Biochemical Pharmacology, Vol. 19, pp. 1867–1877. Pergamon Press. 1970. Printed in Great Britain

INTERFERENCE OF BARBITURATES WITH PYRIMIDINE INCORPORATION—II

STRUCTURAL SPECIFICITY OF THE INHIBITION OF OROTATE UPTAKE IN *BACILLUS CEREUS**

H. GEORGE MANDEL and MARGIT RIIS

Department of Pharmacology, The George Washington University, School of Medicine, Washington, D.C., U.S.A.

(*Received* 5 *August* 1969; *accepted* 5 *September* 1969)

Abstract—The structural specificity of the previously observed selective inhibition by amobarbital of the uptake of 2-^{14}C- or 6-^{14}C-orotate into exponentially growing cells of *Bacillus cereus* was examined. The effect of the drug was specific for the uptake of orotate and was not demonstrated for other labeled organic acids. None of several normal metabolites competed with orotate for transport into the cell. A series of other barbiturates, all at 1 mM, inhibited the orotate uptake system without concomitantly affecting the incorporation of uracil into cellular pyrimidines. Phenobarbital, barbital, thiopental, pentobarbital and amobarbital were most active in the model system, as was the convulsant 5(1,3 dimethylbutyl)-5-ethylbarbituric acid. Barbituric acid itself was inactive and *N*-substitution, as in hexobarbital, reduced activity. Certain substitutions in the phenobarbital molecule led to loss of activity in the system, indicating structural requirements for interference with the uptake system. Attempts to relate the relative loss of activity to the extent of ionization and lipid solubility were only partially successful.

The closely related hydantoin derivative, phenylethylhydantoin, was extremely active. Diphenylhydantoin also interfered with orotate uptake, whereas pheneturide, an open chain derivative, was completely inactive. It is concluded that these drugs, many of which have important sedative or anticonvulsant properties, inhibit the transport system for orotate in the bacteria. The structural components apparently required for activity in the system have been described.

IN A PREVIOUS study we reported on the inhibitory action of amobarbital on the incorporation of 2-^{14}C- or 6-^{14}C-orotate into polynucleotides of *Bacillus cereus*.[1] The effect was unaccompanied by other measurable effects on the cells, such as an alteration in the incorporation of uracil or adenine into polynucleotides, a change in oxygen consumption, or the rate of turbidimetric increase of the culture. By a stepwise examination of the component reactions, it was concluded that the drug effect did not involve *de novo* pyrimidine biosynthesis as such, but apparently was associated with the decreased penetration of orotate into the cell in the presence of amobarbital. This drug effect then led to the diminished utilization of orotate for polynucleotide pyrimidine biosynthesis. The amobarbital-produced antagonism of orotate uptake into *B. cereus* was shown to have the characteristics of competitive inhibition, suggesting the existence of an "orotate permease".

* This work was supported by USPHS Research Grant AI 04264 from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.

It is the purpose of this report to describe the effects of drugs related to amobarbital on the incorporation of radioactivity from 2-^{14}C- or 6-^{14}C-orotate into polynucleotides. A number of barbiturates, structurally related pharmacological agents and other pyrimidines were examined for their ability to interfere with the incorporation of orotate and other organic acids into the bacterial cells. These studies resulted in an understanding of some of the structural and associated physicochemical requirements for inhibition of orotate uptake. Although no close relationship between these results and pharmacological action could be established, it is remarkable that many important drugs affecting the central nervous system (CNS) were active in the test system. A preliminary report on these investigations has been presented.[2]

EXPERIMENTAL

Bacterial growth and sampling. Cultures of *Bacillus cereus* 569H were grown as previously described,[1] and drugs and radioisotopic compounds, dissolved in water or in 0·5% sodium carbonate, added to logarithmic cultures. In a few instances, drugs were dissolved in dimethylsulfoxide, since this solvent at low concentrations did not affect growth or orotate uptake in *B. cereus*. Growth was monitored turbidimetrically at 540 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer. The ^{14}C-orotate concentration was 13×10^{-6} M. For most radioactive compounds, 0·01 to 0·1 μc was added per ml of medium. Uptake of radioisotopes into cells was measured by membrane (B-6 Bac-T-Flow, Schleicher & Schuell, Keene, N. H.) filtration of 2-ml aliquots removed from the bacterial cultures at frequent intervals and treated with trichloroacetic acid to precipitate nucleic acids. Comparisons have been made after equivalent amounts of cell growth of the cultures.[3]

