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PYRIMIDINE SYNTHESIS IN RATS

Robert H. Nott

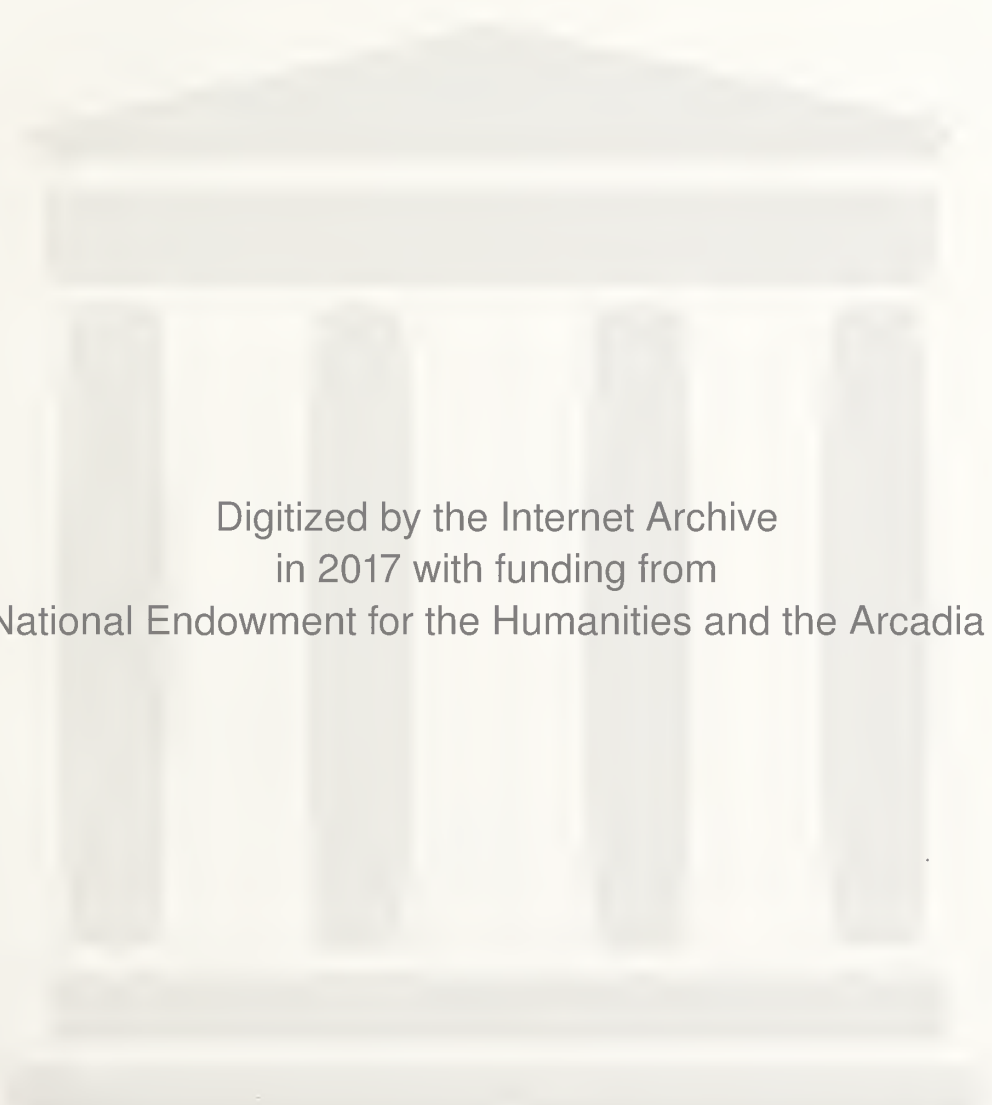
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COMPARISON OF THE EFFECTS OF 5-AZACITIDINE AND 6-AZACITIDINE
ON BEHAVIOR AND BRAIN PYRIMIDINE SYNTHESIS IN RATS

by

Robert Henry Noth

A Thesis Submitted in
Partial Fulfillment
Of the Requirements for the
Degree of

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INTRODUCTION

The administration of 6-azauracil (as-triazine-3,5-dione; 6-AzU) a decade ago to a group of patients with neoplastic disease was complicated by the unexpected appearance of signs of central nervous system toxicity.^{1,2,3} Although this resulted in withdrawal of 6-AzU from clinical use, the cause of the unusual neurological syndrome has continued to be a subject of interest and research.^{4,5} In particular, the question has arisen whether the toxic effects on the central nervous system are inseparably connected to the known antimetabolic action of 6-AzU, or whether they are the result of some unknown action of the as-triazine ring per se.¹¹

In human subjects with neoplastic disease, oral administration of 6-AzU in doses as small as 4.5 mg/kg/day resulted in the appearance of characteristic premonitory changes in the electroencephalogram after several days and lethargy, followed by somnolence, motor abnormalities, psychic disturbances, and coma.⁶ The most common motor abnormality was hyperreflexia, but muscle weakness and twitching, incontinence, choreiform movements, and tremor were noted in some patients. Psychic disturbances occurred in only about half of the patients, and commonly included mental deterioration, hallucinations, and in some patients euphoria and toxic psychoses. About a quarter of the 44 subjects became semicomatose or comatose. All changes were slowly reversible on withdrawal of the drug.

Several patients had convulsive seizures after withdrawal. Additional toxic effects included thrombocytopenia and leukopenia during the course of administration in several of the patients. The ribonucleoside of 6-AzU, 6-azauridine (6-AzUR) is a much more effective cytostatic agent and neurological disturbances have not occurred with intravenous administration of as much as 600 mg/kg/day. However, oral administration of 6-AzUR to patients results in 80% absorption in the form of 6-AzU, presumably because of the action of intestinal flora,^{7,8} and causes the acute onset within a day of the typical encephalopathy usually seen after administration of 6-AzU. Although intravenous 6-AzUR is almost completely without toxicity in patients, only partial remissions have been produced by it in several types of adult acute leukemias; experience with solid tumors has been less encouraging.⁸ Intravenous 6-AzUR and oral administration of triacetyl-6-azauridine (2',3',5'-triacetyl-6-azauridine) have, however, been remarkably successful in producing remissions in severe psoriasis and mycosis fungoides.⁹ Administered orally, triacetyl-6-azauridine results in sustained blood levels of 6-AzUR; 80% of the dose is excreted in the urine as 6-AzUR, 17% as the monoacetyl derivative, and only 3% as the unchanged triacetyl derivative.¹⁰ In a series of 17 patients treated with this drug initially at 270 mg/kg/day for 1 to 3 weeks and then reduced to 135 mg/kg/day and maintained for as long as 6 months, the only toxic manifestation was a mild to moderate anemia.⁹ The neurotoxicity observed in two of these patients receiving triacetyl-6-azauridine by mouth was attributable to an impurity of 6-AzUR (0.6%).

Comparison of these effects in human subjects to those in experimental animals is complicated by differences in route and rate of administration, and by problems of measurement of neurological variables. Some generalizations, however, can be made; the supporting evidence for these will be reviewed subsequently. The general pattern of neurological response to 6-AzU in mice, rats, dogs, and cats resembles that observed in the human subjects; the animals become somnolent and comatose, and appear to develop both motor and behavioral abnormalities. The dose required to elicit these responses, however, is probably several orders of magnitude greater for the laboratory animals. Rats and mice also differ from human subjects in responding to intravenous 6-AzUR with neurological signs indistinguishable from those caused by 6-AzU. From studies using the intraventricular route of administration it appears that the ribonucleotide of 6-AzU, 6-azauridylic acid (6-AzUMP) is also neurologically active.⁴ In both cats and human subjects the effects of small doses of 6-AzU appear to be cumulative over a period of days, being neurologically equivalent to a single large dose, but again, as with the above observations, quantitative aspects have not been studied in detail.

The changes in the electroencephalogram are quite distinctive. Both in cats, mice, and rats and in human subjects these include reduction of fast wave activity, reduction of photic response, and the appearance of large amplitude, spiking slow waves.^{3,4,12} In both groups these precede other neurologic changes. In cats and

rats this pattern appears within an hour after intraperitoneal injection of 1 to 3 gm of 6-AzU/kg. The minimum amount, administered either acutely or in repeated doses, required to produce these changes, however, is probably less. Similar changes in the electroencephalogram of cats results from intraventricular administration of 6-AzUR,⁴ but no change occurs from intravenous 6-AzUR in patients.⁸

Novotny, et al.,⁵ have carefully measured several parameters of central nervous system activity which have been useful in comparing the effects of 6-AzU and 6-AzUR in mice, but which unfortunately are of little help in comparing mice to patients. They found that when 6-AzU was administered in a single intraperitoneal injection, significant depression of exploratory activity occurred after 50 mg/kg; anticonvulsant activity, especially against nicotine appeared at 200 to 400 mg/kg, analgesia at 700 mg/kg, motor incoordination at 900 mg/kg, loss of righting reflex at 1550 mg/kg, and death at 2200 mg/kg. 6-AzUR by this route had the same effects, but at about 2 to 4 times the dose. No effect was noted on deconditioning of avoidance of electric shock. Morris and Glaser¹² have measured the maze running ability of rats made ataxic by 1 gm of 6-AzU/kg and found no significant deficit in speed or accuracy. The observation³ that somnolent patients appeared to be mentally clear after their attention was aroused may be related.

The response to parenteral administration of 6-AzU by cats and dogs is similar to that of mice and rats; 0.7 to 1.2 g/kg caused muscular flaccidity and ataxia without loss of righting reflex.

In monkeys, however, no abnormalities of behavior or changes in the electroencephalogram were observed after oral administration of 180 mg of 6-AzU/kg/day for 4 days. Although this dose is much greater than that causing toxicity in patients, it is small compared to that causing motor incoordination and electroencephalographic changes in mice.

