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INTERFERENCE OF BARBITURATES WITH PYRIMIDINE INCORPORATION—III

STUDIES ON THE MECHANISM OF THE AMOBARBITAL–OROTATE RELATIONSHIP*

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Abstract—The previously observed inhibitory effect of various barbiturates on the uptake of orotate into *Bacillus cereus* was explored in greater detail. The effect was demonstrated for cells incubating at 0° or in the presence of compounds interfering with energy conversion. Although it was not possible to measure intracellular orotate pools, it was concluded that amobarbital prevented the membrane-associated transport of orotate, a process which apparently involved facilitated diffusion. The amobarbital effect was still observed under conditions of hypertonicity, did not include alterations in potassium flux, and appeared to be unrelated to biochemical effects of barbiturates described in mammalian species. Indeed, it was clearly demonstrable only in *B. cereus* or in the closely related *Bacillus subtilis*.

Two chemically unrelated compounds known to affect the functions of cellular membranes also prevented the incorporation of orotate into *B. cereus*. Chlorpromazine and phenethyl alcohol inhibited ^{14}C-orotate uptake into polynucleotides by about 50 per cent, while the corresponding effect on ^{14}C-uracil uptake was about 10 per cent; phenethyl alcohol and amobarbital potentiated each other in their inhibition of orotate uptake. Thus, phenethyl alcohol probably behaves differently from amobarbital and structually related derivatives, even though both appear to interfere with the bacterial membrane orotate transport system.

Radioactivity was associated with cells of *B. cereus* after incubation with ^{14}C-pentobarbital, but no cellular concentration was observed. Orotate did not have a reciprocal effect on the disposition of the barbiturate. Washing with medium readily removed almost all pentobarbital from the cells.

PREVIOUS studies[1] have demonstrated that amobarbital at 10^{-3} M inhibited the incorporation of radioactivity from 2- or 6-^{14}C-orotate into logarithmic cultures of *Bacillus cereus*. The effect took place under conditions of unaltered uptake of ^{14}C-uracil or ^{14}C-aspartate for nucleic acid pyrimidine formation, indicating that the pathways for pyrimidine synthesis and incorporation were functioning normally. In addition, growth, adenine incorporation into nucleic acid purines, the conversion of amino acids into proteins, the utilization of diaminopimelate for cell wall formation or the uptake of oxygen were unchanged by amobarbital. The observed selective effect was localized at the step of uptake of orotate into the cells, since it was demonstrated that in the presence of amobarbital the cellular pool of orotate and anabolites

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was greatly diminished, and since no other biochemical step was shown to be altered by the drug.[1] The observed inhibition of orotate uptake had the properties of competitive inhibition and was reversible when the drug was removed.[1] Structural specificity was demonstrated in that certain other barbiturates and related 5-membered heterocyclics shared this biochemical property, but major quantitative differences resulted following specific structural changes.[2] It was considered most likely that the observed effect involved a bacterial membrane transport system.

In preliminary experiments, two structurally unrelated drugs, chlorpromazine[3] and phenethyl alcohol, also were observed to interfere selectively with orotate uptake. The purpose of the present report is to characterize further the system of orotate uptake and to clarify the action of amobarbital on this step. Attempts have been made to alter quantitatively this drug action by producing changes in tonicity, membrane function or energy availability, or in some other way interfering with orotate transport. At the same time, the disposition of a radioactively labeled barbiturate was followed in the absence and presence of unlabeled orotate to determine whether a simple reciprocal relationship in transport existed. The generality of the drug response was then measured in other biological systems. A preliminary report of this work has been presented.[4]

MATERIALS AND METHODS

Chemicals. Xylosyladenine was kindly furnished by Drs. J. Venditti and H. B. Wood, National Cancer Chemotherapy Service Center, Bethesda, Md. Dr. R. Handschumacher, Yale University, contributed 5-azauracil. All other drugs came from commercial sources.

Radioisotopes. Radiolabeled compounds were purchased from the following suppliers: U-^{14}C-leucine, 6-^{14}C-orotate, 7-^{14}C-orotate, 2-^{14}C-uracil, $^{86}Rb^+$, 2-^{14}C-pentobarbital and carboxyl-^{14}C-inulin, New England Nuclear Corp., Boston, Mass.; 2-^{14}C-orotate and 8-^{14}C-adenine, Calbiochem Co., Los Angeles, Calif.; $^{42}K^+$, Isoserve Corp., Cambridge, Mass.

Microorganisms. B. cereus 569H was a laboratory strain. *Bacillus megaterium JA* came from Dr. Fred E. Hahn, Walter Reed Army Institute for Research. *B. subtilis*, *Serratia marcensis*, *Citrobacter freundii* and *Escherichia coli* were donated by Dr. R. Hugh, Department of Microbiology, The George Washington University. Uracil-requiring auxotrophs of *B. subtilis* MB 1867, MB1887 and 1888, which were developed to use orotate as a pyrimidine source, were contributed by Dr. A. L. Demain, Merck Sharp & Dohme Laboratories. Other mutants, which were furnished by Drs. P. P. Saunders, M. D. Anderson Hospital, and E. Freese, NIH, are gratefully acknowledged.

