cited following methotrexate therapy in both clinical and experimental settings.

Skin:

Van Scott (1963) stated that in the normal process of mitotic reduplication, a cell must double its DNA content during the intermitotic phase. Since folic acid is essential for DNA synthesis, the effect of methotrexate on the epithelium is to prevent or inhibit the cell from reaching the state of mitotic preparedness. This is recognizable by the rate of mitotic figures observed in a section of tissue per unit time. In integument, the process of mitosis is essential to replace those cells

that exfoliate and are lost to the external environment.

In the normal epidermis, the rate of loss of desquamated cells is such as to require a relatively low rate of mitosis in the single layer of germinative cells or basal layer, and histologically, comparatively few mitotic figures are found.

Rothberg et al (1961), using glycine -C^{14} incorporation into the normal stratum corneum as an indicator, found the turnover time of normal epidermis to be approximately 27 days.

Van Scott and Ekel (1963) observed an approximate ninefold increase in the number of germinative cells in the basal cell layers, and a 27-fold mitotic generation of epidermal cells in the hyperplastic epidermis of psoriasis. The skin of the upper back of six patients was chosen and, for comparative study specimens, the inner aspect of the lower lip were obtained in four of the six patients. They had observed that it was uncommon for the oral mucosa to become involved with psoriasis, even though it was continuous with epidermis. They attributed this to the fact that the population of germinative cells of the oral mucosa was normally large or as they termed it "psoriasiform".

Mitoses were found to occur in the three lower most cell layers of oral mucosa which, as in psoriatic epidermis, are often discernible by their deeper stainability. Another similarity of the oral mucosa to psoriatic epidermis was that the germinative cell population per unit surface of oral epithelium was augmented by undulations of the submucosal connective tissue papillae. However, the number of mitoses per unit sur-

face area of mucosa, while it was more than three-fold than in uninvolved epidermis, was still less than that in psoriasis.

Other similar characteristics shared by the oral mucosa and the psoriatic skin were parakeratosis, absence of a granular layer, and permeability for topically applied substances, all of which seem to be correlated with incomplete keratinization secondary to a heightened turnover time of the epithelium.

More recently, an autoradiographic study of the turnover times of normal and psoriatic epidermis was done by Weinstein and Van Scott (1965) using tritiated thymidine injected subepidermally. A total turnover time of 26-27 days for normal epidermis was found versus 4 days for that of psoriatic epidermis.

Van Scott (1963) felt that since the total thickness of the normal epidermis is composed of many cell layers, any interference with the mitotic replacement during an interval of one or a few days would interfere very little with the protective function of this epithelium. Therefore, after moderately large doses of the folic acid antagonists for a limited period, grossly detectable skin changes would be minimal in nature. Clinical changes may occur in skin following large doses, but these did not seem to result primarily from interference with mitotic replacement of the epidermis.

A limited amount of investigation of methotrexate activity on normal epithelium has been performed, such as the study of Robinson et al (1969). However, the majority of studies on methotrexate action have

been reported in treatment of psoriasis. Here the turnover time was found by Van Scott (1963) to be only three to four days. Therefore, he felt that psoriatic epidermis, because of its high turnover dynamics, was well-suited for the investigation of methotrexate action; especially, since this type of epidermis would be exquisitely responsive to the drug activity.

The use of folic acid antagonists in the treatment of psoriasis was introduced by Gubner and August (1951); and the work of Rees and his associates (1955, 1959) established that this treatment was effective and relatively safe.

Van Scott and Reinertson (1959) treated a total of eighteen psoriatic patients, 30-76 years of age, with several different chemotherapeutic agents, both topically and systemically. Six of these were given methotrexate 0.5 mg-5 mg/kg in a single intravenous dose and the epithelium was observed in conjunction with the following laboratory tests: white blood cell count, hemoglobin, platelet count and spermatozoa counts (on 2 patients given aminopterin and in two patients given methotrexate). All tests were repeated during and after therapy. Scalp hair roots were examined during therapy for any presence of dysplasia.

The skin biopsies of the treated patients revealed not a single cell in mitosis twenty-four hours after the initial injection of methotrexate. Within forty-eight hours a few cells in mitosis were found. Since keratohyaline granules could be identified in cells of the upper malpighian layer and the formation of tonofibrils continued during therapy, they concluded that the cytoplasmic activity was undisturbed.

The various laboratory procedures disclosed a lowering of the white blood cell count and platelets in the peripheral blood. In addition, nausea and/or ulceration of the oral mucous membrane occurred. Dysplasia of the roots of anagen, or growing hairs was observed, with or without hair losses.

Failure of topical methotrexate, in this study, to affect psoriasis locally, suggested to them that its pharmacologic action required initiation at a distant systemic site.

An interesting finding during their investigation was that spermatogenesis was depressed. The spermatozoa count depression ranged from 49% to 90%, and the total spermatozoa depression ranged between 63% and 97%. The sperm counts were done twelve to fourteen days after the single intravenous dose of methotrexate or oral aminopterin for several repeated courses. However, the depression was determined not to be permanent, since the two patients who had taken aminopterin, revealed normal counts two and three years later.

Biro et al (1967) in treating psoriatic patients noted that twenty-four hours following a single 50 mg. intramuscular dose of methotrexate, mitotic figures were no longer prominent with a marked decrease in the mitotic index. Pyknotic epidermal cells were found in large numbers in the two or three cell layers adjacent to the basement membrane. They felt that the location and number of pyknotic cells were suggestive of some relationship to the mitotic process. No change was seen in the dermis. After 72 hours the mitotic index returned to pre-injection levels or higher, and pyknotic cells were virtually absent. Epidermal

thickness and acanthosis were measured and no lasting change was produced.

Halperin and Ohkawara (1967) determined the effects of methotrexate on the enzymes of carbohydrate metabolism in psoriatic epithelium. They treated five patients with intramuscular methotrexate once a week. Many of the enzymes involved in carbohydrate metabolism were assayed prior to the treatment and at various times during treatment. They found that most of the enzymes measured decreased after therapy and there were no specific enzymes which were markedly resistant to methotrexate or markedly sensitive to its action. Psoriatic skin and surrounding normal appearance skin shared this suppression of enzyme activities. In the involved areas, the extent of clinical improvement corresponded in general to the measurable decreases in enzyme activities.

From their results, they concluded that methotrexate depressed enzyme activities in a rather nonspecific manner. They felt that since these enzymes are made by the same mechanism as any other cellular protein, the action of methotrexate would seem to depress protein production in general within the cell. They stated that protein formation was a complex process that required activated amino acids, transfer ribonucleic acids, ribosomal RNA, messenger RNA and DNA templates. Also necessary were an adequate supply of the various enzymes for forming these molecules and nucleotides in the form of adenosine or guanosine triphosphate as an energy source.

Since it has been determined that methotrexate binds much more strongly to the enzyme folic reductase than to folic acid itself, they agreed that all these reactions which required tetrahydrofolic acid for

their synthetic pathways would be affected. However, they felt that the decrease in enzyme levels noted in their treated patients were primarily due to a lack of protein synthesis, secondarily to the effect on the DNA and RNA synthesis. Yet, they were unable to substantiate this hypothesis with experimental proof.

Another observation of note in their project was an unexpected drop in enzyme activities in the uninvolved epidermis of a magnitude comparable to that in the involved epidermis. No gross changes were noted in the uninvolved skin clinically.

Gordon et al (1967) treated patients with various agents for psoriasis, giving two of them 20 mg. methotrexate I.V. weekly and one patient methotrexate 2.5 mg orally daily for seven days. Biopsies were taken daily for one week, weekly for one month, and then monthly. Skin from the back was stained with H and E. They classified the results according to the effects noted on each layer of epidermis. The parakeratosis of the stratum corneum disappeared by the sixth day after injection of methotrexate. The stratum granulosum assumed a normal appearance by the fifth day, and a 78% decrease in the stratum malpighii occurred by day four, but then began to increase again.

They suggested that methotrexate acted mainly through mitotic inhibition of the epidermal cells, since there was relatively little change in the inflammatory cell infiltrate or in the blood vessels.

In addition to the histopathological results, they determined that both the epidermal and dermal acid mucopolysaccharides remained consistent and did not show any changes. Only a moderate to minimal reduction

in the dehydrogenases activities were seen.

In the realm of animal experimentation, Robinson and Stoughton (1969) studied the effect of methotrexate on cell division in the epidermis of 4 - 5 day old Sprague-Dawley rats using the mitoses per 1000 interfollicular basal cells as an indicator. A dose of 5 mg/kg was administered subcutaneously on the ventral surface, and animals were sacrificed at 1/2, 1, 2, 3, 4, 15 and 24 hours after injectinn.

They found an obvious difference in the mitotic activity two hours after injection and this depression lasted up to and including the twenty-four hour group. The "t" test was not significant at the 1/2 and one hour times, but the differences were significant at 0.5% from the 2 hour interval and remained so thereafter. There was no significant difference between untreated, water or methotrexate treated groups on the mean percentage distribution of the phase of the mitotic cycle recorded.

In discussingtheir results, they stated that Fukayama (1961) reported the minimum G_2 time to be about six hours. Thus cells with a double complement of DNA should be available for mitosis throughout this period. They suggested that for some reason G_2 cells were unable to enter the mitotic cycle in the presence of this dosage of methotrexate. They proposed that this might be due to the failure of continuous entry of more cells into G_2 from the "S" phase, or to some effect on the little known processes within G_2 itself.

They further mentioned that Pelc (1968) maintained that metabolic DNA may be formed in some organs after the "S" phase and before mitosis.

However, there is no published information on the formation of metabolic DNA in the epidermis of the young rat.

Other theories on the exact reason for the failure of the onset of mitosis with methotrexate include that of Jacobson (1954) in which he felt that a lack of high energy compounds such as adenosine and guanosine nucleotides could lead to a failure of DNA, RNA and protein synthesis, thereby secondarily leading to a failure of mitosis.

Galper and Darnell (1969) found that methotrexate inhibited the synthesis of formylmethionine S-RNA in the mitochondria of HeLa cells, which are of mammalian origin. This observation could give credence to the belief that methotrexate has a direct effect on the protein synthesis of cells as well.

Fry and McMinn (1966) correlated the findings of methotrexate therapy on both the skin and the intestine of psoriatic patients. A dose of 0.5 mg/kg methotrexate was administered intramuscularly once a week. They used the mitotic skin count and that of the intestinal crypt cells for comparison. Ten of twelve patients showed varying degrees of clinical improvement within six weeks. Two of three patients showed a decrease in skin counts at three hours, and 3 out of 6 by the 24 hour interval. Microscopically, the intestinal specimens had normal appearing villi with two of four patients eliciting a decreased count at twenty-four hours.

