Interference of Barbiturates with Pyrimidine Incorporation

I. Amobarbital Inhibition of Orotate Uptake into *Bacillus cereus*

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SUMMARY

Amobarbital, 10^{-3} M, inhibited the conversion of orotate-2-^{14}C into RNA pyrimidines of exponentially growing *Bacillus cereus*. The drug produced no effect on the growth rate or oxygen uptake of the cells. Furthermore, the incorporation of adenine or uracil into polynucleotides, of amino acids into protein, and of diaminopimelate into cell wall was unaltered by amobarbital.

The effect on orotate incorporation was detectable at 10^{-4} M amobarbital, increased with higher concentrations of the barbiturate, and decreased as the concentration of orotate in the medium was raised.

Studies to localize the effect eliminated the conversion to 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 as the drug-sensitive step. It is concluded that amobarbital, probably as the undissociated compound, markedly depressed the uptake of orotate into the cells. The otherwise unaffected conversion of this pyrimidine precursor into nucleic acid pyrimidines was therefore limited. This amobarbital-orotate effect had properties of competitive inhibition.

INTRODUCTION

Previous investigations on the biochemical effects of a variety of growth-inhibitory agents, such as carcinostatic drugs, antibiotics, and other metabolic inhibitors, have been carried out in microbial systems, and have helped to elucidate the mechanisms by which these compounds produce their pharmacological effects on the isolated cell (1). The difficulties in understanding the biochemical mechanisms of action of other drugs prompted the application of some of these techniques to the study of the cellular effects produced by non-growth inhibitory agents. Although drug-induced effects in microorganisms need not reveal the mechanism of the effects in mammalian systems, they may contribute to the understanding of significant and universal features of the interaction of the drug with biological tissue.

The present studies were undertaken when it was observed that amobarbital at non-growth inhibitory concentrations produced a selective effect on the conversion of the pyrimidine precursor, orotic acid, into polynucleotides of *Bacillus cereus* (2). Since amobarbital, like orotic acid, is a pyrimidine derivative, the possibility of some antimetabolic action was explored.

METHODS

Bacterial growth and sampling. Cultures of *Bacillus cereus* 569H were incubated in

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a gyratory shaker at 37° in a medium consisting of 0.026 M potassium phosphate, 2×10^{-3} M magnesium sulfate, 3×10^{-6} M manganese sulfate, 13×10^{-6} M ferrous ammonium sulfate, and 10 g of casamino acids per liter, pH 7.0. Supplementation of the medium with pyrimidines, such as uracil or orotate, exerted no effect on growth. Growth was monitored turbidimetrically at 540 mμ using a Bausch and Lomb Spectronic 20 or Beckman DU spectrophotometer. Amobarbital and radioisotopes dissolved in water or 0.5% sodium carbonate solution were added during the logarithmic phase of growth, usually at a turbidimetric reading (OD_{540}) of 0.2, corresponding to 0.2 mg dry weight per milliliter of bacterial suspension. These supplementations had no effect on the pH of the bacterial medium. For most radioactive compounds 0.01–0.1 μC was added per milliliter of medium, but labeled amino acids usually required up to 1 μC per milliliter of medium because of the diluting effect of the amino acids in the growth medium. Uptake of radioisotopes into cells was measured by membrane (B-6 Bac-T-Flex, Schleicher and Schuell Co., Keene, New Hampshire) filtration of aliquots removed from the bacterial cultures at frequent intervals, and counting the radioactivity of the filtered cells after washing with saline. Corresponding aliquots were precipitated at room temperature in 5% trichloroacetic acid to determine the radioactivity in the acid-insoluble fraction of cells. The difference in radioactivity between saline and trichloroacetic acid-treated cells represented the acid-soluble fraction. Similarly, aliquots were treated with 1 M KOH overnight to degrade RNA without decomposing DNA, followed by filtration (3).

In these studies comparisons are made on a turbidimetric (ΔOD) rather than time basis to allow more carefully for minor differences in initial cell mass among cultures. The microorganisms grew at identical rates in the presence and absence of amobarbital. Previous experiments with various chemotherapeutic agents (4–6) have demonstrated a close relationship between turbidity and cell mass of bacterial cultures even under conditions of growth inhibition.

Oxygen uptake studies. Oxygen uptake was measured by the standard Warburg technique at 37° (7). Cell cultures of known bacterial turbidity were incubated in Warburg vessels, and at the end of the incubation the turbidity was again measured to assure agreement with that of a parallel culture grown in a gyratory shaker.

Chromatography. Descending chromatography on Whatman No. 3 MM paper included these solvent systems:

(a) 170 ml isopropanol, 44 ml concentrated HCl and water to 250 ml (adapted from (8))

(b) 1 M-ammonium acetate pH 7.5–ethanol, 3:7 (9)

(c) isobutanol-ethanol-glacialacetic acid–H_2O, 50:15:5:30

(d) isopropanol–H_2O, 70:30, with conc. ammonia in tank (10)

(e) *n*-butanol–H_2O, 86:14

Isolation and assays of nucleic acid pyrimidines. Logarithmically growing cells received one of the following radioactive supplements: aspartate-4-^{14}C, 0.04 μC/ml medium; aspartate-3-^{14}C, 0.5 μC/ml medium; or orotate-2-^{14}C, 0.6 mC/mMole, 0.008 μC/ml medium. The cultures were subdivided, amobarbital was added to one for a final concentration of 10^{-3} M, the other serving as control. When the turbidity had approximately doubled, the cultures were harvested by mixing with trichloroacetic acid (final concentration 5%), centrifuging, and hydrolyzing the cell residue in 1 N HCl at 100° for 1 hr. The eluate was chromatographed in solvent (a) and specific activities of pyrimidine nucleotides were calculated from direct counts on paper and, after elution, absorbance values at the wavelength of maximal absorption (11). More than 99% of the radioactivity in the trichloroacetic acid-precipitated residue from cells grown in the presence of orotate-2-^{14}C was extractable with hot acid. After chromatography almost all the counts were recovered in the

areas corresponding to uridylate and cytidylate.