Estimation of orotate uptake. Since it was impossible to measure the acid-soluble pool of orotate itself, cells were grown in the presence of ^{14}C-orotate and were treated with trichloroacetic acid. The radioactivity of the cells in the nucleic acid fraction was then measured and used to represent uptake. This procedure had been validated in our previous study, indicating that the various biochemical steps following uptake, such as the incorporation into DNA, the interconversion of RNA pyrimidines, the decarboxylation of orotate, the *de novo* biosynthesis of pyrimidines, and the flavin-mediated interconversion of orotate and dihydroorotate were insensitive to amobarbital. In addition, the reduced content of labeled acid-soluble components derived from ^{14}C-orotate confirmed that measuring incorporation into nucleic acid pyrimidines was a convenient assay of the effect of amobarbital on orotate uptake, since uptake apparently was the limiting step.[1]

Partition coefficients. The partition coefficients of various barbiturates in an aqueous and organic solvent were determined by calculating the distribution of the drugs between an equilibrated mixture of 1 M Tris buffer, pH 8·5, and *n*-butyl chloride at 25°. This solvent had been previously extracted with concentrated sulfuric acid and, after washing, was twice distilled to reduce its ultraviolet light absorbance. About 0·07 mM drug was dissolved in buffer saturated with butyl chloride, and ultraviolet absorbance measured at the absorption maximum (240 or 245 mμ). An equal volume of butyl chloride saturated with buffer was added and the mixture shaken gently for 2 min. After separation of the two layers by centrifugation, the butyl chloride phase was aspirated and absorbance was remeasured in the aqueous phase. The extraction process was then repeated to assure the validity of the analyses. Readings were

corrected for the blank values of drug-free buffer equilibrated with butyl chloride, but these corrections were extremely small. The partition coefficients represent the ratio of the amount of drug extracted by the butyl chloride to the amount remaining in the aqueous phase. Extractions were run in duplicate and were averaged. The results from the successive extractions were in good agreement unless compounds were excessively soluble in either solvent. To correct solubility ratios for pH 8·5 buffer to that of 7·0, the method of Butler[4] based on pK_a values was used.

Ionization exponents. The spectrophotometric procedure of Butler *et al.*[5] was used, and pK_a was determined from shifts in absorbance. Buffers included potassium phosphate (pH 3·6, 4·15, 6·3, 9·15), Tris-HCl, pH 8·5, and sodium borate (pH 10, 11 and 12).

Source of compounds. Radiochemicals were purchased from the following suppliers: 3-^{14}C-pyruvate, 1-^{14}C-lactate, 2-^{14}C-propionate, 7-^{14}C-nicotinate, ^{14}C-bicarbonate, 1,6-^{14}C-adipate, ^{14}C-formate, 6-^{14}C- and 7-^{14}C-orotate, and 2-^{14}C-uracil came from New England Nuclear Corp., Boston, Mass.; 1-^{14}C-succinate and DL-2-^{14}C-glutamate from Tracerlab, Waltham, Mass.; 2-^{14}C-orotate from Calbiochem Co., Los Angeles, Calif.; 6-^{14}C-orotate and 2-^{14}C-uracil from Isotopes Specialties, Burbank, Calif.

Drugs. N,N'-dimethylbarbital, *N*-ethylbarbital, dimethylbarbiturate and *N*-methylamobarbital were obtained from Dr. M. Bush, Vanderbilt University School of Medicine; *N*-phenylbarbital, Burroughs Wellcome Co., Tuckahoe, N. Y.; *N*-methylbarbital, trimethadione and bemegride (Megimide), Dr. H. G. Schoepke, Abbott Labs., North Chicago, Ill.; amobarbital, methohexital, 5(1,3-dimethylbutyl)5-ethylbarbiturate and ethinamate, Eli Lilly & Co., Indianapolis, Ind.; hydroxyamobarbital and chlorpromazine, Drs. Kamm and Van Loon, Smith, Kline & French Laboratories, Philadelphia, Pa.; nikethamide, glutethimide and aminoglutethimide, Dr. A. J. Plummer, Ciba Pharmaceutical Co., Summit, N. J.; compounds 14062 Ba, 11553 Ba, 12277 Ba, and 5-bromoglutethimide, Drs. Keberle and Sury, Ciba Ltd., Basle; cyclobarbital, pheneturide and *N*-methylphenobarbital, Dr. R. O. Clinton, Sterling-Winthrop Research Institute, Rensselaer, N. Y.; meprobamate, Wallace Laboratories, Cranbury, N. J.; methyprylon, Roche Laboratories, Nutley, N. J.; phensuximide and D- and L-2 ethyl-2-phenylsuccinimide, Dr. A. J. Glazko, Parke Davis & Co., Detroit, Mich.; mephenytoin and phenylethylhydantoin (Nirvanol), Dr. J. H. Trapold, Sandoz Pharmaceuticals, Hanover, N. J.; and primidone, 2-deoxymethylphenobarbital and 2-deoxymethyl-1, 2-dihydrophenobarbital, Dr. G. W. Driver and Dr. A. Spinks, Imperial Chemical Industries, Ltd., Macclesfield, England. Other drugs came from commercial sources.