The presence of degenerate cells with pyknotic nuclei (not included in the counts) was regarded by them as evidence of the toxic effect of methotrexate on both epidermal and intestinal cells; this was especially evident twenty-four hours after the first injection.

A marked fall in mitotic index after methotrexate occurred in the intestine and the skin, but unlike the psoriatic epidermis, the count of the intestinal epithelium returned to the preinjection level by the seventh day. They claimed that this observation supported the theory that the "healing" action of methotrexate in psoriasis is primarily a direct one on the premitotic and mitotic cells of the epidermis.

Regarding the utilization of the autoradiographic technique for studying the intestine of humans, they felt that injections of thymidine in suitable dosage form were impractical, aside from the fact of the ethical problem involved in giving a radioactive isotope with a half-life of twelve years to patients with a non-fatal disease.

Intestine

The other suitable tissue in which some work has been done with methotrexate or tritiated thymidine, and which can be related to the oral mucosa, is the intestine. Both animals and humans have been used for investigation.

Loehry and Creamer (1969) have investigated the three-dimensional structure of the rat small intestinal mucosa,both in the normal state and under increased and decreased mitotic conditions. To observe the intestine in a repressed turnover state, they used a single 7.5 mg I.M. dose of methotrexate and studied the effects daily for a period of seven days. In conjunction with mitotic counting, two rats on day five after methotrexate and two normal controls were injected intraperiotoneally with 100 uc of tritiated thymidine. One methotrexate and one normal rat were sacrificed

after eight hours and the other pair 24 hours after the injection. Autoradiograms were prepared on sections of small intestinal mucosa.

The mitotic counts revealed a marked reduction on days one and two with a few mitoses reappearing on day three. By day four to six, a marked increase had occurred, a rate greater than that of the normal. Mitotic activity returned to normal by the seventh day.

The tritiated thymidine was administered during the period of regeneration of the intestinal mucosa, day five, so that the eight hour finding in the control showed labeling of the crypt cells, while the methotrexate treated had occasional labeling at the base of the villi. By the twenty-four hours after ^{3}H-TDT, the control rats showed labeling approximately one third of the way up the villi, whereas, in the methotrexate treated animals the lower two-thirds were labeled. The latter observation was agreeable with the higher than normal mitotic rate found in the regenerating intestine.

The height of the labeled villous cell column at twenty-four hours was a mean of 22 cells in the control rats compared with a mean of 41 cells in the treated group. A standard deviation of 4.3 was present in both groups. The difference between the two groups, according to their findings, was highly significant ($p < 0.001$).

Their investigation showed that a single dose of methotrexate produced a period of diminished cell production for the first three days after injection, followed by a three day period of increased cell production as the mucosa regenerated. Tritiated thymidine was utilized for the "specific documentation"of the regeneration period dynamics.

In the past, Phillips et al (1949, 1950), using lethal doses of aminopterin produced gross cytoplasmic vacuolization of intestinal epithelium in the rat, mouse and dog; the epithelial lining of the intestine became desquamated with quite extensive leukocytic infiltration of the lamina propria.

Dustin (1950) noted striking mitotic inhibition of the mouse crypt epithelial cells associated with nuclear degeneration, within a few hours after lethal doses of aminopterin. A return of mitoses was evident in sixty hours, and by ninety-six hours, mitoses appeared normal.

Vitale et al (1954) administered nonlethal doses of aminopterin to rats and observed a reduction in the number of mitoses, degenerated mitotic figures, nuclear remnants, and disintegrated crypt cells within four and one-half hours after injection. Normal mitoses returned 48 hours later.

Trier (1962) reported on the morphologic alteration in the human proximal small intestine induced by methotrexate therapy of 2-5 mg/kg I.V. in fourteen patients without evidence of gastrointestinal disorders. Within three hours after the injection of methotrexate, mitoses per 1000 cells were greatly reduced compared to baseline levels. By six to twelve hours, mitoses were virtually absent. With this marked inhibition, he noted the appearance of discrete, spherical bodies within the cytoplasm of many crypt cells. These bodies ranged from a fraction of a micron to 4 or 5 u in diameter and varied considerably in structure. Most of these bodies contained some material which stained intensely with the Feulgen technique, indicating DNA probably of nuclear origin. The intracytoplasmic bodies were confined exclusively to the crypt region and were never seen in the

villous epithelium. Trier noted the identical bodies in the crypt cells of a biopsy taken from a patient 20 hours after 100 r of abdominal x-irradiation.

He stated that the mitotic inhibition persisted for approximately 48 hours, with an increased return of mitoses from 48 - 96 hours, at which time they were again normal.

With light microscopy the architecture of the small intestine did not appear to be altered by methotrexate, nor were cytologic changes noted anywhere than in the crypt. However, under the electron microscope Trier (1916) observed striking dilatation of cisternae of the endoplasmic reticulum and Golgi material, swelling with fragmentation of the micro-villi of some mucosal cells, and moderate mitochondrial fragmentation occurring eight to 48 hours after methotrexate. Adjacent to such damaged cells, morphologically normal appearing cells were seen, and at 96 hours, no structural alterations were seen. He concluded that methotrexate in a dose range of 1.5 to 5.0 mg/kg regularly produced extensive but reversible morphologic alterations of the human small intestinal mucosa, by the inhibitory effect on nucleoprotein synthesis. Also, these changes correlated temporally with the period of gastrointestinal toxicity.

Oral Mucosa

Shklar et al (1966) in their investigation of the effect of methotrexate on chemical carcinogenesis of the hamster buccal pouch, had a group of control animals to which only methotrexate was given. A dose of 0.5 - 0.568 mg/kg was injected subcutaneously three times weekly for a period of twelve weeks. They stated that this dosage represented the

maximum tolerated dose for this type of animal for the length of their study. Higher rated dosages of methotrexate were found to result in the deaths of a number of similar experimental animals in observations they carried out prior to this experiment.

They concluded that methotrexate not only appeared to accelerate the formation of tumors in the buccal pouch painted with carcinogenic chemical, but acted to produce or develop a more highly anaplastic type of carcinoma.

The buccal pouches of the methotrexate control groups in their study were found to be free of gross pathology. However, microscopically, there was some evidence of collagen degeneration in the lamina propria of the pouch mucosa of the 12 week group of animals.

Two years thereafter, Shklar (1968) gave 0.8 mg/kg of methotrexate to 20 hamsters six months of age, weighing 115-130 grams. This dose was administered subcutaneously three times weekly for four weeks. Sections of tongue, palate and gingivae were taken then stained with H and E. He found that, although there were no striking gross oral changes visible, microscopic examination of the sections revealed a decrease in the width of the stratum corneum and a notable degeneration of the collagen bundles in the underlying lamina propria, an observation in his earlier project. The collagen bundles were clumped and stained more deeply in comparison with the control specimens. He concluded that the administration of non-lethal dosages of methotrexate resulted in collagen degeneration and epithelial atrophy of the oral mucosa, thereby possibly explaining the occurrence of oral lesions in patients on such therapy. No mitotic counts or other criteria were done in this study, nor has any been noted elsewhere

in the literature on the effect of methotrexate on the oral mucosa.

Drug Application

Farber et al (1948) were the first to report results of aminopterin treatment on five children with acute leukemia. Their study covered a period from November, 1947, to April 15, 1948. The original group contained sixteen children, of which ten showed some transient clinical, hematologic, and pathologic improvement. The remaining six did not respond. Of the five on which they elaborated, one twelve-year old boy developed a stomatitis which interferred with optimal use of the drug. Another twelve-year old boy developed minor ulcerative lesions of the oral mucosa, resulting in dose reduction.

Since this initial report, numerous clinical applications have been investigated. Bertino and Johns (1967) stated that among them are the treatment of neoplastic diseases, which include choriocarcinoma, Burkitt's lymphoma, palliation of patients with epidermoid carcinoma of the head and neck and also of the lung. They mentioned that the use of methotrexate in large intermittent doses has produced objective responses in about 30 to 50 per cent of the patients treated for head and neck carcinoma. However, these responses have been of short duration.

In addition to the above, methotrexate therapy has been utilized in psoriasis (Van Scott, 1964), and more recently, for the suppression of the immune response.

Friedman, Buckler and Baron (1961), using male guinea pigs, revealed that methotrexate prevented the development of a delayed skin-hypersensitivity response to diphtheria toxoid when ovalbumin was given as a single immunizing dose.

Prichard and Hayes (1961) found that no animal receiving both aminopterin and bacilli simultaneously developed hypersensitivity, but that all controls reacted positively.

Tobin and Argano (1967) administered a dose of 4 mg./kg. i.p. to young adult male mice. In addition, a single dose of tanned sheep red cells was given I.V. to the experimental group. Control animals were given antigen but were only injected with distilled water intraperitoneally. Their results indicated that there was significant suppression of antibody production in the treated mice when compared with the control animals. One month later both groups received a second injection of sheep red cells, but no additional methotrexate. In this instance, they found that the antibody titres were equal in both groups. They concluded that in this particular system, methotrexate did not induce a permanent refractory state to the antigen, although it was capable of suppressing the primary immune response. That is to say, methotrexate failed to induce tolerance. From these findings they concluded that methotrexate may have therapeutic implications in the treatment of autoimmune diseases in man.

Of particular note is the work of Broughton (1967) on homologous tooth transplants. He transplanted maxillary and mandibular tooth buds from a 50-day fetal guinea pig into the subcutaneous space in the axillary region of adult guinea pigs. This was according to the method of Fleming and Soni (1965).

The experimental guinea pigs were injected with 5 mg. of metho-

trexate i.p. one day prior to transplantation and daily thereafter for three weeks. Then the injections were given every other day until the animals were sacrificed. However, the control animals were not given injections. On the fifteenth postoperative day and for one month thereafter one guinea pig from each group was sacrificed weekly.

Histologic examination of the sections of recovered tooth germs showed a significant difference between transplants of the two groups. The treated group showed normal formation of predentin, dentin, and enamel. The inflammatory response was negligible and appeared to have little or no effect on the formation of the teeth. However, in the control group there was evidence of a severe inflammatory response, resorption of the dentin, and irregular enamel formation. Therefore, he concluded that methotrexate had suppressed the inflammatory reaction in the treated group.