Preparation of bacterial extract. Bacterial cells were centrifuged at 12,000 *g* for 10 min and resuspended in 0.01 M Tris buffer pH 7.8 containing 0.014 M magnesium acetate and 0.06 M potassium chloride. The suspension was then sonicated in a MSE Model 60 W ultrasonic disintegrator for 5 min at 0°, followed by centrifugation for 10 minutes at 12,000 *g*.

Decarboxylation of orotate-7-^{14}C. Incubations were carried out in 25-ml Erlenmeyer flasks each containing a small test tube suspended over the incubate by means of a wire coil inserted through the stopper. The incubation mixture, all in 1 ml, contained (in micromoles): $MgCl_2$, 2; Tris-HCl, pH 7.0 or 8.6, 200; phosphoribosylpyrophosphate, 0.46; amobarbital, 1, or saline; orotate-7-^{14}C, 0.006 (0.005 μC); and cell-free extract equivalent to 20 μg protein, as determined by the method of Lowry *et al.* (12). The test tube contained 0.5 ml of hyamine to trap CO_2 (13). Incubations which were carried out at 37° and in triplicate were started upon addition of orotate-^{14}C and were terminated at appropriate intervals by the addition of 0.5 ml of 2 *N* $HClO_4$. The flask was shaken for another hour to complete the absorption of CO_2 by the hyamine, which was then transferred into a scintillation vial containing 10 ml of a solution of 3 g of PPO (2,5-diphenyloxazole) and 100 mg of dimethyl POPOP [1,4-bis-2-(4 methyl-5-phenyloxazolyl)benzene] per liter of toluene. A Nuclear-Chicago scintillation spectrometer was used for counting.

Conversion of orotate to dihydroorotate and carbamylaspartate. The incubation mixture, all in 0.6 ml, contained (in μmoles) $MgCl_2$. 1; potassium phosphate buffer, pH 7.2, 50; L-cysteine, 5; NADH, 0.18; orotate-7-^{14}C, 0.04 (0.03 μC); amobarbital, 0.60, or saline; and cell-free extract equivalent to 400 μg protein. After incubation at 37°, 0.1-ml samples were removed at 0, 40, and 80 minutes, spotted on Whatman No. 3 MM paper strips together with 0.2 mg each of orotate, dihydroorotate, and carbamylaspartate. The strip was subjected to electrophoresis (9) for 4 hr at 1000 V (20 V/cm) in 0.5 M formate buffer pH 2.75 and air dried; a radioautogram was made. All the radioactivity was concentrated in three distinct areas which were identified as those of orotate (total mobility 33–35 cm), dihydroorotate (23–25 cm), and carbamylaspartate (14.5 cm). Orotate was located by ultraviolet absorption; dihydroorotate after spraying with 1 M KOH followed in 30 min by a 10% solution of dimethylaminobenzaldehyde in conc. HCl-acetone, (1:4 v/v) (14); and carbamylaspartate by the dimethylaminobenzaldehyde spray without prior alkali treatment. Distribution of radioactivity was calculated from total counts of appropriate areas of the paper using a gas-flow counter.

Synthesis of dihydroorotic acid-2-^{14}C. Orotic acid-2-^{14}C (0.5 mg, 2.1 μC) in 5 ml of 0.001 N HCl plus 12.7 mg 5% rhodium on alumina catalyst (K & K Laboratories, Plainview, New York) was hydrogenated at room temperature and atmospheric pressure for 2 hr (15). Aliquots were removed at intervals to monitor the decrease in ultraviolet light absorption at 280 mμ. The conversion was quantitative, as indicated by the loss of absorbance, and dihydroorotic acid-^{14}C was obtained after centrifugation to remove the catalyst. Paper chromatography in solvent system (a) revealed a single radioactive band at R_F 0.66 characteristic of this compound.

Sources of chemicals. Adenine-8-^{14}C. diaminopimelic acid-2-^{14}C, orotic acid-2-^{14}C from Calbiochem Co., Los Angeles, California; DL-aspartic acid-4-^{14}C from Tracerlab, Waltham, Massachusetts; L-lysine-U-^{14}C and glycine-2-^{14}C from Volk Radiochemical Co., Skokie, Illinois; methionine-^{35}S from Schwarz BioResearch Institute, Orangeburg, New York; DL-phenylalanine-3-^{14}C, DL-aspartic acid-3-^{14}C, and uracil-2-^{14}C from Isotopes Specialties, Burbank, California; orotic acid-6-^{14}C from Research Specialties Co., Richmond, California; orotic acid-7-^{14}C from New England Nuclear Corp., Boston, Massachusetts; amobarbital from Eli Lilly Co., Indianapolis, Indiana; and 6-azauridine

from Dr. Harry B. Wood, Drug Development Branch, Cancer Chemotherapy National Service Center, Bethesda, Maryland.

