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INHIBITION BY BARBITURIC ACID AND ITS DERIVATIVES OF THE ENZYMES IN RAT BRAIN WHICH PARTICIPATE IN THE SYNTHESIS OF PYRIMIDINE RIBOTIDES*

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Abstract—Barbituric acid competitively inhibits dihydro-orotate dehydrogenase, uridine phosphorylase, and (directly or indirectly) orotate phosphoribosyltransferase. In addition, the ribotide of barbituric acid, 6-hydroxyuridine-5′-monophosphate, inhibits uridine monophosphate kinase and is a powerful competitive inhibitor of orotidylate decarboxylase—the final enzyme in the sequence for the *de novo* synthesis of pyrimidine ribotides. Barbituric acid itself causes marked competitive inhibition of decarboxylase activity provided the reaction mixture contains 5-phosphoribosyl-1-pyrophosphate (PRPP). Another barbituric acid derivative, phenobarbital, is a weak non-competitive inhibitor (in the presence of PRPP) of the decarboxylase. One convulsant barbiturate, DMBB [5-(1,3-dimethylbutyl)-5-ethyl-barbituric acid], and bemegride—a stimulant structurally resembling the barbiturates—were found to inhibit rat brain uridine phosphorylase activity, and this same enzyme was also inhibited by isobarbituric acid.

The structure of barbituric acid (2,4,6-trioxohexahydropyrimidine or 6-hydroxyuracil) is shown in Fig. 1. Barbituric acid (BA) is of biological interest in at least two respects. The compound is a catabolite of uracil in *Corynebacterium*, *Mycobacterium* [1], *Nocardia corallina* [2, 3] and in an aerobic soil bacterium [4–6]. In the first three of these organisms, barbituric acid itself is catabolized to malonate and urea. There are four very early papers suggesting the existence of a similar sequence for uracil catabolism in dogs and in man [7–10]. Unfortunately, by modern criteria these latter results are not conclusive. Somewhat more recent experiments performed on rats [11] and man [12] indicate that the majority of label from oral or intravenously administered [^{15}N]uracil is excreted as urea. Moreover, when high doses of [2-^{14}C]uracil are injected into rats, up to one-tenth of the label appears as urea [13]. These results at least raise the possibility that barbituric acid might prove to be a uracil metabolite in one or more mammalian tissues.

In addition, barbituric acid (or its 2-thio-analogue) is the common structural element which is shared by all of the hypnotic and convulsant barbiturates [14]. The substitution of a wide variety of hydrocarbons for each of the two hydrogens on the number 5 carbon atom of barbituric acid converts the molecule to a barbiturate. Nearly any hydrocarbon substitutions, including both alkyl and aryl chains, lead to a compound which, in some way, affects the level of arousal. Hence, there is reason to suspect that much of the structural specificity for these drugs may reside in the ring of barbituric acid.

Barbituric acid has been previously reported to inhibit competitively the dihydro-orotate dehydrogenase (DHO-DHase, EC 1.3.3.1) activity of *Zymobacterium oroticum* [15], of human cultured fibroblasts [16] and of rat liver [17]. This enzyme catalyzes the fourth reaction in the sequence for the *de novo* synthesis of uridine-5′-monophosphate (see Fig. 2). It has also been reported that several barbiturates reduce the amount of [^{14}C]carbamylaspartate incorporated into [^{14}C]orotic acid in rat liver slices [18]. However, the observed inhibition was weak, and no attempt was made to study directly the effect of the barbiturates on the activities of the pyrimidine biosynthetic enzymes, nor was BA itself studied.

In the work to be described below, we have inquired whether barbituric acid or certain of its derivatives inhibit a number of enzymes involved in the *de novo* or salvage synthesis of pyrimidine ribotides.

We have sought to determine whether, in crude extracts of rat brain, barbituric acid or its correspond-

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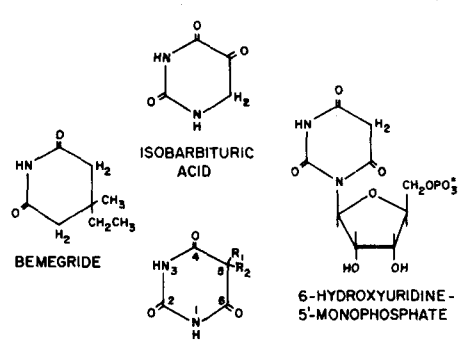

	BARBITURIC ACID	PHENOBARBITAL	CHEB	DMBB
R_1	H	⬡	CH_2CH⬡	CH_3 CH_3 $CHCH_2CHCH_3$
R_2	H	CH_2CH_3	CH_2CH_3	CH_2CH_3

Fig. 1. Structure of barbituric acid and some related compounds and drugs used in this study.

ing riboside (6-hydroxyuridine, 6-OHUR) or ribotide (6-hydroxyuridine-5'-monophosphate, 6-OHUMP) inhibits the following activities:

(1) Carbamyl phosphate synthetase (CPSase, EC 2.7.2.9).

(2) Aspartate transcarbamylase (ATCase, EC 2.1.3.2).

(3) Dihydro-orotase (DHOase, EC 3.5.2.3).

(4) Dihydro-orotate dehydrogenase (DHO-DHase, EC 1.3.3.1).

(5) Orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10).

(6) Orotidine-5'-monophosphate decarboxylase (ODCase, EC 4.1.1.23).

(7) Uridine monophosphate kinase (EC 2.7.4.14).

(8) Uridine phosphorylase (EC 2.4.2.3).

(9) Uridine kinase (EC 2.7.1.48).

The first six enzymes catalyze the respective sequential reactions (in the order given) which effect the *de novo* synthesis of uridine-5'-monophosphate. The last two enzymes catalyze the respective sequential reactions which effect the conversion of uracil to uridine-5'-monophosphate—the so-called "salvage pathway".

We have also measured the effect of a hypnotic barbiturate (phenobarbital), of two convulsant barbiturates (DMBB and CHEB), of bemegride (a stimulant which structurally resembles the barbiturates), and of isobarbituric acid (5-hydroxyuracil, IBA) on most of the rat brain enzyme activities listed above. The structure of all these compounds is given in Fig. 1.