Growth and radioactivity incorporation procedures. B. cereus was grown in Casamino acids medium in the presence of various drugs as indicated, and growth was measured turbidimetrically. The sampling and membrane filter fractionation procedures have been described in detail.[1, 5] Drugs were dissolved in water, 0·5% sodium carbonate or occasionally in dimethylsulfoxide, which had no apparent effect on the bacterial system.

$^{42}K^+$ studies. The incorporation of $^{42}K^+$ into growing cells was measured by the membrane filtration technique. Logarithmic cultures received 1 μc/ml of $^{42}K^+$. Two-ml samples were taken periodically and filtered through Schleicher and Schuell membrane filters (B-6 Bac-T-flex) which, after washing with saline, were dissolved in Bray's

solution for scintillation counting in a Beckman liquid scintillation spectrometer. For the potassium release experiments, cells prelabeled with $^{42}K^+$ were harvested, washed and resuspended in fresh normal nonradioactive medium in the presence or absence of drugs. The cultures were then further incubated at 37° in a bacterial shaker, and samples removed periodically for measurement of bacterial turbidity and radioactivity. The same procedure was followed for $^{86}Rb^+$ experiments.

^{14}C-pentobarbital distribution between cells and medium. Logarithmically growing cells of *B. cereus* were harvested and then subdivided into two flasks with a final volume of 20 ml of fresh medium each, so that about 15 mg wet weight of cells was present per ml of medium. One flask also contained 1 mM orotate. To these bacterial suspensions ^{14}C-pentobarbital was added to a final concentration of 0·025 μc/ml and a drug concentration of 6 μM. The cultures were rapidly mixed and, after incubation at 37°, 10 ml was removed and centrifuged at 8000 rpm in a Servall centrifuge. Replicate aliquots of 0·1 ml of supernatant were placed into ribbed planchets containing up to 2 ml formic acid and were heat-dried prior to counting in a gas-flow Nuclear Chicago counter. The bacterial residue either before or after washing by resuspension in non-radioactive medium was dried in a vacuum desiccator in tared tubes. The weighed drug residue was then dissolved in a few milliliters of concentrated formic acid, and up to 2 ml of this solution (to maintain equal weight of the samples, about 10 mg each) was placed in planchets and counted as above.

^{14}C-inulin or 7-^{14}C-orotate distribution between cells and medium. Experiments were carried out exactly as with ^{14}C-pentobarbital, except that only normal medium was used for resuspension. The radioactivity of the labeled inulin in the bacterial medium was adjusted to have comparable concentrations of radioisotopes as used in the ^{14}C-pentobarbital experiments. Incubation with 7-^{14}C-orotate was at 0°.

Chromatography. To examine the soluble pool of cells, harvested cells were placed in boiling 80% ethanol and the supernatant solution was evaporated *in vacuo*. The extract was subjected to descending chromatography on Whatman 3 MM paper in a system of 70% isopropanol–water, using suitable markers.

RESULTS

Localizing the orotate transport step in B. cereus

To describe the transport of orotate into bacterial cells, it would be desirable to measure intracellular concentrations of orotate, but in spite of many approaches, experiments to carry out such analyses have not been possible. As is characteristic of other biological systems investigated,[6] intracellular orotate was immediately anabolized by phosphoribosyl transferase to orotidylate before decarboxylation to uridylate. The latter then formed various phosphorylated derivatives, since the compounds recovered in the acid-soluble pool after 6-^{14}C-orotate were mainly uridylate and its derivatives, as shown chromatographically. Analyses of this acid-soluble pool after extraction of 6-^{14}C-orotate-labeled *B. cereus* revealed only a trace of orotate, and most of this compound may have been derived from contaminating medium. In addition, the pool of radioactive compounds in the acid-soluble fraction was small; only about 5 per cent of the total radioactivity in the cells was extractable with trichloroacetic acid, the rest being located in the polynucleotide pyrimidines. Corresponding experiments with 7-^{14}C-orotate, which would be expected to lead to the biosynthesis of unlabeled uridylate and derivatives, produced no measurable acid-soluble pool.