RESULTS

Selective effect of amobarbital on orotate utilization. Amobarbital, 10^{-3} M, had no effect on growth of exponential cultures of *Bacillus cereus*, and bacterial morphology was unchanged. As shown in Fig. 1, under these conditions most biochemical effects measured were unaltered. For example, incorporation of adenine-8-^{14}C into nucleic acid purines, of L-lysine-U-^{14}C into proteins (16) and of diaminopimelate-2-^{14}C into cell wall (16) were normal (all comparisons made for similar turbidimetric increases). Similarly, incorporation of methionine-^{35}S, DL-phenylalanine-3-^{14}C or glycine-2-^{14}C proceeded unimpeded in the presence of the drug. No effect on oxygen consumption by the cells could be demonstrated. The formation of nucleic acid pyrimidines from uracil-2-^{14}C as the precursor was unaffected. On the other hand, with orotate-2-^{14}C or -6-^{14}C, amobarbital produced a pronounced selective inhibitory action on incorporation of radioactivity into the cells. As with uracil, almost all the pyrimidine skeleton of the orotate taken up by cells was recovered in the pyrimidine nucleotides of nucleic acids. The effect of amobarbital was immediate and the degree of inhibition remained constant throughout the experiments. Similarly, the incorporation of radioactivity from orotate proceeded without delay and its magnitude was directly related to the increase in cell mass. The drug effect was reversible, and upon subculture in the absence of amobarbital the cells immediately began to incorporate orotate in a manner similar to that in control cells.

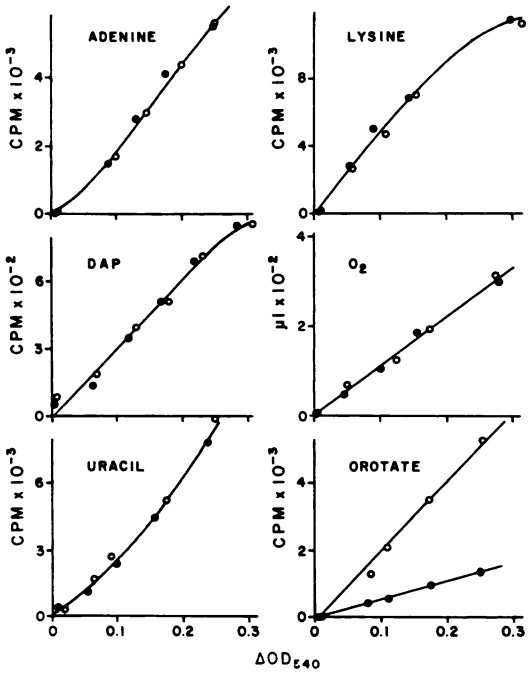

FIG. 1. *Effect of 10^{-3} M amobarbital on biochemical actions of Bacillus cereus*

Cells were grown in the presence and absence of drug, and comparisons were made for similar increases in bacterial turbidity. Oxygen uptake was measured as described in Methods. Other compounds labeled with ^{14}C were allowed to incorporate into cells which were then treated with trichloroacetic acid, filtered, and radioassayed, as described.

Concentration interrelationships of the amobarbital-orotate effect. Concentrations of amobarbital of 10^{-4} M inhibited the uptake of orotate (Fig. 2), and a characteristic dose-response curve was obtained up to the limit of solubility of amobarbital at about 4×10^{-3} when growth was slightly inhibited. Conversely, at a fixed concentration of amobarbital, the concentration of orotate also influenced the degree to which amobarbital depressed the incorporation of the precursor into nucleic acid pyrimidines. As the concentration of orotate was increased, amobarbital exerted less of an inhibitory effect (Fig. 3).

When these data are replotted by the method of Hunter and Downs (17) a straight line results, indicative of competitive inhibition (Fig. 4). From this and four similar experiments, the K_i for amobarbital is $0.24 \pm 0.03 \times 10^{-3}$, while the K_m is calculated as $0.043 \pm 0.008 \times 10^{-3}$ M. The interrelationship between orotate and amobarbital is more complex, however, especially when values for lower concentrations of amobarbital are considered. Although results plotted by the method of

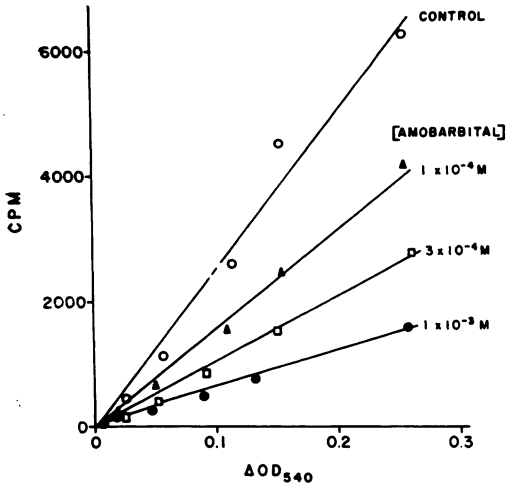

FIG. 2. *Effect of concentration of amobarbital on incorporation of radiocarbon from orotate-2-^{14}C into trichloroacetic acid-treated cells (nucleic acids)*

Amobarbital, ○, 0; ▲, 10^{-4} M; □, 3×10^{-4} M; ●, 10^{-3} M Concentration of 2-^{14}C-orotate, 13×10^{-6} M.

Dixon (18) for 10^{-3} M amobarbital also suggest competitive inhibition, values at lower amobarbital concentrations make such a conclusion uncertain. Examination of data by the method of Lineweaver and Burk (19) reveals that at higher concentrations of orotate the double reciprocal relationship of orotate and radioactivity incorporated is no longer linear.