MATERIALS AND METHODS

Biochemicals and reagents. Components of the chromatographic solvents, magnesium chloride, ethylene glycol monomethyl ether (EGME), and dimethylsulfoxide (DMSO) were obtained from Fisher Scientific Co., Pittsburgh, PA. Scintillation grade toluene, 2,5-diphenyloxazole, and TLA (8 g 2-(4'-τ-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxdiazole, "butyl-PBD", and 0.5 g 2-(4'-biphenyl)-6-phenyl-benzoxazole, "PBBO"/liter of toluene) were from Beckman Instrument Co., Mountainside, NJ. LSC complete scintillation fluid and Hydromix were purchased from Yorktown Research, South Hackensack, NJ. Dithiothreitol (DTT) (Cleland's Reagent), cytidine, and cytidine-5'-monophosphate were obtained from Calbiochem, San Diego, CA. Sodium phenobarbital was from Mallinckrodt Chemical Works, St. Louis, MO. Bemegride sulfate (Megemide) was the gift of Dr. A. O. Geiszler, Abbott Laboratories, Chicago, IL. CHEB [5-(2-cyclohexylidene-ethyl)-5-ethylbarbituric acid] and DMBB [5-(1,3-dimethylbutyl)-5-ethyl-barbituric acid] were gifts of Dr. Hall Downes, University of Oregon Medical School, Portland, OR. Adenosine-5'-triphosphate (dipotassium salt; K-ATP) was a product of P. L. Biochemicals, Milwaukee, WI. All other non-radioactive chemical and biochemical reagents used in the course of these investigations were purchased from Sigma Chemical Co., St. Louis, MO. In every case, the compounds were of the highest grade available.

Radiochemicals. [^{14}C]carbamyl phosphate (7.99 mCi/m-mole), [^{14}C]carbamyl aspartate (4.62 mCi/m-mole), DL-[^{14}C]dihydro-orotic acid (3.74 mCi/m-mole), [6-^{14}C]orotic acid (52.7 mCi/m-mole), [carboxyl-^{14}C]orotic acid (42.4 mCi/m-mole), [2-^{14}C]-uridine (57.0 mCi/m-mole), [2-^{14}C]uracil (56.6 mCi/m-mole) and [carboxyl-^{14}C]orotidine-5'-monophosphate (39.3 mCi/m-mole) were all obtained from New England Nuclear, Boston, MA. [4-^{14}C]uridine-5'-monophosphate (45 mCi/m-mole) was obtained from Amersham Searle, Arlington Heights, IL. [^{14}C]-$KHCO_3$ was prepared by the method of Tatibana and Ito [19] from [^{14}C]Ba_2CO_3 (57.7 mCi/m-mole) obtained from Amersham Searle, Arlington Heights, IL.

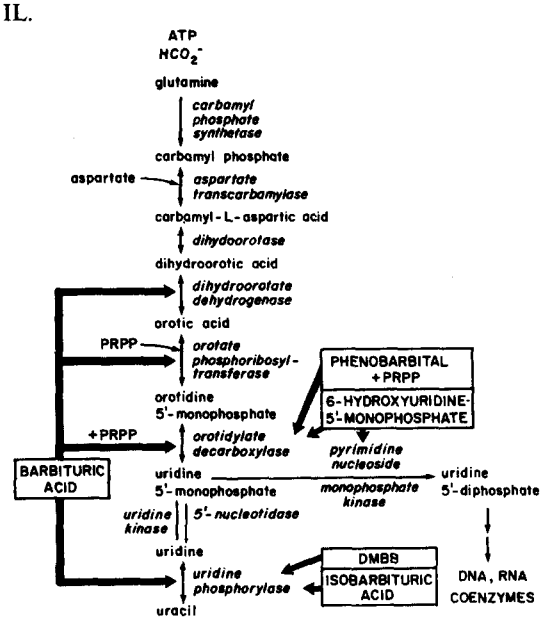

Fig. 2. Pathways for the *de novo* and salvage synthesis of pyrimidine ribotides. Points of inhibition of enzyme activity by barbituric acid and some related compounds and drugs are indicated.

Chromatographic materials. Whatman No. 1, Whatman No. 3MM, and Whatman DE-81 diethylaminoethyl cellulose chromatography paper were obtained from Reeve Angel & Co., Clifton, NJ. Polyethyleneimine (PEI) cellulose thin-layer plates which contained ultraviolet indicators (No. 6610-090-1) were purchased from Brinkmann Instruments, Westbury, NY. Sephadex G-25 medium was a product of Pharmacia, Uppsala, Sweden.

Major equipment. Unless otherwise indicated, enzyme assays were carried out at 37° in a Dubnoff metabolic shaking incubator (GCN Precision Scientific, Chicago, IL). The liquid scintillation samples were counted in a Beckman LS-150 (Beckman Instrument Co., Mountainside, NJ) and centrifugation was performed in a Sorvall RC2-B (Ivan Sorvall, Inc., Norwalk, CT).

Source and authenticity of 6-hydroxyuridine and 6-hydroxyuridine-5'-monophosphate. 6-Hydroxyuridine (1-β-D-ribofuranosyl barbituric acid) and 6-hydroxyuridine-5'-monophosphate (1-β-D-ribofuranosyl barbituric acid 5'-monophosphate) were prepared at our request by organic synthesis by Sigma Chemical Co., St. Louis, MO. The 6-hydroxyuridine (Lots No. 65C-7140 and 65C-7770) was 98 per cent pure as determined by the manufacturer. The 6-hydroxyuridine-5'-monophosphate (sodium salt; Lot No. 35C-7311) was also 98 per cent pure, and was contaminated at a level of about 0.2 per cent with 6-hydroxyuridine. The ultraviolet spectrum of the 6-hydroxyuridine preparation agreed well with previously published analyses [20–22]. As yet there is no published record of the physical characteristics of 6-hydroxyuridine-5'-monophosphate. Hence, we do not have an external standard with which to compare our material.

Enzymes. Ornithine carbamyl transferase (EC 2.1.3.3; 625 units/mg of protein) was obtained from Sigma Chemical Co., St. Louis, MO, and will be designated "OTCase."

Preparation of rat brain for enzymatic assays. Except where otherwise noted, rat brain extracts were prepared from adult male Wistar rats (Camm Research, Wayne, NJ or Fabry Laboratories, New City, NY). The isolated rat brain was dissected free of visible blood vessels, weighed, and minced in either ice-cold 0.9% saline or in an appropriate buffer solution (specified for each assay when other than the saline solution was employed).

Two methods were used to prepare extracts: in method I, a single rat brain was homogenized for 5 min in 10 ml of 0.1 M Tris–HCl buffer, pH 7.4, after which sonic disruption was carried out according to the procedure of Stern and Krooth [23]. The extract was then centrifuged at 12,000 *g* for 15 min and, depending upon the enzyme under study, the supernatant was either assayed immediately or was stored at −70° for future assays. In method II, the rat brain was homogenized over ice in a glass Duall tissue grinder fitted with a glass pestle (Kontes Glass Co., Vineland, NJ). Further details are specified under the procedure for each of the enzyme assays.

Protein determination. The amount of protein in each extract was determined by the method of Lowry *et al.* [24] using bovine serum albumin (Sigma) as the standard. When the extracts were prepared in buffer containing glycerol with DTT or DMSO, appropriate blanks and standards were employed to adjust for the increased optical absorbance resulting from the use of these stabilizing agents.