Injection of 6-AzUR and 6-AzUMP into a lateral or the fourth ventricle allows more direct comparison of their relative activity in brain tissue. With some differences, the resulting neurological syndrome resembles that observed clinically from 6-AzU. Intraventricular administration of these drugs has not been performed in human subjects. The evidence of Novotny, et al.,⁵ suggests that in mice the relative activity of 6-AzUMP is greater than that of 6-AzUR, and that both may be more active than 6-AzU. The effects of direct intraventricular administration of 6-AzU, however, are not reported in the literature. Injection of 50 mg of 6-AzUR into the lateral cerebral ventricle of a cat (of unstated weight) caused motor incoordination. The concentration of 6-AzUR in various regions of the brain was 0.22 to 0.74 micromoles/gm of wet tissue, while the concentration of 6-AzUMP was 3 to 4% of this. Intraventricular injection of doses of 25 to 50 mg of 6-AzUR/kg in cats caused motor incoordination, often preceded by outbursts of stereotyped movements, such as cleaning or shaking of the head, followed in thirty minutes by sleep. In rats a similar syndrome was observed at 5 to 12.5 mg of 6-AzUR/kg; outbursts of activity became progressively incoordinated, finally becoming a series of muscular jerks.

This was elicited by only 1 to 5 mg of 6-Azump. Larger doses of these drugs usually caused only general depression. The early period of activity observed after intraventricular, but not intraperitoneal, injection may just be a function of the region of the brain effected. Administration of 1 or 10 mg of 6-AzUR into the fourth ventricle of cats each day for three days caused the appearance of lethargy, unsteadiness on the feet, and after two days nystagmus, extensor hypertonia, and myoclonic jerks. Whether or not this occurred at both dosages is unclear.^{4,14} In these cats the characteristic changes in the electroencephalogram developed later; this may be related to the particular site of injection which is some distance from midbrain and forebrain structures.¹⁴ The response of these animals also closely resembles the response of patients to 6-AzU in that there was evidence of hyperactivity (myoclonic jerks) accompanied by lethargy and incoordination. In the above experiment assuming that 30 mg of 6-AzUR was administered during the 3 days, and that all of it remained in brain tissue as 6-AzUR or 6-AzUMP, the concentration of triazine would have been roughly 0.5 micromoles/gm. An equivalent of 6-AzU entering the brain by the vascular route in rats would probably have caused only lethargy and a measurable decrease in exploratory activity. This suggests that 6-AzUR or 6-AzUMP may be more active neurologically than 6-AzU. It may also be that particular centers are more important in producing the neurological effects, and that they are markedly effected by the ventricular administration.

An unexplained observation in both patients and laboratory

animals is that younger subjects are more sensitive to the triazines.³ This may in part be caused by the relatively smaller amount of body tissue as compared to brain tissue in young animals, or may be related to some biochemical or neurological difference in the developing nervous system.

The anti-neoplastic activity of 6-AzUR¹⁵⁻¹⁹ has been shown to result from conversion in situ to 6-AzUMP which competitively inhibits orotidylic decarboxylase.¹⁹⁻²² This same conversion and inhibition occurs after administration of 6-AzUR in brain tissue of cats and rats.⁴ Koenig, et al.,⁴ have shown that in cat brain 6-AzUR is several times more rapidly converted to the nucleotide than is 6-AzU. Skold²³ has measured the activity of the two enzymes responsible for these conversions, uridine kinase and uridine phosphorylase, in extracts of mouse brain and has found a similar pattern, the uridine kinase being about three times more active. There was some variation in the ratio of the activities of these two enzymes in comparing other tissues of mice and rats, and extrapolation of the above three to one ratio to rat brain and human brain may be inaccurate. Further evidence of species variation is shown by the complete absence of the uridine kinase in the protozoa Trypanosoma equiperdum.²⁴

Other actions of the triazines need only be mentioned. The inhibition of polynucleotide phosphorylase by 6-AzUDP is of unknown physiological importance.^{25,26} In cat brain, however, the percentage of the nucleotide which is phosphorylated to di- or tri-phosphates is vanishingly small.⁴ The inhibition of the incorporation of amino acids into proteins reported to occur in pancreatic homogenates

is likewise of unknown significance.²⁶

The relative uptake of the base and the ribonucleoside into brain tissue from the bloodstream under various conditions has not been extensively studied. In cats, after intravenous infusion of the test solution to achieve constant blood levels, the cerebrospinal fluid to blood ratio during perfusion of the lateral ventricle with artificial cerebrospinal fluid was 0.013 for 6-AzUR, 0.43 for 6-AzU, and 0.57 for glucose.²⁷ Comparison of the concentration of 6-AzU and 6-AzUR in the brain after intraperitoneal administration of these drugs is not possible from old data because of the very low uptake of the riboside by brain tissue.²² Blood levels of 6-AzUR decrease very rapidly because of rapid excretion by the kidney.²⁸ This is probably another factor contributing to the low uptake of the riboside by brain tissue.

The metabolic fate of orotic acid is of central importance in this study. The biosynthesis de novo of orotic acid was first extensively studied in bacteria by Kornberg and Liebermann over a decade ago.³³⁻³⁶ The pathway in mammalian tissue is probably the same, except that carboxymethyl hydantoin is not formed (Figure 1).³⁷⁻⁴⁰

Whether the orotic acid pathway is a major supplier of acid-soluble nucleotides in brain is obviously of importance; it is not the only source. The therapeutic effectiveness of the triazines probably depends on the wide variation of various tissues with respect to their dependence on the synthesis of pyrimidines de novo. Specifically, human neoplastic leukocytes and normal canine leukocytes are particularly sensitive to 6-AzUMP. In these tissues, the activity

of orotidylic acid decarboxylase is high. Probably the most convincing evidence of the degree of dependence of brain tissue on synthesis de novo is the previously mentioned study of Koenig, et al.,⁴ in which 1 or 10 mg of 6-AzUR was administered into the fourth ventricle of two cats for 4 days and 0.112 micromoles of 6-¹⁴C-orotic acid was given three hours before sacrifice; this caused the incorporation of the radioactive orotic acid into acid-soluble pyrimidines of the hindbrain to diminish by 78 and 97%. Most significantly, the acid-soluble pyrimidine pool had been decreased by about 50%. Since the 6-AzUR was administered intraventricularly, the inhibition of pyrimidine synthesis in the liver was probably not significant, and exogenous pyrimidines were probably available to the brain. That the neurological syndrome caused by the administration of 6-AzUR may itself cause depletion of the pool of acid-soluble nucleotides in the brain cannot at present be ruled out, but the changes in pyrimidine metabolism are consistent with the known mechanism of action of 6-AzUR in other tissues. An unexplained finding in Koenig's study was that despite 75 to 98% inhibition of the incorporation of radioactive orotic acid into RNA, the RNA pool size unchanged after 4 days. This suggests the possibility of preferential incorporation of pyrimidines, perhaps from the liver, into brain RNA. Alternatively, the apparent sparing of brain RNA may be a reflection of a relatively slow rate of turnover of the RNA. Other studies by Koenig^{41,42} show that radioactive orotic acid is actively incorporated into the RNA of neurons and oligodendroglia. Adams⁴³ has compared incorporation of radioactive

orotic acid and radioactive uracil into the RNA of the brains of untreated young adult rats and concludes that these brains utilize pyrimidines synthesized de novo for RNA synthesis.

Although it is probably the case that acid-soluble pyrimidine nucleotides in the brain are formed largely from orotic acid, there are other potential sources. Radioactive uridine is rapidly incorporated into uridylic acid and its derivatives in cat brain when orotidylic acid decarboxylase is inhibited by 6-AzUR.⁴ Geiger, et al.,^{44,45} have reported that the electrical activity of isolated, perfused brains of cats is prolonged by the addition of uridine and cytidine to the perfusion medium. In hereditary orotic aciduria, the genetic equivalent of 6-AzUMP blockade in human subjects, the neurological, as well as hematological, symptoms are markedly improved by administration of uridine, though not by uracil.³² It has been shown in rats that both uridine kinase and uridine phosphorylase, which convert uridine to uridylic acid and uracil to uridine respectively, are present in brain, but as in bone marrow and spleen, in very low concentration compared to intestine.²³ The local concentration of these enzymes in the intestine, which receives exogenous uracil and uridine from the diet, may be related to the good tolerance of the intestine during administration of 6-AzUR.⁴⁶ The ability of convert orotic acid to uridylic acid, on the other hand, was greatest in the spleen. In mice receiving 250 mg of 6-AzUR/day for a month the spleen became smaller, the number of lymphocytes and lymphoblasts decreased, and atypical mitotic activity appeared.⁴⁷ However, in this group of mice, there were also marked changes in

the intestinal mucosa, and it is therefore obviously unsafe to generalize that tissues with active synthesis of pyrimidines from orotic acid and with relatively low uridine kinase activity are more severely effected by 6-AzUR. Although uracil incorporation into the acid-soluble pyrimidine nucleotides of rat brain is relatively slow, this may be an artifact of measurement caused by the relatively large pool of uracil, as pointed out by Canellakis in his studies with rat liver.²⁹

In order to estimate the degree of blockade of orotidylic acid decarboxylase using the method described below, it is important to know that the only pathway utilized for the metabolism the radioactive orotic acid is toward synthesis of uridylic acid. The equilibrium of the reaction catalyzed by purified dihydroorotic acid dehydrogenase of Zymobacterium oroticum strongly favors formation of dihydroorotic acid.³⁴ A number of observations suggest, nevertheless, that this pathway is not used by mammalian tissues in vivo:⁴⁸ in liver, carbamyl-L-aspartate is catabolized only one-fifth as rapidly as orotate; both 5-azaorotic acid and 6-AzUMP can almost completely block catabolism of orotic acid; in tissue homogenate studies in vitro, 5-phosphoribosyl-1-pyrophosphate in the medium is necessary for any catabolism of orotic acid. In the brain of the cat it is also possible to decrease the incorporation of radioactive orotic acid into acid-soluble derivatives by as much as 98% by administration of 6-AzUR. If alternative pathways were available for the metabolism of orotic acid, radioactivity should have appeared in the acid-soluble fraction. On the other hand,

one study has shown that the specific activity of uracil as compared to uridylic acid in rat brain (but not in rat liver) is significantly increased after the administration of radioactive orotic acid.⁴⁹ This suggests either that there is a heterogenous pool of UMP in the brain, or that orotic acid is directly converted to uracil. The amount of radioactive uracil derived directly from orotic acid was probably quantitatively unimportant in the present experiment (see below); however, the amount of uracil which was rapidly lost from brain tissue or rapidly converted into uridine, uridylic acid, or β -alanine was not measured.