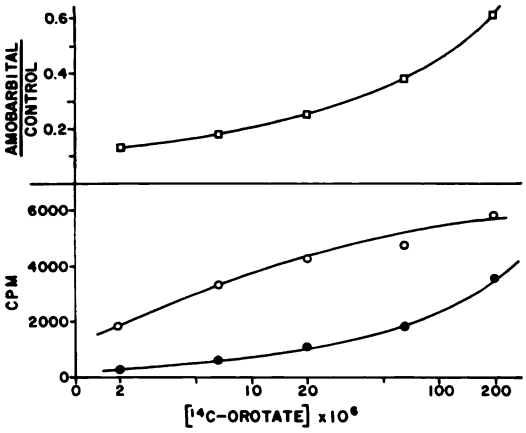

FIG. 3. *Effect of concentration of orotate-2-^{14}C on incorporation of radiocarbon into cell nucleic acids in the presence (●) and absence (○) of 10^{-3} M amobarbital*

Bottom, incorporation values; top, ratio of incorporation in the two cultures.

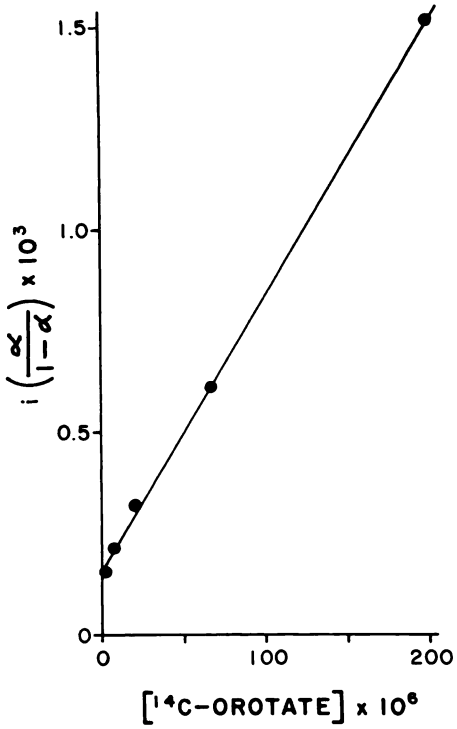

FIG. 4. *Plot of orotate incorporation data from Fig. 3 by the method of Hunter and Downs (17)*

$\alpha = v_i/v$, where v and v_i represent counts per minute incorporated into control and amobarbital cultures, respectively, and i is concentration of amobarbital.

Amobarbital effect on orotate incorporation at various pH values. Increasing the pH of the bacterial medium from 7 to 8 produced a 40% decrease in incorporation of orotate, whereas lowering the pH to 5 produced little if any reduction of the incorporation. Amobarbital potently inhibited incorporation of orotate at all the pH values tested, although the effect at pH 8 was slightly diminished (Table 1).

Localization of the Amobarbital Effect

Possibility of complex formation between amobarbital and orotate. The unlikely possibility of a direct chemical interaction between amobarbital and orotate-^{14}C leading to a diminished supply of orotate-^{14}C for incorporation was tested by

TABLE 1

Effect of pH on amobarbital-induced inhibition of orotate-2-^{14}C incorporation

Cultures of *Bacillus cereus* were grown in media of different pH containing orotate-2-^{14}C (13×10^{-6} M) for similar increases in bacterial turbidity in the presence and absence of 10^{-3} M amobarbital. Control incorporation approximately 5000 cpm/2 ml of bacterial suspension.

pH	Relative incorporation	Amobarbital-induced incorporation % of control	
		Expt. 1	Expt. 2
5	102	—	31
6	104	29	—
7	(100)	21	24
8	52; 71	43	44

two procedures. (a) Chromatograms (solvent systems (a), (d) and (e)) of bacterial medium containing orotate-^{14}C had identical distribution of radiocarbon in the presence or absence of amobarbital. (b) The partition ratio of amobarbital in an *n*-butyl chloride–pH 8.5 Tris buffer system was unaltered by the presence of orotate, even when orotate was present at twice the molar concentration of amobarbital.

Incorporation into DNA. In this and all subsequent experiments the concentration of orotate-^{14}C was 13×10^{-6} M unless otherwise specified. Following growth of *B. cereus* in the presence of orotate-^{14}C, less than 10% of the radioactivity in the cell fraction insoluble in cold trichloroacetic acid resisted solubilization by KOH. This residual fraction has been concluded to contain all the DNA (3). Amobarbital reduced the incorporation of radiocarbon into the KOH-insoluble fraction to the same extent as that into the acid-insoluble fraction. The magnitude of the amobarbital effect observed in the acid-washed cells as well as the lack of a particular effect on the alkali-treated cells ruled out any specific drug effect on incorporation into DNA.

Formation of RNA pyrimidines. The total conversion of exogenous orotate-^{14}C into the pyrimidine nucleotides of RNA was measured by calculating their specific activities (Table 2). Although the activity values were sharply diminished when cells were grown to the same final turbidity in the presence of 10^{-3} M amobarbital, the syntheses of RNA cytidylate and uridylate from orotate-^{14}C were decreased by similar extents, indicating that both pathways were depressed equally. From the molar activities of the isolated cytidylate and the orotate-^{14}C used for labeling (141 cpm per mμmole), and the relative growth of cells before and after the addition of the isotope, it is calculated that only about 15% of pyrimidines synthesized were de-

TABLE 2

Effect of amobarbital on pyrimidine biosynthesis from different labeled precursors

Relative molar activities of pyrimidine ribomononucleotides from RNA after growing *Bacillus cereus* with appropriate supplements. For details see Methods.