Blocking of subsequent biochemical reactions. Attempts at selectively interfering with the anabolism of intracellular orotate by preventing the phosphoribosyl transferase reaction were unsuccessful. Adenine (2 mM),[7] xylosyladenine (1 mM)[8] or 5-azauracil (1 mM)[9, 10] compounds which have been reported to compete with or inhibit the conversion of orotate to orotidylate in other biological systems, did not reduce the incorporation of exogenous ^{14}C-orotate into nucleic acid pyrimidines of *B. cereus*. 6-Azauridine, which inhibits the decarboxylation of orotidylate,[11] allowed the intracellular accumulation of orotidylate and orotate following growth of *B. cereus* with 7-^{14}C-orotate. Radioactivity in this pool was diminished when cells had been grown in the presence of amobarbital. However, various additional unidentified products resulted from the reutilization of released $^{14}CO_2$, and the radioactivity in the acid-soluble pool fraction became extremely variable after brief periods of growth.[1]

Differential alterations in rates of biosynthetic and transport reactions by reducing temperature. The pool size of radioactive intermediates from 6-^{14}C-orotate could be increased from 5 per cent at 37° to almost 90 per cent of the total cellular radioactivity by incubating cells in an ice bath. Under these conditions (160 mμc/ml of medium, 13 $\times$ 10^{-6} M orotate) there was very little incorporation into polynucleotides, whereas penetration into the cell was less affected. Chromatographic analysis of the isolated labeled pool, after washing of cells, showed that even at almost 0° very little of the radioactivity was due to orotate, but uridylate was again present in large amounts. Repeated washing of the cells did not alter the quantity of radioactivity in the pool, suggesting that most of the compounds were phosphorylated derivatives. It should be noted that amobarbital reduced the radioactivity pool under these experimental conditions (Fig. 1).

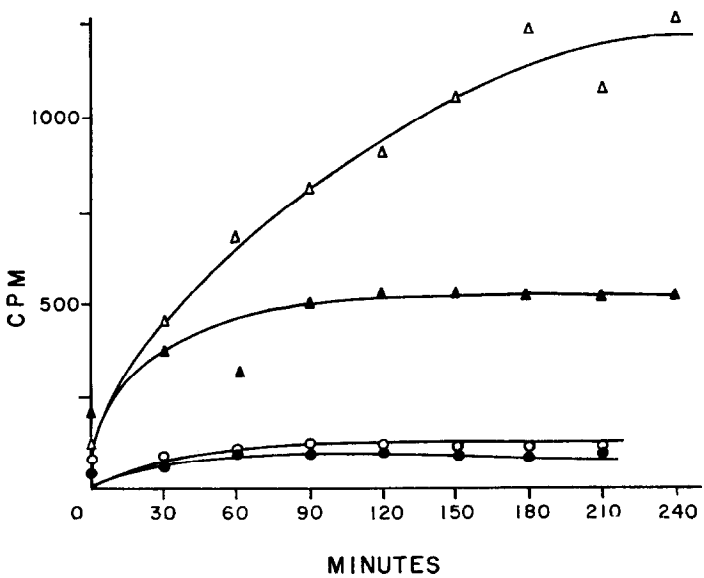

FIG. 1. Effect of amobarbital, 1 mM, on incorporation of radioactivity from 6-^{14}C-orotate into *B. cereus* at 0°. Radioactivity in cells per ml of culture after wash with fresh medium to measure ^{14}C in whole cell (△, ▲); and after wash with trichloroacetic acid to measure ^{14}C in nucleic acids (○, ●). Control culture, open symbols; amobarbital culture, solid symbols.

When this experiment was carried out with 7-^{14}C-orotate (40 mμc/ml, 13×10^{-6} M orotate) to measure orotate more specifically, no radioactivity was present in the pool after one wash of the cells, indicating the small size of the orotate pool, or suggesting that orotate could be readily washed out, or both. Measurements with batches of unwashed cells, although crude, indicated that the ratio of radioactivity from 7-^{14}C-orotate associated with cells to that in the medium was not very different from that using ^{14}C-inulin (orotate: 0·83 $\pm$ 0·15, n = 5; inulin, 0·76 $\pm$ 0·11, n = 4), which is usually distributed extracellularly only. Thus, the intracellular pool of orotate in *B. cereus*, even before washing, was too small to measure by these techniques.

Alterations in bioenergetics

Although the bacterial cells concentrate orotate from the medium against a concentration gradient, the influx of orotate need not be energy-dependent. Even though it was calculated that more than 50 per cent of the orotate in 1 ml of medium was incorporated into 2 mg wet weight of cells within two doublings of bacterial turbidity, the energy dependence of subsequent steps could encourage influx of orotate, by non-energy-dependent mechanisms. It therefore became of interest to observe whether the amobarbital effect could be prevented by interfering with energy availability.