Carbamyl phosphate synthetase. The activity of this enzyme was measured using a modification of the method of Tatibana and Ito [19]. The reaction mixture contained 100 mM Tris–HCl (pH 7.5), 20 mM glutamine, 20 mM K-ATP (adjusted to pH 7.2), 28 mM $MgCl_2$, 20 mM ornithine, 5 units of purified OTCase, 10 mM [^{14}C]$KHCO_3$ (diluted with cold $KHCO_3$ to about 2000 cpm/nmole), H_2O, and 0.05 ml extract (containing 1.5 to 2.0 mg protein) in a total volume of 0.5 ml. The extract for this assay was prepared using 1 to 2-week-old rats according to method II. The rat brains were minced and then homogenized in an equal volume (w/v) of the stabilizing buffer system previously described by Forman and Grisolia [25]. The reaction was initiated by the addition of the 25,000 *g* 30-min supernatant fraction of the extract. The complete reaction mixture was then incubated for 60 min at 37°. The reaction was terminated by the addition of 0.05 ml of 50% trichloroacetic acid (TCA). Precipitated protein was sedimented by centrifugation and discarded. $^{14}CO_2$ was removed from the supernatant by the repeated addition of small amounts of crushed dry ice. The entire sample was counted in 10 ml Hydromix.

Aspartate transcarbamylase. Enzyme activity was measured using a modification of the method of Bethell *et al.* [26]. The reaction mixture contained 100 mM Tris–HCl buffer (pH 8.5), 3 mM potassium aspartate (adjusted to pH 8.5), 0.4 mM [^{14}C]carbamyl phosphate (diluted with cold carbamyl phosphate to about 3400 cpm/nmole), H_2O and 0.05 ml extract (containing 0.9 mg protein) in a total volume of 0.5 ml. For this assay the brain extract was prepared according to method II in 3 vols (w/v) of 0.05 M Tris–HCl (pH 7.5) containing 10% (w/v) glycerol. A 12,000 *g* 10-min supernatant fraction of the extract was used. The reaction was initiated by the addition of extract, incubated for 15 min at 37°, and stopped by the addition of 0.5 ml of 0.5 N perchloric acid. Precipitated protein was removed by centrifugation, and the resulting supernatants were heated in a boiling water bath for 5 min. Removal of $^{14}CO_2$ and scintillation counting were as described for the carbamyl phosphate synthetase assay.

Dihydro-orotase. Velocity in the forward direction was assayed in a reaction mixture which contained 100 mM sodium phosphate buffer (pH 6.5), 0.5 mM [^{14}C]carbamyl aspartate, H_2O, and 0.05 ml extract (containing 0.9 mg protein) in a final volume of 0.25 ml. Extract for this assay was prepared according to method II as described for aspartate transcarbamylase except that the buffer used was 10 mM sodium phosphate (pH 7.0) containing 10% (w/v) glycerol. The reaction was initiated by the addition of extract, incubated for 60 min at 37°, and terminated by the addition of 25 μl of 70% perchloric acid. The reaction mixture was then neutralized by the addition of 50 μl of 4 N KOH and 25 μl of 1 M $KHCO_3$. Precipitated protein and perchlorate were removed by centrifugation. Samples (10–20 μl) were spotted on chromatography paper. Substrate ([^{14}C]carbamyl aspartate) and products ([^{14}C]dihydro-orotate and [^{14}C]orot-

ate) were separated by ascending chromatography in either 0.6 N formic acid [27] on Whatman DE-81 paper or in 44:44:11:0.26 tertiary butanol–methyl ethyl ketone–water–formic acid [28] on Whatman No. 1 paper. The strips were dried, cut into 1-cm sections and counted in LSC scintillation fluid. Per cent conversion of substrate to products was calculated and used to determine specific DHOase activity.

Dihydro-orotate dehydrogenase. The activity of this enzyme was determined using a modification of the method of Matsuura and Jones [29]. The reaction mixture contained 100 mM Tris–HCl (pH 8.6), 0.1 mM or 0.5 mM [^{14}C]dihydro-orotic acid, H_2O, and 0.05 ml extract (containing 0.7 to 0.9 mg protein) in a total volume of 0.1 ml. Extract for this assay was prepared according to method II in 0.05 M Tris–HCl buffer (pH 7.5) containing 10% (w/v) glycerol. The crude (uncentrifuged) homogenate was used for this assay because of the reported association of the DHO-DHase with mitochondria [17, 29]. The reaction was initiated by the addition of extract, incubated for 30 min at 37°, and stopped by the addition of 5 μl of 70% perchloric acid. After neutralizing the reaction mixture by adding 10 μl of 4 N KOH and 5 μl of 1 M $KHCO_3$, precipitated protein and perchlorate were removed by centrifugation. Samples of each reaction mixture were then spotted on DE-81 chromatography paper. Substrate ([^{14}C]dihydro-orotic acid) was separated from product ([^{14}C]orotate) by ascending chromatography in a 1:1:1 methanol–formic acid–water system [27]. The strips were dried, cut and counted as described for the dihydro-orotase assay. Per cent conversion of substrate to product was then calculated and used to determine specific DHO-DHase activity.

Orotate phosphoribosyltransferase. This enzyme was measured by a modification of the method of Tax *et al.* [30]. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.4), 1 mM tetrasodium 5-phosphoribosyl-1-pyrophosphate (PRPP), 25 mM $MgCl_2$, 0.1 mM [carboxyl-^{14}C]orotic acid, 0.05 ml extract (containing 0.4 to 0.5 mg protein), and H_2O in a total volume of 0.25 ml. In some assays the concentration of PRPP was raised to 12.5 mM and the concentration of orotic acid was lowered to 5 μM in order to look for inhibition, which is competitive with respect to orotic acid rather than with respect to PRPP. Extract for these assays was prepared according to method II in 3 vols (w/v) of 0.05 M Tris–HCl (pH 7.5) containing 10% (w/v) glycerol and 2 mM dithiothreitol, and the 12,000 *g* 10-min supernatant was used. Endogenous rat brain orotidine-5′-monophosphate decarboxylase was employed to convert the [carboxyl-^{14}C]orotidine-5′-monophosphate into $^{14}CO_2$ and UMP. The reaction was initiated by the addition of extract, incubated for 60 min at 37°, and stopped by the addition of 0.35 ml of 60% perchloric acid. The methods for trapping and measuring $^{14}CO_2$ have been previously described [31].