Expt. No.	Precursor	Amobarbital 10^{-3} M	Orotate 1.3×10^{-4} M	RNA cytidylate (cpm/mμmole)	^{14}C-Pyrimidines isolated, relative activities	
					Cytidylate	Uridylate
1	Orotate-2-^{14}C	−		8.8	(100)	85
		+			23	23
2	Orotate-2-^{14}C	−		13.0	(100)	80
		+			23	19
3	Aspartate-3-^{14}C	−	−	16.5	(100)	100
		+	−		99	105
		−	+		97	105
4	Aspartate-4-^{14}C	−	−	1.4	(100)	100
		+	−		93	96

rived from exogenous orotate. Since uridine 5′-phosphate (UMP) is believed to be the major intermediate in the conversion of orotate to the two RNA pyrimidines, the block by amobarbital, therefore, must have preceded the formation of that intermediate.

Formation of UMP from orotate. The conversion of orotate-2-^{14}C to UMP was examined in a cell-free system of *B. cereus* in which UMP and its catabolite, uridine, were isolated by chromatography, using solvent system (b). Although amobarbital did not affect the formation of UMP plus uridine from orotate, the possibility remained that the relatively high concentrations of orotate necessitated by this technique might reverse any amobarbital effect, as has been shown in Fig. 3.

In more critical experiments, therefore, orotate-7-^{14}C was incubated with cell-free extract in the presence or absence of 10^{-3} M amobarbital, and decarboxylation was measured by trapping and counting $^{14}CO_2$. It is evident from Fig. 5 that amobarbital produced no inhibition of the rate of decarboxylation, whether measured at pH 8.6, where the reaction was optimal, or at 7.0, at which the cells were normally grown.

Possible interconversion of orotate and dihydroorotate. Giuditta and Strecker (20) have demonstrated that amobarbital and other barbiturates are capable of inhibiting brain NADH dehydrogenase and that this inhibition is competitive with NADH (21). The sensitivity of flavoprotein enzymes to barbiturates (22) and the reported inhibition by barbituric acid of dihydroorotic dehydrogenase (23, 24), another flavoprotein, suggested that the latter enzyme might be involved in the uptake of exogenous orotate into cells for the subsequent metabolism into cellular pyrimidines. The reduction of orotate to dihydroorotate is a reversible reaction which has been demonstrated in aerobic bacteria (25), and the conversion appears essential when growth of cells is based on orotate as the carbon source (24). Even though there is no evidence that this reversible reaction is involved in biosynthesis from exogenous orotate, it was necessary to rule out the possibility that amobarbital partially blocked the NADH-mediated reduction to dihydroorotate, and that less dihydroorotate was therefore available for direct conversion to nucleic acid pyrimidines. Since carbamylaspartate does not serve as a pyrimidine precursor of growing *B. cereus* cells (11) two types of studies were carried out: (a) the rate of conversion of orotate to dihydroorotate and carbamylaspartate

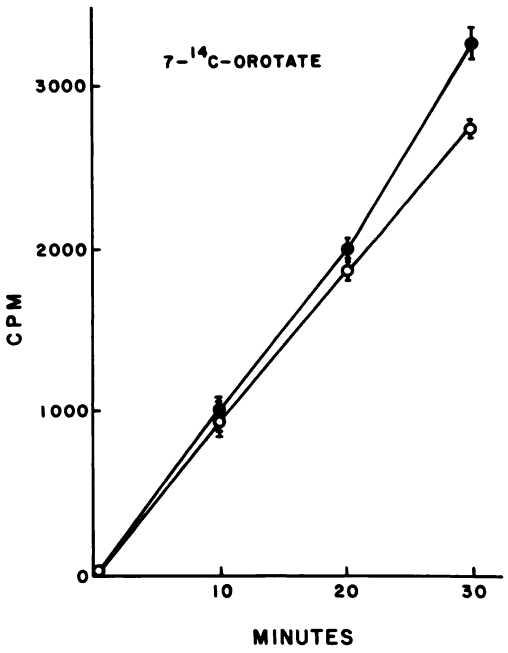

FIG. 5. *Effect of amobarbital on in vitro decarboxylation of orotate-7-^{14}C*

See Methods for details; ○, control incubation mixture; ●, 10^{-3} M amobarbital-supplemented incubation mixture.

by cell-free extracts was measured and found to be unaffected by the presence of 10^{-3} M amobarbital (Table 3); (b) dihydroorotate-2-^{14}C was synthesized and its incorporation into cells was measured. As shown in Fig. 6, the inhibitory effect of amobarbital on the incorporation of dihydroorotate was similar to that of orotate. It was concluded, therefore, that the amobarbital-induced inhibition of the conversion of orotate to nucleic acid pyrimidines was not localized at the dihydroorotic dehydrogenase step.

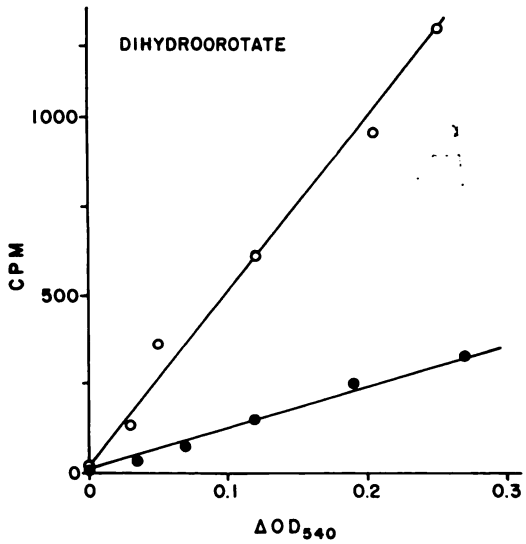

FIG. 6. *Effect of amobarbital on incorporation of radioactivity from dihydroorotate-2-^{14}C into cells, followed by trichloroacetic acid treatment*

○; control culture; ●, 10^{-3} M amobarbital culture.