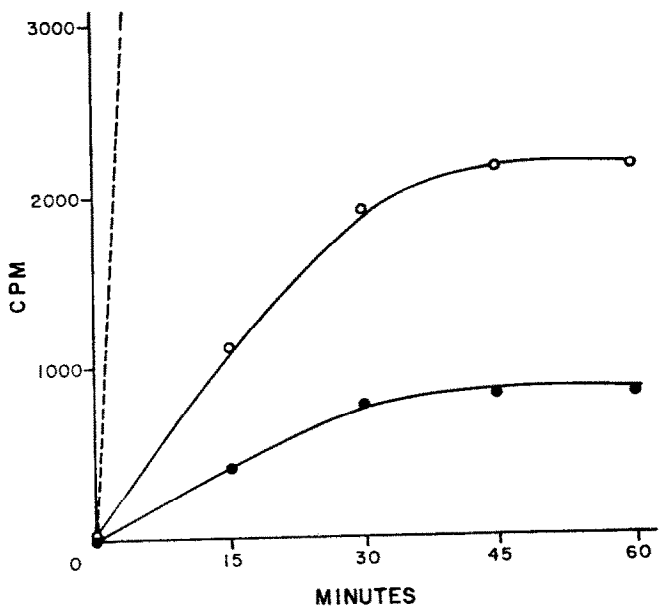

FIG. 2. Effect of amobarbital, 1 mM, on incorporation of radioactivity from 6-^{14}C-orotate into *B. cereus* at 37° in presence of 1 mM arsenite. Radioactivity in cells per ml of culture after wash with trichloroacetic acid. Culture with 1 mM arsenite (○—○); culture with 1 mM arsenite and 1 mM amobarbital (●—●); dotted line represents control culture without arsenite.

Arsenite has been shown to inhibit biosynthetic reactions in *B. cereus* while blocking growth, and it was concluded that this inhibition acted at an early step involving energy production or utilization.[12] Although 1 mM arsenite severely slowed the incorporation of 6-^{14}C-orotate (Fig. 2) or 7-^{14}C-orotate, the amobarbital inhibitory effect was still clearly evident under these conditions.

Furthermore, dinitrophenol did not eliminate the amobarbital effect on orotate uptake. When 0·2 mM or 0·5 mM dinitrophenol was added to cultures incorporating 6-^{14}C-orotate, amobarbital still depressed the uptake of orotate to 38 and 43 per cent of control values, whereas in the absence of dinitrophenol the depression was 28 per cent of control. This small difference is of dubious significance, considering the magnitude of the effects produced by dinitrophenol on the growing cell, some of which are shown in Table 1. It appeared, therefore, that the amobarbital effect was independent of energy requirements.

Orotate uptake in hypertonic medium

When the tonicity of the medium was altered by addition of sucrose to a final concentration of 30 per cent, the rates of incorporation of ^{14}C-leucine, ^{14}C-uracil or ^{14}C-orotate which paralleled growth were only about 40 per cent compared to growth

TABLE 1. CORRELATION OF DRUG RESPONSES WITH OROTATE UPTAKE*

Drug	Concn ($\times 10^3$ M)	Growth doubling time (control/ drug-treated)	O_2 uptake (% of control)	2-^{14}C-uracil uptake (% of control)	2-^{14}C-orotate uptake (% of control)
Sodium cyanide	0·05	94	85		90
Sodium cyanide	0·10	79	94		90
Sodium cyanide	0·30	73	75		98
Sodium azide	0·5	100		86	91
Sodium azide	1·0	71			91
Sodium azide	1·5	61	152	72	88
Dinitrophenol	0·2	93	100	78	85
Dinitrophenol	0·3	82	126		
Dinitrophenol	0·5	74	142	56	73
Amobarbital	1·0	97	96	100	28

* All comparisons were made for similar increases in bacterial turbidity. Usual values for control cultures: doubling time, 45 min; O_2 uptake, 150 μl/3 ml/one doubling time, Δ O.D.$_{540}$ of 0·15; after ^{14}C-uracil and ^{14}C-orotate, 3000 and 2000 cpm, respectively, in trichloroacetic acid-precipitated cells per ml of medium.

TABLE 2. EFFECT OF AMOBARBITAL ON 6-^{14}C-OROTATE INCORPORATION INTO *B. CEREUS* IN NORMAL AND HYPERTONIC MEDIA*

Time of sampling (min)	30% Sucrose	1 mM Amobarbital†		% of control
		−	+	
50	−	1460	380	26
66	−	2260	600	27
76	−	2680	780	29
90	−	4100	1080	27
		−	+	
70	+	900	250	28
100	+	1800	530	29
134	+	3950	1050	27
170	+	7100	2000	28

* Hypertonicity was produced by supplementation of usual growth medium with sucrose to make 30 per cent final concentration. Radioactivity in cells was measured by membrane filtration in the usual manner.

† Values are expressed as cpm in 2 ml of cells.

in normal medium. However, the effect of amobarbital in inhibiting orotate incorporation was still apparent after the addition of sucrose, when comparisons were made at various time points in normal or sucrose media (Table 2). Thus, alterations in bacterial shape resulting from the hypertonicity, which could also be observed microscopically, did not influence the action of amobarbital.