RESULTS

Effect of amobarbital on uptake of organic anions other than orotate into Bacillus cereus

In the previous study,[1] we reported that 1 mM amobarbital selectively decreased the incorporation of radioactivity from exogenous 6-^{14}C- or 2-^{14}C-orotate into cells of *B. cereus*. Since orotic acid, with a pK_a of 2·4, exists entirely as the anion at the pH of the bacterial medium (7·0), the possibility existed that amobarbital had produced a conformational change in the lipoprotein of the bacterial membrane which nonspecifically reduced the penetration of organic anions. Several organic acids were therefore substituted for orotate, and their penetration or incorporation into *B. cereus* was measured in the presence and absence of amobarbital.

Radioactivity from 7-^{14}C-nicotinate and 1,6-^{14}C-adipate was localized primarily in the acid-soluble fraction of the cells, using the membrane fractionation technique.[3] Most of the radioactivity from 2-^{14}C-glutamate, 1-^{14}C-succinate, ^{14}C-formate, 3-^{14}C-pyruvate, and ^{14}C-lactate was recovered upon precipitation with cold trichloroacetic acid, indicating incorporation into cellular macromolecules. Radioactivity from ^{14}C-bicarbonate and ^{14}C-propionate was present in both the acid-soluble and acid-insoluble fractions. In all cases, however, amobarbital exerted no significant effect on the uptake or incorporation of these radioactive compounds. It was concluded, therefore, that the amobarbital effect on ^{14}C-orotate uptake was a response specifically associated with orotate rather than a generalized decrease in the penetration of organic acids.

Tests of specificity of the transport system for normal metabolites other than orotate

Since *B. cereus* has no requirements for exogenous pyrimidines, it was possible that any system for transport of orotate might actually be shared by another metabolite of greater importance to the cell than orotate. Such metabolites would then be expected to reduce orotate incorporation by competition. In spite of certain possible structural resemblances to orotate, however, 1 mM concentrations of thiamine, biotin, *p*-aminobenzoate, pyruvate, lactose, nicotinate, riboflavin, proline and 0·01 mM vitamin B-12 or 0·01 mM folate produced no effect on orotate uptake.

Effect of other barbiturates on orotate uptake

In comparing the effects produced by related chemicals in the bacterial test system in the present experiments, it was considered important to ascertain that the drugs exerted their actions by the same mechanism as had been demonstrated for amobarbital. Accordingly, studies were carried out to eliminate the other relatively likely drug responses which might affect incorporation of orotate into nucleic acids. The following precautionary measures were therefore considered necessary to assure the specificity of the measured reaction: (1) The drugs were added at concentrations which did not alter the growth rate of *B. cereus*, since growth inhibition produced by the drugs might result in secondary responses which might then affect orotate incorporation into the cells indirectly. (2) Since some of the drugs required alkali to become soluble, the bacterial medium was adjusted to a final pH near 7, if necessary. Major shifts in pH have been found to alter the incorporation of ^{14}C-orotate.[1] (3) The effect of the drugs on the incorporation of ^{14}C-uracil into polynucleotides was determined concurrently. Amobarbital had produced its effect selectively on the incorporation of orotate, not of uracil. A drug-induced diminution of uracil incorporation, probably indicating a reduction of nucleic acid formation, would be expected to lead to the nonspecific inhibition of orotate incorporation into nucleic acids. (4) The conversion by cell extracts of 7-^{14}C-orotate into uridylic acid was tested, measuring decarboxylation by trapping $^{14}CO_2$. These reactions are required for nucleic acid pyrimidine formation from orotate. If the drugs inhibited at such a site, incorporation of orotate into RNA would be reduced. However, although all of the compounds could not be tested, none of those examined interfered with this reaction. The few drugs which produced responses on growth rate or uracil incorporation into polynucleotides are indicated.