Toxicity in Humans and Animals

The folic acid antagonists primarily affect the more rapidly growing tissues. Bertino and Johns (1967) stated that the therapeutic index in humans is narrow and, in most instances, toxicity of a significant degree may result even when the antimetabolite is utilized in therapeutic doses. For example, the bone marrow may be depressed and clinical signs of weakness, infection and bleeding may be present. Each of these is related respectively to the anemia, granulocytopenia, and thrombocytopenia seen with this depression. Pharyngitis, as well as ulcerations of the oral and intestinal mucosa may occur.

Delmonte and Jukes (1962) noted that buccal lesions could precede or accompany other symptoms of intolerance. These buccal lesions were described as small, shallow, painful, white or yellow, red-edged lesions on the lips, tongue, or other oral mucosa. Progression to ulcerative lesions followed if therapy was continued.

The gastrointestinal symptoms of methotrexate toxicity include anorexia, abdominal cramps, nausea, and vomiting. Diarrhea, when it occurs, is generally a more severe manifestation of drug toxicity. Delmonte and Jukes (1962) stated that this usually coincided with the development of extensive areas of atrophic degeneration and denudation along the entirety of the lower gastrointestinal tract. They also stated that if drug therapy continued after these signs appeared, severe toxicity resulting in death could follow.

Several minor signs of toxicity with no ulterior sequelae detrimental

to the patient are also possible. Among them are brownish skin pigmentation, erythematous or exfoliative dermatitis, especially on the hands and feet, and weight loss. Delmonte and Jukes (1962) felt that this weight loss attributed by some to the drug therapy, was probably more a consequence of the tumor patient's cachexia and poor physical condition.

Van Scott (1957, 1959) stated that transitory alopecia may occur with therapy, due to reversible atrophy of anagenetic hair bulbs.

One of the most interesting aspects of the antifolic compounds is their cumulative effect. Rall (1963) stated that while the LD_{50} of rats given methotrexate in a single dose is about 100mg./kg., the LD_{50} for a period of fifteen days of administration is only about 0.25mg./kg.--almost a thirty-fold difference in total dose.

Philips et al (1950) concluded that on a dose/kg. weight basis, methotrexate was tolerated much better in mice, and slightly better in dogs, than either aminopterin or dichloromethotrexate--other members of the folic acid antagonist family.

Sullivan (1961) postulated that drug toxicity occurred mainly because of tissue absorption and prolonged contact of the drug within the tissue, and not as a result of drug concentration in the blood.

Freeman and Narrod (1961) found that following oral and intravenous methotrexate, toxicity was related directly to the rate of drug absorption rather than to the rate of its excretion. They felt that the rate of excretion was wholly a consequence of the delayed drug absorption.

Burchenal (1951) demonstrated that the toxic effects of methotrexate

overdosage were reversible. He recommended that 3mg. to 12mg. of citrovorum factor, the most effective antidote, be given intramuscularly immediately or within four hours of the onset of toxic symptoms. When this technique is utilized, the therapeutic effect of the agent is also neutralized.

The effect of methotrexate on the normal mouse leukocytes has been documented by Burchenal et al (1950). They used 20 gram young male mice and administered 3.0 mg./kg. of the drug intraperitoneally three times a week for ten doses. The mice were weighed weekly and the dosage adjusted on a weight basis. Control leukocyte and differential counts were done before treatment, and repeated every four days on the surviving mice. In order that the counts could be done on free-flowing blood, the mice were warmed for ten minutes under an electric lamp. Then, a small cut was made in a tail vein with a razor blade and the studies done on the blood obtained in that manner. Blood smears were stained with Wright's stain.

They revealed that methotrexate appeared to cause only a slight leukopenia in normal mice, with no alteration of the differential count. This slight leukopenia was replaced by normal leukocyte counts around the nineteenth day. These normal counts in the treated animals remained relatively stable for the duration of the thirty day experimental period.

Van Scott et al (1964) have provided evidence that treatment of psoriasis with methotrexate 50 mg. intravenously once a week was effective and would be less toxic than oral therapy of 2.5 mg. daily for six days, the recommended dosage schedule. They further stated that oral

mucosal ulceration following administration of folic acid antagonists seemed to be a direct result of mitotic inhibition where cells normally desquamated were not replaced.

On the other hand, Ryan et al (1964) felt that with low dosage schedules of methotrexate mouth ulceration was no more common or consistent than the appearance of mouth ulcers in patients not on folic acid antagonists. Once ulcers appeared they may have failed to heal unless the folic acid antagonists were discontinued. They proposed further that until the cause of the increased turnover of epidermal cells in psoriasis was determined, it could not be assumed that the demands for folic acid were any greater for nucleic acid synthesis than for some of the other enzyme reactions subserving dermal tissue function, and which could indirectly lead to the increase in mitosis. The as yet undetermined amount of folic acid required for the increased vasomotor or neuronal activity was also believed to play a role in the etiology of psoriasis.

Van Scott (1963) stated that the eruptions of an erythematous nature that may occur during treatment of psoriasis with methotrexate probably were due to effects of the drug on the small blood vessels.

As far as the hematologic effect, Vogel (1961) used a dose range of 0.2 to 2.25 mg./kg. of methotrexate in his investigation of several antineoplastic compounds in determining their tumor-marrow index. He defined the latter as that dose causing 50 per cent destruction of the marrow divided by that dose causing 80 per cent suppression of tumor

growth in the same animal. He utilized the 6C3HED lymphosarcoma in mice for his observations. His obtained value of 3.2 mg./kg. of methotrexate was the dose which would cause 50 per cent marrow destruction.

Sloboda (1960) compared the effect of several folic acid antagonists upon mice bearing the 6C3HED lymphosarcoma to establish a quantitative means of comparing these compounds. He used the carcinostatic index, which he described as the toxic dose/effective dose, to effectively and quickly summarize the attributes of a compound as to its effect upon the tumor-bearing mice. He found that a dose of 0.705 mg./kg. of methotrexate produced an efficacy ratio (control/treated) of 2, while 2.10 mg./kg. was the dose which produced a differential weight change of 3.4 grams between the control and drug treated animals.

B. Autoradiography

The method of autoradiography has been described by Fitzgerald (1953) as dependent upon the fact that radioactive isotopes may be localized to a given area in a histologic section, as well as to individual cells. The tissue under study is placed subadjacent to a photographic emulsion for an adequate time to allow exposure of the emulsion. The emulsion is then developed as in ordinary photography and the processed emulsion is then referred to as an autoradiogram. It consists of accumulations of silver granules which appear black and which overlie the areas in the tissue containing the radioisotope. The ionization of the silver bromide crystals contained in the photo-

graphic emulsion is produced by the rapidly moving charged particles emitted from the radioisotopes, thereby permitting the observation of the presence of the radioisotope (Schoenheider, 1960).

Schoenheider (1960) stated that the chemical, as well as the biological behavior of a substance labeled with a radioisotope were identical to that of its stable counterpart provided that the amount of radioactivity that was contained in it was small enough not to have a significant radiochemical effect and that the amount of material injected was small enough not to produce a significant increase in the amount of the substance in circulation. Thus, the labeled substance could be considered a true "tracer" of normal in vivo metabolism.

Hughes et al (1958) stated that radioactive tritium was an excellent substance to utilize for a label, since very high resolution could be obtained; yet, it has very weak energy and a short range of beta rays. In tissue the maximum distance a beta ray will travel is six microns, and half of the particles will travel less than one micron. Therefore, the majority of the activated silver grains of an autoradiogram should lie within one micron of their source in the tissue.

The use of tritium as a tracer substance in autoradiograms is a useful adjunct in studying the presence of a chemical substance, such as thymidine, or its metabolites in a histologic tissue section.

Lajtha, Phil, and Oliver (1959) concluded that thymidine was a specific component of DNA, since it labeled only DNA. Tritium was determined to have a half life of 12.26 years and a disintegration rate of 0.006% per day.

Messier and Leblond (1960) stated that the synthesis of DNA is known to occur before mitosis. Therefore, if the radioactive precursor were given at that time, the nucleus would become radioactive and, using radioautography, could be noted even before mitosis occurs. These nuclei retained the label of radioactivity and passed it on to the daughter cells during mitosis. Therefore, tritiated thymidine is a valuable compound for the detection of newly formed DNA by the method of autoradiography.

In their study they reported on the labeled nuclei in the mouse tongue and epidermis after eight hours, twenty-four hours, and seventy-two hours from the initial tritiated thymidine injection. They investigated the various rat tissues more extensively with additional detailed time interval observations. Since methotrexate is known to interfere with DNA synthesis, the autoradiographic method is a most accurate one for the determination of the specific effect of this drug on the oral mucosa.

CHAPTER III

MATERIALS AND METHODS

Fifty-two adult male mice, Swiss-Webster strain,weighing an average of 25 grams, and on a diet of Purina Chow for mouse or rat, were used. Food and water were given ad libitum. Blood for initial and final white blood cell counts, and differential Wright's blood smears were obtained by cutting off the tip of the tail of each mouse and aspirating blood into 1 cc. disposable syringe. Weights were taken initially, periodically during the six-day injection period, and at the time of sacrifice. Methotrexate dosage was adjusted according to a weight basis.

The experimental mice, 26 in number, were injected intraperitoneally with a therapeutic dose of 2.5 mg/kg of a 1.5% starch solution of Methotrexate Sodium (supplied by Lederle Laboratories, Adolph E. Sloboda, Ph.D., Pearl River, N.Y.) daily for a period of six days. Twenty-six control mice were injected intraperiotoneally with 0.4 cc. sterile water daily for the same period. Then, on the seventh day both groups were injected intraperitoneally with 50 uc. of tritiated thymidine (supplied by Schwarz Bioresearch, Orangeburg, N.Y.) and were sacrificed at intervals of 15, 30, 45 and 60 minutes, 2, 3, 4, 5, 6, 7,8,9, 10, 12, 14, 16, 18, 20, 24, and 48 hours following the injection.

The tongue and palate were dissected out on each mouse, fixed in formalin, dehydrated, and embedded in paraffin.

Sections of the tongue and palate, cut at 5 microns were prepared. Three slides from each sacrifice period were selected for autoradiography. Additional sections of each tissue from each time interval were stained by the Hematoxylin and eosin method.

Autoradiographic Technique

The autoradiographic technique used was modified from that described by Fitzgerald (1961). Under darkroom conditions, a Wratten #2, Red Safelight 25 watt bulb was used. A humidity of 70% and below was sustained. The slides were dipped in Kodak NTB3 liquid emulsion (supplied by Eastman Kodak, Rochester, N.Y.) and were air dried for 10 minutes. Ten slides were placed in a black lightproof exposure box, section sides up. Lithium chloride was placed in the box for maitaining a low humidity, and black plastic tape was used to seal the box. It was exposed for 10 days at low humidity and temperature. During the exposure time, the box must be maintained in a position which keeps the sections upright. After the 10 day period, the slides were placed in a staining rack and developed for five minutes at $60^{\circ}F$ ($18^{\circ}C$) in Kodak D19 developer. The slides were rinsed in distilled water for 30 seconds and then placed in acid fixer for 10 minutes. Following this, the slides were rinsed in running tap water for 30 minutes. Both the staining dish and slides were covered with tissue paper to prevent dust from settling on them and allowed to dry in a stream of air. The processed autoradiograms were then stained with nuclear fast red and counterstained with indigo carmine.