Assays of rat brain OPRTase used to construct Lineweaver–Burk plots were performed as follows. The reaction mixture contained 200 mM Tris–HCl buffer (pH 7.8), 12.5 mM $MgCl_2$, 10 mM 5-phosphoribosyl-1-pyrophosphate, tetrasodium salt; [6-^{14}C]orotic acid at concentrations in the range of 0.59 μM to 0.59 mM, H_2O, and 0.02 ml of rat brain extract in a final volume of 200 μl. Extract for this assay was prepared by method I. The reaction was initiated by the addition of the radiolabeled orotic acid, and incubated for 60 min at 37°. The reaction was stopped by boiling for 3 min, and the reaction mixture was then centrifuged to remove precipitated protein. After centrifugation, a 20-μl aliquot of the supernatant was spotted onto Whatman No. 1 paper strips. Ascending chromatography was performed for 18 hr in 80:18:2 parts of saturated ammonium sulfate–1 M sodium acetate–isopropanol [32]. The radiolabeled orotic acid (R_f = 0.35) was separated from the radiolabeled products: orotidine-5′-monophosphate (OMP) and uridine-5′-monophosphate (both R_f = 0.74), and orotidine and uridine (both R_f = 0.58). Uracil was not usually formed, but if present it would have been recognizable since its R_f was 0.50. After chromatography, the paper strips were dried and the location of radioactive peaks was determined by scanning them with an Actigraph III (Nuclear Chicago) strip scanner. The peaks were then cut out and counted in LSC Complete Scintillation fluid (Yorktown Research).

Orotidylate decarboxylase. The orotidylate decarboxylase procedure is a modification of assay I, described by Krooth *et al.* [31]. The reaction mixture contained 100 mM Tris–HCl (pH 7.4), 10 μM [carboxyl-^{14}C]orotidine-5′-monophosphate, 0.05 ml extract (containing 0.4 to 0.5 mg protein) and H_2O in a final volume of 0.25 to 1.0 ml (depending on the experiment). Extract for this assay was prepared as described for the first (coupled) OPRTase procedure. The other details of this ODCase assay were also identical to those used in the OPRTase assay.

Uridine phosphorylase (uridine: orthophosphate ribosyltransferase). The synthesis of uridine from uracil plus ribose-1-phosphate was measured by a modification of the method of Bose and Yamada [33]. Rat brain homogenates were prepared by method II in 1 volume (w/v) of 50 mM Tris–HCl (pH 7.5). The homogenate was clarified by centrifugation at 12,000 *g* for 30 min and assayed immediately for uridine phosphorylase activity. The reaction mixture consisted of 50 mM Tris–HCl (pH 7.5), 2 mM α-D-ribose-1-phosphate (dicyclohexyl-ammonium salt), [2-^{14}C]uracil (56.6 mCi/m-mole), in concentrations varying from 0.3 to 0.0125 mM, and approximately 0.5 mg of rat brain protein in a total volume of 100 μl. The reaction was initiated by the addition of extract and was incubated for 15 min, at which time it was terminated by boiling for 3 min. The tubes were cooled and coagulated protein was sedimented by centrifugation at 3000 *g* for 10 min. Ten μl of the supernatant solution and 10 μl of a 50 mM solution of uridine were spotted on sheets of Whatman No. 3MM chromatography paper. After drying, the sheets were submitted to descending chromatography for 16 hr in the upper phase of a solvent consisting of 60:35:5 parts of ethyl acetate–water–formic acid [34]. In this system, the R_f of the product uridine was 0.06, while that of the substrate uracil was 0.21. After development, the sheets were dried and the position of the uridine marker was determined under u.v. light. A 4 by 2.5 cm strip including the uridine marker was cut out and counted in 10 ml of LSC scintillation fluid at a counting efficiency of 82 per cent. Chroma-

tography in three solvent systems showed that uridine was the only labeled product generated by the reaction. Uridine phosphorylase activity is expressed as nmoles uridine formed/hr/mg of rat brain protein.

Uridine kinase (ATP: uridine 5'-phosphotransferase). For the assay of uridine kinase, extracts of rat brain were prepared by method II in 2.5 vols (w/v) of a buffer composed of 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$ and 5 mM dithiothreitol. The homogenate was clarified by centrifugation at 12,000 *g* for 30 min. All the extracts were assayed immediately; however, it was found that when extracts were stored for several weeks at −70° prior to assay the loss in activity due to storage was negligible. The assay employed was a modification of the method of Liacouras and Anderson [35]. The reaction mixture consisted of 50 mM Tris–HCl (pH 7.4), 5 mM adenosine-5'-triphosphate (disodium salt), 10 mM D(−)3-phosphoglyceric acid (sodium salt), [2-^{14}C]uridine (51 mCi/m-mole) in concentrations varying from 0.2 to 0.01 mM, and approximately 0.25 mg of rat brain protein in a total volume of 100 μl. Solutions of ATP and phosphoglyceric acid were freshly prepared and neutralized to pH 7.4 prior to assay. The reaction was initiated by the addition of extract and was allowed to proceed for 20 min, after which it was terminated by boiling for 3 min. After cooling, the coagulated protein was sedimented by centrifugation at 3000 *g* for 10 min. A 10-μl aliquot of the resulting supernatant solution and 5 μl of a 2 mg/ml solution of UMP (10 μg) were spotted on thin-layer PEI cellulose plates which had been pre-washed by chromatography in water, air dried and stored at −20° until use. The plates were developed in water using a standard t.l.c. developing tank (Brinkmann) or a sandwich developer (Eastman Kodak). In this system, all phosphorylated products of uridine remain at the origin while the substrate migrates with the solvent front. Since the rat brain extract also contained the activities capable of further phosphorylation of UMP, the amount of UDP and UTP formed was determined by an additional development of plates that had been spotted with 15 μg each of UMP, UDP and UTP in 1.2 M LiCl, which separates all three products. After development the plates were dried, the position of marker compounds was determined under u.v. light and the corresponding areas were cut out and counted in 10 ml of TLA scintillation fluid at a counting efficiency of approximately 75 per cent. Enzyme activity is expressed as nmoles of all uridine ribonucleotides formed/hr/mg of rat brain protein.

Uridine-5'-monophosphate kinase (dCMP: ATP phosphotransferase). The procedure for the preparation of rat brain extracts is the same as that described under the section for uridine kinase. The 12,000 *g* supernatant could be stored at −70° with no loss in activity provided a reactivation procedure was carried out prior to the assay. In this procedure, the rat brain extract was diluted approximately 100-fold with buffer and incubated with 50 mM dithiothreitol at 37° for 1 hr to reactivate the enzyme [36]. The concentrations in the reaction mixture of Tris–HCl, ATP, phosphoglyceric acid and dithiothreitol (in addition to the amount of this compound routinely added to the extract during the reactivation step) were the same as in the assay for uridine kinase.