The mechanisms controlling the metabolism of orotic acid have been studied in microorganisms and mammals. When Escherichia coli mutants with a block in pyrimidine synthesis are grown in a medium limited in pyrimidines, there is an increase in the production of the enzymes catalyzing the synthesis of pyrimidines,³² probably because of the release of end-product repression. A mechanism of control known to be active in rat liver is inhibition of the activity of aspartate transcarbamylase by various uridine and cytidine derivatives, deoxyribonucleosides, and purine deoxyribonucleotides.⁵⁰ Dihydroorotase is also subject to some degree of inhibition by these products.⁵² Orotidylic acid decarboxylase is inhibited by uridylic acid. An indication of the effectiveness of these mechanisms is the increase in the excretion of orotic acid from one milligram to more than 8 grams within several days in patients treated with 6-AzUR. The accumulation of endogenous orotic acid in tissues after blockade by 6-AzUR would complicate the interpretation of studies

utilizing radioactive orotic acid as a precursor. A large amount of endogenous orotic acid would dilute the radioactivity of the administered orotic acid, and the inhibition of the metabolism of orotic acid would appear falsely high. On the other hand, the mass effect of an increased amount of orotic acid would tend to accelerate its metabolism and to compete with the inhibitor, and the effectiveness of the inhibitor would appear falsely low. The concentration of orotidylic acid, the immediate precursor of uridylic acid, may also be similarly increased, since orotidine excretion in the urine of patients treated with 6-AzUR is also markedly increased. The problem of dilution is probably not encountered in this study, however, since the amount of radioactive orotic acid is probably relatively great, and the time course short (see discussion below).

Since brain tissue shows considerable dependence on the metabolism of orotic acid for its supply of pyrimidines, and since these reactions are strongly inhibited by administration of 6-AzU, it is reasonable to connect the behavioral effects with this inhibition.^{4,5} Nevertheless, another dose-dependent effect of the triazines unrelated to their antimetabolic activity is difficult to rule out. Because of the structural and pharmacological similarities of the triazines to barbiturates, a barbiturate-like effect has been postulated.^{6,11} Evidence suggesting this comparison will be discussed later. The approach taken in the following experiment is the much easier task of demonstrating that equivalent inhibition of the metabolism of orotic acid (and therefore of pyrimidine synthesis) in the brain by another pyrimidine analog is not associated with

neurological signs. Another structural analog of uracil, 5-azauracil (sym-triazine-2,4-dione; 5-AzU), which has been shown to have anti-tumor and antibacterial activity,^{17,54,55} is also capable of inhibiting the metabolism of orotic acid.⁵⁶ Although less extensively studied, 5-AzU does not appear to cause abnormalities in the function of the central nervous system.

The action of this symmetrical triazine is probably inhibition of both orotidylic acid decarboxylase and orotidylic acid pyrophosphorylase. Using a particle-free extract of mouse liver, Handschumacher⁵⁶ has shown that release of $^{14}\text{CO}_2$ from 7- ^{14}C -orotic acid was inhibited 78% by $2 \times 10^{-3}\text{M}$ 5-AzU, and that at the same concentration of 5-AzU release of $^{14}\text{CO}_2$ from 7- ^{14}C -orotidylic acid was also inhibited, but by only 47%. The mechanism of this action may be complex, however, since 5-AzU is to some extent metabolized like 6-AzU,⁵⁷ and in addition, the ring structure is unstable. In a pH 7.4 buffer at 37° the base is hydrolyzed to N-formylbiuret and biuret spontaneously.⁵⁸ The N-formylbiuret is known to inhibit dihydroorotase in Escherichia coli and to have significant antibacterial and anti-mitotic effects.⁵⁹ Another action of 5-AzU is inhibition of uridine phosphorylase and deoxyuridine phosphorylase.⁶⁰ During the three hour course of the following experiments, however, non-enzymatic degradation of 5-AzU would have been only 10 to 15%, and the concentrations of the metabolites required for inhibition are large.

MATERIALS

The solvents used in paper chromatography were prepared as follows: (1) n-butanol, acetic acid, and water, 5:1:2; (2) n-butanol, pyridine, and water, 1:1:1; (3) iso-butyric acid, 2N ammonium hydroxide, and water, 85:1.3:15; (4) n-butanol and water, 86:14; and (5) ethanol, concentrated ammonium hydroxide, and water, 75:5:25. A descending solvent front of 40 to 50 cm on Whatman #3 paper was used for all studies.

The 5-AzU and 6-AzU were obtained from the Cancer Chemotherapy National Service Center. Chromatography in solvent systems 1, 2, and 3 showed no ultraviolet-absorbing impurities. The 2-¹⁴C-5-azaurotic acid was prepared by Dr. P. K. Chang, the 2-¹⁴C-uronic acid was obtained from New England Nuclear Corp., Boston, Mass., and the 2-¹⁴C-6-azauracil was obtained from Volk Radiochemical Company, Burbank, Calif. Chromatography in solvent systems 1 and 2 indicated that the purity of these radioactive compounds was at least 97 to 98%.

Radioactivity of solutions and papers was assayed by liquid scintillation as previously described.⁶¹

METHODS

General

All experimental animals were Charles River Sprague-Dawley white male rats weighing between 55 and 70 gm. In all experiments the freshly-prepared, warm solutions of 5-AzU or 6-AzU were administered by intraperitoneal injection of three equally divided doses one-half hour apart, totaling 2.0 gm/kg of body weight. For purposes of discussion, the time elapsing after injection is always figured from the final injection. The pK_a 's of 5-AzU and 6-AzU are 6.7 and 7.0 and by calculation the isotonic pH 7.4 solutions contain 19.1 and 20.4 mg of triazine/ml, and 0.141 and 0.129 ml of 1N NaOH/ml respectively. These solutions were prepared and their pH adjusted to 7.4 with small amounts of NaOH. Peritoneal washings showed less than 10% of either drug remaining free in the peritoneum one hour after the injections. Control animals received warm 0.9% saline, 200 ml/kg of body weight in three divided doses one-half hour apart.

Behavioral parameter

The 6-azauracil, 5-azauracil, and saline were administered above to six animals and the righting reflex was measured every 15 minutes. Loss of the righting reflex was considered complete when animals did not right themselves within 30 seconds after being placed back down on a hard surface. Activity, muscle tone, and coordination

were observed at 10 minute intervals but not quantitated in the three groups.

Tissue distribution

(a) 5-AzU. 2-¹⁴C-5-azauracil was prepared from 2-¹⁴C-5-azarotic acid by treatment with 0.5 N HCl for 30 minutes at 50°. The main product of the reaction was shown to have the same Rf as 5-AzU in solvent systems 2,4, and 5. It was purified by isotopic dilution with 5-azauracil and recrystallization from boiling methanol. Chromatographic analysis in solvent 1 showed 99.1% of the radioactivity in the ultraviolet-absorbing 5-azauracil spot, and less than 0.3% in the regions known to contain the decomposition products of 5-azauracil. Isotonic pH 7.4 solutions containing the ¹⁴C-labelled compounds were prepared and administered as described above. The animals were sacrificed at 1,2, and 4 hours by withdrawal of blood from the abdominal aorta under ether anaesthesia. Solid tissues were homogenized with 5 volume of 0.5 N perchloric acid and blood homogenized with 3 volumes of 5% trichloroacetic acid. After centrifugation for 15 minutes at 4000 x g the radioactivity of the supernatant solution was measured. Respiratory gases were analyzed for ¹⁴CO₂ in one animal for 4 hours. Urine was collected and analyzed by paper chromatography with solvents 1 and 5.

(b) 6-AzU. The procedure was identical to the above for 5-AzU except with the four animals sacrificed at 2 hours. From these animals a pH 7.0 extract of whole brain was prepared as described below, using 5 volumes of 5 N perchloric acid at 0°. This was lyophilized, recon-

stituted with sufficient water to dissolve the solids at 40°, and aliquots containing about 1700 counts per minute were spotted on paper. Paper chromatography in solvent systems 1,2, and 3 and gradient elution chromatography using Dowex 1 x 4 200 mesh columns with a 2 N ammonium formate eluant was also performed. The single radioactive peak from this column was lyophilized, the resulting white solid dissolved in water, and its identity with 6-AzU confirmed using solvents 1,2, and 3. The acid-insoluble residue containing the RNA was washed an additional 6 times with 5 volumes of 0.5 N perchloric acid. Both the fifth and sixth wash were free of radioactivity. Following successive dehydration with 10 volumes of 95% ethanol, absolute alcohol, a boiling mixture of 1 part absolute alcohol and 3 parts ether, and finally anhydrous ether, a fine white powder was obtained. This was dissolved in 3.0 ml of 0.3 N NaOH/100 milligrams of powder and incubated at 37° for 24 hours. Neutralization with a slight excess of 12 N HCl to about pH 6, addition of 1 volume of absolute alcohol, and centrifugation in the cold yielded a clear supernatant fluid which was assayed for radioactivity (150 minutes for each sample) and ultraviolet absorption at 260 and 280 millimicrons.

Tissue slices

Untreated rats were anaesthetized with ether, exsanguinated via the abdominal aorta, and the brain rapidly excised. Tissue slices were taken from the cerebral cortex such that each hemisphere yielded about 4 slices of gray tissue weighing 125 mg. The slices were immediately transferred to rubber capped center-wall flasks containing

0.1 micromoles of 7-¹⁴C-*orotic acid*, 8×10^5 counts per minute per ml, and Krebs III solution⁶² to a final volume of 2.0 ml. The slices from one hemisphere served as a control; 5-AzU, 6-AzU, or 6-AzUR were added to the other. The center-well contained 0.2 ml of 2 N NaOH. Flasks were incubated with shaking for 30 minutes at 37° and the reaction stopped by injection of 0.5 ml 6 N perchloric acid. The flasks were shaken for an additional 15 minutes to assure the complete release of ¹⁴CO₂. Reagent blanks were treated similarly. The NaOH in the center well was assayed for radioactivity. If incubations were performed for 60 minutes instead of 30 minutes, the release of ¹⁴CO₂ was increased by only 50%, thus indicating a decreased reaction rate with longer incubation periods. Two rats received the standard pretreatment with 5-AzU and 6-AzU, and after 2 hours tissue slices from each hemisphere were incubated as above to determine the effect of treatment in vivo on activity in vitro.

