Effects of Barbital on Amino Acid Metabolism in *Escherichia coli*

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SUMMARY

Barbital (0.02 M) inhibits growth of *E. coli* B in a glycerol medium. The inhibition is partially overcome by addition of amino acids.

L-Alanine oxidation by intact cells is inhibited by barbital, but washed cells previously grown in barbital have an enhanced ability to oxidize alanine. The same effects are seen with cell-free extracts. Barbital does not inhibit alanine uptake or pyruvate oxidation.

Conversion of L-alanine to pyruvate is apparently accomplished by alanine racemase and a D-alanine oxidase. Barbital inhibits the latter competitively, but has no direct effect on the racemase. Cells grown in barbital have increased racemase and D-alanine oxidase activity. Both enzymes are also induced by L-alanine.

This *E. coli* system is considered as a model for studying effects of prolonged drug administration, such as the development of tolerance, addiction, and the withdrawal syndrome. It is proposed that enzyme inhibition by a drug may be followed by derepression of synthesis of the same enzyme. The resulting increase in amount of the enzyme, despite its partial inhibition, could restore normal enzyme activity. Withdrawal symptoms could appear when the derepressed enzyme is suddenly released from drug inhibition.

INTRODUCTION

Control of enzyme reactions by end-product inhibition (1) and by repression (2) is now well known, particularly in bacteria. As it becomes increasingly clear that similar control mechanisms operate in mammalian cells (3–8), new possibilities for mechanisms of drug action are evident. Some drugs may act by "false feedback inhibition" (9), i.e., they may mimic the action of those natural metabolites which inhibit enzymes in their own synthetic pathways. Such a drug would cause an immediate fall in metabolite level. Disruption of the slower-acting process of enzyme repression may explain some long-term drug effects, such as the development of tolerance. In this case, changes in end-product concentration occur only after existing enzymes decay, or (in derepression) after synthesis of new enzyme molecules.

Moyed (10) has demonstrated how enzyme derepression can be the result of false feedback inhibition. In *E. coli*, a histidine analog inhibits an enzyme in the histidine biosynthetic pathway. The consequent scarcity of histidine derepresses the whole pathway so that the initial drug effect is overcome. Similar processes in the brain would lead to tolerance and addiction, as proposed in a preliminary account of this work (11) and by Shuster (12). If a drug inhibits a certain enzyme whose product is essential for normal brain function, a primary drug effect will occur rapidly, as soon as effective drug levels are established. Then the low level of product may allow derepression of enzyme synthesis. Larger drug doses will then be necessary to produce the desired effect or even to maintain a normal state. Addiction thus represents a balance of inhibition versus increased enzyme

synthesis, and, with opiates at least, this balance may be successful in that brain function is normal as long as the drug is present. On withdrawal, the excess enzyme is rapidly released from inhibition, causing symptoms generally opposite to the primary drug effect.

In the initial approach to the problem, here presented, bacteria have been used as a model system and barbital was chosen as the prototype drug. A search was made for an enzyme system inhibited by the drug (primary drug effect). Then cells were grown in the presence of the drug (prolonged administration) and were washed (withdrawal). If the enzyme synthesis were derepressed during growth, enzyme activity would be higher than normal in the washed, barbital-grown cells. Such an effect was seen in the enzyme system converting L-alanine to pyruvate.

METHODS

Cells. Escherichia coli, strain B.

Media and growth. Cells were grown at 37° with shaking, in a mineral medium containing Na_2SO_4 0.001 M, $MgSO_4$ 0.0004 M, KCl 0.01 M, NH_4Cl 0.01 M, in 0.06 M sodium phosphate buffer pH 7.0. Included in the medium in various experiments were glycerol 0.05 M, casein hydrolyzate (Difco Casamino Acids) 0.2%, barbital 0.02 M, and L- or D-alanine 0.05 M. Barbital Sodium, Merck (sodium diethylbarbiturate) was recrystallized before use.[1] Bacterial density (milligrams dry weight of cells per milliliter) was determined turbidimetrically with a Klett-Summerson colorimeter.

Standard conditions for growth were as follows: Inoculating cultures, grown overnight in mineral medium with glycerol[2] or with glycerol plus casein hydrolyzate, were diluted at least 20-fold into mineral medium with glycerol and casein hydrolyzate. The cultures were allowed to grow to 10 times the initial mass and were harvested during logarithmic growth. This required about 2½ hr for normal cultures, and 5–7 hr if barbital was present.

Oxidation rates were measured manometrically; washed intact cells in 0.1 M phosphate buffer, pH 7.0, were used. Barbital (final concentration 0.02 M) was added to the cell suspension. After equilibration, 4 μmoles of substrate was tilted in from the sidearm. The initial rate of O_2 uptake at 37° was determined in duplicate vessels and was corrected for endogenous O_2 uptake. The results are expressed as micromoles of O_2 per hour per milligram dry weight of cells.

Alanine uptake. Three cell suspensions of bacterial density 0.5 were prepared: (a) normal washed cells, (b) the same, plus 0.02 M barbital, (c) washed cells grown in the presence of 0.02 M barbital. At 37°, L-alanine-1-^{14}C was added to a concentration of 2.7 μmoles/ml and 0.02 μc/ml. At intervals up to 5 min, samples were quickly pipetted into frozen buffer and centrifuged. The cells were dissolved in 0.5 ml 0.1 N NaOH; 0.5 ml of Hydroxide of Hyamine (Packard Instrument Co.) and 15 ml of a scintillation mixture were added. The total ^{14}C content of the cells was determined in the Tri-Carb liquid scintillation counter.

Sonic extracts. Cells were harvested in the centrifuge or on Millipore filters and suspended in 0.1 M phosphate buffer pH 7.0 or 7.5. They were disrupted in the Mullard sonic oscillator for 3 min, in an ice bath. Debris and unbroken cells were removed by centrifuging at 12,000 *g* for 10 min. The extracts, usually containing about 12 mg protein per milliliter, were dialyzed overnight against 100 volumes of cold distilled water. Enzyme activities were measured with freshly prepared extracts. Added pyruvate was stable in the presence of these

[1] Lactic dehydrogenase, catalase, and all coenzymes were purchased from Calbiochem. Hog kidney D-amino acid oxidase was a product of Worthington Biochemical Corporation. D-Cycloserine (Seromycin) was a gift of the Lilly Research Laboratories, Eli Lilly and Company.

[2] Glycerol was used instead of glucose in the growth medium because glycerol has a weaker "catabolite repression" effect (13) and is thus less likely to mask any effects of barbital on enzyme synthesis.

extracts, indicating that no appreciable amount of intact cells remained.

Soluble and particulate fractions were prepared by centrifuging the extract for 1 hr at 150,000 *g* in the Spinco model L ultracentrifuge.