Table 1 lists the barbiturate derivatives examined and their inhibitory effect on

incorporation of radioactivity from 2-^{14}C-orotate into polynucleotides, expressed as the percentage of the radioactivity incorporated into control cells grown to the same turbidity. 6-^{14}C-orotate could be used interchangeably with the 2-^{14}C-compound. The values usually represent the average of at least three separate incorporation experiments and, in each case, are based on at least six samples removed sequentially during the growth of the cultures. Although reproducibility in different experiments was remarkably close, values greater than 85 per cent of control were not considered of

TABLE 1. PROPERTIES OF BARBITURATES*

Compound	Orotate incorporation (% of control)	pK_a Found	Partition coefficients butylchloride: buffer	
			Calculated (pH 7·0)	Found (pH 8·5)
Barbituric acid	90 ± 6·2	4·03	0·02†	0·02
N,N'-disubstituted				
N,N'-dimethylbarbital	88 ± 8·0			
N-substituted				
N-ethylbarbital	97 ± 2·3	8·18	11·3	3·9
N-methylbarbital	89 ± 5·4	8·21	2·36	0·85
N-phenylbarbital	87 ± 4·1	7·61	8·8	1·25
N-methylphenobarbital	77 ± 4·5	7·95	8·2	2·0
Hexobarbital	73 ± 5·1	8·23	7·0	2·6
Methohexital	57 ± 3·9	8·10	82·0	25·2
N-methylamobarbital‡	38 ± 3·2			
N,N'-unsubstituted, 5,5-dimethyl substituted				
Dimethyl barbituric acid	69 ± 3·5	8·15	0·21	0·07
N,N'-unsubstituted; 5-ethyl substituted				
Phenobarbital	55 ± 3·5	7·27	0·24	0·02
Barbital	50 ± 6·9	7·70	0·24	0·04
Cyclobarbital	48 ± 3·6	7·49	0·60	0·07
Thiopental§	42 ± 5·2			
Pentobarbital	38 ± 3·6	7·86	1·69	0·36
5(1,3 dimethylbutyl)-5-ethyl barbituric acid	30 ± 1·8	7·86	5·65	1·20
Amobarbital	28 ± 3·9	7·87	1·76	0·38
Miscellaneous				
5-Nitrobarbituric acid	106 ± 3·0			
5-(3-hydroxyisoamyl)-5-ethylbarbituric acid	72 ± 2·0			

* Structure–activity relationships among barbiturates, all at 1 mM. Orotate incorporation is reported as per cent of control in absence of drug ± S.E. Partition coefficients were measured at pH 8·5 and calculated for pH 7. The pK_a values were determined spectrophotometrically. For details, see Experimental.

† Determined at pH 7.

‡ Approximately 10 per cent inhibition of growth; uracil incorporation normal.

§ Approximately 15 per cent inhibition of growth and of uracil incorporation.

biological importance. In all cases, the incorporation of radioactivity into the cells, when plotted against changes in turbidity of the suspension, was linear, as had been observed previously with amobarbital. The compounds are grouped in an effort to draw possible conclusions regarding structure–activity relationships.

As was observed previously,[1] with increasing concentrations of amobarbital the effect on orotate uptake was enhanced, but solubility limitations and onset of other drug effects presented complications. For example, amobarbital at 4 mM inhibited

growth; for similar increases in bacterial turbidity, the incorporation of orotate into the cell was only 10 per cent of control, whereas no effect on ^{14}C-uracil incorporation was observed even under these conditions. Barbital depressed growth at 10 mM; at that concentration uracil incorporation was still essentially unchanged, while that of orotate was 14 per cent of control values. Concentrations of hexobarbital greater than 2 mM were inhibitory to growth, but even at 4 mM drug, orotate uptake was still 72 per cent of control. For these reasons, a concentration of 1 mM was used as the standard drug concentration throughout these investigations.