Attempts to antagonize cellular binding of ^{14}C-pentobarbital by orotate

The apparently competitive inhibition by amobarbital of orotate uptake[1] implied that barbiturates interacted with the carrier system which transports orotate into cells. If this process consisted of a simple single-step transport, orotate might then interfere with the binding of barbiturates to the carrier, assuming that the binding took place at few sites other than those which are involved in orotate uptake. Figure 3 demonstrates

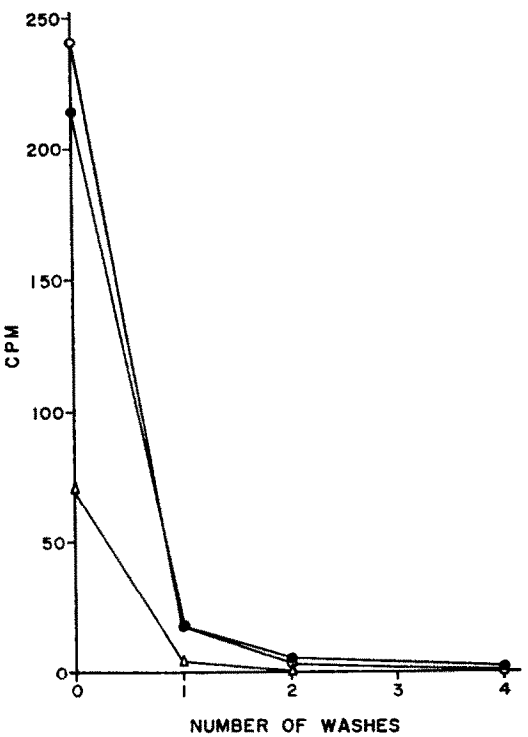

FIG. 3. Radioactivity content per mg dry weight of bacteria as a function of number of cell washings. *B. cereus* cells were suspended in normal medium containing 1 mM orotate, where appropriate, and ^{14}C-pentobarbital or ^{14}C-inulin was then added. Incubation was continued for 5 min at 37°, followed by harvesting and desiccation of cells, as described in Methods. Radioactivity content of medium was identical in all cases. Control medium containing ^{14}C-pentobarbital (○—○); medium containing 1 mM orotate plus ^{14}C-pentobarbital (●—●); control medium containing ^{14}C-inulin (△—△).

that "binding" of radioactivity to *B. cereus* was unaffected when a concentration of orotate as large as 1 mM (70 times the usual concentration) had been added to the medium before a 10-min incubation with ^{14}C-pentobarbital. When the cells were washed once, almost all their radioactivity was lost, and with additional washing no

drug remained associated with the cells. Results were identical when incubation with labeled pentobarbital was tested for periods of from 1 to 40 min.

Since the cell sediment before washing contained entrained medium, some of the radioactivity associated with the bacterial cells represented ^{14}C-pentobarbital dissolved in the extracellular water space. To distinguish between binding to the cells and the entrained drug, an identical experiment was conducted in which ^{14}C-inulin replaced the ^{14}C-pentobarbital. After adjusting the concentrations of the two compounds to provide for identical amounts of radioactivity per milliliter of medium, significantly less radioactivity was associated with the cells when inulin was used as the labeled drug (Fig. 3). In four such experiments, 3·3 $\pm$ 0·6 times as much radioactivity was bound to the cells after ^{14}C-pentobarbital than after ^{14}C-inulin before washing, indicating that the pentobarbital binding to the cells was real and represented an interaction between the drug and the cells. The ready removal by washing implied that this association was weak. The failure to prevent pentobarbital binding indicated that the barbiturate and orotate did not compete at all steps of the orotate transport system, unless indiscriminate binding of pentobarbital masked the effect.

Testing of other compounds on orotate uptake in B. cereus

Several other compounds were tested to determine whether they either altered orotate uptake or influenced the amobarbital-produced effect on orotate uptake. These compounds were selected because they are known to affect barbiturate or orotate actions in mammalian systems. Some of these compounds affect ion movements across membranes,[13–15] compete with the transport of acids,[16, 17] or increase cellular penetration.[18] As is reported in the list in Table 3, however, none of these compounds

TABLE 3. COMPOUNDS WHICH DID NOT AFFECT ^{14}C-OROTATE UPTAKE OR THE DEPRESSION OF ^{14}C-OROTATE UPTAKE PRODUCED BY 1 mM AMOBARBITAL*

		Concn (mM)
Mammalian membrane-active	Acetylcholine	10
	Epinephrine	1
	Histamine	1
	Norepinephrine	1
	Ouabain	1
Ions	Calcium	1
	Nickel	1
	Potassium	125
	Sodium	150
Acids	*p*-Aminohippuric	1
	Benzoic	1
	Phenylacetic	1
	Probenecid	1
	Uric	1
Bases	Aniline	1
	Putrescine	1
	Spermine	1
Penetration aids	Edathamil	1
	Dimethylsulfoxide	100

* Cultures of *B. cereus* were grown: (1) with ^{14}C-orotate only; (2) with supplements as indicated plus ^{14}C-orotate; (3) with 1 mM amobarbital and ^{14}C-orotate; and (4) with supplements plus 1 mM amobarbital plus ^{14}C-orotate. Radioactivity in cultures (1) and (2) was found to be identical; radioactivity in cultures (3) and (4) also was identical, but was only about 30 per cent of that in the absence of amobarbital.

affected orotate uptake or the amobarbital actions on it. The amobarbital effect also was unrelated to the reported actions of barbiturates on oxidation or oxidative phosphorylation, since sodium cyanide, sodium azide and dinitrophenol had no specific effect on orotate uptake (Table 1).