Metabolism of 2-¹⁴C-*orotic acid in vivo*

The following procedure was performed four times, each time on a group of six rats treated with 5-azauracil, 6-azauracil, or saline. One hour after the third injection of the triazine or saline each rat was lightly anaesthetized with ether and 0.05 ml + 0.002 ml of 2-¹⁴C-*orotic acid*, 9.0 micromoles/ml, 30 microcuries/ml was injected into the cisterna magna through a #27 gauge needle fitted with a metal disk 3/16 of an inch from the tip. Animals treated with 5-AzU or saline rapidly awakened after or during the twenty second injection period, but those treated with 6-AzU, responding

only sluggishly to the noxious stimulus of the ether in the first place, remained unresponsive and tachypneic until the time of sacrifice one hour later. In nine of ten control animals an injection of Evans Blue dye was distributed throughout the subarachnoid space. In the radioactivity studies, of the twenty four animals injected with radioactive orotic acid, three were excluded, one because of leakage at the injection site, and two because the total recovery of acid-soluble radioactivity was over 5 standard deviations lower than the mean for their group.

One hour after the injection of radioactive orotic acid, the rats were anaesthetized with ether and rapidly exsanguinated via the abdominal aorta. The brain was removed immediately, cut into six pieces to open the ventricles, washed three times in ten volumes of ice-cold 0.9% saline, blotted, weighed, homogenized in two volumes of 0.5 N perchloric acid at 0°, centrifuged for fifteen minutes at 4000 x g at 0°, and the clear supernatant fluid removed. A second supernatant fraction was similarly prepared from the residue, combined with the first, and assayed as the acid-soluble fraction. This was neutralized with 5 N potassium hydroxide, weighed in tared tubes to determine the volume, and the total radioactivity determined by scintillation counting. The acid-insoluble residue was washed at 0° five times with 10 volumes of water (first two groups) or 10 volumes of 0.5 N perchloric acid (second two groups). In three of the four groups of 6 animals the final wash contained no radioactivity; one group was discarded because of inadequate washing. The dried, defatted residue containing the DNA, RNA and protein

was hydrolyzed with sodium hydroxide as described above. The amount of incorporation of radioactivity into RNA per mg of dried, defatted tissue was calculated, and compared to the control in each group. To measure incorporation into DNA, the residue from the first hydrolysis was rehydrolyzed with the same volume of 0.3 N sodium hydroxide for four hours at 37^o, and reprecipitated again by neutralization and addition of alcohol. This residue was washed three times with 1% trichloroacetic acid at 0^o and heated at 85^o for 30 minutes in 5% trichloroacetic acid. After centrifugation, the radioactivity of the supernatant solution was measured.

The liver was homogenized in 2 volumes of cold 0.5 N perchloric acid, centrifuged at 4000 x g for 15 minutes at 0^o, and the radioactivity of the supernatant fraction measured. Blood was treated similarly except that 3 volumes of 5% trichloroacetic acid was used for extraction. Urine was collected under wire screens at the bottom of beakers and the radioactivity determined.

Aliquots of the acid-soluble extract from the brain were analyzed in two ways; by isotopic dilution with carrier orotic acid after hydrolysis with acid to convert orotidine and orotidylic acid to orotic acid; and by paper chromatography.

(a) Isotopic dilution. One ml aliquots were treated with 6 N hydrochloric acid at 100^o for 15 minutes in stoppered tubes to completely hydrolyze orotidylic acid and orotidine to orotic acid. The kinetics of this hydrolysis were studied using a Spectrocord 500 recording spectrophotometer. Orotic acid (50 mg) was added, dissolved by the addition of NaOH, and precipitated again by the

addition of HCl. The precipitate was crystallized twice from boiling water, yielding 15 to 20 mg of fine white powder. This purified orotic acid was dissolved in 1 N sodium hydroxide and aliquots were taken for determination of radioactivity and absorption of ultraviolet light at 260 millimicrons and 280 millimicrons. From these data the total concentration of orotic acid, orotidine, and orotidylic acid in the perchloric acid extract could be calculated. The purity of the orotic acid was checked in one group by a third recrystallization; the specific activity remained unchanged. Paper chromatography with solvent systems 1 and 2 showed 90% of the radioactivity contained in the wide ultraviolet-absorbing band of orotic acid and 10% non-specifically distributed.

(b) Paper chromatography. A total of twelve samples from the four groups was chosen for the more extensive analysis. The acid-soluble extract after adjustment to pH 7 was resolved satisfactorily by two chromatographic systems, 1 and 2. In addition, aliquots were hydrolyzed by treatment with 6 N HCl at 100° for 15 minutes and also by treatment with 1 N HCl for 30 minutes. The hydrolyzed extracts were evaporated to dryness in vacuo at 40°, reconstituted with water, and analyzed by chromatography with solvent 2. The consistency of these results was confirmed for one sample in solvent system 3. For each chromatogram, 50 microliters of the sample was mixed with 25 microliters of a solution containing orotidylic acid, orotidine, orotic acid, uridylic acid, uridine, and uracil at sufficient concentrations to be detectable by their ultraviolet absorption. The chromatograms were cut into 10 to 15

pieces, based on the positions of the authentic compounds. The radioactivity of the sections was determined by scintillation counting as previously described.

Except when otherwise indicated, the standard deviation of the data is reported with each result.

RESULTS AND DISCUSSION

Behavioral parameter

After administration of 2 gm of 6-AzU/kg by intraperitoneal injection, rats lost the righting reflex within 30 to 60 minutes (after the third divided dose) and became unresponsive to corneal stimulation or to pinching of the foot. However, in later experiments, it was noticed that these rats did show some sluggish responses to anaesthetization with ether. The range of time for complete recovery of the righting reflex was two to seven hours. In the rats treated with saline or 5-AzU there was no ataxia, flaccidity, decrease in activity, or slowing of the righting reflex as was observed in those receiving 6-AzU.

Tissue distribution

In comparison with 6-AzU, the 5-AzU was very rapidly eliminated from the blood stream and achieved lower levels in the central nervous system (Table 2). The concentration of 5-AzU in tissues other than the brain dropped precipitously after the first hour. One hour after receiving the third divided dose of 2-¹⁴C-6-azauracil the concentration of radioactivity in the acid-soluble fraction of the brain was 3.1 micromoles/gm while that of the animals receiving 2-¹⁴C-5-azauracil was only 0.5 micromoles/gm. Of the radioactivity recovered from the animals receiving 6-AzU, most was unchanged 2-¹⁴C-6-AzU (see below). Because of the low specific activity, no attempt was made to determine the extent of the metabolism of radioactive 5-AzU.

The poor penetration of 5-AzU into brain tissue may be partly explained by its acidity. At pH 7.4 only 17% of the 5-AzU is present in the uncharged acid form, compared to 29% of 6-AzU. The marked drop in the blood concentration of 5-AzU during the second hour to about 10% of the one hour level may explain why the brain concentration failed to rise during this time. Plasma levels were not significantly higher than those of whole blood, suggesting that the compound freely permeates erythrocytes. Urine output seemed markedly less in the animals receiving 6-AzU than in the others, and was often not detectable. The total excretion of the radioactivity, presumably as 2-¹⁴C-6-AzU,²⁸ varied widely from 3% to 44% of the injected dose after 2 hours. Although the concentrations of 6-AzU in the brain in the animals sacrificed after 2 hours only varied by 8%, the range of concentrations in other tissues was as great as 50%. The urinary excretion of 5-AzU after 2 hours was only 42% of the injected radioactivity, but the bladder urine was not recovered from this animal. Chromatography of this urine using solvent system 1 showed that 60% of the 5-AzU was excreted unchanged. The total recovery of the respiratory ¹⁴CO₂ from the animal treated with 5-AzU was less than 0.4% of the injected dose after 2 hours.

The results from both paper and column chromatography of the perchloric acid extract of the brain of one of the four animals sacrificed after 2 hours indicate that the rate of metabolism of the administered 6-AzU is very slow in brain tissue. Chromatographic analysis of the acid-soluble extract in solvent systems 1 and 3 allowed good resolution of 6-AzU, 6-AzUR, 6-AzUMP, and glyoxylic

acid semicarbazone, the primary metabolites of 6-AzU,²⁸ and revealed a single radioactive peak. The main peak of the acid-soluble extract was split in solvent 2. This splitting, however, was caused by the interference of salts and other compounds in the neutralized acid-soluble extract, since when the single peaks from the other two chromatograms were eluted and analyzed using solvent system 2, they moved as a single peak. This major peak contained 97.5% of the total radioactivity of the perchloric acid extract and had Rf's identical with those of 6-AzU. The other metabolites were present in low concentration: 6-AzUR, $0.7\% \pm 0.2\%$; 6-AzUMP, $0.5\% \pm 0.2\%$; glyoxylic acid semicarbazone, $0.4\% \pm 0.2\%$. (The mean and the range of the two values from chromatographic analysis in solvent systems 1 and 3 are indicated). These values represent upper limits of activity for the respective metabolites. Recovery of radioactivity after column chromatography was 98%; only one peak was detected. Paper chromatography in solvent systems 1, 2, and 3 showed $98.0 \pm 1.7\%$ of this activity was 6-AzU; there were no other significant peaks.

Incorporation of radioactivity from 6-AzU and 6-AzUR into brain RNA has been reported by Koenig, et al.,⁴ but with the conditions in this experiment, incorporation of radioactivity was not significant. Although radioactivity was detected in one of the RNA hydrolysates, the combined data for the four rats showed only 0.0012 ± 0.0039 micromoles of the label from 2-¹⁴C-6-azauracil in the RNA recovered per 1 gm of wet brain tissue. Compared with the total

recovery of acid-soluble radioactivity from the brain, this is less than 0.1% incorporation into RNA.