Our assay procedure is a modification of the one published by Maness and Orengo [36]. Ten μM [4-^{14}C]UMP (45 mCi/m-mole) was added as substrate, and each reaction mixture contained approximately 2.5 μg of reactivated rat brain protein in a total volume of 100 μl. The reaction was initiated by the addition of extract and allowed to proceed for a period of 10 min, after which it was terminated by boiling for 3 min. After cooling, the coagulated protein was sedimented by centrifugation at 3000 *g* for 10 min. Ten μl of the supernatant solution and 5 μl of a solution containing 3 mg/ml each of UMP, UDP and UTP were spotted on PEI cellulose thin-layer plates pretreated in the manner described under the procedure for uridine kinase. The plates were developed in 0.3 M or 1.2 M LiCl. Since our rat brain extracts convert UMP to UDP, and UDP to UTP, the amount of radioactivity in both UDP and UTP was measured. Development in 0.3 M LiCl leaves UDP and UTP at the origin, while UMP migrates to a position corresponding to an R_f of 0.26. Development in 1.2 M LiCl separates all three compounds. Under our assay conditions, over 95 per cent of the radioactivity in product was in the form of UTP. The procedure for location of marker compounds and scintillation counting is the same as described earlier for uridine kinase. Activity is expressed as nmoles of UDP plus UTP formed/hr/mg of rat brain protein.

RESULTS

In the present study, we have employed rat brain extracts and radiochemical assays to look for inhibition of each of six sequentially acting enzyme activities. These activities collectively effect the *de novo* synthesis of UMP. We have also looked for inhibition, in these extracts, of the two sequentially acting enzymes which convert uracil to UMP. Finally, we have performed similar experiments on the kinases which convert UMP to UTP via UDP. The substances tested for possible inhibitory effects on these various enzyme activities were: BA, IBA, 6-OHUR, 5-OHUR, 6-OHUMP, phenobarbital, bemegride, CHEB and DMBB. All these compounds include within their structure a ring which is similar, or identical, to BA (cf. Fig. 1).

Each of the enzymes of the *de novo* pathway was tested with each of the possible inhibitors listed above. The remaining enzymes were tested with only those compounds which appeared to resemble structurally the natural substrate.

Studies on the inhibition of the enzymes of the de novo *pyrimidine pathway.* The activities of the first three enzymes of the pathway (CPSase, ATCase and DHOase) were unaffected by any of the substances tested (Table 1). In addition, at the concentrations shown in Table 1, CHEB, DMBB, bemegride, IBA, 5-OHUR and phenobarbital proved to have no significant effect on the activities of the final three enzymes of the pathway: DHO-DHase, OPRTase and ODCase (Table 1).

Barbituric acid inhibited DHO-DHase (Table 1), and the inhibition was found to be competitive. The dehydrogenase had a K_m of 4.02×10^{-4} M and the K_i for BA was 4.12×10^{-4} M. BA also competitively inhibited OPRTase activity (Table 1 and Fig. 3). The

Table 1. Relative activities*

Addition to reaction mixture	CPSase	ATCase	DHOase	DHO-DHase		OPRTase		ODCase
Control	100†	100‡	100§	100‖	100¶	100**	100††	100‡‡
BA	102	95	107	28	61	2	14	53
6-OHUR	112	107	77	98	84	1	60	60
6-OHUMP	107	87	104		92	0		0
IBA	113	112	91	100	85	126	105	104
5-OHUR	110	104	93		99	95	99	91
Bemegride	102	100	94	98		100	109	96
DMBB	95	90	99	100		91	82	96
CHEB	93	85	109	100		104	102	97
Phenobarbital	100	102	110	95	98	89	98	98

* Each column footnote applies to the vertical column of data.

† Substrate concentrations: $MgCl_2$, 28 mM; K-ATP, 20 mM; glutamine, 20 mM; and $KHCO_3$, 10 mM. Inhibitor concentration: 1.0 mM. Specific activity of the control = 4.1 nmoles/hr/mg of protein.

‡ Substrate concentrations: potassium aspartate, 3.0 mM; and carbamyl phosphate, 0.4 mM. Inhibitor concentration: 6.0 mM. Specific activity of the control = 37.7 nmoles/hr/mg of protein.

§ Chromatographic assay. Substrate (carbamyl aspartate) concentration: 0.5 mM. Inhibitor concentrations: BA and 6-OHUR, 15.0 mM; all others, 3.0 mM. Specific activity of the control = 5.5 nmoles/hr/mg of protein.

‖ Substrate (dihydro-orotic acid) concentration: 0.1 mM. Inhibitor concentration: 1.0 mM. Specific activity of the control = 3.0 nmoles/hr/mg of protein.

¶ Substrate dihydro-orotic acid) concentration: 0.5 mM. Inhibitor concentration: 1.0 mM. Specific activity of the control = 11.1 nmoles/hr/mg or protein.

** Substrate concentrations: orotic acid, 0.1 mM; PRPP, 1.0 mM; and $MgCl_2$, 25.0 mM. Inhibitor concentrations: CHEB, 2.0 mM; all others, 4.0 mM. Specific activity of the control = 4.96 nmoles/hr/mg of protein.

†† Substrate concentrations: orotic acid, 5.0 μM; PRPP, 12.5 mM; and $MgCl_2$, 25.0 mM. Inhibitor concentration: 1.0 mM. Specific activity of the control = 2.46 nmoles/hr/mg of protein.

‡‡ Substrate (orotidine-5'-monophosphate) concentration: 10.0 μM. Inhibitor concentrations: CHEB, 0.5 mM; all others, 1.0 mM. Specific activity of the control = 8.90 nmoles/hr/mg of protein.

K_m for orotic acid and the K_i for barbituric acid (in competition with orotic acid) were 1.5×10^{-6} M and 1.8×10^{-5} M respectively. BA was found to be a weak inhibitor of the ODCase (Table 1), but when Mg^{2+} and PRPP were added to the reaction mixture the inhibition became striking and proved to be competitive with substrate (Fig. 4). When the extract was preincubated at 37° with BA, Mg^{2+} and PRPP (prior to the addition of substrate), the level of inhibition increased. Moreover, the per cent inhibition was a linear function of the duration of preincubation for at least 60 min. Preincubation of extracts with either BA or PRPP (plus Mg^{2+}) alone caused little or no inhibition of the ODCase, and the inhibition, when measurable, did not increase appreciably with the duration of preincubation. Similarly under our conditions, preincubation of extract alone (for up to 180 min prior to assay) had only a negligible effect on brain ODCase activity. The K_m of the ODCase for OMP and the K_i for BA were, respectively, 1.6×10^{-5} M and 7.5×10^{-5} M. These values were obtained in the presence of Mg^{2+} and PRPP and under the conditions described in the legend to Fig. 4. We shall subsequently discuss possible mechanisms by which barbituric acid inhibits OPRTase and ODCase activities.

The riboside and ribotide of barbituric acid (6-OHUR and 6-OHUMP, respectively) had little or no effect on the first four enzyme activities of the *de novo* pathway (Table 1). 6-OHUMP, however, was found to be a powerful competitive inhibitor of ODCase (Table 1), with a K_i of 4.1×10^{-9} M. 6-OHUR proved to be a weak inhibitor of the ODCase. Both 6-OHUR and 6-OHUMP inhibited OPRTase but, as we shall subsequently explain, the mechanism need not involve a direct competition between each of these compounds and either (or both) of the substrates of OPRTase.