Enzyme assays. L-Alanine oxidation rates in extracts were measured in a reaction mixture containing 50 μmoles of pyrophosphate buffer pH 8.3, 0.01 μmole FAD, 0.1 μmole pyridoxal phosphate, 50 μmoles L-alanine, and cell extract containing about 1 mg protein, in a total volume of 1.0 ml. One sample was boiled immediately after mixing. Three others were incubated for 10, 20, and 40 min at 37°, and the reaction was stopped by immersing the tubes in boiling water for 5 min. The precipitate was centrifuged off and the pyruvate concentration in the supernatant was determined as described below. The rate of pyruvate formation increased during the incubation; estimates of L-alanine oxidation were based on the maximum observed rate.

For assay of D-alanine oxidase, the reaction mixture contained 50 μmoles of Tris-acetate buffer pH 7.5, 0.01 μmole FAD, 50 μmoles D-alanine, and cell extract containing about 0.5 mg protein. The incubation and the pyruvate assay were the same as for L-alanine oxidation. The rate of D-alanine oxidation was constant during the incubation and was proportional to extract concentration.

A unit of D- or L-alanine oxidase is defined as the amount of enzyme forming 1 μmole of pyruvate per hour.

Alanine-glutamic transaminase was measured in a system similar to that used for L-alanine oxidation. The rate of pyruvate formation in the standard mixture (minus pyridoxal phosphate) was compared to the rate with added pyridoxal phosphate and α-ketoglutarate (50 μmoles). The transaminase was taken to be the difference in the rate of pyruvate formation in the two mixtures.

Pyruvate was measured by DPNH utilization in the presence of lactic dehydrogenase. DPNH was added to supernatants to a concentration of 2×10^{-4} M, and the optical density at 340 mμ was determined in the Beckman DK-2 spectrophotometer. The solution was decanted into a tube containing 5 μg (0.1 ml) of crystalline lactic dehydrogenase. After more than 20 min at room temperature, the optical density was read again. Pyruvate concentration was computed from the difference in optical density. The pyruvate assay was unaffected by barbital or cycloserine.

Alanine racemase was assayed by anaerobic incubation of extracts with L-alanine; the resulting D-alanine was measured with D-amino acid oxidase. The incubation was carried out at 37° in Warburg vessels containing 100 μmoles of pyrophosphate buffer pH 8.3, 0.2 μmole of pyridoxal phosphate and cell extract, in a total volume of 2.0 ml. L-Alanine (100 μmoles) was added from the sidearm after gassing with nitrogen. At intervals, the reaction was stopped by placing a vessel in boiling water for 5 min. D-Alanine was usually assayed manometrically by adding 20 mg of crude hog kidney D-amino acid oxidase in pyrophosphate buffer pH 8.3 to the sidearm. KOH was added to the center well. After equilibration at 37°, the enzyme was tilted in and O_2 uptake was followed to completion. One-half mole of O_2 is used per mole of D-alanine. The rate of D-alanine formation in the anaerobic incubation was linear with time up to at least 10 μmoles of D-alanine and was proportional to the amount of extract. Barbital and cycloserine did not affect the manometric assay of D-alanine.

In some experiments, D-alanine was assayed by a more sensitive method under conditions where D-alanine concentration determines the *rate* of the D-amino acid oxidase reaction, and the latter is coupled to lactic dehydrogenase. The reaction mixture contained an aliquot of the Warburg vessel contents, 2×10^{-4} M DPNH, 0.15 mg crystalline lactic dehydrogenase, and 0.6 mg of crystalline catalase. The initial rate of decrease in optical density at 340 mμ was followed in the Beckman DK-2 recording spectrophotometer on addition of 100 μl of D-amino acid oxidase. Partially purified D-amino acid oxidase was used, prepared by calcium phosphate gel negative adsorption and a single precipitation with ammonium

sulfate, according to DeLuca *et al.* (14). The enzyme preparation contained 10^{-4} M added FAD. D-Alanine standards in the range 0.5–2.0 μmoles were included in each run and were used for calibration. The rate of DPNH oxidation was proportional to D-alanine concentration in this range.

A unit of racemase activity is defined as the amount of enzyme forming 1 μmole of D-alanine per hour.

Protein was determined by the method of Lowry *et al.* (15).

RESULTS

Inhibition of growth by barbital. In mineral medium containing either glycerol or casein hydrolyzate as carbon source, 0.02 M barbital caused about 80% inhibition of growth rate (Table 1). However, inhibition was partially overcome by inclusion of *both* glycerol and casein hydrolyzate in the medium. This preliminary observation suggested an effect of the drug on amino acid metabolism, especially the interconversion of amino acids and carbohydrates.

Addition of vitamins, purines, and pyrimidines did not affect the growth rate inhibition in either glycerol or glycerol–amino acid medium.[3] Only small changes in growth rate inhibition occurred when individual amino acids were tested, either by adding them singly to the glycerol medium or by omitting them one at a time from the complete glycerol–amino acid mixture. This indicated that the metabolism of more than one amino acid was affected.

Barbituric acid (which lacks the typical pharmacologic actions of the substituted barbiturates) at 0.02 M did not inhibit growth on glycerol.

Oxidation of amino acids by intact cells. Manometric studies with L-amino acids showed that glutamate, glutamine, aspartate, asparagine, alanine, serine, and threonine were oxidized at appreciable rates. Most strikingly affected by barbital were alanine, glutamate, and aspartate oxidation.

TABLE 1
Barbital effect on growth rate

Cells from an overnight culture were inoculated into mineral medium, with additions as shown. The logarithmic growth rate at 37° was determined in the presence or absence of 0.02 M barbital. Vitamin-free casein hydrolyzate was used here; addition of tryptophan had no effect.

Additions to mineral medium	Growth rate, doublings per hour		Per cent inhibition
	Control	Barbital	
Glycerol 0.04 M	0.90 ± 0.03[a] (7)	0.21 ± 0.03 (7)	77
Casein hydrolyzate 0.16%	1.1 ± 0.06 (3)	0.17 ± 0.01 (3)	85
Glycerol 0.04 M plus casein hydrolyzate 0.16%	1.6 ± 0.03 (6)	0.80 ± 0.05 (6)	50

[a] Standard error of the mean, based on the number of experiments shown in parentheses.

Table 2 shows, in columns (a) and (b), the direct effect of 0.02 M barbital, namely inhibition of alanine oxidation by 72% and stimulation of glutamate oxidation to 170% of the control. Aspartate oxidation was unaffected. For study of the indirect effect, cells grown in glycerol–casein hydrolyzate medium containing barbital were washed and tested without the drug (column c). In these cells, alanine was oxidized at twice the normal rate, but glutamate oxidation was severely depressed and aspartate oxidation almost eliminated.

[3] The vitamin-purine-pyrimidine mixture had the following final concentrations per milliliter of medium: 0.8 μg each of thiamine, pyridoxamine, nicotinamide, pantothenic acid, biotin, folic acid, and choline; 0.08 μg of riboflavin; 0.16 μg of inositol; and 1 μmole each of adenine sulfate, guanine HCl, cytosine, uracil, and thymine.