Cell Population

An area of 100 basal cells in length was determined and the corresponding numbers of total epithelial cells it represented was found. This was then adjusted for a total population of 1000 epithelial cells. For each time interval, 2-4 slides were utilized.

Percentile of Labeled Nuclei

Under high dry magnification 500 basal cell fields were counted. The number of labelled nuclei observed in an area of 1000 cells were recorded, and from this, the mean and standard deviation were determined. The percentages were converted to $\log_{10}$ for the preparation of a curve (Figs. 4-5).

CHAPTER IV

FINDINGS

Within one hour after injection of tritiated thymidine, an average of 9.83% and 10.55% of the stratified squamous epithelial cells in the tongue and palate, respectively, were labeled in the control animals. The experimental mice showed an average of 15.60% labeled nuclei in the tongue and 24.24% in the palate during the same interval. (Tables I,II)

The number of labeled nuclei in the tongue doubled from an average of 9.83% to 19.40% by the six hour period in the control animals. The experimental group showed a doubling of 15.60% to 32.75% in the number of epithelial cells in the tongue an hour later (7 hours). (Table I)

In the palate of the control group the number of labeled nuclei doubled from 10.55% to 27.50% by the sixth hour after injection of the tritiated thymidine. The experimental group showed doubling of labeled cells from 24.24% to 45.30% by the eight hour period. (Table II)

The labeled cell values continued to climb and reached a peak of 73.80% and 67.30% in the control tongue and palate, respectively, during the 10-14 hour interval.

In the experimental animals the level of labeled cells reached a plateau around 27% in the tongue and 43% in the palate within the 10-14 hour period.

During the 12-24 hour interval the control group showed a gradual decline in the number of labeled epithelial cells, while that of the experimental group showed a recovery by maintaining an average of 25% labeled cells in the tongue and 34% in the palate. (Fig. 4-5) By the end of 24 hours the total number of labeled nuclei in both control and experimental groups were comparable.

The total white blood cell counts of the control group showed only interpersonal variation and did not increase or decrease significantly during the experimental period. (Table III) On the other hand, the counts of the treated group were found significantly to decrease 19% during the six day treatment period. (Table IV) The differential white blood cell count did not vary from the normal.

Upon comparison of the initial white blood cell counts of the control and experimental animals a "t" value of 0.113 was obtained. A similar comparison of the initial and final control counts gave a "t" value of 0.484. (Table III) However, when the initial and final white blood cell counts of the experimental animals were analyzed, a "t" value of 1.783 at 50° levels of freedom was found to be significant (0.05 P 0.025). (Table IV)

Throughout the six-day treatment period no significant weight loss was noted. (Table V)

CHAPTER V

DISCUSSION

Stratified squamous epithelial tissues from the tongue and palate of the mouse have been investigated in regard to the specific effect of methotrexate upon these tissues.

During the first hour after injection of tritiated thymidine, the control animals showed an average of 10% labeled epithelial nuclei versus 16% and 24% in the experimental animals. This indicated that there was initially a greater residue of inhibited cells in the methotrexate treated animals; that is, many cells in the experimental group were unable to proceed with DNA synthesis and continue into mitosis, since they were thymidine deficient. However, upon injection of the tritiated thymidine, these inhibited cells did accept the thymidine, but still were unable to proceed immediately into mitosis, as was evident by the delay in doubling time of one hour in the tongue and two hours in the palate.

The actual time of doubling occurred between 6 - 14 hours in the control group and 7 -14 hours in the experimental group. This is evident since the labeled cell values continued to climb, especially in the control group, until a peak was reached during the 10 - 14 hour interval. (Fig. 4-5) However, at 10 hours only 23% of the experimental cells were labeled as compared to 47% of the control group. This showed that the control animal

cells were free to continue mitosis while the experimental cells were suppressed. Therefore, methotrexate at this therapeutic dosage was effective in inhibiting the epithelial cells since they were not labeled, and a resultant delay into mitosis was observed.

The actual doubling time, as derived from the continued increase in labeled values after 6 hours in the control group, agrees with the findings of Schoenheider (1960) who reported a doubling time of eleven hours in the stratified squamous epithelium of the ventral surface of the tongue in the mouse. Dhawan (1964) also found a peak of labeled epithelial cells in the ventral surface of the tongue at 10 hours. He reported a doubling time of 8 - 10 hours in the epithelium of the palate in mice. Joglekar (1964) agreed with these findings in her study of the normal mouse interdental papillae.

The delay in doubling time of one to two hours found in the tongue and palate is supported by previous investigations of methotrexate inhibition upon DNA synthesis and mitosis in the epidermis and intestinal mucosa of both humans and animals. Robinson and Stoughton (1969) observed depressed mitotic activity in the epidermis within two hours after a dose of 5 mg/kg of methotrexate in 4 - 5 day old rats. Fry and McMinn (1966) noted a marked decrease in the mitotic counts of the skin and intestine of psoriatic patients as early as three hours after methotrexate administration. Trier (1962) reported greatly reduced mitotic counts in human intestine within three hours after drug injection.

The fact that the epithelial cells of the experimental animals in this study elicited a recovery of mitotic activity during the 12 - 24 hour

interval might be explained by Werkheiser (1963) who maintained that as long as a sufficient level of methotrexate was present, all the available folic reductase enzyme would be bound. However, as time progressed, the concentration of the drug diminishes and newly formed enzyme could be utilized to continue DNA synthetic pathways. The formation of new enzyme can occur since it is essentially protein synthesis, which is not greatly reduced by the presence of methotrexate.

During the six day treatment period no significant weight loss was noted (Table 5). This is in contrast to Sloboda (1960) who stated that 2.10 mg/kg of methotrexate for six days produced a differential weight change of 3.4 gms between control and treated animals. However, his study also involved the 6C3HED lymphosarcoma in the mice, and it may be assumed that the weight change was due to influencing factors other than methotrexate, as also stated by Delmonte and Jukes (1962).

The depression of the total white blood cell count by 19% found in this investigation demonstrated that 2.5 mg/kg of methotrexate is a therapeutic dosage and is agreeable with the findings of Vogel (1961) who reported some marrow destruction from a comparable dosage of methotrexate.

The fact that no significant change occurred in the differential count of the experimental animals coincides with the results of Burchenal et al (1950). They concluded that with a 3.0 mg/kg dose of methotrexate given three times a week to mice, no alteration in the differential count occurred.

The present investigation has shown that tritiated thymidine administration may serve as a model for studying the effect of a therapeutic dosage

of methotrexate for a period of six days upon the tongue and palate of the mouse.

CHAPTER VI

SUMMARY

Twenty-six adult male mice, Swiss-Webster strain, were maintained on a therapeutic dose of methotrexate sodium, 2.5 mg/kg, daily for a period of six days. Twenty-six similar control mice were injected intraperitoneally with 0.5 cc. sterile water for injection daily for the same period.

On the seventh day both groups were injected with 50 uc. of tritiated thymidine and were sacrificed at twenty intervals between fifteen minutes and forty-eight hours.

The tongue and palate were sectioned at 5 microns, and the sections were stained with hematoxylin and eosin. Additional mounted sections also were used to expose a coating of Kodak nuclear track emulsion NTB3 for ten days, followed by development of the sections and staining with nuclear fast red and counterstaining with indigo carmine.

The number of labeled nuclei in the basal and subsequent layers were counted for each time interval.

At fifteen minutes labeled nuclei were observed in the epithelium of the ventral surface of the tongue and of the palate in a random distribution. Within one hour an average of 10% of the control group tongue and palate were labeled as compared to 16% and 24% in the methotrexate treated tongue and palate, respectively. Through mitosis the cells had doubled

in number within six hours in the control animal tongue and palate. In the experimental animals the doubling occurred at seven hours in the tongue and eight hours in the palate.

The total white blood cell counts of the treated group decreased significantly by 19%

Throughout the six day treatment period no significant weight loss was noted.

CHAPTER VII

CONCLUSIONS

Ten per cent of the cells in the tongue and palate of the control animals were labeled within one hour. In the experimental group an average of 15.60% of the cells in the tongue and 24.24% of those in the palate were labeled within the first hour after injection.

The basal cell population which had undergone DNA synthesis doubled in number in six hours in the control tongue and palate. Doubling in the experimental tongue and palate occurred in seven and eight hours, respectively.

Methotrexate at a therapeutic dosage of 2.5 mg/kg was shown to inhibit the epithelial cells in the mouse tongue and palate, since at 10 hours only an average of 23% of the experimental cells were labeled as compared to 47% in the control group tissues.

The white blood cell differential count did not change significantly in the experimental animals, while the total white blood cell count did decrease by 19%.

BIBLIOGRAPHY

1. Altman, P.L. and Dittmer, D.S.: Biology Data Book, (Federation of American Societies for Experimental Biology, Washington, D.C.), 1964, p. 274.

2. Angier, R.B.; Boothe, J.H.; Hutchings, B.L.; Mowat, J.H.; Semb, J.; Stokstad, E.L.R.; Subbarow, Y.; Cosulich, D.B.; Farhrenback, M.J.; Hultquist, M.E.; Kich, E.; Northey, E.H.; Sieger, D.R.; Sekels, J.P.; and Smith, J.M.: The structure and synthesis of the liver L. casei factor, Science, 103:667-669, 1946.

3. Berlin, N.I.; Rall, D.; Mead, J.A.R.; Freireich, E.J.; Van Scott, E.J.; Hertz, R. and Lipsett, M.B.: Folic acid antagonists, effects on the cell and the patient. Combined clinical staff conference at the National Institutes of Health, Ann. Intern. Med. 59:931-956, 1963.

4. Bertino, J.R. and Johns, D.G.: Folate antagonists, Ann Rev of Medicine, 18:27-34, 1967.

5. Bessey, O.A.; Lowe, H.J. and Salomon, L.L.: Water-soluble vitamins, Ann Rev Biochem, 22:555-563, 1953.

6. Binkley, S.B.; Bird, O.D.; Bloom, E.S.; Brown, R.A.; Calkins, D.G.; Campbell, C.J.; Emmett, A.D.; and Pfiffner, J.J.: Science, 100: 36-37, 1944.