Inspection of Table 1 reveals that the effect on orotate uptake into *B. cereus* by amobarbital was shared by many other barbiturate derivatives. Barbituric acid was essentially inactive in the system, and substitution in the C-5 position was required for activity. The nature of the substituents in the C-5 position was of significance, as revealed by the increasing inhibitory activity going from barbiturate to dimethylbarbiturate and to diethylbarbiturate (barbital). Of all the compounds tested, amobarbital was the most inhibitory at the concentrations tested. Substitution of the H on the N atom greatly decreased potency (barbital vs. the *N*-ethyl, *N*-methyl or *N*-phenyl derivatives; phenobarbital vs. *N*-methylphenobarbital). Hydrophilic substituents, such as hydroxy or nitro groups, decreased activity. The CNS stimulant, 5(1,3 dimethylbutyl)-5-ethylbarbiturate,[6] which differs from pentobarbital only by one additional methyl group in the chain, also was very active in the system, confirming that branching near the chain end conferred additional activity. A thio group could replace the ketone in C-2, but thiopental differed from pentobarbital in being slightly inhibitory to growth (85 per cent of control) and reducing uracil incorporation (about 90 per cent of control). *N*-methylamobarbital also was slightly growth-inhibitory, but no effect on uracil incorporation was noted.

Attempts were made to associate changes in structure of the drugs with physicochemical alterations, such as the ionization exponents (pK_a) or the partition coefficients of the compounds between a nonpolar and a polar solvent. These values were determined spectrophotometrically (described in Experimental). The partition values which needed to be measured at pH 8·5 were converted to the expected values for pH 7 to simulate the conditions of the drug interaction with the growing cells.

Values for the pK_a of the derivatives differed only slightly from each other, and no overall relationship between biochemical activity and concentrations of dissociated or undissociated species was evident. Among the *N,N'*-unsubstituted 5-ethyl barbiturates, however, an increase in pK_a was associated with an increase in inhibition of orotate uptake. The progressive increase in concentration of the undissociated species of these drugs at pH 7 is consistent with the previous observation that the effect of amobarbital on orotate uptake apparently was linked to the uncharged form of the drug.[1] The pK_a of barbituric acid differed appreciably from that of all the substituted compounds, and the concentration of the undissociated form probably was too minimal at pH 7 to produce a biological effect.

Similarly, partition coefficients offered no consistent association with biochemical activity. Among the *N,N'*-unsubstituted 5-ethylbarbiturates, however, an increase in relative lipid solubility enhanced activity. On the other hand, *N*-substituted compounds with high partition coefficients exhibited little biochemical activity in the orotate test system. It is important to recall that *N*-substitution, in addition to changes in the partition coefficients, also reduces keto-enol tautomerism and planarity.

In summarizing these structure–activity studies, it is apparent that the previously observed effect of amobarbital on inhibiting the incorporation of orotate into the cell is not unique for that barbiturate only. The stereochemistry of the compounds appeared to be paramount in establishing the extent of the biochemical activity. The concomitant changes in physicochemical properties relating to partition coefficient and the concentration of the undissociated form of the drugs undoubtedly also contributed to the relative responses.

Effect of related heterocyclic compounds on orotate uptake

In view of the stereospecificity of the compounds inhibiting the uptake of orotate additional compounds with specific structural alteration were examined (Fig. 1). Loss

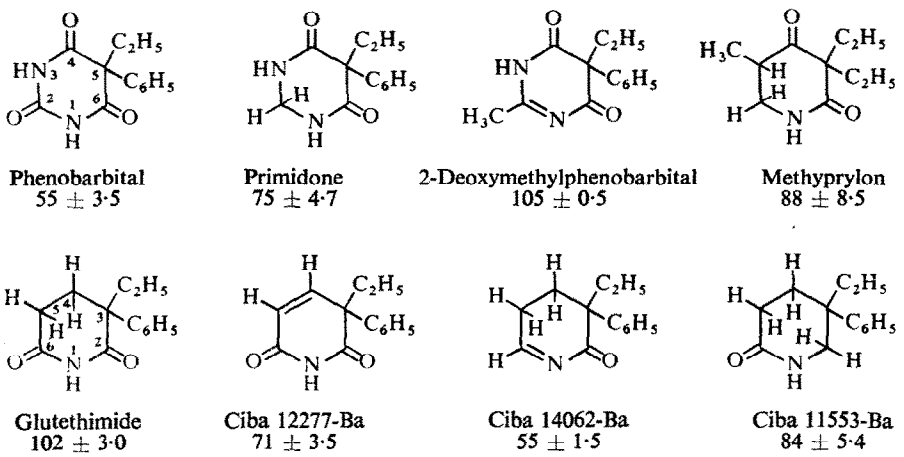
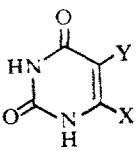
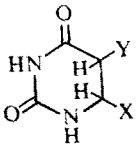