TABLE 2

Oxidation of amino acids by normal, barbital-treated, and barbital-grown cells

Oxygen uptake was measured manometrically, using (a) washed cells grown in glycerol–casein hydrolyzate medium, (b) the same normal cells with barbital 0.02 M added to the Warburg vessels, and (c) cells grown in glycerol–casein hydrolyzate–barbital medium, then washed and tested without the drug. Rates are expressed as micromoles of oxygen taken up per hour per milligram dry weight of cells.

	Oxygen uptake				
		(b) Barbital-treated		(c) Barbital-grown	
Substrate	(a) Control rate	Rate	% of control	Rate	% of control
L-Alanine	1.7 ± 0.2[a] (4)	0.48 ± 0.19 (3)	28	3.5 ± 0.4 (3)	206
L-Glutamic acid	1.9 ± 0.3 (2)	3.2 ± 0.2 (2)	170	0.22 ± 0.15 (2)	12
L-Aspartic acid	1.0 ± 0.3 (2)	1.1 ± 0.1 (2)	110	0.02 ± 0.02 (2)	2

[a] Standard error of the mean, based on the number of experiments shown in parentheses (each with duplicate vessels).

The barbital effects on glutamate and aspartate oxidation have not yet been investigated. Attention was focused on the changes in alanine metabolism, with which the remainder of this paper is concerned.

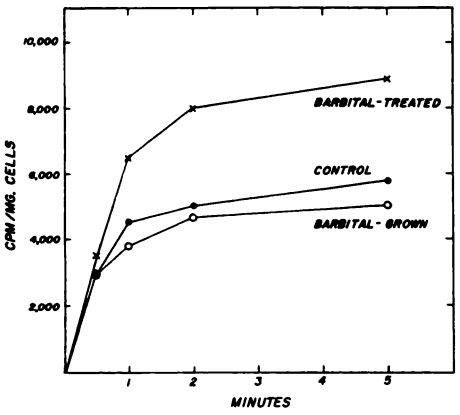

FIG. 1. L-*Alanine uptake*

L-Alanine-1-^{14}C was added to normal cells in the presence or absence of barbital (0.02 M) and to barbital-grown cells, as described under Methods. The total internal ^{14}C was determined by liquid scintillation counting.

L-*Alanine uptake.* Figure 1 shows that barbital did not inhibit L-alanine uptake. On the contrary, the internal alanine concentration was higher in barbital-treated cells than in either normal or barbital-grown cells, when measured after brief exposure of the cells to ^{14}C-alanine. The barbital-affected step was clearly in the utilization, not the uptake, of alanine.

Pyruvate oxidation. Figure 2 compares the barbital effects (primary and long term) on alanine and pyruvate oxidation by intact cells. Alanine oxidation was inhibited by barbital but was increased in barbital-grown cells, as described above. Pyruvate oxidation was unaffected by the drug, which must therefore act on the conversion of alanine to pyruvate. This step must be rate limiting in alanine oxidation (with or without barbital) since the cells can oxidize pyruvate much faster than alanine.

CONVERSION OF L-ALANINE TO PYRUVATE BY CELL-FREE PREPARATIONS

Barbital inhibited L-alanine oxidation by extracts of normal cells, but extracts of cells grown in the presence of barbital oxidized alanine abnormally fast. Since no permeability barrier is involved and the extracts do not oxidize pyruvate, this confirms the localization of the barbital effects at the alanine-to-pyruvate step. Possible mechanisms for this reaction include transamination with α-ketoglutarate, direct oxidation of L-alanine to pyruvate, and race-

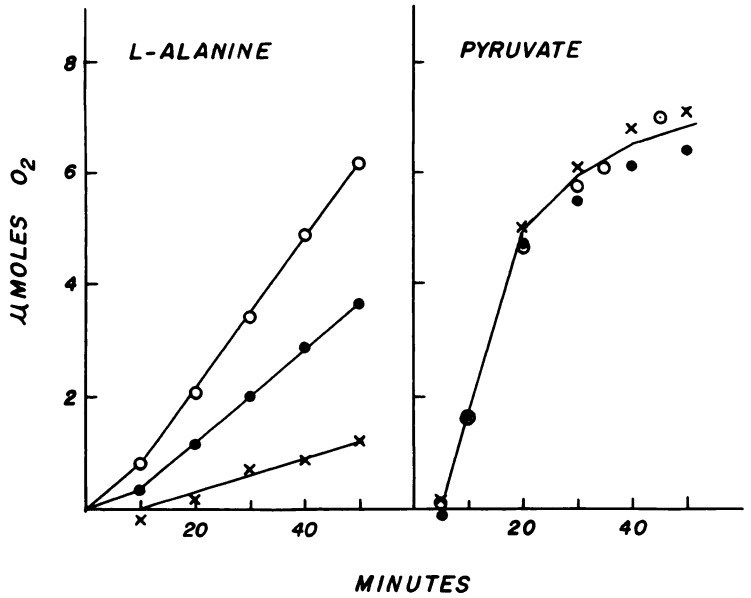

FIG. 2. *Oxidation of* L-*alanine and pyruvate by normal, barbital-treated, and barbital-grown cells*

Cell suspensions were prepared as described in Table 2. Each vessel contained 2 mg dry weight of intact cells. At time zero, 4 μmoles of L-alanine or pyruvate was added. ●——●, normal cells; ×——×, barbital-treated; ○——○, barbital-grown.

mization to D-alanine followed by D-alanine oxidation. These systems have been examined in normal cell extracts, in extracts treated with barbital, and in extracts of cells grown in barbital.

A. Normal Cell Extracts

Transamination of L-alanine with α-ketoglutarate was measurable as an increase in the rate of pyruvate formation from L-alanine on addition of α-ketoglutarate and pyridoxal phosphate. The transaminase activity was about one-third of the L-alanine oxidation rate. Pyridoxal phosphate alone had a small effect. The transaminase activity was not inhibited by barbital, nor was it abnormal in barbital-grown cells. It was not considered further.

Alanine dehydrogenase, a pyridine nucleotide-dependent enzyme has been reported in various microorganisms (16–18). *E. coli* extracts and fractions precipitated with ammonium sulfate did not reduce DPN or TPN when incubated with alanine. However, no fractions thus tested were free of DPNH oxidase activity. The rate of pyruvate formation from alanine by dialyzed extracts, described below, is unaffected by added DPN.

Alanine racemase was measured under anaerobic conditions to avoid oxidative destruction of L- or D-alanine. A very active racemase was present, as shown by Wood and Gunsalus (19). The level in normal extracts was 60 units per milligram protein N. When the extract was centrifuged for 1 hour at 150,000 *g*, 98–99% of the activity remained in the supernatant fraction. The enzyme had a pH optimum of about 8.4 (Fig. 3A). Pyridoxal phosphate gave only a slight stimulation in various preparations, but it was routinely included in racemase assays.