Tissue slices

The above data raised the question whether the concentrations of either 5-AzU or 6-AzUMP in the brain were sufficient to inhibit synthesis of uridylic acid. A somewhat equivocal answer was obtained by measuring the release of radioactive CO₂ from 7-¹⁴C-orotic acid by slices of cerebral cortex (Figure 2). This assay is sensitive to inhibition of orotidylic acid pyrophosphorylase or orotidylic acid decarboxylase. Although in this system both 5-AzU and 6-AzUR did inhibit decarboxylation, the later was 100 times more effective. At the observed concentration of 5-AzU in brain tissue, inhibition was only about 20%. 6-AzUR inhibited decarboxylation by 50% at 3×10^{-5} M concentration. At 30 times this concentration 6-AzU was ineffective, suggesting a much slower rate of conversion to 6-AzUMP.

Metabolism of 2-¹⁴C-orotic acid in vivo

5-AzU, 6-AzU, or saline were administered to rats in the usual manner, and at the time when the animals in the group which was given 6-AzU became comatose (that is, after 1 hour), 0.5 micromoles of 2-¹⁴C-orotic acid was injected into the cisterna magna of all animals (the details of this procedure are presented under "Methods"). The interpretation of the results requires consideration of several incompletely understood variables.

Since both triazines inhibit the activity of the enzymes between orotic acid and uridylic acid (Figur 1), the parameter of interest is

the rate of conversion of orotic acid to uridylic acid. A completely controlled system for comparison of the inhibition of this conversion by 5-AzU or 6-AzUMP have (1) just the two enzymes and co-factors, (2) a large (and therefore constant) amount of nonradioactive uridylic acid, and (3) 2-¹⁴C-orotic acid, and (4) inhibitor. Although accurate determination of this parameter requires more than one measurement, an approximation can be made by measuring the extent of the (partial) conversion of the radioactive orotic acid to radioactive uridylic acid in a given period of time. Isotopic dilution with carrier orotic acid is a convenient method for measuring the amount of radioactive orotic acid which has not been metabolized during the reaction period. Acid hydrolysis of the reaction mixture before isotopic dilution will add a small amount of radioactivity by converting orotidine and orotidylic acid to orotic acid; by subtraction of this value from the total radioactivity of the reaction mixture, the amount of uridylic acid formed may be calculated. The reaction from orotidylic acid to uridylic acid is essentially irreversible.⁴

The first two of the above conditions are clearly applicable to the situation in the experimental animals. Probably only one enzyme, orotidylic acid pyrophosphorylase, is responsible for the metabolism of orotic acid in the brain. (see Introduction). There is no evidence that orotidine or orotidylic acid are metabolized to any product other than uridylic acid or orotic acid. Although chromatographic studies did not resolve and identify all the metabolic products of 2-¹⁴C-orotic acid, activity associated with

dihydroorotic acid was not above background, and there were no areas of unexplainable radioactivity. The possibility that orotic acid is directly decarboxylated to uracil has been raised. However, uracil was only about 20% of the imperfectly resolved peak designated as uridine plus uracil. Unless uracil was very rapidly lost from brain tissue, any direct contribution from orotic acid not passing through uridylic acid would have been quantitatively insignificant. The results of the chromatographic analysis of the urine of 3 animals in this study support the idea that uracil does not enter the general circulation to a significant extent in one hour. Over 97% of the radioactivity in the urine of the animals administered 5-AzU or 6-AzU was identified as unmetabolized 2-¹⁴C-orotic acid in solvent systems 1 and 2. If the excretion of radioactive uracil in the urine of these animals had occurred to the extent of 1/20th of the amount of the sum of uracil and uridine recovered in the brain (see figure 2), it would have been detectable. On the other hand, only 70 to 75% of the radioactivity in the urine of the control animal was 2-¹⁴C-orotic acid, and 25 to 30% was detected in unidentified metabolites, an amount about equal to only about 1/5th of the amount of the sum of uridine and uracil recovered from the brain in this animal. In the liver, the pool of uracil is relatively large²⁹ and significant incorporation of radioactivity into uridine and uridylic acid from uracil would not be anticipated. If the pool of uracil in the brain is also large, then the relative increase of the uracil plus uridine peak in comparison with that of the uridylic acid and higher phosphate and sugar derivatives in the

animals given either triazine was largely attributable to a relative increase of uridine, not uracil (Figure 3).

It is also obvious that uridylic acid was much more abundant than 2-¹⁴C-ototic acid since the concentration of all radioactive compounds in the brain was probably only about 0.03 micromoles/gm on the average, and the amount of 2-¹⁴C-ototic acid at any time was probably less. The concentration of uridylic acid should have been at least 10times this.⁴ Furthermore, there is probably little change in the pool of uridylic acid during the experiment, since in Koenig's cats, 80% inhibition of ototic acid metabolism by 6-AzUR for 3 days resulted in only about a 50% reduction in pool size.

The third condition presents the most perplexing problem since the concentration of endogenous ototic acid, while of considerable theoretical importance, is unknown. Although it seems worthwhile to consider some of the theoretical effects that this unknown variable might have on the comparison of the effects of 5-AzU and 6-AzU, in fact there is evidence that the amount of endogenous ototic acid is negligible compared to the amount of administered radioactive ototic acid. Attempts to measure the pool size of ototic acid in various tissues have been unsuccessful; the results of work in this lab indicate that it is less than 0.1 micromoles/gm in liver.³¹ Isotopic dilution studies using radioactive ototic acid have provided indirect evidence that the pools of ototic acid in plasma and in tissues are probably small. In one study with rats,³⁸ the specific activity of radioactive ototic acid in the urine was 90% of the intraperitoneally injected dose (5.75 micromoles). Equilibration

with tissue orotic acid, however, was probably only partial. Although orotic acid can enter the general circulation from the tissues, as shown by the massive orotic aciduria after blockade of orotidylic acid decarboxylase by 6-AzUR in human subjects, the amount which normally enters is apparently very small. Plasma levels of orotic acid are estimated to be 5×10^{-5} micromoles/ml, and urinary excretion is only 1.4 mg/day in human subjects.³² Since blockade of orotidylic acid decarboxylase occurs before the administration of radioactive orotic acid, the rate of formation of orotic acid in tissues may be of more importance in this study than its normal concentration. In comparison with the amount of radioactive orotic acid injected into the rats in this study, however, the production of endogenous orotic acid is probably very small. The previously mentioned study of Koenig, et al.,⁴ indicated that a relatively low rate of synthesis of uridylic acid would maintain the pool size in the brain. The concentration of uridylic acid and its higher phosphate derivatives decreased from 0.25 to 0.14 micromoles/gm in the brain of an animal treated for 3 days with 1 mg of 6-AzUR per day by intraventricular injection. This amounts to only 0.0015 micromoles/hour, despite the 75 to 87% blockade of the metabolism of radioactive orotic acid. The rate of synthesis of pyrimidine bases from orotic acid in the adult human subject has been estimated to be only 600 mg/day,^{32,53} a comparatively small amount. Although it is probably the case that accumulation of endogenous orotic acid before or after the administration of radioactive orotic acid is not significant, several points may in addition be considered as

weighing against a significantly different accumulation of orotic acid in the two treatment groups. First, the inhibition of its metabolism is in either case incomplete, being at most 52% (see below). Second, a somewhat circular but still pertinent argument is that if the blockade by 6-AzU (after conversion to 6-AzUMP) became significantly greater than that by 5-AzU during the hour after the injection of radioactive orotic acid, then an equivalent and compensating accumulation (causing dilution of the radioactive orotic acid) would have occurred during this hour as occurred in the hour previous to labelling in the 5-AzU treated animals. In conclusion, then, orotic acid in fact probably represents a pharmacological dose, and not a tracer dose.

Assuming, then, that the model situation applies to rat brain, isotopic dilution with orotic acid provides the most important information about the action of these two inhibitors. Preliminary studies showed that orotidylic acid and orotidine are completely converted to orotic acid by the acid hydrolysis, and that no significant decomposition of orotic acid occurred during similar treatment for one hour.

One possible explanation of the 60% greater recovery of acid-soluble activity from the animals treated with 6-AzU (Figure 3) is found in the markedly different distribution of radioactivity in these animals. (Table 2). Not only did they produce only a few drops of urine, or none, but they also excreted very little of the radioactivity in this urine. The animals treated with 5-AzU or saline produced at least several milliliters of urine; the animals

receiving saline also excreted very little of the radioactivity, probably because of the rapid metabolism of orotic acid to nucleotides in the liver. Respiratory elimination of the ¹⁴C label would not have been extensive since the 2-carbon is not released until metabolism of uracil to β -alanine. In the animals treated with 6-AzU the conversion of orotic acid into uridine nucleotides in the liver was also blocked. With the major routes of elimination and metabolism blocked, the blood and probably cerebrospinal fluid levels were increased. It seems likely that hypotension with resulting poor kidney perfusion could explain the decreased urinary excretion by these comatose rats, although specific tubular damage such as observed from chronic administration of 1 gm/kg of 6-AzUR⁴⁷ may also have been a factor.

The results summarized in the first two columns of Figure 3 show that in control animals the ratio of the sum of orotic acid, orotidine, and orotidylic acid to the sum of uridylic acid and all other derivatives was 0.17 as compared to 1.1 in rats treated with 5-AzU and 1.4 in those treated with 6-AzU. Expressed as a percentage of radioactive orotic acid undergoing decarboxylation to uridylic acid, these values represent $85\% \pm 4\%$, $47\% \pm 3\%$ and $41\% \pm 5\%$ respectively. Inhibition of this conversion was therefore 45% by 5-AzU, and 52% by 6-AzU.

The results from paper chromatography did not differ significantly from those above in the 12 animals studied (Tables 3 and 4). Uridylic acid and derivatives converted to uridylic acid by acid hydrolysis were easily distinguishable from uracil and uridine.

Chromatographic studies showed that the conditions of the 1 N hydrolysis, known to hydrolyze uridine triphosphate and uridine diphosphate to the monophosphate, ^{63,64} caused no significant hydrolysis of uridylic acid or uridine. The higher phosphate derivatives and the various sugar derivatives together were present in the original extract in an amount approximately equal to the amount of uridylic acid. The fraction of radioactivity contained in orotidine and orotidylic acid together was obtained by subtraction of the amount found in orotic acid before hydrolysis from that found after hydrolysis and was $0.7\% \pm 0.5\%$ in control animals, $8.3\% \pm 3.1\%$ and $2.4\% \pm 1.8\%$ in animals treated with 5-AzU and 6-AzU. These results, though not highly significant statistically, suggest that the orotidylic acid decarboxylase is inhibited by 5-AzU, and that the formation of orotic acid from orotidylic acid may also be inhibited.