7. Biro, L.; Carriere, R.; Frank, L.; Minkowitz, S.; and Petrou, P.: Morphologic changes induced by methotrexate, J. Invest Derm, 48: 429-437, 1967.

8. Broughton, W.L.: Effects of methotrexate on homologous tooth transplants, J Dent Res, 46,3:627, 1967.

9. Brown, G.M.; Weisman, R.A.; and Molnar, D.A.: The biosynthesis of folic acid, J Biol Chem, 236:2534-2543, 1961.

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

11. Burchenal, J.H.; Biedler, J.L.; Nutting, B.A.; and Stobbe, G.D.: The effects of 4-amino-N^{10}-methyl-pteroylglutamic acid on the leukocytes of the normal and leukemic mouse, Blood, 5:167-176, 1950.

12. Burchenal, J.R.; Coley, V.; and Kaufman, R.: Effects of dosage technique on prevention of methotrexate toxicity by end products of the reactions inhibited, Proc Am Assoc Cancer Res, 3:213, 1961.

13. Cherache, S.; Condit, P.T.; and Humphreys, S.R.: Studies on the folic acid vitamins. IV. The persistence of amethopterin in mammalian tissues, Cancer, 13:236-240,1960.

14. Cohen, SS.; and Barner, H.D.: Studies on the induction of thymine deficiency and on effects of thymine and thymidine analogs in Escherichia coli, J Bact, 71:588-597, May, 1956.

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

16. Condit, P.T.: Multiple sites of action of folic acid antagonists, Proc Am Assoc Cancer Res, 6:12, 1965.

17. Condit, P.T.; and Yoshino, T.: Persistence, displacement, and metabolic fate of ^{3}H-methotrexate in mice, Proc Am Assoc Cancer Res, 9:12, March, 1968.

18. Delmonte, L.; and Jukes, T.H.: Folic acid antagonists in cancer chemotherapy, Pharmacol Rev, 14:91-135, 1962.

19. Dietrich, L.S.; Monson, W.J.; Gwoh, H.; and Elvehjem, C.A.: Determination of folic acid and citrovorum factor in animal tissue, J Biol Chem, 194:549-553, 1952.

20. Dustin, B., Jr.: Lesions cellulaires provoquees par les acides 4-aminopteroyglutamiques chez la souris, Rev Hemat, 5:603-617, 1950.

21. Farber, S.; Diamond, L.K.; Mercer, R.D.; Sylvester, R.F., Jr.; and Wolff, J.A.: Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-amopteroylglutamic acid (aminopterin), New Eng J Med, 238:787-793, 1948.

22. Fischer, G.A.; Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y leukemic cells, Biochem Pharmacol, 11:1288-1234, 1962.

23. Fitzgerald, P.J.; Simmel, E.B.; Weinstein, J.; and Martin, C.: Radioautography--Theory, technic, and applications, Lab Invest, 2:181-182, 1953.

24. Fleming, . and Soni, .: Heterotransplantation of tooth germs, J Dent Res, 44:1035-1039, 1965.

25. Fountain, J.R.; Hutchison, D.J.; Waring, G.B.; and Burchenal, J.H.: Persistence of amethopterin in normal mouse tissues, Proc Soc Exp Biol & Med, 83:369-373, 1953.

26. Freeman, M.V.: The fluorometric measurement of the absorption, distribution, and excretion of single doses of 4-amino-10-methyl pteroylglutamic acid (amethopterin) in man, J Pharmacol Exp Therap, 122:154-162, 1958.

27. Freeman, M.: in Symposium--Methotrexate in the treatment of cancer, Brit Med J, 2:954-955, 1969.

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

29. Fry, L.; and McMinn, R.H.M.: Action of methotrexate on skin and intestinal epithelium in psoriasis, Arch Derm, 93:726-730, 1966.

30. Fukuyama, K.; and Bernastein, I.A.: Autoradiographic studies of the incorporation of thymidine-H^3 into deoxyribonucleic acid in the skin of young rats, J Invest Derm, 36:321-326, 1961.

31. Galper, J.; and Darnell, J.: The presence of N-formyl-methionyl-t-RNA in HeLa cell mitochondria, Biochem Biophys Res Commun, 34:205-214, 1969.

32. Gordon, M.; Johnson, W.C.; and Burgoon, C.F., Jr.: Histopathology and histochemistry of psoriasis. II. Dynamics of lesions during treatment, Arch Path, 84:443-450, 1967.

33. Grant, P.: The influence of folic acid analogs on development and nucleic acid metabolism in Rana pipiens embryos, Devl Biol, 2:197-251, 1960.

34. Grant, P.: The synthesis of deoxyribonucleic acid during early embryonic development of Rana pipiens, J Cell Comp Physiol, 52:227-247, 1958.

35. Green, E.L.: Biology of the laboratory mouse, (Mc Graw-Hill Book Company, New York), 1956, p. 253-367.

36. Gubner, R.; August, S.; and Ginsberg, V.: Therapeutic suppression of tissue reactivity. II. Effect of aminopterin in rheumatoid arthritis and psoriasis, Amer J Med Sci, 221:176-182, 1951.

37. Halprin, K.M. and Ohkawara, A.: Methotrexate effects on the enzymes of carbohydrate metabolism in psoriasis, Arch Derm, 96:565-571, 1967.

38. Hartmann, K.U.; and Heidelberger, C.: Studies on fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase, J Biol Chem, 236:3006-3013, 1961.

39. Henderson, E.S.; Adamson, R.H.; Denham, C.; Oliverio, V.T.: The metabolic fate of tritiated methotrexate. I. Absorption, excretion, and distribution in mice, rats, dogs, and monkeys, Cancer Res, 24:1008-1017, 1965.

40. Huennekens, F.M.; and Osborn, M.J.: Folic acid coenzymes and one-carbon metabolism, M J Adv Enzymology, 21:369-446, 1959.

41. Hughes, W.L.; Bond, V.P.; Brecher, G.; Cronkite, E.P.; Painter, R.B.; Quastler, H.; and Sherman, F.G.: Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine, Proc Nat Acad Sci, 44:476-483, 1958.

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

43. Johns, D.G.; Iannotti, A.T.; Sartorelli, A.C.; Booth, B.A.; and Bertino, J.R.: Enzymic oxidation of methotrexate and aminopterin, Life Sci, 3:1383-1388, 1964.

44. Johns, D.G.; Sperti, S.; and Burgen, A.S.V.: The metabolism of tritiated folic acid in man, J Clin Invest, 40:1684-95, 1961.

45. Lajtha, L.G.; Phil, D.; and Oliver, R.: The applications of autoradiography in the study of nucleic acid metabolism, Lab Invest, 8(1):214-224, 1959.

46. Larrabee, A.R.; Rosental, S.; Cathou, R.E.; Buchanan, J.M.: A methylated derivative of tetrahydrofolate as an intermediate of methionine biosynthesis, J Amer Chem Soc, 83:4094-4095, 1961.

47. Loehry, C.A.; and Creamer, B.: Three-dimensional structure of the rat small intestinal mucosa related to mucosal dynamics, GUT, 10:112-120, 1969.

48. McDougall, B.M.; and Blakeley, R.L.: The biosynthesis of thymedylic acid. I. Preliminary studies with extracts of Streptococcus faecalis-R., J Biol Chem, 236:832-837, 1961.

49. Mandel, H.G.: The physiological disposition of some anticancer agents, Pharmacol Rev, 11:789-799, 1959.

50. Medak, Herman: Histodifferentiation of oral epithelium in the adult mouse, Ph.D. Thesis, University of Illinois, 1959.

51. Messier, B.; and Leblond, C.P.: Cell proliferation and migration as revealed by radioautography after injection of thymidine-H^3 into male rats and mice, Amer J Anat, 106:247-284, 1960.

52. Nichol, C.A.: On the metabolic alteration of pteroylglutamic acid, Proc Soc Exp Biol Med, 83:167-170, 1953.

53. Oliverio, V.T.: Chromatographic separation and purification of folic acid analogs, Anal Chem, 33:263-265, 1961.

54. Osborn, M.J.; Freeman, M.; and Hennekens, F.M.: Inhibition of dihydrofolic reductase by aminopterin and amethopterin, Proc Soc Exp Biol Med, 97:429-431, 1958.

55. Osborn, M.J.; and Huennekens, F.M.: Enzymatic reduction of dihydrofolic acid, J. Biol Chem, 233:969-974, 1958.

56. Pelc, S.R.: Turnover of DNA and function, Nature, 219:162-163, 1968.

57. Peters, J.M.; and Greenberg, D.M.: Dihydrofolic acid reductase, J Amer Chem Soc, 80:6679-6682, 1958.

58. Pfiffner, J.J.; Binkley, S.B.; Bloom, E.S.; Brown, R.A.; Bird, O.D.; Emmett, A.D.; Hogan, A.G.; and O'Dell, B.L.: Isolation of the anti-anemia factor in crystalline form from liver, Science, 97:404-405, 1943.

59. Philips, F.S. and Thiersch, F.B.: Studies of the action of 4-amino-pteroylglutamic acid in rats and mice, J Pharmacol Exp Ther, 95: 303-334, 1949.

60. Philips, F.S.; Thiersch, J.B.; and Ferguson, F.C.: Studies on the action of 4-aminopteroylglutamic acid and its congeners in mammals, Ann. N.Y. Acad. Sci, 52:1349-1359, 1950.

61. Rees, R.B.; Bennett, J.H.; and Bostick, W.L.: Aminopterin for psoriasis, AMA Arch Derm, 72:133-143, 1955.

62. Robinson, T.W.E. and Stoughton, R.B.: The effect of methotrexate on cell division in the epidermis of the young rat, J Invest Derm, 53:223-227, 1969.

63. Rothberg, S.; Crounse, R.G.; and Lee, J.L.: Glycine-C^{14} incorporation into the proteins of normal stratum corneum and the abnormal stratum corneum of psoriasis, J Invest Derm, 37:497-505, 1961.

64. Ryan, T.J.; Vickers, H.R.; Salem, S.N.; Callender, S.T.; and Badenoch, J.: The treatment of psoriasis with folic acid antagonists, Brit J Derm, 76:556-564, 1964.

65. Schoenheider, W.A., Jr.: D.N.A. Metabolism of the basal cell layer of the epithelium of the tongue in the mouse, M.S. thesis, Loyola University, 1960.

66. Seeger, D.R.; Cosulich, D.B.; Smith, J.M., Jr.; Hultquist, M.E.: Analogs of pteroylglutamic acid. III. 4-amino-derivatives, J Amer Chem Soc, 71:1753-1758, 1949.