D-*Alanine oxidase* activity was present in normal extracts at a level of about 5 units per milligram protein N. The enzyme had a pH optimum of about 7.5 and was slightly more active in Tris than in phosphate buffers. FAD stimulated some preparations slightly (10–20%), but no sample was entirely dependent on FAD. Part of the activity was precipitated by centrifuging at

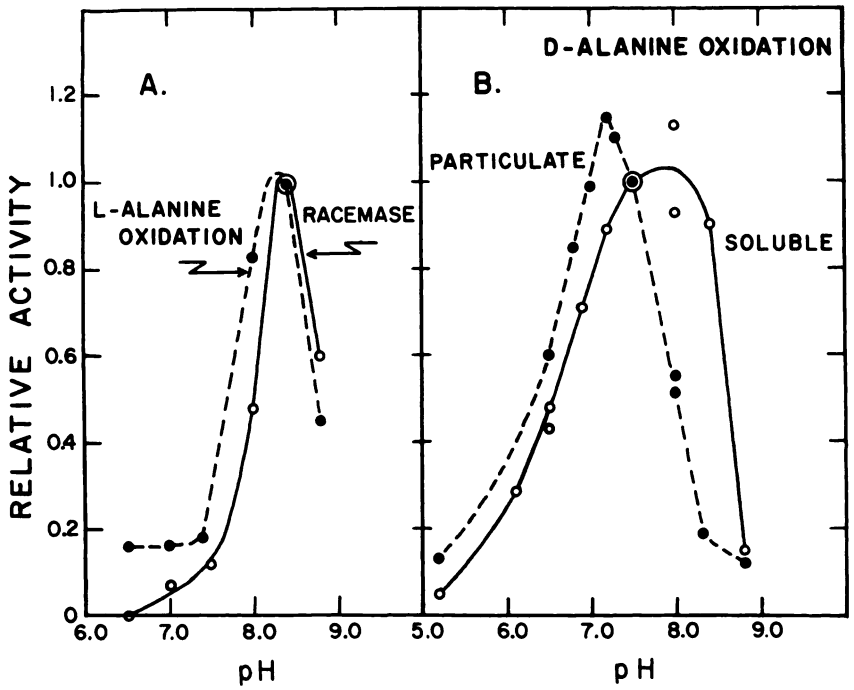

FIG. 3. *pH dependence of alanine enzymes*

A. Alanine racemase and L-alanine oxidation activity were determined in Tris-acetate buffers. For racemase, the supernatant from a high-speed centrifugation of a normal extract was used. D-Alanine formation was measured in a system where the rate of added D-amino acid oxidase action is determined by the D-alanine concentration (see Methods). Separate D-alanine standard curves at each pH were used for calibration. L-Alanine oxidation was measured in whole extract by assay of pyruvate production. Both activities are shown as relative to the rate at pH 8.4. ○——○, racemase; ●- - -●, L-alanine oxidation.

B. D-Alanine oxidation was measured in Tris-acetate buffers, using the soluble and particulate fractions from a high-speed centrifugation of dialyzed extract. The figure shows the combined results from two soluble and two particulate preparations, the units being expressed as activity relative to that at pH 7.5. ○——○, soluble enzyme; ●- - -●, particulate enzyme.

150,000 *g* for 1 hr. Although the ratio of soluble to particulate enzyme varied in different experiments, all the activity of the whole extract was recovered in the two fractions.

The soluble D-alanine oxidase was partially purified by ammonium sulfate precipitation, as shown in Table 3. The fraction precipitated by ammonium sulfate 35–45% saturated was free of measurable racemase activity and had a negligible capacity to oxidize L-alanine. This fraction was used for further study of the properties of the D-alanine oxidase.

The stoichiometry of the D-alanine oxidase was studied in order to determine whether peroxide is formed as a product of the reaction, as is the case with mammalian D-amino acid oxidase. The two mechanisms to be considered are summarized in reactions (1) and (2).

$$(1)\ CH_3CHNH_2COOH + \tfrac{1}{2}O_2 \xrightarrow{\text{oxidase}} CH_3COCOOH + NH_3$$

$$(2)\ a.\ CH_3CHNH_2COOH + O_2 + H_2O \xrightarrow{\text{oxidase}} CH_3COCOOH + NH_3 + H_2O_2$$

$$b.\ H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2$$

$$\text{Sum: } CH_3CHNH_2COOH + \tfrac{1}{2}O_2 \longrightarrow CH_3COCOOH + NH_3$$

Note that 0.5 mole O_2 is utilized per mole of pyruvate produced in both cases if catalase is present, as it is in *E. coli* and in crude hog kidney extracts (20). The two

TABLE 3
Separation of racemase and D-alanine oxidase activities

A 15L culture, growing logarithmically in glycerol–casein hydrolyzate medium was harvested in the Szent-György–Blum continuous centrifuge. The cells were suspended in buffer, disrupted in the Mullard sonic oscillator, and centrifuged at 12,000 *g* for 10 min. Magnesium acetate, 10^{-2} M final concentration, was added to the supernatant, and it was centrifuged for 1 hr at 150,000 *g* in the Spinco model L ultracentrifuge. The pellet, suspended in buffer, was assayed without further purification. The supernatant was fractionated with ammonium sulfate. The precipitate formed at 0.35 saturation with ammonium sulfate was inactive. Precipitates on further addition of ammonium sulfate to 0.45 and then to 0.8 saturation were suspended in buffer, dialyzed overnight against distilled water, and stored frozen in Tris-acetate buffer pH 7.5.

		D-Alanine oxidase			Alanine racemase		
Fraction	Total protein (mg N)	Total units	Units per mg N	Recovery	Total units	Units per mg N	Recovery
Whole extract	130	268	2.06	(100)	2550[a]	—	—
Particulate fraction	12.5	102	8.17	38	Nil[a]	—	—
Soluble fraction	110	150	1.36	56	2550	23.2	(100)
$(NH_4)_2SO_4$ 0.35–0.45	7.0	83	11.8	31	Nil	Nil	Nil
$(NH_4)_2SO_4$ 0.45–0.80	46	11	0.24	4	790	17.2	31

[a] These fractions were not assayed in this experiment. Previous experiments showed that all the racemase of whole extracts is soluble.