FIG. 1. Effect of phenobarbital derivatives (1 mM) on uptake of orotate into *B. cereus*. Values represent per cent of control incorporation ± S.E.

of the oxygen or sulfur atom on C-2 greatly decreased activity, and primidone was much less active than phenobarbital. A methyl group in that position completely abolished the biochemical effect (2-deoxymethylphenobarbital). The additional replacement of the N-3 imino group by alkyl further reduced activity (methyprylon). Exchange of the —NH—CO— group in positions 3 and 4 of phenobarbital by an ethyl group, which reduced the planarity of the ring, led to complete loss of activity (glutethimide); on the other hand, unsaturation in this replacement group, which corresponds to the 4, 5 position of glutethimide, restored aromatization and resulted in the return of partial activity (Ciba 12277 Ba). A double bond adjacent to the nitrogen atom, conjugated with the ketone (Ciba 14062 Ba) provided a compound with considerable activity, whereas compound Ciba 11553 Ba had little activity. Aminoglutethimide (Elipten) and 5-bromoglutethimide were also inactive, like glutethimide, and 2-deoxymethyl-1,2-dihydrophenobarbital produced no effect on orotate uptake.

Pyrimidines without alkyl substituents (see Fig. 2) such as dihydrouracil (IV), 4,6-dihydroxy-2-amino-pyrimidine (92 ± 4·3 per cent of control) or 2,6-dihydroxy-4-amino-pyrimidine (96 ± 2·0 per cent) were essentially devoid of activity, as was thymine (V)and dihydrothymine (VI). On the other hand, 6-methyluracil (I) and 6-





I. X = Me Y = H 79 ± 0·5
III. X = H Y = H (20)
V. X = H Y = Me 93 ± 2·5

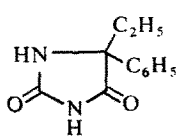
II. X = Me Y = H 82 ± 0·6
IV. X = H Y = H 95 ± 5·7
VI. X = H Y = Me 92 ± 1·5

FIG. 2. Effect of uracil derivatives (1 mM) on uptake of orotate into *B. cereus*. Values represent per cent of control incorporation ± S.E. I, 6-methyluracil; II, 6-methyldihydrouracil; III, uracil (represents special case, see text); IV, dihydrouracil; V, thymine; VI, dihydrothymine.

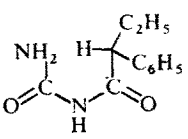
methyldihydrouracil (II) showed slight but reproducible inhibition of orotate uptake, suggesting that to retain inhibitory activity the C-6 position must be substituted either with a methyl group (6-methyluracil), or a ketone (amobarbital, etc.) in place of the carboxyl of orotate. Compounds such as uracil-6-sulfonate were not tested because these growth-inhibitory drugs are inhibitors of orotidylic pyrophosphorylase.[7]

Uracil (III, Fig. 2) was extremely effective in inhibiting the incorporation of orotate, but its mechanism undoubtedly was related to its ability to serve as an excellent source of nucleic acid pyrimidines in *B. cereus*, thus reducing the utilization of orotate. With 1 mM uracil, inhibition of orotate incorporation into nucleic acids to 20 per cent of that of control cells was observed. When amobarbital was introduced, together with uracil, incorporation could be reduced to as little as 10 per cent of control values. Dihydroorotate (1 mM) depressed the utilization of ^{14}C-orotate to 13 per cent of control, apparently because the hydrogenated derivative competes with orotate as a source of nucleic acid pyrimidines. The utilization of dihydroorotate by *B. cereus* was depressed by amobarbital similarly to that of orotate.[1] Purines, including adenine, xanthine and hypoxanthine, and urate showed no activity on the uptake of orotate.