Recovery of radioactivity from 2-¹⁴C-orotic acid in the hydrolyzed RNA fraction varied widely from about 1% to 15%, depending on the wash procedure and drug treatment. However, by taking the average value of the two control animals in each group, and relating the other values to this, the relative incorporation can be calculated. Normalizing the data to 1.0 ± 0.05 for the control animals, 5-AzU and 6-AzU treated animals were 0.43 ± 0.07 and 0.57 ± 0.05 respectively. These compare only approximately to the relative activity of uridylic acid and all derivatives which were 1.0 ± 0.07 , 0.42 ± 0.02 and 0.75 ± 0.09 for controls, 5-AzU, and 6-AzU treated animals respectively. Incorporation into DNA appeared to be less than 5% of that into RNA.

CONCLUSIONS

The experiments described above were designed to test the hypothesis that the neurological syndrome caused by 6-AzU is the result of "pyrimidine starvation" as a consequence of the inhibition of the synthesis of uridylic acid from orotic acid in the brain. The supporting evidence for this hypothesis has come from showing considerable interdependence of the neurological and pharmacological effects of the drug. The work of Koenig, et al.,⁴ and Novotny, et al.,⁵ in support of this hypothesis has been presented and will be discussed in more detail below. It is proposed, however, that the hypothesis is incorrect if equivalent inhibition of the synthesis of pyrimidines de novo from orotic acid can be demonstrated in the absence of neurological effects. Demonstration of the converse is less conclusive, since the neurological effects are less easily definable.

In young white rats, the acute effect of 5-AzU was to create a metabolic blockade approximately equivalent to that of 6-AzU, but without causing observable neurological abnormalities. The drugs were administered by the same route; both are known to act principally the conversion of orotic acid to uridylic acid in the brain. Inhibition of this conversion at the time of complete loss of righting reflex in the rats treated with 6-AzU was marked, and was approximately equivalent with both drugs. The completeness of the blockade,

however, is difficult to estimate, not knowing the certainty of the concentration of orotic acid in normal or treated animals. If in fact the injected radioactive orotic acid did contribute significantly to production of uridylic acid, then the degree of "pyrimidine starvation" was greater in the animals treated with 5AzU than in those treated with 6-AzU. In addition, the 60% greater amount of radioactivity in the brains of the animals treated with 6-AzU may have tended to overcome the blockade, making inhibition by 6-AzU appear falsely high. There is no evidence to suggest, therefore, that 5-AzU is any less effective an inhibitor of orotic acid metabolism in the brain than 6-AzU under the present conditions. The hypothesis that the neurological effects result from inhibition of orotic acid metabolism or pyrimidine starvation is judged to be incorrect in this particular situation.

One of the central issues is whether the short term (hours) and the long term (days) neurological effects are a result of the same mechanism. Certainly the basic signs of somnolence, motor abnormalities, coma, and characteristic changes in the electroencephalogram observed both chronically and acutely in rats, cats, and human subjects are basically similar. In adults receiving large doses of 6-AzU acutely because of intestinal breakdown of 6-AzUR, the neurological effects developed rapidly (within a day).³¹ Although it cannot be assumed from this that the mechanisms of long term and short term toxicity are the same, it remains a possibility.

The unusual sensitivity of the human central nervous system to very small doses of 6-AzU is not explained adequately by any theory. The wide variability of the responses and the doses of 6-AzU required to elicit them seems to be a phenomenon limited to human subjects. This may in part be attributable to different schedules of administration and to different underlying disease states. Psychological variability may in part explain some of these, but it is difficult to explain how personality structure per se could cause variation in such signs as hyperreflexia and coma, or electroencephalographic changes. Human subjects differ from animals not only in their increased sensitivity to 6-AzU but probably also in a decreased sensitivity to the central effects of 6-AzUR. The latter effect may possibly be explainable on the basis of poor penetration into brain tissue, but it is unlikely that 6-AzUR could be more active than 6-AzU in brain tissue of man such as may be the case in animals. One would certainly hesitate, however, to administer this drug intrathecally in patients.

Although long-term administration of the as-triazines causes more marked changes in pyrimidine metabolism than does short-term administration, the effect of such interference on brain metabolism in general is not known. Koenig, et al.,⁴ suggested that interference with the role of uridine and cytidine nucleotides as co-factors in sugar, polysaccharide, and phospholipid metabolism might be the mechanism causing the disturbance in brain function. It is certainly conceivable that the long term depletion of these pools might be detrimental to the functioning of the central nervous system, but in

what way is not known. There is no report of anatomic lesions in the brains of animals treated with 6-AzU. In dogs administered 120 mg of 6-AzU/day death occurred by the ninth day, but no lesions of the central nervous system were found on autopsy.^{11,31} In this connection, it is also of interest that uridine and cytidine in doses of as much as 2.5 gm in human subjects failed to reverse the neurological effects of electroencephalographic changes in two patients with signs of neurological toxicity from 6-AzU. The role of RNA is probably less important both neurologically and pharmacologically. Koenig, et al.,⁴ found that the pool of RNA in the brain was spared despite the 50% decrease in other pyrimidine pools. Incorporation of the radioactivity from 2-¹⁴C-6-AzU over short periods of time was not detectable, and incorporation during longer periods did not appear to be extensive. Furthermore, inhibition of the synthesis of RNA by 95% with actinomycin D in brain appears to have no neurological consequences.⁶⁵

The studies by Novotny, et al.,⁵ clearly indicate that the neurological activity of 6-AzUR is similar to and probably greater than that of 6-AzU in mice. To what extent the "antineoplastic" activities and the neurological activities of 6-AzU and 6-AzUR correspond is a question which deserves further consideration. The similarity of the behavioral changes noted after the intraperitoneal injection of 6-AzUR to those caused by one-half to one-quarter the dose of 6-AzU administered by the same route are certainly convincing, and furthermore, the 6-AzUR unquestionably penetrates very poorly into brain tissue. That it achieves a concentration in the brain only one

thirty-sixth as great as that of 6-AzU, however, seems unlikely. With doses of 6-AzUR as great as 10 gm/kg, one might anticipate slower elimination by the kidneys and maintenance of high blood levels, and consequently relatively greater penetration into the brain than the smaller doses of 6-AzU. In addition, the assumption by Novotny, et al.,⁵ that because 6-AzUR is 20 times more effective as an anti-neoplastic agent than 6-AzU it must also cause 20 times as great an inhibition of orotidylic acid decarboxylase in rat brain is probably unjustified. Nevertheless, there is little doubt that if only one-tenth as much 6-AzUR reached the brain, instead of one thirty-sixth, its activity would still be greater than that of 6-AzU in mice. There is also no doubt that it would be a more active inhibitor of orotidylic acid decarboxylase since 6-AzU must be converted to 6-AzUR before conversion to 6-AzUMP. Injection of 6-AzUR intracisternally into a cat caused motor incoordination when the concentration of 6-AzUR in brain tissue was 0.22 to 0.74 micromoles/gm. The concentration of 6-AzU in the brains of the comatose rats in this present experiment was 3.1 micromoles/gm; comparison with the above result suggests that the activities of 6-AzU and 6-AzUR are at least of the same order of magnitude.

Although the results of this study suggest no new hypotheses, they should by exclusion refocus attention on the barbiturate-like properties of the as-triazines. There is no problem finding both similarities and differences in the neurological and pharmacological effects of these drugs; for that matter, the barbiturates differ among themselves.

Structurally 6-AzU resembles the barbiturates. The series of

6-alkyl substituted homologs of 6-AzU are even more similar, These homologs produce somnolence, and in higher doses, analgesia and coma;⁶⁶ the motor and psychic abnormalities caused by 6-AzU in patients, however, are not seen with the barbiturates. The relationships among the lipid solubility, the duration of action, the rapidity of metabolism, the length of the alkyl side chain, and the hypnotic potency seen in a series of barbiturate homologs⁶⁹ are also observed with the 5-alkyl substituted triazines (Table 6).⁶⁷ Although lengthening the alkyl substituent increases the hypnotic potency of homologs of 6-AzU, none of the homologs has significant anti-tumor activity,³¹ and presumably none inhibits orotidylic acid decarboxylase. A structural difference between the as-triazines and the barbiturates, however, is that the barbiturates require both substituents on the 5-carbon for neurological activity whereas homologs of 6-AzU are active only if there is a single substituent.⁶⁶ The metabolism of 5-ethyl-6-azauracil is predominantly by oxidation to 5-(1-hydroxyethyl)-6-azauracil, and therefore resembles barbiturate inactivation.⁶⁸ Only 1 to 3% of the metabolites recovered in the urine are in the form of the ribonucleoside. It is tempting to suggest that the increase in neurological activity that occurs with the addition of the ribose moiety to 6-AzU is a barbiturate-like property. However, the increase in the activity of the barbiturates with moderately long alkyl substituents in the 5-position is probably a result of increased lipid solubility which increases the amount of the drug distributed to the brain. Addition of ribose to 6-AzU, however, decreases its penetration into the brain, and presumably,

its lipid solubility. The structure of 5-AzU differs from that of 6-AzU in having its free carbon atom situated between two nitrogen atoms and in this respect is superficially less like the barbiturates. Because of the low concentration of 5-AzU in brain tissues, however, an accurate estimation of its potential for neurotoxicity cannot be made.

The importance of some of the dissimilarities has probably been over-stressed, though certainly there are differences. In particular, the electroencephalogram in animals treated with 6-AzU and with barbiturates has important similarities as well as differences. Neither resembles sleep patterns, and the unusual feature of the electroencephalogram after administration of 6-AzU is its resemblance to that seen in soma or encephalitis, but at a time when no behavioral effect is observable. These changes precede somnolence by several days in patients. The onset of electroencephalographic changes after barbiturate administration accompanies the somnolence. The changes in the electroencephalogram after high doses of barbiturates resemble those after 6-AzU administration; both drugs cause large-amplitude random slow waves, depression of photic and acoustic responses, depression of fast activity, and burst-suppression patterns.^{12,69} Since most barbiturates have no anti-convulsant activity it is difficult to assess the significance of the antagonism of 6-AzU to nicotine as opposed to pentamethylenetetraazole antagonism seen with barbiturates.⁵

Although the effects of these drugs may be similar in many respects, comparison will be inconclusive as long as the mechanism of action of barbiturates is not understood.