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

68. Shklar, G.: The effect of 4-amino-N^{10}-methyl-pteroylglutamic acid on oral mucosa of experimental animals, J Oral Ther Pharm, 4:374-377, 1968.

69. Shklar, G.; Cataldo, E.; and Figzgerald, A.L.: The effect of methotrexate on chemical carcinogenesis of hamster buccal pouch, Cancer Res, 26:2218-2224, 1966.

70. Sloboda, A.E.: Amethod for the quantitative comparison of anticancer agents with special reference to a series of folic acid antagonists, J Pharm Exp Therap, 128:419-427, 1960.

71. Stokstad, E.L.R.: The biochemistry of the water-soluble vitamins, Ann Rev Biochem, 31:462-472, 1962.

72. Subbarow, Y.: Folic acid, Ann N.Y. Acad Sci, 48:257-349, 1947.

73. Sullivan, R.D.; Miller, E.; and Duff, J.K.: Effects of continous intra-arterial methotrexate with intermittent intramuscular citrovorum factor therapy in human cancer, Proc Am Assn Cancer Res, 3:271, 1961.

74. Thiersch, J.B. and Philips, F.S.: Effects of 4-aminopteroylglutamic acid in dogs with special reference to megaloblastosis, Proc Soc Exp Biol Med, 71:484-490, 1949.

75. Timmis, G.M. and Williams, D.C.: Chemotherapy of Cancer, Cancer Monograph Series, (Butterworth, New York), 1967, p. 121-156.

76. Tobin, M.S. and Argano, S.A.: The effect of methotrexate on the immune response of the mouse, Clin Res, 15,2:301, 1967.

77. Trier, J.S.: Morphologic changes in human small intestinal mucosa induced by methotrexate, Proc Am Assoc Cancer Res, 3:273, 1961.

78. Trier, J.S.: Morphologic alterations induced by methotrexate in the mucosa of human proximal intestine, Gastroenterology, 42:295-305,1962.

79. Van Scott, E.J.; Auerbach, R.; and Weinstein, G.D.: Parenteral methotrexate in psoriasis, Arch Derm, 89:550-556, 1964.

80. Van Scott, E.J.and Ekel, T.M.: Kinetics of hyperplasia in psoriasis, Arch Derm, 88:373-381, 1963.

81. Van Scott, E.J. and Reinertson, R.P.: Morphologic and physiologic effects of chemotherapeutic agents in psoriasis, J Invest Derm, 33:357-364, 1959.

82. Van Scott, E.J.; Reinertson, R.P.; and Steinmueller, R.: The growing hair roots of the human scalp and morphologic changes therein following amethopterin therapy, J Invest Derm, 29:197-203, 1957.

83. Vitale, J.J.; Zamcheck, N.; Di Giorgio, J.; and Hegsted, D.M.: Effects of aminopterin administration on the respiration and morphology of the gastrointestinal mucosa of rats, J Lab Clin Med, 43:583-594, 1954.

84. Vogel, A.W.: Tumor-marrow index: A means of laboratory evaluation of antineoplastic compounds, Cancer Res, 21,10:1450-1454, 1961.

85. Wahba, A.J. and Friedkin, M.: Direct spectrophotometric evidence for the oxidation of tetrahydrofolate during the enzymatic synthesis of thymidylate, J Biol Chem, 236:PC11, 1961.

86. Weinstein, G.D. and Van Scott, E.J.: Autoradiographic analysis of turnover times of normal and psoriatic epidermis, J Invest Derm,45: 257-262, 1965.

87. Welch, A. and Nichol, CA.: Water-soluble vitamins concerned with one and two-carbon intermediates, Ann Rev Biochem, 21:633-686, 1952.

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

89. Werkheiser, W.C.: The relation of folic acid reductase to aminopterin toxicity, J Pharmacol Exp Ther, 137:167-72, 1962.

90. Werkheiser, W.C.: Selective permeability and the response of tissues and tumors to the 4-amino-folic acid antagonists, Proc Am Assoc Cancer Res, 3:371, 1962.

91. Werkheiser, W.C.: The biochemical, cellular, and pharmacological action and effects of the folic acid antagonists, Cancer Res, 23,pt.2: 1277-1285, 1963.

92. Werkheiser, W.C.; Law, L.W., Roosa, R.A.; and Nichol, C.A.: Further evidence that selective uptake modifies cellular response to the 4-amino folate antagonists, Proc Am Assoc Cancer Res, 4:71, 1963.

93. Williams, A.D.; Slater, G.G.; and Winzler, R.J.: The effect of Amethopterin on formate-C^{14} incorporation by mouse leukemias *in vitro*, *Cancer Res*, 15:532=536, 1955.

94. Willis, L; Contab, M.A.; and Lond, B.S.: Treatment of Pernicious anemia of pregnancy and tropical anemia, *Brit Med J*, 1:1059-1064, 1931.

95. Wood, R.C.; Ferone, R.; and Hitchings, G.H.: The relationship of cellular permeability to the degree of inhibition by amethopterin and pyrimethamine in several species of bacteria, *Biochem Pharmacol*, 6:113-124, 1961.

96. Zakrzewski, S.F.: Purification and properties of folic acid reductase from chicken liver, *J Biol Chem*, 235:1776-1779, 1960.

APPENDIX

TABLE I

PERCENTILE OF LABELED CELLS IN STRATIFIED SQUAMOUS EPITHELIUM OF THE VENTRAL SURFACE OF THE TONGUE IN ADULT MICE

Time Interval	Control Percent	Log Percent	Experimental Percent	Log Percent
15 minutes	12.35	1.092	13.25	1.125
30 minutes	8.10	0.909	17.15	1.233
45 minutes	11.77	1.075	14.75	1.167
1 hour	7.12	0.853	17.25	1.236
2 hours	14.30	1.155	14.37	1.155
3 hours	7.87	0.896	17.00	1.230
4 hours	6.00	0.778	16.25	1.210
5 hours	--	--	5.40	0.073
6 hours	19.40	1.288	--	--
7 hours	16.25	1.210	32.75	1.515
8 hours	19.50	1.290	22.00	1.342
10 hours	43.00	1.634	24.00	1.380
12 hours	73.80	1.868	--	--
14 hours	39.00	1.591	27.50	1.439
16 hours	--	--	16.25	1.212
18 hours	--	--	30.50	1.484
20 hours	11.20	1.049	2.00	0.031
24 hours	51.60	1.713	--	--
	Mean 22.75		Mean 18.03	
	Standard Deviation 19.21		Standard Deviation 16.73	

"t" = 0.692

$0.25 > p > 0.20$

TABLE II

PERCENTILE OF LABELED CELLS IN STRATIFIED SQUAMOUS EPITHELIUM OF THE PALATE IN ADULT MICE

Time Interval	Control Percent	Log Percent	Experimental Percent	Log Percent
15 minutes	12.12	1.097	28.30	1.447
30 minutes	2.60	0.042	18.95	1.277
45 minutes	18.45	1.265	5.30	0.074
1 hour	9.00	0.095	25.48	1.407
2 hours	15.80	1.199	26.98	1.430
3 hours	5.65	0.075	14.14	1.155
4 hours	5.60	0.075	9.30	0.097
5 hours	--	--	14.60	1.165
6 hours	27.50	1.439	--	--
7 hours	25.00	1.398	28.60	1.447
8 hours	5.30	0.072	45.30	1.653
10 hours	51.30	1.708	21.67	1.322
12 hours	67.30	1.826	--	--
14 hours	44.60	1.644	43.30	1.637
16 hours	--	--	22.00	1.342
18 hours	--	--	36.00	1.556
20 hours	4.67	0.067	6.00	0.078
24 hours	88.40	1.945	--	--
	Mean 25.56		Mean 23.06	
	Standard Deviation 25.16		Standard Deviation 11.87	

"t" = 0.335

$0.35 > p > 0.30$

TABLE III

TOTAL WHITE BLOOD CELL COUNTS IN THE CONTROL MICE

Time Interval	Initial (C_i) cu/mm blood	Final (C_f) cu/mm blood
15 minutes	8,400	12,560
	9,256	6,400
30 minutes	14,220	9,000
	10,950	15,300
	22,750	14,650
45 minutes	14,800	15,350
	9,600	11,700
1 hour	13,250	8,550
	16,000	9,700
2 hours	9,250	11,500
	7,600	5,400
3 hours	8,500	13,150
	7,900	12,400
4 hours	11,474	19,850
5 hours	10,474	5,800
6 hours	10,300	14,250
7 hours	5,200	12,500
8 hours	9,374	36,750
10 hours	12,220	8,450
12 hours	19,320	9,600
14 hours	14,420	13,200
16 hours	45,600	19,200
18 hours	6,920	15,300
20 hours	20,150	19,850
24 hours	14,420	10,550
48 hours	26,350	15,000
	Mean 13,796	Mean 13,306
	Standard Deviation 8078	Standard Deviation 6080

C_i vs. C_f : "t" = 0.0484

$0.4875 > p > 0.475$

C_i vs. F_i : "t" = 0.113 $0.475 > p > 0.45$

TABLE IV

TOTAL WHITE BLOOD CELL COUNTS IN THE EXPERIMENTAL MICE

Time Interval	Initial(E_i) cu/mm blood	Final(E_f) cu/mm blood
15 minutes	12,500	20,700
	19,250	15,900
30 minutes	21,000	7,600
	11,450	7,950
	19,300	14,750
45 minutes	8,900	7,700
	22,150	9,050
1 hour	13,300	8,500
	10,950	10,050
2 hours	8,150	19,800
	12,200	24,600
3 hours	17,200	10,450
	6,000	9,700
4 hours	7,150	2,350
5 hours	27,850	7,550
6 hours	11,750	14,150
7 hours	14,500	10,650
8 hours	17,850	8,100
10 hours	7,450	11,250
12 hours	11,150	8,000
14 hours	15,950	10,850
16 hours	10,400	8,050
18 hours	17,200	16,750
20 hours	9,750	4,600
24 hours	9,850	9,500
48 hours	9,850	7,700
	Mean 13,578	Mean 10,992
	Standard Deviation 5,245	Standard Deviation 5,007