Experiments with other membrane-active drugs

We have briefly reported that 3×10^{-5} M chlorpromazine produced a selective inhibitory effect on ^{14}C-orotate uptake while having very little action on ^{14}C-uracil incorporation.[3] This information is provided in Fig. 4. Approximately a 50 per cent

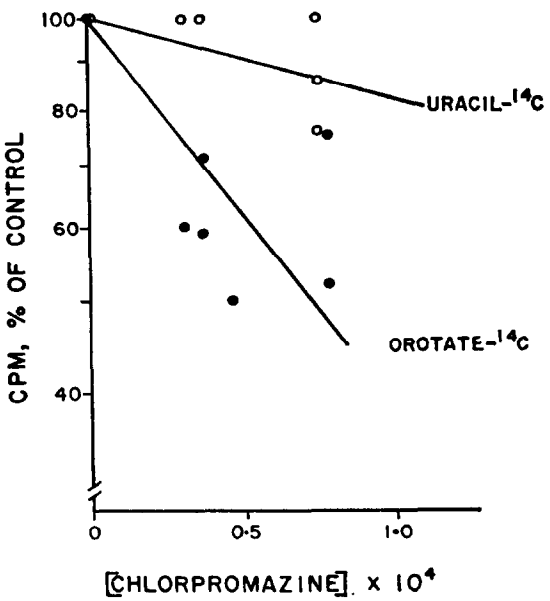

FIG. 4. Effect of chlorpromazine on incorporation of ^{14}C-uracil (○) and ^{14}C-orotate (●) into nucleic acid pyrimidines. *B. cereus* cultures were grown for equivalent change in bacterial turbidity and about seven samples were serially removed from each growing culture for measurement of radioactivity in cells, after washing with trichloroacetic acid.

reduction of orotate uptake was observed at concentrations of this drug which depressed the utilization of uracil usually by about 10 per cent or less. In these experiments, considerable variation in the results of different incorporation experiments was noted, partially because concentrations of the drug greater than 7×10^{-5} M produced growth inhibition, and with somewhat higher concentrations, cell lysis.

Phenethyl alcohol, on the other hand, produced a more reproducible effect at non-growth-inhibitory concentrations (Fig. 5). Again the drug's action on ^{14}C-uracil incorporation was far less pronounced. Phenethyl alcohol produced no selective effect on the incorporation of uracil into DNA of *B. cereus*, even though such an effect has been described for gram-negative bacteria.[19] Since phenethyl alcohol is known to interfere with the uptake of amino acids in microorganisms[20, 21] and apparently accelerates that of acriflavine,[22] this compound has been postulated to produce reversible alterations of bacterial cell membranes.

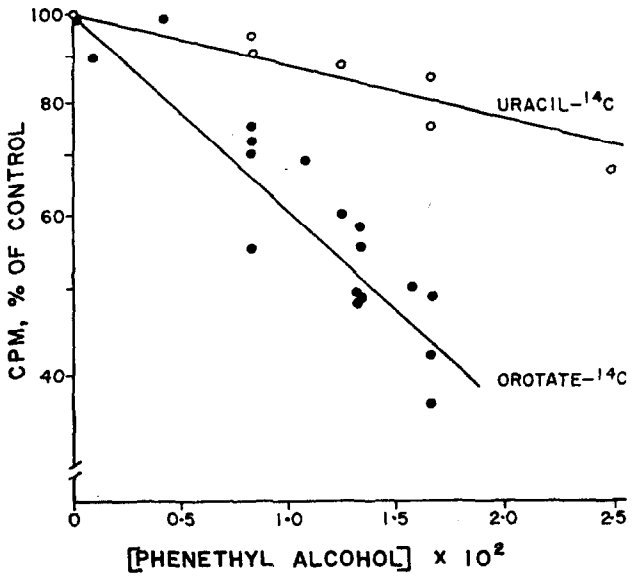

FIG. 5. Effect of phenethyl alcohol on incorporation of ^{14}C-uracil (○) and ^{14}C-orotate (●) into nucleic acid pyrimidines. See legend for Fig. 4.

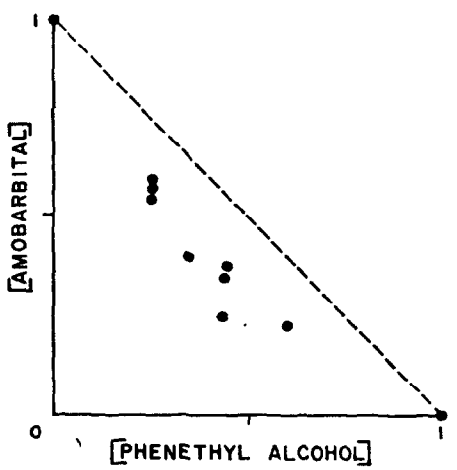

FIG. 6. Plot according to Elion *et al.*[23] to indicate drug potentiation. Values represent concentrations of drug(s) producing 50 per cent inhibition of incorporation of ^{14}C-orotate into *B. cereus*. For amobarbital, $ID_{50} = 1$ is approximately 0·25 mM; for phenethyl alcohol, 13·5 mM.