mechanisms could be distinguished by D-alanine:O_2 ratios in purified preparations free of catalase. (Pyruvate production could not then be used in the assay because pyruvate would be destroyed by peroxide in the absence of the protective catalase.) A simpler approach is offered by the observation of Keilin and Hartree (20) that addition of a suitable acceptor (e.g., ethanol) to a coupled D-amino acid oxidase–catalase system will double the O_2 uptake. Catalase must be present in high concentration. It will then utilize the peroxide from the primary oxidation to carry out a secondary oxidation of ethanol. Then the reaction is as follows:

$$\text{(3) a. } CH_3CHNH_2COOH + O_2 \xrightarrow{+H_2O \text{ oxidase}} CH_3COCOOH + NH_3 + H_2O_2$$

$$\text{b. } CH_3CH_2OH + H_2O_2 \xrightarrow{\text{catalase}} CH_3CHO + 2H_2O$$

$$\text{Sum: } CH_3CHNH_2COOH + O_2 + CH_3CH_2OH \longrightarrow CH_3COCOOH + NH_3 + CH_3CHO + H_2O$$

Addition of ethanol and excess catalase to an oxidase proceeding by reaction (1) would have no effect since no peroxide is present, but the same additions to a peroxide-producing oxidation would double the O_2 uptake. Table 4 shows that the bacterial D-alanine oxidase, whether soluble or particulate, uses 0.5 mole of O_2 in the presence or absence of ethanol and excess catalase; i.e., it does not produce peroxide. A similar experiment with hog kidney D-amino acid oxidase is included for comparison. Here the O_2 uptake per mole of pyruvate is doubled by addition of ethanol, as expected. Norton *et al.* (21) recently reported a similar D-alanine oxidase in cell membranes of *Pseudomonas aeruginosa;* it does not produce peroxide.

The pH curves for the particulate and the purified soluble enzyme were different (Fig. 3B). The soluble enzyme had a pH optimum of about 7.8 as against 7.2 for the particulate form.

As shown in Fig. 4, the Michaelis constant for D-alanine was 0.02 M for the soluble enzyme (after ammonium sulfate precipitation) and 0.04 M for the particulate enzyme. Both values are an order of magnitude higher than those reported for the mammalian enzyme (22).

L-Alanine oxidation by cell extracts may now be examined to see whether it could be the combined effect of the observed race-

TABLE 4
Stoichiometry of D-alanine oxidases

For the bacterial enzymes, each Warburg vessel contained 100 μmoles of buffer pH 7.5, 20 μmoles FAD, 0.2 mg crystalline catalase, 50 or 100 μmoles D-alanine, and the enzyme preparation. The particulate enzyme was a washed precipitate from a 150,000 *g* centrifugation of crude extract, 2.2 mg protein per vessel. The soluble enzyme was the partially purified ammonium sulfate fraction (0.35–0.45 saturated) described in Table 3; 1.0 mg protein per vessel. Ethanol (100 μmoles) was added as indicated. The center well contained 0.2 ml 20% KOH. The reaction was started by tilting in enzyme or D-alanine from the sidearm and was stopped by immersing the vessels in boiling water. Pyruvate was determined on aliquots by the usual method. In the case of hog kidney D-amino acid oxidase, the procedure was the same except that the pH was 8.3, and no FAD was added; 10 mg crude enzyme was used. Each figure is the mean of duplicate vessels. In the absence of D-alanine there was no oxygen uptake or pyruvate production.

Enzyme	Additions	μMoles per vessel		O_2: pyruvate
		Pyruvate	Oxygen	
Particulate	D-Alanine	6.3	3.3	0.52
	D-Alanine + ethanol	6.2	3.2	0.52
Soluble	D-Alanine	5.3	2.7	0.51
	D-Alanine + ethanol	5.3	2.9	0.55
Hog kidney	D-Alanine	16.0	7.0	0.44
	D-Alanine + ethanol	12.9	11.4	0.88

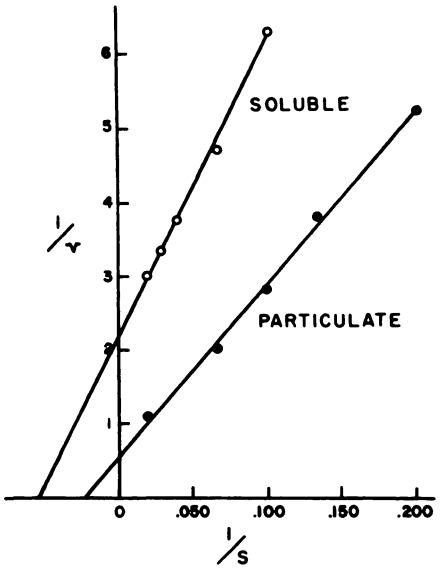

FIG. 4. *Effect of substrate concentration on D-alanine oxidases*

D-Alanine oxidase activity was determined at various substrate concentrations. Upper curve, a partially purified sample of soluble enzyme, precipitated with ammonium sulfate 0.35–0.45 saturated, as described in Table 3. Lower curve, the particulate enzyme, i.e., the resuspended pellet from a 150,000 *g* centrifugation of crude extract. v = μmoles of pyruvate produced per hour; S = D-alanine (mM). Lines are drawn by the method of least squares.

mase and D-alanine oxidase activities. The maximum observed rate of L-alanine oxidation is only half that of D-alanine oxidation; it would be necessary to postulate a separate L-alanine oxidase only if L-alanine were utilized faster than the D-isomer. The pH curve of L-alanine oxidation is similar to that of alanine racemase (Fig. 3A). Experiments were designed to separate racemase from D-alanine oxidase activity by (a) enzyme purification and (b) selective inhibition, to show whether both enzymes are needed for L-alanine oxidation. (Attempts to isolate a racemaseless mutant for this purpose have failed.)

(a) After high-speed centrifugation of whole extract, the soluble fraction had about two-thirds of the original L-alanine oxidizing activity and the pellet was inactive. Recombination of pellet and supernatant restored the activity almost completely, as shown in the upper half of Table 5. Boiled particulate fraction did not stimulate the soluble activity.

Further fractionation of the soluble moiety with ammonium sulfate gave a fraction (I) containing D-alanine oxidase with only a small amount of racemase, and a fraction (II) lacking any measurable D-alanine oxidase, but rich in racemase. Preparation of these fractions is described in the

TABLE 5

L-Alanine oxidation by fractionated extracts

A sonic extract of a 15L culture was prepared as described in Table 3. After removal of debris, it was dialyzed overnight and then centrifuged for 1 hr at 150,000 *g*. The supernatant was fractionated with ammonium sulfate at 0–0.40 and 0.40–0.62 saturation. The precipitates, fractions I and II, respectively, were dissolved in distilled water and assayed for oxidases and alanine racemase as described under Methods. In the upper half of the table, the L-alanine oxidation activity of whole extract is compared to that of the soluble and particulate fractions. The lower half compares a reconstituted extract with activities of the individual fractions.