E_i vs. E_f : "t" = 1.783

$0.05 > p > 0.025$

TABLE V

WEIGHTS OF CONTROL AND EXPERIMENTAL ANIMALS

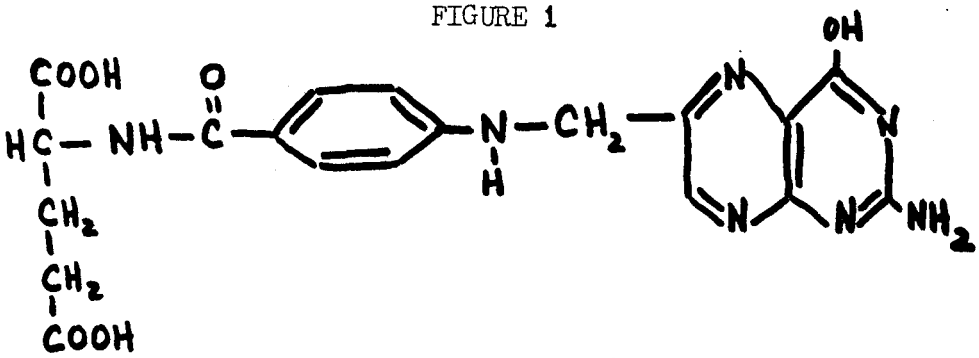
Time Interval	Control		Experimental	
	Initial Gms.	Final Gms.	Initial Gms.	Final Gms.
15 minutes	23.0	24.7	27.7	27.8
	25.6	24.1	23.5	20.7
30 minutes	27.3	27.5	23.9	23.3
	22.1	21.5	24.4	22.5
	24.5	27.2	27.0	26.6
45 minutes	27.5	26.6	28.3	28.9
	22.5	20.9	24.9	25.6
1 hour	25.6	26.0	21.9	21.7
	23.2	21.5	29.3	28.3
2 hours	27.9	28.4	24.5	23.4
	22.4	22.4	24.0	21.7
3 hours	24.7	26.5	23.2	23.6
	28.3	26.6	25.8	27.8
4 hours	25.7	27.2	27.7	22.3
5 hours	24.8	22.1	21.6	19.5
6 hours	22.7	22.3	31.7	30.3
7 hours	25.3	25.0	26.6	26.9
8 hours	25.1	25.5	28.6	29.4
10 hours	23.2	23.2	28.7	23.2
12 hours	22.0	22.1	22.7	21.3
14 hours	22.2	21.0	25.8	24.8
16 hours	23.1	23.2	24.5	23.2
18 hours	26.8	24.4	27.1	27.3
20 hours	23.4	22.2	27.1	27.3
24 hours	25.1	25.1	24.6	23.6
48 hours	24.0	21.9	22.0	22.6
	Mean 24.5	24.2	Mean 25.6	23.9
	Standard Deviation 1.87	2.26	Standard Deviation 2.51	5.54

C vs. E final weights : "t" = 0.288

$0.40 > p > 0.35$

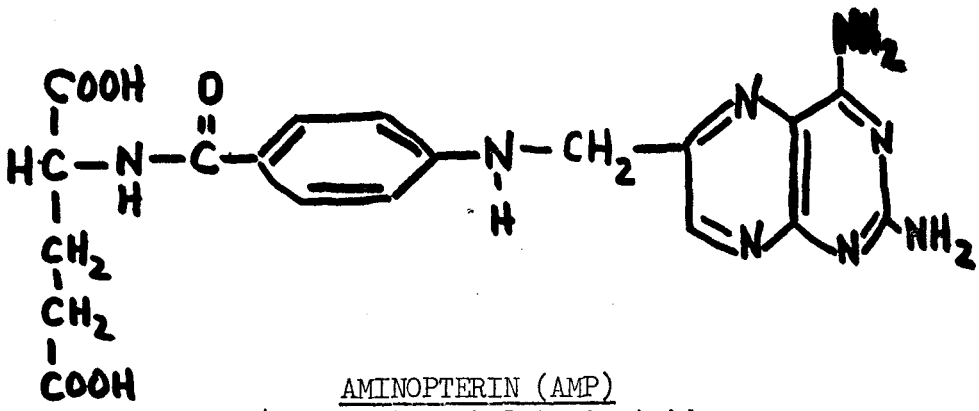
FIGURE 1

a)



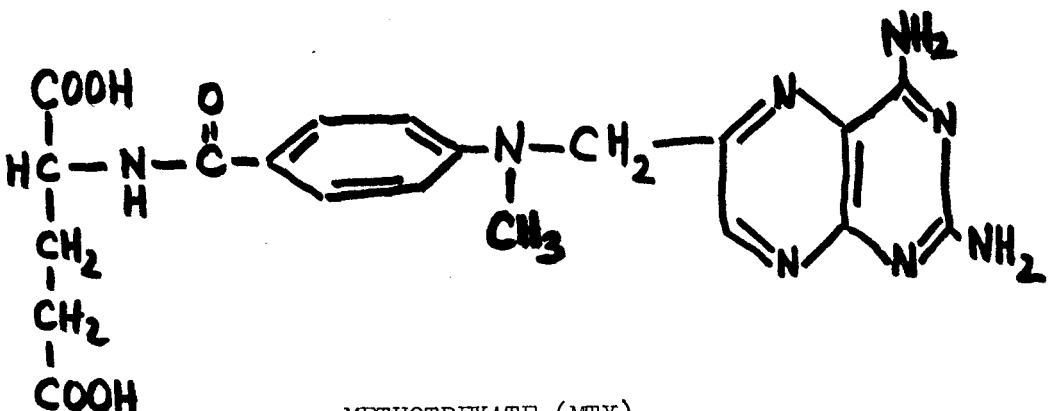
FOLIC ACID (PGA)
Pteroylglutamic Acid

b)



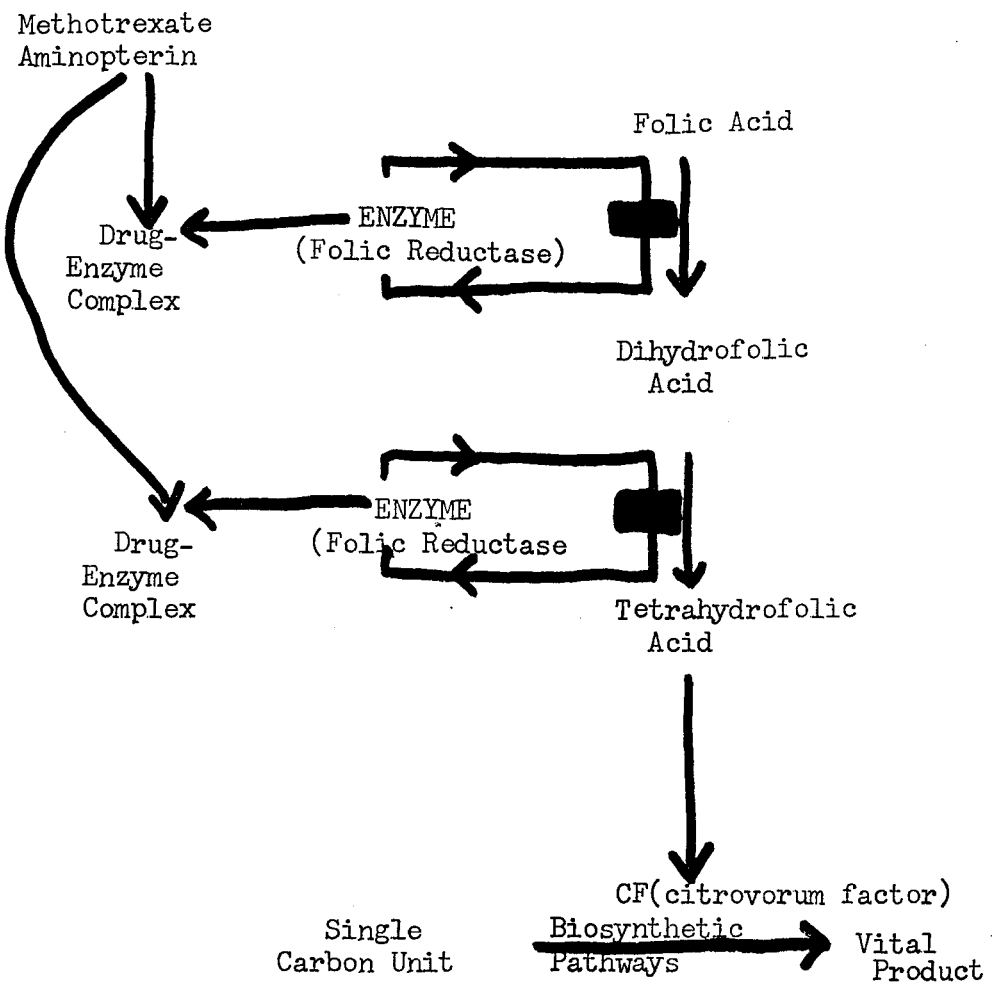
AMINOPTERIN (AMP)
4-aminopteroylglutamic Acid

c)