Although 1 mM phenobarbital failed to affect ODCase activity (Table 1), phenobarbital concentrations of 10–50 mM did cause a weak inhibition of the enzyme. As can be seen from Table 2, ODCase inhibition by phenobarbital was dependent upon the presence of PRPP and Mg^{2+} in the reaction mixture. Table 2 also shows that the inhibition due to phenobarbital does not appear to be competitive with substrate. The data in the table reveal that the inhibition is demonstrable over a 50-fold range of substrate concentrations, but, over this entire range, the per cent inhibition ascribable to phenobarbital (in the presence of PRPP) does not change with the concentration of substrate.

We have also found that the level of inhibition caused by phenobarbital increases with the duration of preincubation of the extract with phenobarbital, Mg^{2+} and PRPP (prior to the addition of substrate). In this circumstance, a progressive fall in enzyme activity can be observed over at least 3 hr of preincubation. However, we noted little or no decline in enzyme activity in control flasks in which extract was preincubated (for up to 4 hr) without additives or with either phenobarbital or PRPP (plus Mg^{2+}) alone. These results are of course analogous to those just described for BA, except that with phenobarbital the inhibition

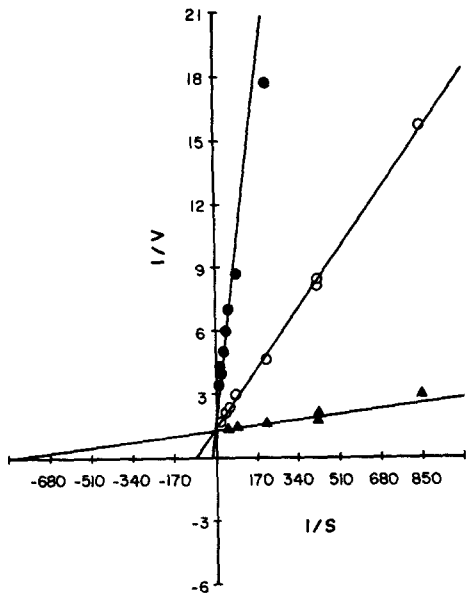

Fig. 3. Double reciprocal plot of the inhibition of OPRTase by barbituric acid. The units of reaction velocity (V) are nmoles product/hr/mg of protein. The units of concentration (S) are m-moles/l, and S refers to the concentration of orotic acid. Key: (▲——▲) control; (○——○) 0.1 mM BA; and (●——●) 0.5 mM BA.

was invariably weak. Less than 50 per cent inhibition was obtained even when the concentration of phenobarbital was 50 mM, and even when the preincubation period was extended to 4 hr.

Studies on the inhibition of enzymes involved in the salvage synthesis of UMP. In mammals the salvage pathway for the biosynthesis of UMP from uracil involves two steps (Fig. 2). First, uracil reacts with ribose-1-phosphate to form uridine. This step is catalyzed by uridine phosphorylase. Second, uridine reacts with ATP to yield UMP in a reaction catalyzed by uridine kinase.

Uridine phosphorylase activity was consistently inhibited by BA, IBA and DMBB (Table 3). It was also inhibited very weakly by bemegride. BA proved to be the most potent of these inhibitors. Double reciprocal plots relating velocity to substrate concentration revealed that the inhibition was competitive. The apparent K_m of the phosphorylase for uracil was 4.2×10^{-4} M and the apparent K_i for barbituric acid was 7.86×10^{-4} M.

The uridine kinase activity of rat brain extracts was unaffected by the inclusion of 5 mM 6-OHUR in the reaction mixture. In the same experiments, however, 5 mM cytidine, which is an alternative substrate and a potent competitive inhibitor of uridine phosphorylation [35], caused almost total inhibition of enzyme activity.

We also inquired whether phenobarbital alone or in combination with other reagents causes inhibition of uridine kinase. Rat brain extracts were preincubated in the presence or absence of phenobarbital. In both cases, the preincubation mixture either contained no further additive or one of the following: 10 mM PRPP, 10 mM ribose-5-phosphate, 10 mM ribose-1-phosphate, or 10 mM ribose-1-phosphate plus 5 mM ATP. After preincubation periods of 10 and 60 min, the extracts were assayed for uridine kinase activity at a uridine concentration of 12.5 μM. None of the combinations listed above produced significant inhibition of uridine kinase activity.

Studies on the inhibition of uridine-5'-monophosphate kinase. Uridine-5'-monophosphate kinase catalyzes the reaction of UMP with ATP to yield UDP. It also catalyzes the analogous reaction employing CMP or dCMP in place of UMP [36].

6-OHUMP proved to be a weak inhibitor of uridine-5'-monophosphate kinase activity. When the UMP concentration was 10 μM, 5 mM 6-OHUMP caused, on the average, a 35 per cent inhibition of the kinase. If the concentration of 6-OHUMP was decreased 5-fold, resulting in a substrate to inhibitor ratio of 1:100, significant inhibition of enzyme activity no longer occurred. CMP is a known competitive inhibitor of purified rat liver UMP kinase [36]. When we substituted 5 mM CMP for 5 mM 6-OHUMP in our reaction flasks, UMP kinase activity was completely inhibited.

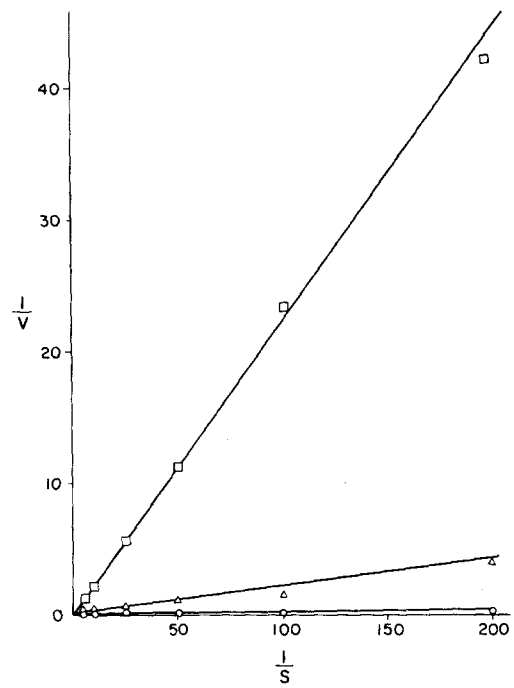

Fig. 4. Inhibition of rat brain ODCase activity by BA in the presence of 12.5 mM Mg^{2+} and PRPP. The assays were performed in a total volume of 1.0 ml. All the ingredients except for substrate were added to the reaction flasks. At this point the flasks contained 800 μl solution. The flasks were then preincubated at 37° for 120 min. After the preincubation period, substrate (in a volume of 200 μl) was added, and the reaction was allowed to proceed for 60 min. The assay procedure was as described under Materials and Methods except that the final concentration of Tris buffer (after addition of substrate in 200 μl) was 0.25 M. Reaction velocity (V) and substrate concentration (S) are expressed in the same units as in Fig. 3. Key: (○——○) control; (△——△) 0.5 mM BA; and (□——□) 5.0 mM BA.