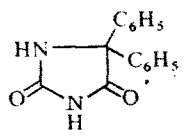
Of the 5-membered ring heterocyclics, the most active compound of any tested was phenylethylhydantoin (Nirvanol) (Fig. 3). This drug reduced uracil incorporation to a



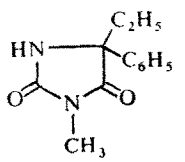
Phenylethylhydantoin†
19 ± 1·1



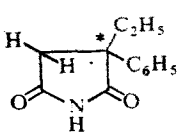
Pheneturide
95 ± 2·0



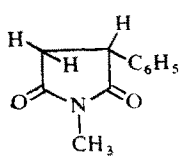
Diphenylhydantoin‡
60 ± 4·6



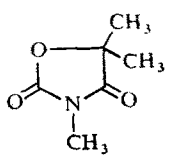
Mephenytoin
74 ± 3·4



2-Ethyl-2-phenylsuccinimide
D: 56 ± 0
L: 63 ± 4·5



Phensuximide
78 ± 4·8



Trimethadione
109 ± 8·5

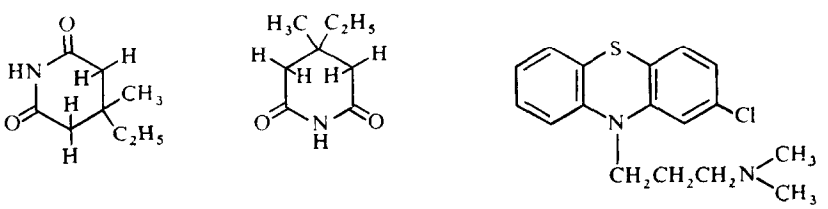
FIG. 3. Effect of hydantoins and related drugs (1 mM) on orotate uptake into *B. cereus*. Values represent per cent of control incorporation ± S.E. †Phenylethylhydantoin produced 15 per cent depression of uracil uptake, 10 per cent depression of growth. ‡ Diphenylhydantoin produced 25 per cent depression of uracil uptake, but normal growth.

small but significant degree (85 per cent of control). Diphenylhydantoin had 60 per cent of control activity, but also depressed uracil incorporation. Again, as in the case of the barbiturates, substitution on N reduced activity (mephenytoin). 2-Ethyl-2-phenylsuccinimide was active, indicating that a nitrogen was replaceable by a methylene group. Testing the available optical isomers revealed that stereospecificity around the asymmetric carbon was not pertinent for biochemical activity. Phensuximide had less activity and trimethadione was inactive, as might be anticipated from the presence of alkyl substituents.

It is of interest that pheneturide, the open chain analog of phenylethylhydantoin, was completely inactive, indicating the requirement of a ring structure for the effect on orotate uptake.

Effect of other CNS drugs on orotate uptake

Since so many of the compounds inhibitory to orotate uptake happened to be important CNS depressant or anticonvulsant drugs, several other compounds with pharmacological effects on the CNS were examined. Nikethamide, chloroform, procaine, ethinamate and meprobamate, all at 1 mM, were completely inactive. On the other hand, bemegride did produce activity in the orotate test system (Fig. 4). This



Bemegride
66 ± 3·8

Chlorpromazine
60

FIG. 4. Other drugs which affected orotate uptake: bemegride, 1 mM; chlorpromazine, 0·05 mM. Values represent per cent of control incorporation ± S.E.

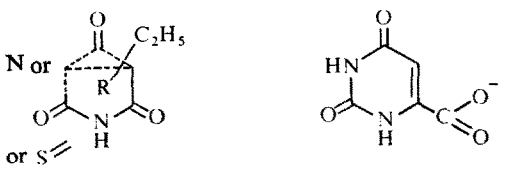
drug, which can be written either as an analog of dihydrouracil (II, Fig. 2) or of glutethimide, was not expected to possess the observed activity. Perhaps the bulky substituents of the barbiturate need not be in position C-5, but this question cannot be answered at the present time.

In these studies, two other and unrelated compounds also depressed the incorporation of orotate selectively, without producing corresponding effects on uracil incorporation. Both of these compounds, chlorpromazine[8] and phenethyl alcohol, are known to produce alterations in membrane function.[9–11] The effects of these agents are considered in the subsequent paper[12] dealing with a more detailed analysis of the mechanism of the effects of drugs on orotate incorporation.