De novo pyrimidine biosynthesis. In order to evaluate the observed decrease of incorporation of orotate with respect to total nucleic acid pyrimidine biosynthesis, experiments were performed using aspartate-^{14}C as the source of labeled RNA pyrimidines. Table 2 demonstrates that amobarbital had no effect on the conversion of aspartate to RNA cytidylate or uridylate, implying that pyrimidines were being synthesized normally in the presence of amobarbital. Surprisingly, however, exogenous orotate (even at 10 times the usual concentration) did not diminish the conversion of aspartate to pyrimidines either (Table 2). Although the remote possibility of separate pathways for the two pyrimidine precursors has not been excluded for the *B. cereus* system, in contrast to that of *Escherichia coli* (26), the relatively inefficient conversion of orotate to pyrimidines probably accounts for the inability by orotate to exert feedback inhibition following its anabolism or to dilute the *de novo* pathway. In any case, the major discrepancy between these results and those when the ^{14}C source was orotate (Table 2) suggested that the observed drug effect was not related to pyrimidine biosynthesis but to the impeded entry of exogenous orotate into the cell, which thus limited the otherwise unaffected conversion to nucleic acid pyrimidines.

Penetration of orotate into cells. The content of radioactivity in the acid-soluble fraction of cells growing in orotate-2-^{14}C medium in the presence and absence of amobarbital was examined next. Figure 7 indicates that the total radioactivity in this fraction was sharply diminished by drug treatment. Examination of these pool fractions by paper chromatography (solvent system (b) revealed mainly compounds of uracil and cytosine (11) and none of orotate. The relative distribution of radioactivity among the components was un-

TABLE 3

Conversion of orotate-^{14}C to dihydroorotate and carbamylaspartate

Cell-free extracts of *Bacillus cereus* were incubated with orotate-7-^{14}C. At 0, 40, and 80 minutes of incubation samples were removed and fractionated by paper electrophoresis. Distribution of radioactivity in three bands corresponding to orotate, dihydroorotate, and carbamylaspartate is reported as percentage of total recovery (about 5000 cpm in each instance). For details of incubation and assay see Methods.

Amobarbital 10^{-3} M	Percent of radioactivity								
	As orotate			As dihydroorotate			As carbamylaspartate		
	0′	40′	80′	0′	40′	80′	0′	40′	80′
−	97.6	75.4	72.7	2.4	22.7	24.1	0	1.9	3.2
−	97.7	76.6	65.1	2.3	21.5	30.6	0	1.9	4.3
+	96.9	76.6	62.9	2.7	21.7	33.0	0.4	1.7	4.1
+	97.2	75.9	62.0	2.0	22.2	25.9	0.8	1.9	12.1

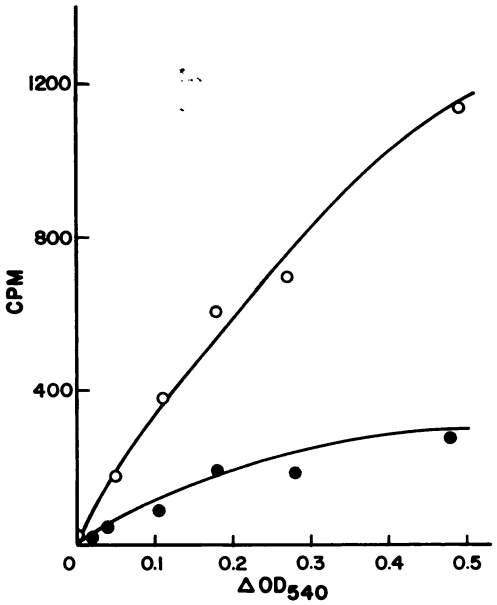

FIG. 7. *Effect of amobarbital on incorporation of radioactivity from orotate-2-^{14}C into acid-soluble pool fraction of cells*

Each value represents the difference between radioactivity in cell samples washed with saline and with trichloroacetic acid. ○, control culture; ●, 10^{-3} M amobarbital culture.

affected by growth in the presence of amobarbital.

To further localize the effect of amobarbital at the step of entry of orotate into the cell rather than during the subsequent reactions, experiments were carried out using orotate-7-^{14}C to preclude labeling of uracil derivatives in the cell following decarboxylation. However, after growth of cells with their labeled compound, most of the radioactivity associated with the microorganisms was not in the acid-soluble fraction where orotate would be expected. Instead, the radioactivity was precipitable with trichloroacetic acid (Fig. 8) apparently because $^{14}CO_2$ liberated by decarboxylation immediately labeled the growing cells. Nevertheless, Fig. 8 reveals that much less radioactivity from orotate-7-^{14}C was present in cells grown in the presence of amobarbital than in its absence. The addition of sodium bicarbonate to the bacterial suspensions to dilute any $^{14}CO_2$ released into the medium lowered the incorporation of radioactivity into the acid-insoluble fraction without appreciably affecting that in the acid-soluble fraction.