Table 2. PRPP-dependent inhibition of ODCase activity by phenobarbital*

Phenobarbital (43 mM)	PRPP and Mg^{2+} (both at 12.5 mM)	Symbol	Concentration of OMP (μM) 1	0.2	0.02
0	0	A	0.732	0.196	0.019
	+	B	0.830	0.195	0.019
+	0	C	0.662	0.179	0.019
	+	D	0.502	0.119	0.011
% Inhibition in presence of PRPP			39.5	39.0	39.4

* Assay conditions and protocol are identical to those given in the legend to Fig. 4, except that the flasks were preincubated for 90 min at 37° (rather than 120 min), and that sodium phosphate buffer (pH 6.6) at a concentration of 0.25 M was employed in place of Tris buffer. Similar results (after preincubation for 180 min) have been observed with Tris buffer (pH 7.4) at 0.25 M. All concentrations described in the table and in this legend are the final ones, i.e. the concentrations which prevailed after the addition of substrate. The numbers in the body of the table are specific activities, expressed as nmoles $^{14}CO_2$ released from [carboxyl-^{14}C]OMP/hr/mg of rat brain extract. The per cent inhibition, ascribable to phenobarbital, in the presence of PRPP is defined as: (B–D)/B.

Table 3. Effect of additives on uridine phosphorylase, uridine kinase and uridine monophosphate kinase activity

Enzyme	Additive	Concentration of additive (mM)	Enzyme activity expressed as per cent of control containing no additive
Uridine phosphorylase*	None (control)		100
	Barbituric acid	5	3
	Isobarbituric acid	5	49
	Phenobarbital	5	111
	DMBB	5	19
	CHEB	5	158
	Bemegride	5	77
Uridine kinase†	None (control)		100
	6-Hydroxyuridine	5	109
	Cytidine	5	1
Uridine monophosphate kinase‡	None (control)		100
	6-Hydroxyuridine-5′-monophosphate	5	65
	6-Hydroxyuridine-5′-monophosphate	1	104
	Cytidine-5′-monophosphate	5	0

* Substrate ([2-^{14}C]uracil) concentration: 50 μM. Specific activity of the control = 8.78 nmoles/hr/mg of protein.
† Substrate ([2-^{14}C]uridine) concentration: 12.5 μM. Specific activity of the control = 10.27 nmoles/hr/mg of protein.
‡ Substrate ([4-^{14}C]uridine-5′-monophosphate) concentration: 10 μM. Specific activity of the control = 1304.45 nmoles/hr/mg of protein.

We also examined the effect on enzyme activity of preincubation of extract with either phenobarbital alone or with various possible cofactors (employing the compounds listed earlier in the description of our results for uridine kinase). No significant inhibition of UMP kinase activity was observed with any of the substances or combinations of substances tested.

DISCUSSION

Interpretation of results. We have shown that, in extracts of rat brain, BA competitively inhibits three enzyme activities which catalyze, respectively, the final three reactions of the sequence for the *de novo* synthesis of UMP (Fig. 2): DHO-DHase, OPRTase and ODCase. We have also found that BA competitively inhibits uridine phosphorylase, which is the first of the two sequentially acting enzymes (Fig. 2) that effect the conversion of uracil to UMP.

We shall consider the enzymes of the *de novo* pathway first. Striking inhibition of ODCase activity by BA occurred only when the reaction mixture contained Mg^{2+} and PRPP. Therefore, it seemed likely that the inhibition of ODCase by BA involved an interaction between BA and PRPP. Moreover, our results suggested that the interaction was time-dependent. The nature of the interaction is presently unknown, and is now under investigation. One possibility—though by no means the only one—is that BA

and PRPP (or a catabolite of PRPP) react with one another to form an inhibitory ribotide.*

In this connection, we have examined the inhibition of ODCase by one particular ribotide of BA, 6-hydroxyuridine-5'-monophosphate (6-OHUMP). This compound was prepared for us by a commercial supplier and proved to be an extremely potent competitive inhibitor of ODCase. The corresponding riboside, 6-hydroxyuridine (6-OHUR), was found to be a weak inhibitor of ODCase. The inhibitory effect of 6-OHUR might have reflected the synthesis, by our extracts, of a small amount of ribotide from the riboside. Further experiments on this question are planned.

The inhibition of OPRTase by BA was competitive with orotic acid. The mechanism responsible for the inhibition is again unknown, and indeed our experiments are considerably more ambiguous, with respect to mechanism, than they might at first appear. The simplest and perhaps the most plausible possibility is that BA competes with orotic acid for the catalytic site on OPRTase. Another explanation for the inhibition, however, is related to the inclusion of PRPP in our reaction mixture for the OPRTase assay. Since PRPP is a co-substrate of OPRTase, enzyme activity cannot be assayed in the absence of PRPP. The inhibition might have involved not a direct effect of BA, but rather an interaction between BA and PRPP—perhaps with the formation of a ribotide—as just described in the case of ODCase. It is worth emphasizing, incidentally, that we do not yet know whether BA will be found to be competitive with PRPP as well as with orotic acid. Table 1 suggests that the inhibition of OPRTase by BA may be greater when PRPP (column designated by superscript**) is rate-limiting than when orotic acid (column designated by superscript††) is rate-limiting. However, more experiments must be performed to establish this point securely. Yet another possibility is related to the fact that OPRTase is competitively inhibited [39] by its own product (OMP). Hence, the mechanism for inhibition could follow from the accumulation of OMP due to the inhibition, by BA in the presence of PRPP, of the ODCase activity in our extracts (*vide supra*). Alternatively, the mechanism might involve the simulation of OMP by a product formed as a result of the interaction of BA and PRPP.

The weak inhibition of OPRTase by 6-OHUR (Table 1) in the presence of limiting orotic acid might follow from the formation of 6-OHUMP (as discussed earlier in the case of ODCase) or, conceivably, from the enzymatic release of BA. Moreover, the assay for OPRTase shown in Table 1 (as distinct from the assay shown in Fig. 3) depends upon the endogenous ODCase activity of our extracts. This assay directly confounds inhibition of OPRTase activity with inhibition of ODCase activity.