When phenethyl alcohol was added to bacterial cultures together with amobarbital, the effects on orotate uptake were greater than those resulting from either drug alone. Figure 6 provides evidence of potentiation of the drug combination. In accordance with the method of Elion *et al.*,[23] the dotted line represents the expected values if the actions of phenethyl alcohol and amobarbital were additive, and values inside the triangular area signify potentiation. One would conclude from these results that

amobarbital and phenethyl alcohol produced their effects on orotate uptake by different mechanisms. Such a result would also be anticipated from the lack of structural resemblance of the two compounds. It was observed previously[2] that the effect of barbiturates and related compounds on orotate uptake is associated with great structural specificity.

Silver and Wendt[21] have shown that *E. coli* cells lost $^{42}K^+$ more readily in the presence of phenethyl alcohol, although no such effect could be established for *B. cereus*. Cellular uptake and release of $^{42}K^+$ were also uninfluenced by amobarbital (Fig. 7). Similar results were obtained with $^{86}Rb^+$.

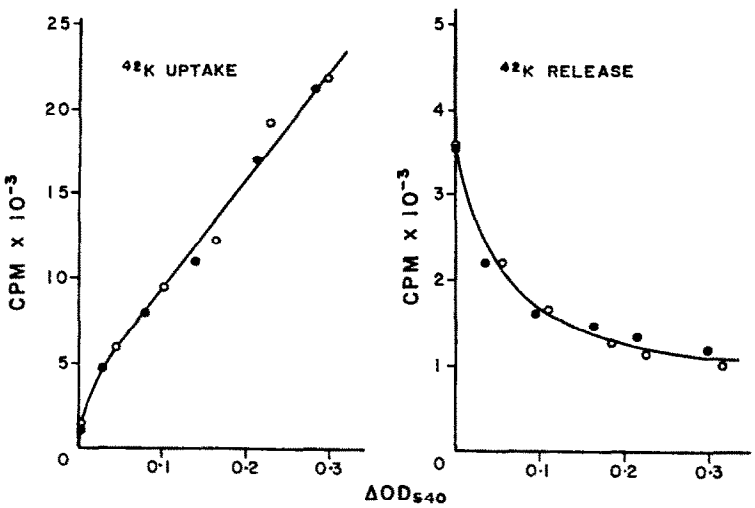

FIG. 7. Lack of effect of 1 mM amobarbital on uptake (left) and release (right) of $^{42}K^+$ of logarithmically growing *B. cereus* cultures. Uptake was measured by the usual membrane filtration technique (see Methods). For release, prelabeled normal cells were washed and resuspended in normal growth medium (○) or in medium containing 1 mM amobarbital (●).

Other biological systems

Microorganisms. Experiments with other microorganisms to detect an effect of 1 mM amobarbital on orotate incorporation were consistent only with *B. subtilis*, which behaved like the closely related *B. cereus*. Amobarbital reduced the uptake of 6-^{14}C-orotate, whereas that of 2-^{14}C-adenine was unchanged (Fig. 8). However, growth of three *B. subtilis* auxotrophs using orotate to partially satisfy their pyrimidine requirements was not prevented by amobarbital, perhaps because high concentrations of orotate were required for growth and the requirement was not absolute. Although amobarbital usually produced a 30 per cent decrease in orotate uptake of *B. megaterium JA*, incorporation of other nucleic acid precursors, such as uracil, guanine or adenine, frequently was similarly depressed. Amobarbital produced only a minor and irregular depression of orotate uptake in *Citrobacter freundii*, *Serratia marcensis* and *E. coli*.

Mammalian systems. No transport system for orotate was found resembling that described for uracil in the small intestine of rats.[24, 25] Amobarbital had no selective effect on incorporation of ^{14}C-orotate into rat-liver slices, and brain slices took up only

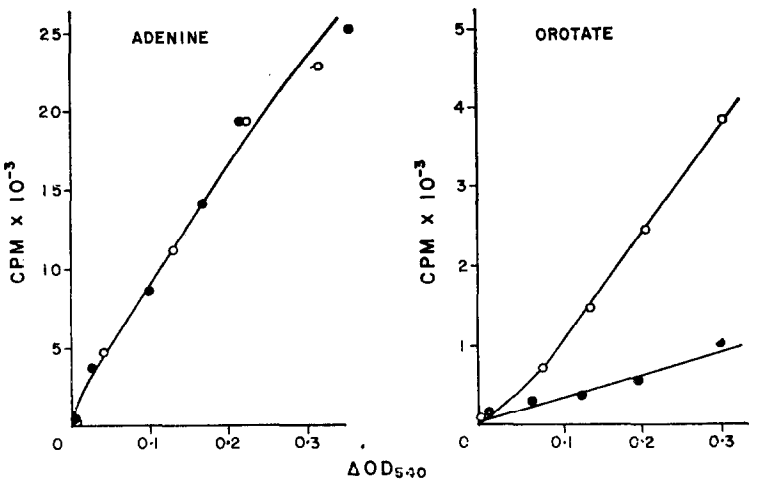

FIG. 8. Effect of amobarbital on incorporation of ^{14}C-adenine (left) and ^{14}C-orotate (right) into *B. subtilis*. Control culture (○); 1 mM amobarbital culture (●).