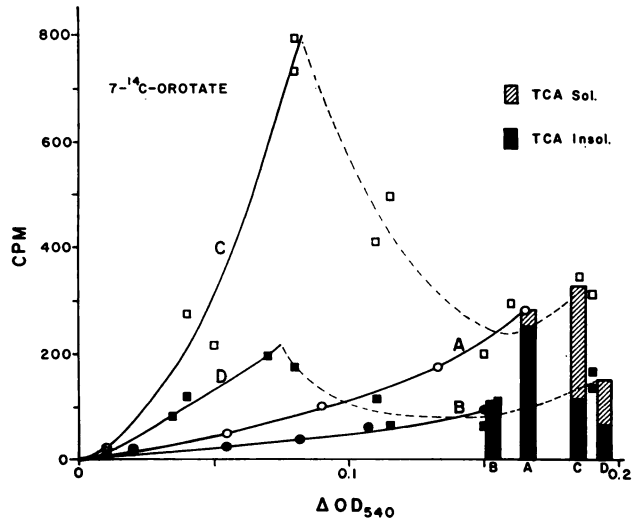

FIG. 8. *Effect of amobarbital on incorporation of radioactivity from orotate-7-^{14}C into cells washed with saline*

Cultures A and B were grown in the usual procedure. Cultures C and D received 6-azauridine to inhibit decarboxylation of orotidylate. Vertical bars represent distribution of radioactivity between trichloroacetic acid-soluble and insoluble fractions; ○, control culture; ●, 10^{-3} M amobarbital culture; □, 0.2×10^{-3} M 6-azauridine culture; ■, 0.2×10^{-3} M 6-azauridine plus 10^{-3} M amobarbital culture.

However, frequent sporadic results limited the usefulness of this procedure.

The normally extremely small orotate pool of the cells could be enhanced by the use of 6-azauridine, a pyrimidine analog which prevents decarboxylation of orotidylate to uridylate (27). The cells accumulated radioactivity when grown in the presence of orotate-7-^{14}C plus 6-azauridine, and under these conditions most of the radioactivity was recovered in the acid-soluble fraction. Chromatography (solvent (c)) of extracts from washed cells grown in this manner revealed that most of the recovered label was associated with orotate and orotidine. After about 20 min of growth in the presence of azauridine, however, cells suddenly but consistently lost radioactivity, and thereafter the content of labeled compounds fluctuated considerably. It is clear from the results of Fig. 8, making use of the azauridine procedure, that the intracellular pool of orotate compounds was greatly diminished by amobarbital treatment. Since amobarbital has been shown not to affect conversion of orotate to orotidylate or to uridylate (see Fig. 5) it can be concluded that amobarbital must have impeded specifically the step(s) of penetration of orotate from the medium into the acid-soluble fraction of the cell.

DISCUSSION

Microbial systems may serve a useful role in the development of model systems for the study of pharmacological action of nonchemotherapeutic agents. Recent studies by Simon and Van Praag (28) have shown that high concentrations of the narcotic analgesic, levorphanol, depress the synthesis of RNA in *Escherichia coli* cultures. Goldstein (29) has found in this bacterial system that inhibition of D-alanine oxidase by barbital is followed by derepression of this enzyme, and has suggested the use of the microorganism as a model for studying addiction, or upon washout of barbital, withdrawal.

The present observation of a specific effect of amobarbital on the incorporation of orotate into nucleic acids of growing bacterial cells was considered of interest for several reasons. The coincidence of a pyrimidine drug, amobarbital, affecting the metabolism of an intermediate in pyrimidine biosynthesis, orotate, suggested a potential antimetabolic role of the barbiturate which might provide basic information on its biochemical actions. It is also known that another pyrimidine derivative, 6-azauracil, as a ribomononucleotide, interferes with the synthesis of pyrimidines from orotate (27, 30), and produces central nervous system depression in various species (31). The concentration of amobarbital required to elicit the response on orotate uptake in *B. cereus* (Fig. 2) is close to that in brain during anesthesia (0.5 mM) (32). There is little information as to a possible role of orotate in brain pyrimidine biosynthesis, but it is doubtful whether this highly ionized molecule ($pK_a = 2.4$) can pass the blood brain barrier. When injected intracisternally or intraventricularly into rats or cats, however, it effectively serves as a precursor for brain pyrimidines (30, 33).

Our present study was undertaken to localize the observed drug effect at a biochemical level. From the evidence presented, it appears that the drug does not affect pyrimidine biosynthesis as such. The possible interference of amobarbital with dihydroorotic dehydrogenase, a NAD-linked flavoprotein system sensitive to high concentrations of barbituric acid (23), was specifically excluded. Furthermore, these authors had found no sensitivity to barbital in this system. The lack of growth inhibition by amobarbital, the normal incorporation of uracil into nucleic acids by a microorganism that has no pyrimidine requirements, the unaltered conversion of aspartate to RNA polynucleotides, and the lack of sensitivity to amobarbital of the various intermediary reactions indicate that the drug must act at an earlier stage in the incorporation of orotate. Indeed, the cells' uptake of orotate-2-^{14}C was found to be inhibited by amobarbital (Fig. 7) although none of the radioactivity in the acid-soluble pool fraction was present as orotate. The usual experiments to measure cell penetration of a compound were not

applicable because of the insignificant pool of orotate *per se*. The use of orotate-7-^{14}C in combination with 6-azauridine to block orotidylate decarboxylase (27) confirmed that the amobarbital effect was localized at the step of entry of orotate from the medium into the cell.

It is quite possible that the penetration of orotate into the cell is mediated by a permease system associated with the bacterial membrane (34). Such a permease system has stereospecificity, and compounds with closely related configurations as, for instance, amobarbital, may compete for the carrier system. Such a permease system would be constitutive rather than inducible since the incorporation of radioactivity from ^{14}C-orotate begins immediately and quantitatively is directly related to the cell mass.