METHOTREXATE (MTX)
4-amino-N^{10}-methylpteroylglutamic Acid

FIGURE 2

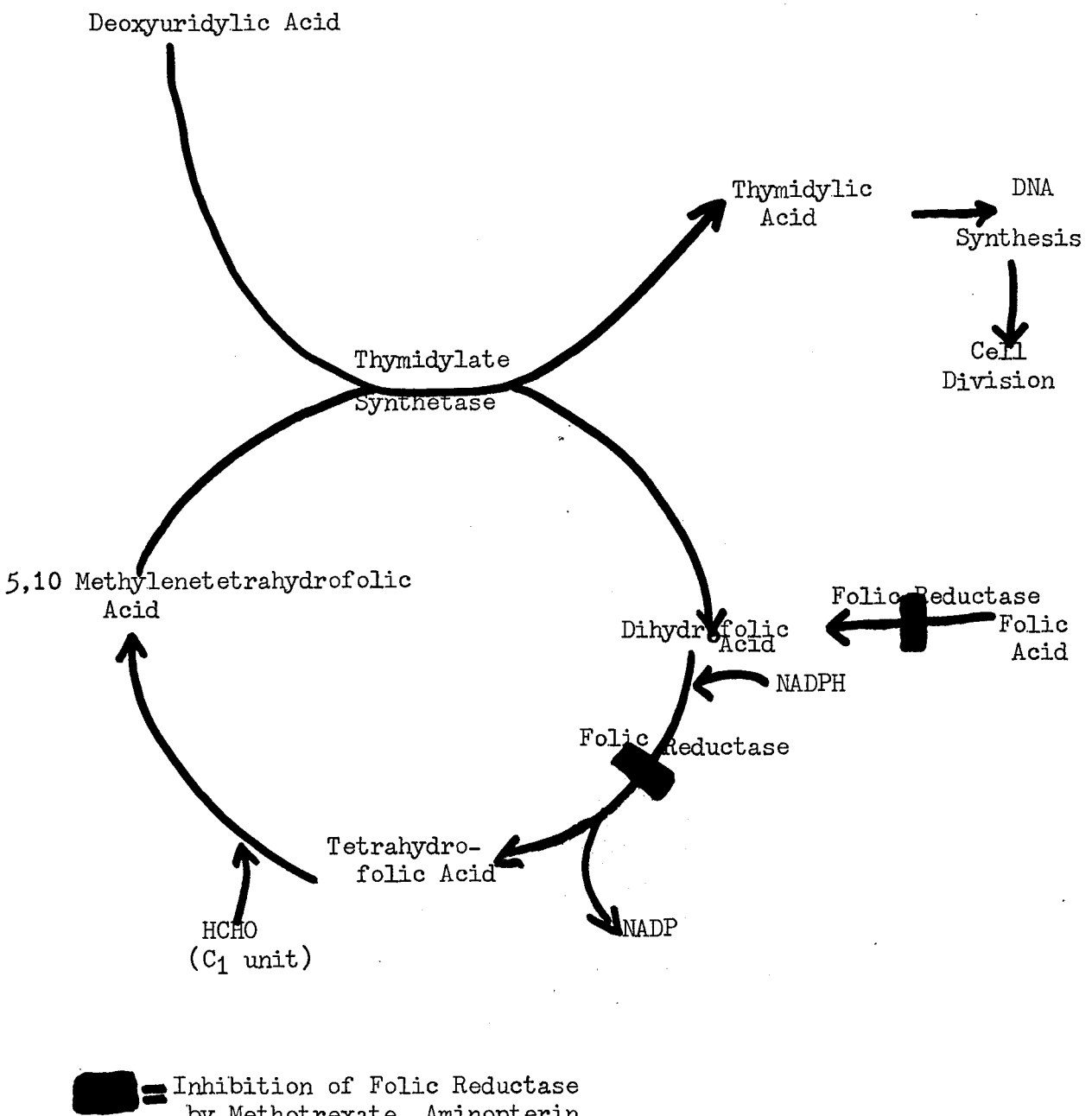


■ = Blockage by Methotrexate, Aminopterin

INHIBITION OF ENZYME FOLIC REDUCTASE
BY METHOTREXATE, AMINOPTERIN *

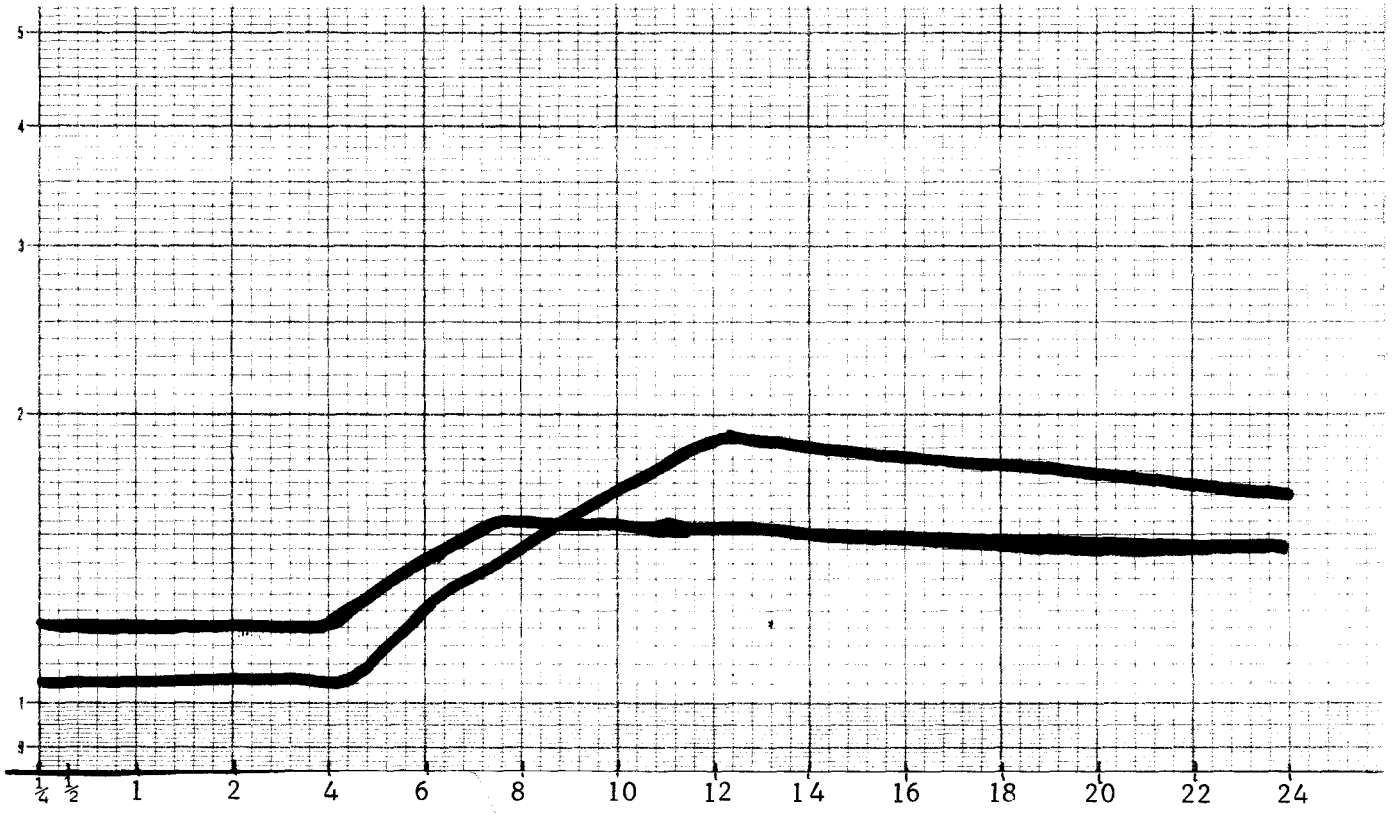
*Adapted from: Mandel (1959), p. 796.

FIGURE 3



BIOSYNTHESIS OF THYMIDYLIC ACID:
Role of tetrahydrofolate coenzymes *

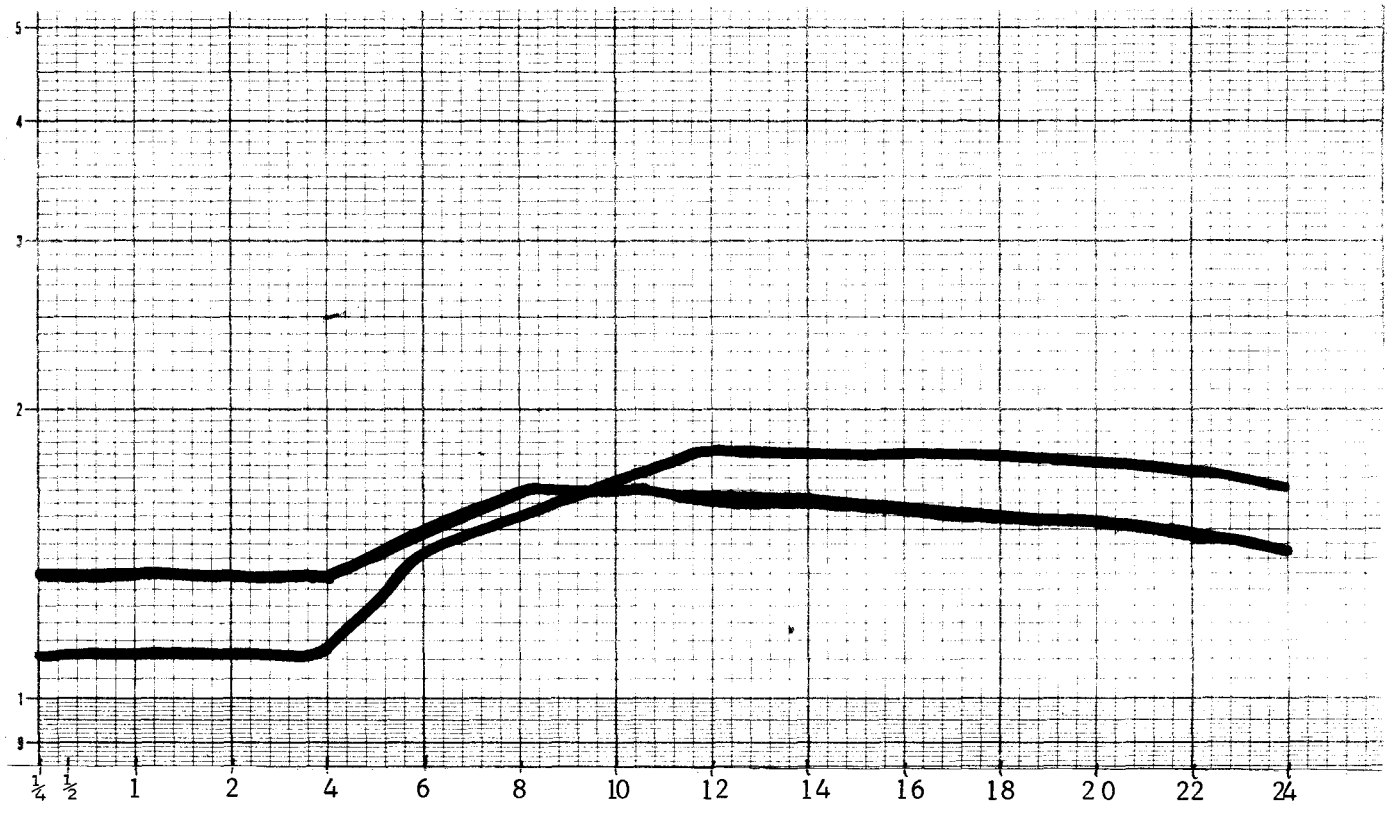
*Adapted from: Mead (1963), p. 937.



PERCENTAGE DISTRIBUTION OF LABELED STRATIFIED SQUAMOUS EPITHELIAL CELLS OF THE VENTRAL SURFACE OF THE TONGUE

Horizontal scale is Time in Hours. Vertical scale is Log % of Labeled Epithelial Cells. Black line denotes control animals, while red line represents the experimental animals.

FIGURE 4



PERCENTAGE DISTRIBUTION OF LABELED STRATIFIED SQUAMOUS EPITHELIAL CELLS OF THE PALATE

Units, labels, and scales are identical to those used in Figure 4.

FIGURE 5

APPROVAL SHEET

The Thesis submitted by Jack S. Litz has been read and approved by two members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 25 1970
DATE

SIGNATURE OF ADVISOR