SUMMARY

Intraperitoneal injection of 6-azauracil (2 gm/kg) into young rats caused complete loss of the righting reflex in about one hour while an equimolar amount of 5-azauracil did not appear to effect behavior. The concentration of 6-azauracil in the brain one hour after the loss of righting reflex was six times that of 5-azauracil and metabolites during the equivalent period. At this time the concentration of 6-azauridine in the brain was only about 0.7% that of the 6-azauracil, and 6-azauridylic acid and glyoxylic acid semicarbazone were even less. Incorporation of the radioactivity from 2-¹⁴C-6-azauracil into the RNA of the brain was not significant, and was probably less than 0.03% of the total radioactivity in the brain. The rate of synthesis of pyrimidine nucleotides by slices of cerebral cortex was measured by the release of ¹⁴CO₂ from 7-¹⁴C-orotic acid. Concentrations of 5-azauracil in the incubation medium equivalent to those observed in vivo significantly slowed the release of ¹⁴CO₂ but 6-azauracil did not. On a molar basis, however, 6-azauridine was about one hundred times more effective as an inhibitor of orotic acid metabolism than 5-azauracil. The rate of synthesis of pyrimidine nucleotides in the brain in vivo was estimated from the extent to which ring-labelled orotic acid was converted into uridylic acid and its metabolites. Intracisternal injection of the radioactive orotic acid was made at the time of complete loss of the righting



reflex or its equivalent time in the animals administered 5-azauracil or saline. In the animals receiving 5-AzU or saline about 10% of the injected radioactivity was recovered in an acid extract of the brain one hour later; the recovery from the animals receiving 6-AzU was about 16%. Analysis of the acid-soluble extract of the brain indicated that uracil, uridine, uridylic acid, and sugar and higher phosphate derivatives of uridylic acid constituted 85% of the recovered radioactivity in control animals, but only 41 and 47% in those administered 6-azauracil and 5-azauracil, respectively. These results suggest that the loss of the righting reflex, and probably other temporally related neurological effects of 6-azauracil are independent of its inhibition of the synthesis de novo of pyrimidines in the brain. The neurological and pharmacological properties of 6-azauracil and barbiturates are compared and the possibility of a barbiturate-like action of 6-azauracil is discussed.

TABLE 1. THE CONCENTRATION OF 2-¹⁴C-5-AZARACIL AND 2-¹⁴C-6-AZARACIL AND THEIR METABOLITES IN THE TISSUES OF THE RAT AFTER INTRAPERITONEAL ADMINISTRATION

Tissue	Micromoles of drug/gm wet tissue				
	6-azauracil		5-azauracil		
	hours after injection*				
	1 (2)	2 (4)	1 (1)	2 (1)	4 (1)
whole blood	10.9	9.3	15.1	1.2	0.1
plasma	12.2	11.2	----	1.7	0.2
whole brain	3.1	3.8	0.6	0.6	0.2
liver	10.7	7.8	2.8	1.2	0.1
kidney	12.8	13.0	13.0	4.5	0.5
intestine	9.2	11.9	2.8	1.1	0.1
spleen	9.1	8.6	3.1	1.1	0.1
thymus	7.5	6.1	2.5	1.2	0.2
muscle	3.6	5.8	1.8	0.7	0.1

The procedure is described in detail under "Methods."

*The numbers in parentheses indicate the number of rats in each group.

TABLE 2. THE EFFECT OF PREVIOUS ADMINISTRATION OF 5-AZAUACIL AND 6-AZAUACIL ON THE TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER INTRACISTERNAL INJECTION OF 2-¹⁴C-OROTIC ACID

Pretreatment	Radioactivity			
	Total cpm x 10 ⁻³		cpm/ml x 10 ⁻³	
	Brain	Liver	Urine	Blood
Saline	352 ± 55	1140 ± 290	108 ± 53	10.4 ± 1.2
5-azauracil	318 ± 24	322 ± 20	1050 ± 200	13.4 ± 2.0
6-azauracil	583 ± 100	286 ± 25	50 ± 44	18.8 ± 4.0

The animals were administered 0.5 micromoles of 2-¹⁴C-ototic acid, 3.3 x 10⁶ cpm, by intracisternal injection 1 hour before sacrifice. The procedure is described more fully under "Methods."

TABLE 3. RESOLUTION OF SELECTED PYRIMIDINES AND TRIAZINES
IN THREE SOLVENT SYSTEMS

Compound	Rf x 100		
	Solvent systems		
	(1)	(2)	(3)
6-Azauracil	59	71	63
6-Azauridine	40	63	50
6-Azauridylic acid	13	14	28
Glyoxylic acid semicarbazone	52	36	41
5-Azauracil	43	63	--
Dihydroorotic acid	34	35	--
Orotic acid	25	50	30
Orotidine	18	42	29
Orotidylic acid	8	12	14
Uridylic acid	16	20	30
Uridine diphosphate	9	17	--
Uridine triphosphate	6	14	--
Uridine diphosphate glucose	6	24	12
Uridine diphosphate glucosamine	6	28	16
Uridine	42	73	60
Uracil	54	70	69

The solvent systems are: (1) n-butanol, glacial acetic acid, and water, 5:1:2; (2) n-butanol, pyridine, and water, 1:1:1; and (3) iso-butyric acid, 2 N ammonium hydroxide, and water, 85:1.3:15.

TABLE 4. COMPARISON OF THE DATA OBTAINED BY PAPER CHROMATOGRAPHY (TABLE 4) TO THOSE OBTAINED BY ISOTOPIC DILUTION

Pretreatment	Percentage of the total radioactivity of the acid extract of brain present as orotic acid after acid hydrolysis.		
	By paper chromatography (average)	By isotopic dilution	
Saline	14.7 ± 4.9 (4)	14.3 ± 3.5 (4)	14.9 ± 4.4 (6)
5-Azauracil	54.7 ± 5.0 (4)	55.1 ± 2.0 (4)	52.8 ± 3.3 (8)
6-Azauracil	60.6 ± 2.6 (4)	60.9 ± 3.7 (4)	58.5 ± 4.7 (7)

The numbers in parentheses indicate the number of animals in each group; the data in the first two columns are from the same animals; the third column shows the result for all 21 animals.

TABLE 5. COMPARISON OF HYPNOTIC POTENCY AND PHARMACOLOGICAL PROPERTIES OF 6-AZAUACIL AND ALKYL DERIVATIVES*

Compound	Relative molar potency in mice	Time of onset of hypnosis in min.	Relative distribution coefficient EtAc:pH 7.3 buffer	Duration of hypnosis in hours	Percent Metabolized in 24 hours
6-Azauracil	1.0	45-60	1.0	4-8	0-10
5-Methyl-6-azauracil	2.7	20-30	3.1	2-3	20
5-Ethyl-6-azauracil	5.0	18-20	7.9	$\frac{1}{2}$ -1	73
5-Propyl-6-azauracil	8.2	8-12	12.1	$\frac{1}{2}$ -2	87

*Unpublished data of R. E. Handschumacher.

Figure 1. Summary of pyrimidine metabolism in mammals and the action of 6-azauracil. Abbreviations: USA, ureidosuccinic acid; DHOA, dihydroorotic acid; OA, orotic acid; OR, orotidine; OMP, orotidylic acid; PRPP, 5-phosphoribosyl-1-pyrophosphate; PP, inorganic pyrophosphate; UMP, uridylic acid; UR, uridylic acid; U, uracil; UDP, uridine diphosphate; dUDP, deoxyuridine diphosphate; dUMP, deoxyuridine monophosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; dCDP, deoxycytidine diphosphate; dCTP, deoxycytidine triphosphate; TMP, thymidine monophosphate; TDP, thymidine diphosphate; TTP, thymidine triphosphate; UDPX, uridine diphosphate sugar derivatives; CDPX, cytidine diphosphate sugar derivatives.