Enzyme preparation	Enzyme units per 0.1 ml whole extract: L-Alanine oxidation	D-Alanine oxidase[a]	Alanine racemase
Whole dialyzed extract	0.58	0.52	12
Complete = soluble + particulate	0.55	0.55	12[b]
Omit particulate	0.37	0.53	12[b]
Omit soluble	0.01	0.07	Nil[b]
Soluble + boiled particulate	0.27	—	—
	Enzyme units per sample, equivalent to 0.26 ml whole extract		
Complete = I + II + particulate	0.61 (0.11)[c]	(1.6)[c]	(7.4)[c]
Omit particulate	0.41 (0.09)	(1.3)	(7.4)
Omit I	0.26 (0.04)	(0.3)	(6.0)
Omit II	0.20 (0.10)	(1.6)	(1.4)
Particulate alone	0.024	0.3	Nil[b]
I alone	0.072	1.3	1.4
II alone	0.014	Nil	6.0

[a] D-Alanine oxidase was measured as usual at pH 7.5. For comparison with L-alanine oxidation rates, the D-alanine oxidation data have been corrected to show how much activity would exist at pH 8.3. The corrections are based on the pH-dependence curves shown in Fig. 3B, for the soluble and particulate enzymes.

[b] Previous experiments showed all racemase is soluble. Racemase activity in the particulate fraction was not measured in this experiment.

[c] Data in parentheses are sums of activities of individual fractions. See text. Combined fractions were assayed for L-alanine oxidase only.

caption to Table 5, and their activity is shown at the bottom of the table. The complete system included the soluble fractions I and II plus the particulate fraction. Each component had very low L-alanine oxidizing activity when tested alone. Combinations of fractions always gave more activity than that calculated from the sum of individual components. These calculated sums are shown in parentheses in Table 5. The activity of the complete system was six times the sum of its parts; i.e., it was 0.6 unit as against 0.1 unit in the sum of fractions I, II, and the particulate fraction.

D-Alanine oxidase and racemase activities are included in Table 5 for comparison with L-alanine oxidation. L-Alanine was oxidized only by those fractions or combinations which contained an adequate supply of both D-alanine oxidase and racemase.

(b) D-Cycloserine has been shown to inhibit alanine racemase (23). It did not inhibit either soluble or particulate D-alanine oxidase. The effect of D-cycloserine on racemase and on L-alanine oxidation is shown in Table 6. Crude preparations (intact cells or fresh, undialyzed extracts) were used here, since the object was to show whether the *whole* activity of the cells was inhibited when racemase was inhibited. Table 6 shows that the inhibition of racemase and of L-alanine oxidation was similar at various doses of D-cycloserine.

Alanine racemase and D-alanine oxidase are induced by L-alanine. This effect will be discussed below, in connection with a similar effect of barbital.

B. Barbital-Treated Extracts

Barbital inhibited oxidation of both L- and D-alanine. Fifty per cent inhibition of L-alanine oxidation was produced by 6×10^{-4} M barbital, whereas the same inhibition of D-alanine oxidation required five times more barbital, as shown in Fig. 5. This does not mean that different enzymes oxidize the two isomers. Clearly the substrate concentration for D-alanine oxidase is higher in the D-alanine oxidase assay system than in the L-alanine system, where D-alanine is formed by racemase. Thus the greater sensitivity of L-alanine oxidation suggested a competition

TABLE 6

Inhibition of L-alanine oxidation and of racemase by cycloserine in intact cells and fresh extracts

Washed intact cells, 8.2 mg dry weight, suspended in 0.1 M phosphate buffer pH 8.0, were incubated in Warburg vessels with 100 μmoles of L-alanine. One set of vessels, containing cycloserine 0 to 10^{-3} M, was incubated anaerobically for racemase estimation. After 30 min at 37°, the reaction was stopped by boiling, and the D-alanine formed was assayed manometrically. The second set, with cycloserine at the same concentrations, was incubated aerobically and the initial rate of oxygen uptake was measured.

A fresh, undialyzed extract, minus debris and unbroken cells, was diluted in pyrophosphate buffer pH 8.3, to 2.4 mg protein per sample. Racemase was measured as above, except that the linear rate of D-alanine formation was followed in 4 vessels with each cycloserine concentration; the total incubation time varied from 20 to 60 min, depending on the expected activity. L-Alanine oxidation in the extract was measured by the standard method, i.e., by rate of formation of pyruvate.

Preparation	Activity	Control activity[a]	% Inhibition by cycloserine, final conc., M		
			10^{-5}	10^{-4}	10^{-3}
Intact cells	Racemase	11.6	35	66	83
	L-Alanine oxidation	9.9	50	81	86
Extracts	Racemase	27.2	21	29	90
	L-Alanine oxidation	2.7	15	33	84

[a] Racemase activities are expressed as micromoles D-alanine formed per hour per sample. L-Alanine activity units are, for intact cells, micromoles oxygen taken up per hour per sample; and for extracts, micromoles pyruvate formed per hour per sample.

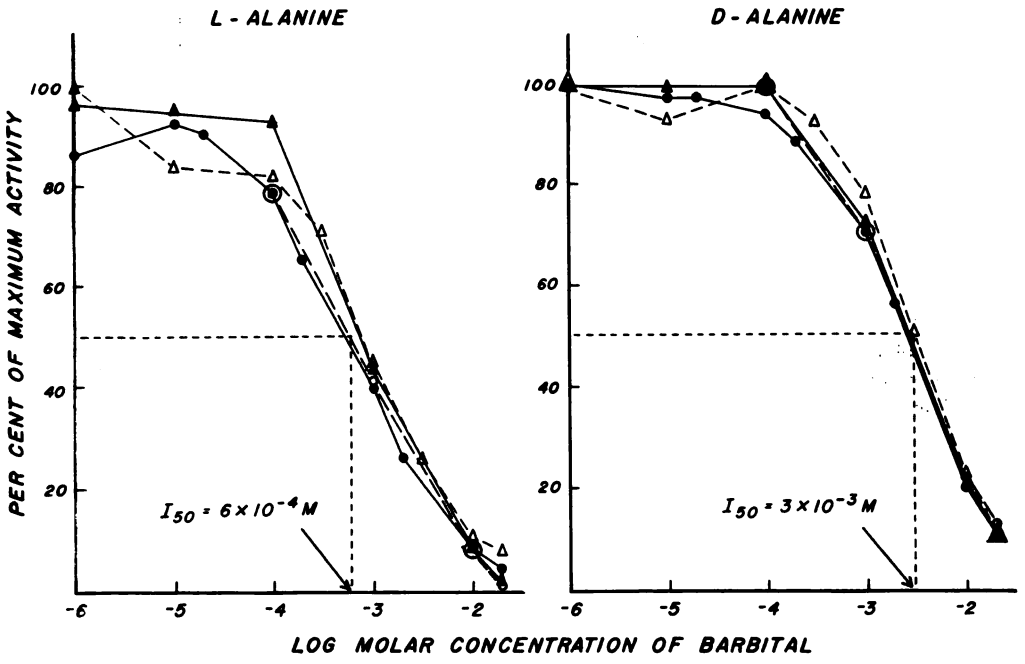

FIG. 5. *Barbital effect on conversion of alanine to pyruvate*

Extracts were incubated for 30 min with L- or D-alanine (0.025 M) with different concentrations of barbital. Pyruvate formation was measured as described under Methods. Closed symbols (●——●, ▲——▲) represent extracts of normal cells; open symbols (○---○, △---△) represent extracts of cells grown in the presence of 0.02 M barbital. The lines are drawn to connect points obtained in one experiment.

of barbital with D-alanine. A Lineweaver-Burk plot of barbital inhibition of D-alanine oxidase at varying substrate concentrations (Fig. 6) confirmed the prediction. D-Amino acid oxidase from hog kidney, although it was less sensitive to barbital, also showed the kinetics of competitive inhibition. The observed decrease in affinity for D-alanine in the presence of barbital is not necessarily due to competition at the substrate site, as the same kinetics are seen when an inhibitor combines with an allosteric site.