minimal quantities of orotate. Amobarbital reduced the incorporation into polynucleotides of ^{14}C-orotate into Ehrlich ascites cells incubating *in vitro* no more than that of ^{14}C-adenine or ^{3}H-uridine.

Experiments with mice made it doubtful that amobarbital interfered with the availability of brain pyrimidines. Massive amounts of orotate (285 mg/kg) or of uridine plus cytidine (200 mg/kg each) when injected intravenously (which had no effect by themselves) did not counteract the effects of an intravenous dose of amobarbital just sufficient to make control mice lose their righting reflex (30 mg/kg).

DISCUSSION

The unavailability of a method for measuring orotate pool sizes complicates the elucidation of the inhibitory effect of amobarbital on the incorporation of orotate into *B. cereus*. The evidence presented certainly points to an action involving uptake of orotate into the cell. An interaction *in vitro* between orotate and amobarbital has not been observed,[1] and other steps in pyrimidine biosynthesis have been shown to be insensitive to the barbiturate. The evidence strongly suggests that amobarbital inhibits a transport mechanism, undoubtedly associated with the bacterial membrane, which quite specifically permits orotate to enter *B. cereus* cells. The process permits the cellular entry of the highly charged orotate ($pK_a = 2{\cdot}4$) ion, is structurally specific,[2] is reversible,[1] shows signs of competition,[1] can be saturated,[1] and is independent of the availability of energy, as demonstrated in the presence of amobarbital in the present experiments. Thus, it meets the criteria of facilitated diffusion. It is known from previous work that chloramphenicol and 8-azaguanine, which inhibit protein synthesis in *B. cereus*, produced no inhibitory effect on orotate uptake.[26, 27] Thus, if the system involves a protein "permease", it must have a relatively long half-life.

The amobarbital effect was also established for a wild strain of *B. subtilis*, but could not be readily demonstrated for other microorganisms or mammalian cells. In contrast to *B. cereus* or *B. subtilis*, in these systems barbiturates produced additional

biochemical effects. An inhibitory effect of various barbiturates on several enzymes of the pyrimidine pathway of rat liver slices has been reported, but has not been specifically localized.[28] An effect of barbiturates on DNA synthesis of ascites cells has been reported.[29]

It is remarkable that in addition to various structurally related drugs, only chlorpromazine and phenethyl-alcohol, of a large group of drugs examined, selectively inhibited orotate uptake in *B. cereus*. Chlorpromazine, among numerous other biochemical actions, is known to stabilize membranes, to prevent red cell lysis[30] and to alter permeability of oxalacetate in microorganisms.[31] The selective effect of this drug on orotate uptake may be related to effects on membranes. Similarly, phenethyl alcohol is known to affect permeability of bacterial membranes.[20–22] The potentiation observed, as concluded from Fig. 6, suggests that the mechanism of the effect on the membrane differs from that of amobarbital, and probably explains why this structurally unrelated drug superficially appears to share the normally structurally specific effects on orotate uptake. In any case, it appears that orotate uptake may be useful as an index of membrane alteration in *B. cereus*.

Relatively little information is available on the intracellular distribution of barbiturates. These drugs are known to bind to albumin *in vitro*,[32] but the relative order of affinities is unrelated to the ranking of various barbiturates in inhibiting orotate transport.[2] Amobarbital did not bind firmly to mitochondria and was readily recovered by washing.[33] Phenobarbital was found to bind strongly to liver microsomes and appeared to be quantitatively related to the microsomal content of cytochrome P-450.[34] Soyka[35] has reported binding of ^{14}C-pentobarbital to liver microsomes, with a smaller extent of binding to nuclei, mitochondria and the soluble fraction. This drug association resisted trichloroacetic acid treatment and displacement by excess unlabeled pentobarbital. Washing with sucrose displaced most of the binding from the mitochondria.

Some pentobarbital was bound to or penetrated bacterial cells in these studies, and was readily removed by washing. The quantity of drug associated with unwashed cells does not differ appreciably from that calculated for an equivalent volume of medium. After allowing for extracellular water space from the inulin experiment, 1 mg dry weight of bacteria (or 4 mg wet weight) contained 150 cpm of ^{14}C-pentobarbital, or about 37 cpm/mg wet weight; the medium itself assayed as 28 cpm/μl, suggesting very little if any selective concentration of pentobarbital in the cells.

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