Aspartate + Carbamylphosphate

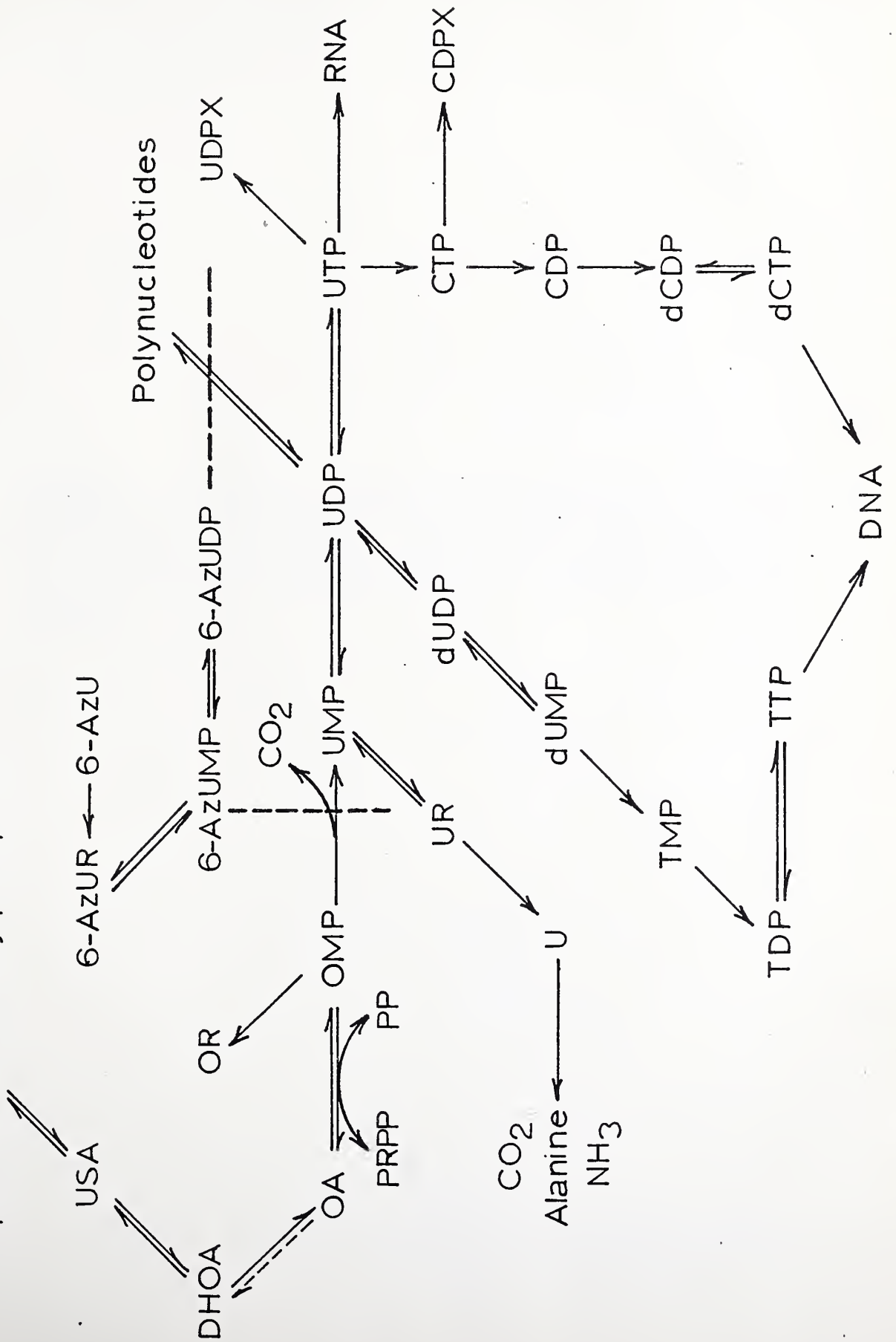


Figure 2. The effect of 5-azauracil, 6-azauracil, and 6-azauridine on the release of $^{14}\text{CO}_2$ from 7- ^{14}C -orotic acid, 0.05 micromoles/ml, by tissue slices from the cerebral cortex of rats, 30 min. incubation. Each symbol represents a single determination. The mean value and standard deviation is indicated for 9 control animals.

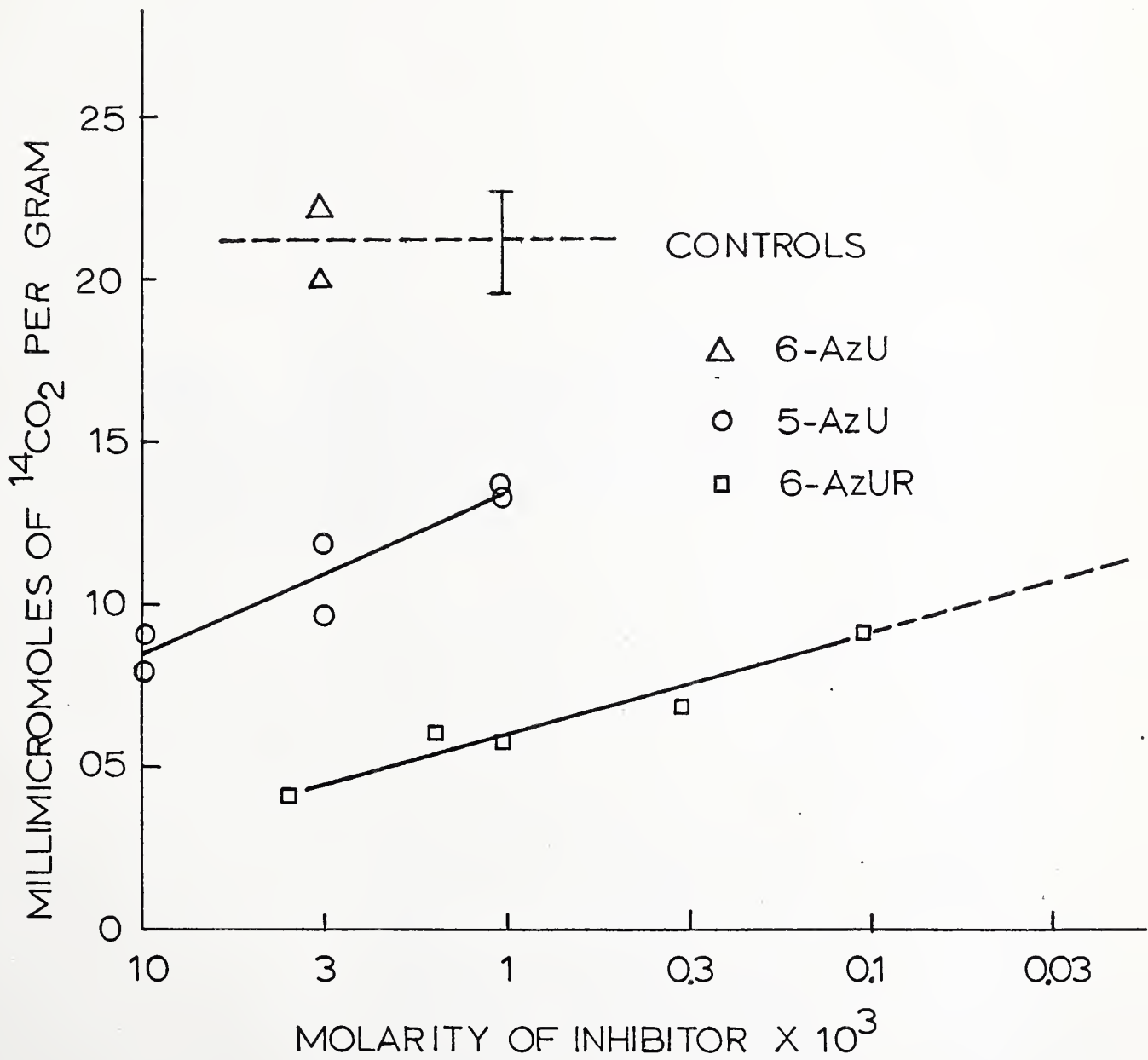
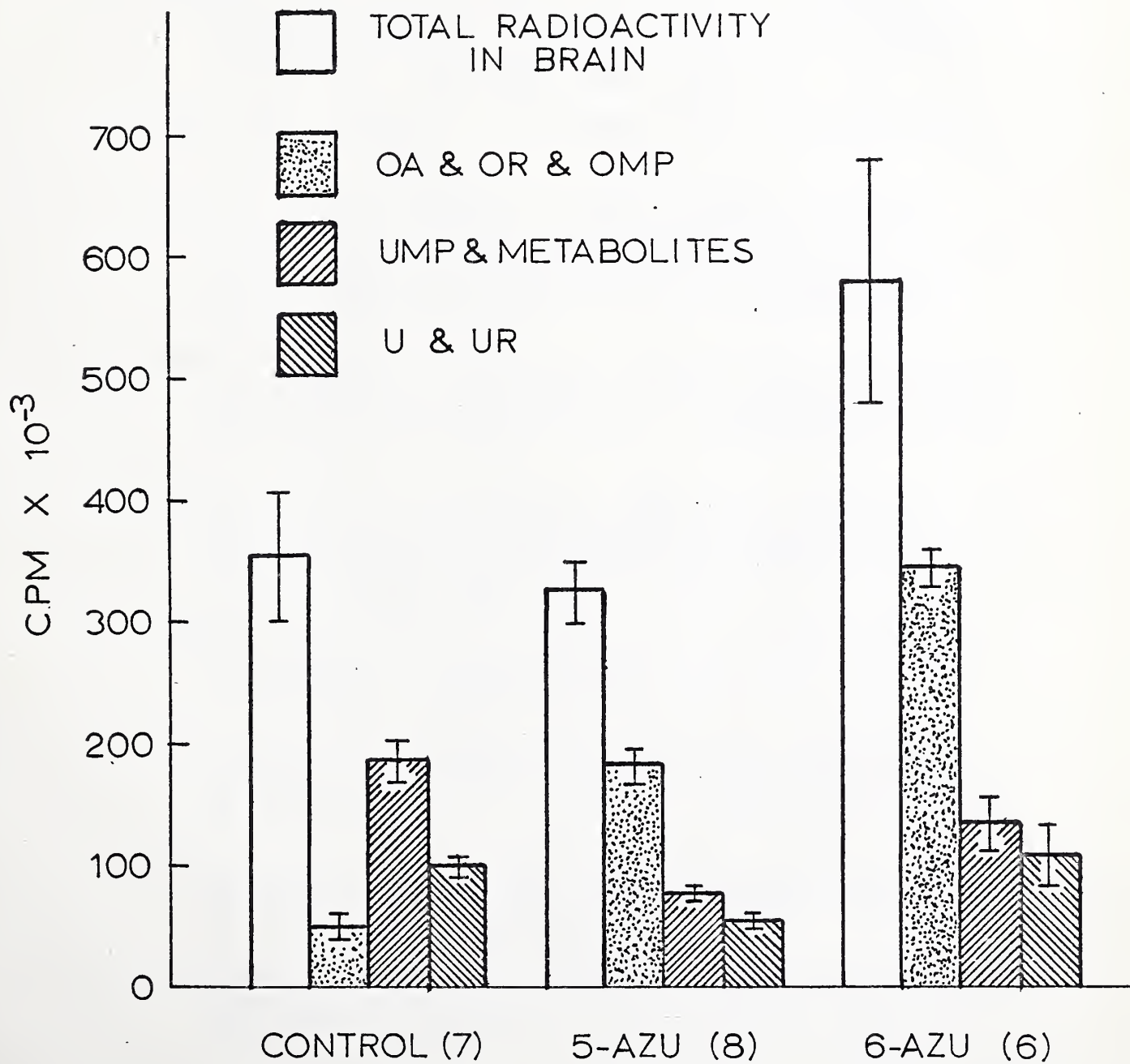


Figure 3. The effect of 5-azauracil, 2g/kg, and 6-azauracil, 2g/kg, on the metabolism of 7-¹⁴C-orotic acid (0.45 micromoles, 3.3×10^8 cpm) administered into the cisterna magna of rats. The numbers in parentheses indicate the number of animals in each group. Abbreviations are defined on p. 15. The procedure is described under "Methods."



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