The onset of the amobarbital effect is immediate, and the response does not increase with time. Thus, it is unlikely that any metabolite of amobarbital has been produced by the bacteria which is responsible for the observed drug effect. However, some catabolism of barbituric acid and barbital may take place in bacterial cultures (35–37).

The change in pH of the medium (Table 1) produces several effects which cannot be evaluated clearly. Because of the low pK_a of orotate, less than 1% of that compound would be present as the undissociated form even at pH 5. Amobarbital, however, with a pK_a of 7.9, would become appreciably more ionized as the pH of the medium approached 8, and the reduced utilization of orotate incorporation suggests that it is only the undissociated form of amobarbital which is active. However, growth of cells at pH 8 may also alter other biochemical functions of the microorganisms, as demonstrated by the decreased incorporation of orotate.

The partial reversal of the drug effect by increasing concentrations of orotate reveals a competitive relationship between the reactants. As has been mentioned, this interaction is more complicated, however. Although the various methods of kinetic analysis were designed for a pure enzyme system, their extension for the present purpose would be reasonable if amobarbital inhibits only at one step which then becomes rate-limiting. Unfortunately, the use of whole cells, as well as the multistep reaction sequence measured do not fulfill this rigid requirement.

It appears, therefore, that amobarbital specifically antagonizes the process which allows orotate to enter the cell. The structural resemblance between the two pyrimidines probably accounts for this antagonism, and will be the subject of the next report.

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REFERENCES

1. H. G. Mandel, *in* "Isotopes in Experimental Pharmacology" (L. J. Roth, ed.), p. 439. Univ. of Chicago, Chicago, Illinois, 1965.
2. H. G. Mandel and H. M. Oliver, *Pharmacologist* **7**, 172 (1965).
3. D. B. Roodyn and H. G. Mandel, *Biochim. Biophys. Acta* **41**, 80 (1960).
4. H. G. Mandel, *J. Biol. Chem.* **225**, 137 (1957).
5. H. G. Mandel, R. G. Latimer and M. Riis, *Biochem. Pharmacol.* **14**, 661 (1965).
6. M. Reich and H. G. Mandel, *J. Bacteriol.* **91**, 517 (1966).
7. W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques," 3rd ed. Burgess, Minneapolis, Minnesota, 1957.
8. G. R. Wyatt, *Biochem. J.* **48**, 584 (1951).
9. R. Markham and J. D. Smith, *Biochem. J.* **52**, 552 (1952).
10. A. C. Paladini and L. F. Leloir, *Biochem. J.* **51**, 426 (1952).
11. H. G. Mandel, *J. Pharmacol.* **133**, 141 (1961).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
13. D. Cuppy and L. Crevasse, *Anal. Biochem.* **5**, 462 (1963).
14. R. M. Fink, R. E. Cline and H. M. G. Koch, *Federation Proc.* **13**, 207 (1954).
15. M. Green and S. S. Cohen, *J. Biol. Chem.* **225**, 397 (1957).
16. D. B. Roodyn and H. G. Mandel, *J. Biol. Chem.* **235**, 2036 (1960).

17. A. Hunter and C. E. Downs, *J. Biol. Chem.* **157,** 427 (1945).
18. M. Dixon, *Biochem. J.* **55,** 170 (1953).
19. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56,** 658 (1934).
20. A. Giuditta and H. J. Strecker, *Biochem. Biophys. Res. Commun.* **2,** 159 (1960).
21. A. Giuditta and H. J. Strecker, *Nature* **193,** 979 (1962).
22. L. Casola, A. Giuditta and E. Rocca, *Arch. Biochem. Biophys.* **107,** 57 (1964).
23. H. C. Friedmann and B. Vennesland, *J. Biol. Chem.* **233,** 1398 (1958).
24. S. Ukada and B. Vennesland, *J. Biol. Chem.* **237,** 2018 (1962).
25. E. S. Reynolds, I. Lieberman and A. Kornberg, *J. Bacteriol.* **69,** 250 (1955).
26. R. A. Yates and A. B. Pardee, *J. Biol. Chem.* **221,** 743 (1956).
27. R. E. Handschumacher and C. A. Pasternak, *Biochim. Biophys. Acta* **30,** 451 (1958).
28. E. J. Simon and D. Van Praag, *Proc. Natl. Acad. Sci. U.S.* **51,** 1151 (1964).
29. D. B. Goldstein, *Mol. Pharmacol.* **1,** 31 (1965).
30. W. Wells, D. Gaines and H. Koenig, *J. Neurochem.* **10,** 709 (1963).
31. B. I. Shnider, E. Frei, III, J. H. Tuohy, J. Gorman, E. J. Freireich, C. O. Brindley, Jr. and J. Clements, *Cancer Res.* **20,** 28 (1960).
32. P. J. Heald, *in* "Phosphorus Metabolism of Brain," p. 138. Pergamon Press, London, 1960.
33. D. H. Adams, *J. Neurochem.* **12,** 783 (1965).
34. G. N. Cohen and J. Monod, *Bacteriol. Rev.* **21,** 169 (1957).
35. O. Hayaishi and A. Kornberg, *J. Biol. Chem.* **197,** 717 (1952).
36. F. J. S. Lara, *J. Bacteriol.* **64,** 279 (1952).
37. D. Kluepfel, G. C. Lancini and G. Sartori, *Appl. Microbiol.* **13,** 600 (1965).