Alanine racemase, on the other hand, was not inhibited by barbital. In a normal extract, 8.8 μmoles of D-alanine was formed in 40 min. In the presence of 0.02 M barbital (added to the extract before addition of L-alanine) the rate was 9.3 μmoles in 40 min.

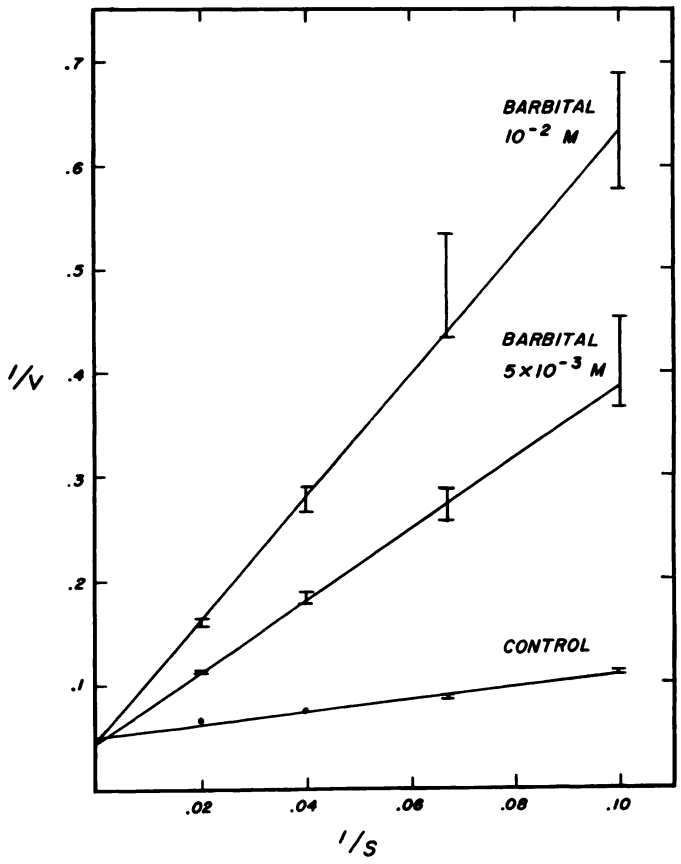

FIG. 6. *Barbital inhibition of* D-*alanine oxidase*

Aliquots of an extract of normal cells (1.1 mg protein per milliliter) were incubated for 20 min with varying amounts of D-alanine, and at two barbital concentrations. Pyruvate production was measured as described under Methods. The range shown at each point is the mean ± one standard error for the triplicate assays. v = μmoles of pyruvate produced per hour per milliliter of extract; S = D-alanine (mM).

C. Extracts of Barbital-Grown Cells

Cells grown in the presence of barbital had 2.5 times the normal level of alanine racemase, as shown in the top half of Table 7. D-Alanine oxidase was also increased, but to a smaller extent. The increased enzyme activities are not attributable to changes in coenzyme levels, since the enzyme assay mixtures included excess coenzyme (pyridoxal phosphate or FAD). These changes presumably explain the increased rate of

TABLE 7

Enzyme levels after growth in alanine or barbital

Cells were grown for three generations in mineral medium plus 0.05 M glycerol and 0.2% casein hydrolyzate, with additions as shown. They were harvested by Millipore filtration during logarithmic growth. Sonic extracts were prepared, as described under Methods, and were dialyzed overnight. Racemase (with manometric D-alanine assay) and D-alanine oxidase were measured by standard procedures. The data are means of three experiments (or four for growth rates in control, L-alanine, and barbital), ± the standard error of the mean.

Additions to growth medium	Growth rate (doublings/hr)	Racemase (units/mg N)	D-Alanine oxidase (units/mg N)
None	1.57 ± 0.03	57 ± 2	4.6 ± 0.3
Barbital, 0.02 M	0.68 ± 0.04	145 ± 8	7.0 ± 0.6
L-Alanine, 0.05 M	1.34 ± 0.03	104 ± 13	14.0 ± 0.7
D-Alanine, 0.05 M	1.38 ± 0.07	52 ± 4	7.5 ± 0.6

oxidation of L-alanine by barbital-grown cells and extracts.

The enzymes from barbital-grown cells retained normal sensitivity to barbital inhibition. As was shown in Fig. 5, the dose-response curves for L- or D-alanine oxidation are the same for extracts of normal or barbital-grown cells.

Could the increased enzyme levels be the result of direct inhibition of D-alanine oxidase by barbital? Such inhibition should increase intracellular pools of both L- and D-alanine. (Figure 1 showed a rise in L-alanine in cells briefly exposed to barbital.) Then an increase in *external* L- or D-alanine might also increase racemase and D-alanine oxidase levels. Table 7 shows that external L-alanine had this effect. (The added L-alanine is about 100 times the concentration present in the casein hydrolyzate.) Rather surprisingly, D-alanine did not induce racemase and had only a small effect on D-alanine oxidase. The slight depression of growth rate with either isomer indicates that both penetrated the cells to some extent.

Since there remained a possibility that the increase in racemase was due to selection of high-racemase mutants, enzyme levels were followed at intervals during growth in normal and barbital-containing media and in a culture returned to normal medium after growth in barbital. The results are shown in Fig. 7. The inoculum, a full-grown normal culture, had a low racemase level. This increased about 3-fold during the lag phase, then leveled off or decreased slightly in logarithmic growth. When a portion of the same inoculum was grown in barbital, enzyme levels increased much more rapidly and continued to rise during logarithmic growth to 7 times the initial level. In the stationary phase, racemase levels were lower in both media. Normal cultures had 65 units per milligram N in log phase, but this fell to 24 units in the stationary phase. In barbital media, the level of 175 units in log phase fell to 68 units after overnight growth. When cells grown overnight in barbital were inoculated into normal medium, the racemase level was initially at the level which a normal culture would attain in log phase. During growth it decreased slightly, remaining similar to the racemase level of cells which had never been exposed to barbital.