DISCUSSION

These studies indicate clearly that the previously observed effect of amobarbital on the incorporation of radioactivity from ^{14}C-orotate into *B. cereus* is shared by other barbiturate drugs, as well as other structurally closely related compounds. Many of the active derivatives are drugs with important CNS properties in man, and very little

information on the biochemical actions of these drugs has been reported in the literature. Although a single biochemical model for central nervous system activity would be most desirable for predicting drug action, a clear association between pharmacological activity and an effect on orotate uptake could not be established. It is important to emphasize that the observed effect was quite specific for ^{14}C-orotate, since the drugs produced no such effect on the incorporation of ^{14}C-uracil into *B. cereus*. This microorganism does not require orotate for growth, but apparently can utilize the compound for pyrimidine biosynthesis. The biological importance of the orotate uptake system is still unclear, except that the transport of orotate is undoubtedly associated with the bacterial membrane. The present report indicates that the transport system has structural specificity. This specificity required a cyclic structure, such as a 5- or 6-membered ring, preferably with considerable aromaticity. In addition, a carboxyureido group and bulky substituents in the adjacent position were most effective in the test system. The nitrogen atoms were unsubstituted by alkyl groups for maximal activity. Figure 5 represents the most important structural features of the



Active inhibitor Orotate

FIG. 5. Structure of orotate and structural specificity of compounds inhibiting its uptake into cells of *B. cereus*. R should be ethyl, phenyl or preferably a branched alkyl group.

active inhibitor of orotate uptake. Structural modifications altered the relative activity of the drugs and apparently steric relationships were of greater significance than degree of dissociation or partition coefficients.

Structure–activity studies of barbiturates using purified enzyme systems have been carried out by other investigators. Methylation of ring nitrogens increased the potency of inhibiting NAD(P)H dehydrogenase, and a variety of noncyclic amide derivatives also showed activity in this system.[13, 14] A good correlation has been noted between inhibitory effects of barbiturates on mitochondrial NADH cytochrome C oxidoreductase and their chloroform buffer partition ratios.[15] *D*-Aspartate oxidoreductase inhibition was markedly decreased by ring methylation of barbiturates, and open chain ureides showed little if any effect;[16] barbituric acid was not inhibitory, and increasing the size of the C-5 substituent group generally increased inhibitory action. The order of potency of the barbiturates was altered depending on whether potassium ferricyanide or 2,6-dichlorophenolindophenol was the electron acceptor. Although there were some similarities in relative activities of derivatives, an exact correlation for inhibition of the D-aspartate oxidoreductase and orotate uptake did not exist.

REFERENCES

1. H. G. MANDEL, H. M. OLIVER and M. RIIS, *Molec. Pharmac.* **3**, 537 (1967).
2. H. G. MANDEL, S. TRIESTER, P. J. BARNES and D. SZAPÁRY, *Pharmacologist* **11**, 284 (1969).
3. D. B. ROODYN and H. G. MANDEL, *Biochim. biophys. Acta* **41**, 80 (1960).
4. T. C. BUTLER, *J. Pharmac. exp. Ther.* **108**, 11 (1953).
5. T. C. BUTLER, J. M. RUTH and G. T. TUCKER, JR., *J. Am. chem. Soc.* **77**, 1486 (1955).

6. E. E. SWANSON, *Proc. Soc. exp. Biol. Med.* **31**, 963 (1934).
7. W. L. HOLMES, *J. biol. Chem.* **223**, 677 (1956).
8. P. KLUBES, G. S. TABOR and H. G. MANDEL, *Pharmacologist* **9**, 193 (1967).
9. M. A. SPIRTES and P. S. GUTH, *Biochem. Pharmac.* **12**, 37 (1963).
10. G. LESTER, *J. Bact.* **90**, 29 (1965).
11. S. SILVER and L. WENDT, *J. Bact.* **93**, 560 (1967).
12. H. G. MANDEL, S. TRIESTER and D. SZAPÁRY, *Biochem. Pharmac.* **19**, 1879 (1970).
13. A. GIUDITTA and G. DIPRISCO, *Biochim. biophys. Acta* **77**, 394 (1963).
14. M. L. COWGER, R. F. LABBE and B. MACKLER, *Archs Biochem. Biophys.* **96**, 583 (1962).
15. H. E. SPIEGEL and W. W. WAINIO, *J. Pharmac. exp. Ther.* **165**, 23 (1969).
16. L. CASOLA, A. GIUDITTA and E. ROCCA, *Archs Biochem. Biophys.* **107**, 57 (1964).