Further experiments must be done to distinguish between the primary inhibition of OPRTase and inhibition of OPRTase secondary to inhibition of ODCase. Ideally, these experiments should employ a chromatographic assay (as in Fig. 3) and OPRTase which has been purified free of ODCase activity. Unfortunately, it is already clear from previous literature [40–42] that purification of mammalian OPRTase, in this sense, is likely to prove extremely difficult—perhaps impossible. The known obstacles are the lability of OPRTase [40–42] and the fact that mammalian OPRTase and ODCase appear to be physically associated in a bienzyme complex [42–44]. Despite numerous attempts, there is only one report describing the separation of OPRTase and ODCase activities [40]. The physical association of the two enzymes, incidentally, imposes yet another level of possible complexity on the mechanisms by which BA and/or its metabolites might inhibit either activity. Conceivably, molecules interacting with the OPRTase active site might lead secondarily to changes in the catalytic activity of the ODCase site, and vice versa.

Thus, although we have shown inhibition of ODCase and OPRTase activity by BA, the molecular mechanisms have not been unambiguously established. Moreover, the elucidation of these mechanisms may require a great deal of further work.

The competitive inhibition of rat brain DHO-DHase activity by BA is consistent with earlier observations. Similar inhibition has been found in the case of the DHO-DHase activity purified from *Z. oroticum* [15], and with the DHO-DHase activity of several species of *Plasmodium* [45], of cultured human skin fibroblasts [16], and of rat liver [17].

With two exceptions, the barbiturates tested had little or no inhibitory effect on the enzyme activities under study. The exceptions were, first, the weak inhibition of ODCase activity by the hypnotic barbiturate phenobarbital, and second, the somewhat stronger inhibition of uridine phosphorylase by the convulsant barbiturate DMBB. In the case of phenobarbital and ODCase, significant inhibition was demonstrable only in the presence of PRPP. Moreover, the level of inhibition increased markedly when the extract was preincubated, prior to the addition of substrate, with phenobarbital and PRPP (plus Mg^{2+}). Preincubation of extract with either phenobarbital or PRPP (plus Mg^{2+}) alone caused little or no inhibition. In these respects, the inhibition of ODCase activity caused by phenobarbital was similar to the inhibition caused by BA. However, phenobarbital, unlike BA, led to an inhibition which was relatively weak and was non-competitive with substrate (Table 2).

The inhibition of uridine phosphorylase by DMBB was considerably less than that caused by BA (Table 3), but it was nonetheless appreciable. In the case of DMBB, we do not yet know whether the inhibition is competitive with substrate. The much stronger inhibition of uridine phosphorylase by DMBB than by the other barbiturates tested was (for us) an unexpected result. It raises the interesting possibility that the structure of the substituted hydrocarbons might play a role in the recognition of barbiturates by this enzyme. The structure of the hydrocarbons is known

* When cultured human cells are incubated with any one of several compounds, the cells develop increased levels of ODCase activity [37, 38]. The compounds are: 6-azauridine, 5-azaorotic acid, barbituric acid (BA) or oxipurinol. All these compounds, except for BA, are known, or surmised on the basis of substantial evidence, to be converted by the cells to ribotides which competitively inhibit ODCase [37, 38]. Our present results indicate that in fact BA is unlikely to be an exception to this generalization, since in the presence of Mg^{2+} and PRPP, BA competitively inhibits ODCase.

to be one of the major factors in determining whether a barbiturate acts as an excitant or a depressant of the level of arousal (cf. Ref. 46 *inter alia*).

Our results with the barbiturates are also of possible interest in two other respects. First, as noted earlier, most barbiturates may be described as a molecule of BA in which hydrocarbons are substituted for the two hydrogens linked to the number 5 carbon atom of BA (Fig. 1). Hydrocarbon substitutions for both hydrogens have proven to be essential if a derivative of BA is to influence the level of arousal. At least in the case of the barbiturates we have studied, these hydrocarbon substitutions appear greatly to reduce, or abolish, the potency of BA as an inhibitor of the enzymes of pyrimidine synthesis in rat brain.* Hence, it is conceivable that one pharmacologic function subserved by the hydrocarbon substitutions is to divest BA of its inhibitory effect on pyrimidine synthesis.

Second, since two of the barbiturates we have studied effect weak inhibition of one or another of the enzymes of pyrimidine metabolism, one might surmise that, at least in these drugs, the BA ring is sufficiently exposed to permit enzymatic recognition. In fact, space-filling models of a number of barbiturates (including phenobarbital) and structurally similar drugs [46] suggest that five out of six atoms in the BA ring are indeed exposed.† On the other hand, the inhibition of ODCase which we observed with phenobarbital did not appear to be competitive with substrate (Table 2). Hence, one could argue that there is really no persuasive evidence that the BA ring itself is involved in mediating the inhibition. However, the fact that phenobarbital inhibited only in the presence of PRPP and that the interaction between the two molecules appeared to be time-dependent makes us suspect that the BA ring is indeed involved. We think it a little unlikely that the phenyl and ethyl moieties of phenobarbital could, in themselves, account for the interaction, particularly since a similar interaction was observed in the case of BA.

Possible general significance of the observations. We suspect that these observations, when pursued by further experiments, might eventually prove of interest in several respects. For one thing it is noteworthy that BA, a catabolite of uracil in certain organisms, is a powerful inhibitor of three enzyme activities which participate in *de novo* pyrimidine synthesis. Hence, it is conceivable that BA might be involved in the mediation of cellular control over the velocity of pyrimidine synthesis in some species. It would be of considerable interest to determine whether one or more mammalian tissues synthesize BA from uracil.

The potency of BA as an inhibitor of the terminal enzymes of UMP synthesis raises the possibility that BA or its derivatives may be found useful in the experimental chemotherapy of neoplasia and infection. There is one report suggesting that BA has antibacterial activity [50]. However, in human diploid cell strains, BA itself (even at 1 mM) does not impose a nutritional requirement for uridine or cause an inhibition of growth [37]—perhaps because BA does not readily enter the cell. On the other hand, the cell might be more permeable to the riboside (or to other derivatives of BA). We should add that it is not yet known how, or whether, 6-OHUR is metabolized by cells.

Finally, as indicated earlier, our observations might bear on the mode of action of the barbiturates. They provide *prima facie* evidence that these drugs do not act by decelerating *de novo* pyrimidine synthesis. Indeed, we have suggested that one function subserved by the two hydrocarbon substitutions, which form part of all barbiturate molecules, may be to divest BA of its potency as an intracellular inhibitor of the enzymatic synthesis of UMP.

* It is of interest that about twenty years ago Friedman and Vennesland [15] noted that, while BA inhibits purified bacterial DHO-DHase, the hypnotic barbiturate, barbital (5,5-diethylbarbituric acid) does not. More recently, Mandel *et al.* [47] have shown that amobarbital (5,5-ethyl-isopentylbarbituric acid) does not inhibit the decarboxylation of labeled orotic acid by *Bacillus cereus*.

† In addition, amobarbital, phenytoin and a number of structurally similar compounds have been shown to inhibit the uptake of orotic acid by *B. cereus* [47, 48]. A similar inhibition was not, however, observed with mammalian cells and tissues [49] (which had only minimal ability to incorporate orotic acid).

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