DISCUSSION

Barbiturates affect many metabolic reactions, notably oxidative phosphorylation (24–26) and flavin-dependent oxidases (27–29). Barbiturates are among the agents that increase liver microsomal enzyme levels (30, 31). Enzymes catalyzing metabolism of some barbiturates are affected (32). Inhibition of DL-alanine oxidation by mammalian kidney was shown many years ago by Quastel (33).

In *E. coli*, barbital affects amino acid metabolism. The observed effects are specific for individual amino acids, so they do not suggest a general disruption of oxidative

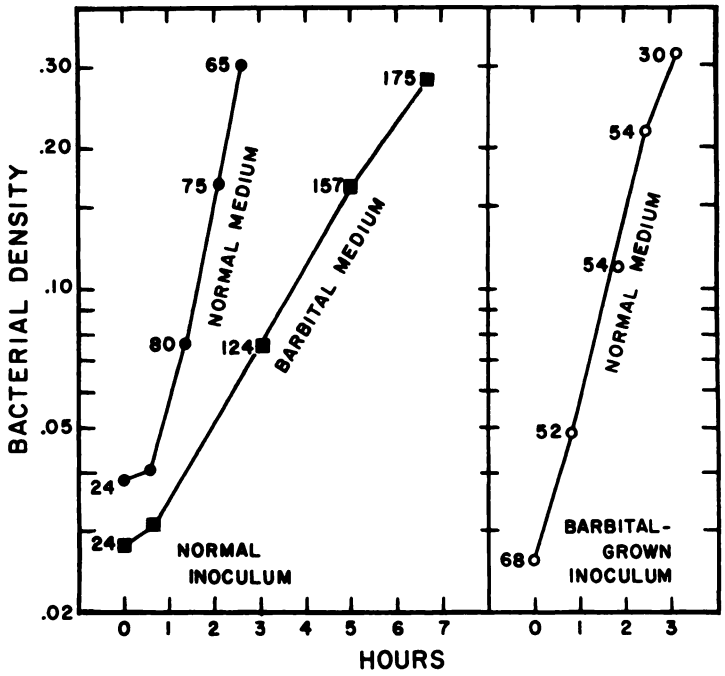

FIG. 7. *Time course of changes in racemase level*

An overnight culture in glycerol-casein hydrolyzate medium was used as inoculum for the 2 cultures shown at the left of the figure. These cells were grown in "normal medium," i.e., glycerol-casein hydrolyzate, or in "barbital medium" which contained glycerol, casein hydrolyzate, and 0.02 M barbital. The culture shown at the right was grown in normal medium, using an inoculum of cells grown overnight in barbital medium. The growth curves are plotted on a logarithmic scale, as bacterial density in milligrams dry weight per milliliter. Samples taken at intervals from all the cultures, including the inocula, were harvested on Millipore filters. Extracts were prepared as usual and were dialyzed overnight to remove barbital if present. Racemase levels, expressed as units per milligram protein N, are written next to the corresponding points on the growth curve.

phosphorylation, membrane permeability, etc. In two cases (alanine and glutamate), the primary and long-term effects of the drug are in opposite directions, indicating that some compensatory adjustment occurs when cells are grown in the presence of the drug.

Data presented above suggest that L-alanine is normally oxidized via D-alanine in *E. coli*. The evidence is as follows:

1. L-Alanine oxidation required more than one component of fractionated cell extracts. Activity was seen only in subfractions containing D-alanine oxidase *and* racemase. If a separate oxidase for L-alanine exists, it must be soluble but must be stimulated by some component of the particulate fraction, possibly by cytochromes. In this case, its disappearance on further purification might be due to instability.

2. Selective inhibition of either racemase or D-alanine oxidase diminished L-alanine oxidation. Cycloserine inhibited L-alanine oxidation and alanine racemase to the same extent, with no effect on D-alanine oxidase. Barbital inhibited L-alanine oxidation and D-alanine oxidase, with no inhibition of racemase. L-Alanine oxidation was the more sensitive to barbital—this is explained by the finding that inhibition of D-alanine oxidase was stronger at low substrate concentrations. If a separate oxidase for L-alanine exists, it must be sensitive to both inhibitors and its sensitivity to cycloserine (at least) must be the same as that of the racemase.

3. The pH curve of L-alanine oxidation

was consistent with a combination of racemase and D-alanine oxidase.

Oxidation of L-alanine by *E. coli* is thus similar to the oxidation of L-hydroxyproline in *Pseudomonas* (34–36), which involves a soluble epimerase and a particulate D-allohydroxyproline oxidase.

The following mechanism of action of barbital, with respect to alanine catabolism, is proposed. The primary drug effect is inhibition of D-alanine oxidase. When cells are grown in the presence of barbital, the L-alanine of the medium enters the cells without difficulty. Some of it is converted to D-alanine, as the racemase is not inhibited, but further metabolism of D-alanine is blocked. Pool sizes of both isomers should therefore increase. The D-form is a poor agent for modifying enzyme synthesis, at least when it is externally supplied. L-Alanine, on the other hand, induces racemase. It is not known whether alanine uptake or alanine racemase is the rate-limiting step in the formation of D-alanine. If racemase is rate limiting, racemase induction will further expand the pool of D-alanine. L-Alanine also induces D-alanine oxidase. Barbital inhibition of D-alanine oxidase is thus overcome in two ways; the amount of enzyme per cell is increased and so is the level of its substrate. When barbital is withdrawn, inhibition of D-alanine oxidase is relieved, and the existing high levels of racemase and of D-alanine oxidase allow the cell to oxidize L-alanine at a rate much above normal.

This work was undertaken as a model system for study of long-term drug effects. We were led to a study of D-amino acid metabolism, which is of importance to bacteria but not, so far as is known, to brain. The possible existence of brain enzymes similar to those described in *E. coli* is currently under investigation. We have found low levels of D-amino acid oxidase in rat brain homogenates, using either D-alanine or D-allohydroxyproline (37) as substrate. This confirms previous results of others (38, 39). The barbital concentrations effective in bacterial alanine metabolism are similar to reported brain concentrations of barbital in animals. In *E. coli*, L-alanine oxidation is inhibited 50% by 0.6 mM; induction of sleep in rats and mice occurs at brain concentrations of 0.4 mM (40) to 1.1 mM (41). When bacteria are chronically exposed to barbital, the effectiveness of the drug is diminished by increased synthesis of enzymes in the affected pathway. A similar mechanism in brain would confer tolerance. The resulting high activity on removal of barbital may be analogous to the withdrawal syndrome in animals.

Levels of amino acids and related amines in the brain undoubtedly affect its function. These levels may be controlled in the brain, as in bacteria, by repression and derepression of enzyme synthesis. The effects of drugs upon enzymes so regulated should be examined further. Drugs that inhibit repressed enzymes of amino acid metabolism may be addicting drugs.

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