The Induction of Alanine Dehydrogenase

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The specific activity of L-alanine dehydrogenase is very low when a (transformable) strain of *B. subtilis* is grown in glucose minimal medium with vigorous aeration. This activity rapidly increases when L- or D-alanine or various alanine analogs are added to the medium. Both chloramphenicol and actinomycin D inhibit the increase, which indicates that alanine dehydrogenase induction requires *de novo* protein synthesis as well as the formation of a specific messenger RNA. When actinomycin D is added later than the inducer, enzyme synthesis continues for a few minutes, the curve being parallel to that of general protein synthesis. Most but not all inducers of alanine dehydrogenase also inhibit growth. All known inducers have an affinity for alanine dehydrogenase (as substrates or inhibitors) but not all complexing agents are inducers. When the bacteria are grown in glucose minimal medium either with little aeration or at a low concentration of phosphate, the specific activity of alanine dehydrogenase is much higher than in the control.

The synthesis of biosynthetic enzymes is usually repressed by the end-product of the metabolic pathway (e.g., Vogel, 1961). In contrast, the synthesis of enzymes which convert a compound into components of the general carbohydrate pool is usually induced by this compound (e.g., Magasanik, 1961). Some enzymes, however, may serve both "biosynthetic" and "catabolic" functions, depending on the physiological state of the cell. An example is alanine dehydrogenase (AlD),[1] which catalyzes the reaction:

$$\text{pyruvate} + NH_4^+ + \text{NADH} \rightleftharpoons \text{L-alanine} + H_2O + NAD^+$$

This reaction could be utilized from left to right to assimilate ammonia and in the opposite direction to supply the energy source pyruvate when L-alanine is present in the medium. The latter process seems to be utilized during the germination of bacilli, which is triggered by L-alanine, (O'Connor and Halvorson, 1961b).

This paper shows that the specific activity of AlD in *B. subtilis* is low in minimal medium and is rapidly induced upon the addition of L-alanine. In order to unravel the molecular nature of this control mechanism the spontaneous level of AlD has been measured under several physiological conditions and many analogs of alanine have been tested for their effect on enzyme induction and repression.

Materials and Methods

Bacteria.—*B. subtilis* SB 19 (Romig) = prototroph, and P8 (Anagnostopoulos) = indole$^-$, alkaline phosphatase$^-$. SB 19 was used except when specifically stated.

Bacterial Growth.—Bacteria grown overnight on plates with tryptose blood agar base (Difco) at 37° were inoculated into the minimal-glucose medium of Anagnostopoulos and Spizizen (1961) (supplemented with 50 μg/ml L-tryptophan in the case of P8), aerated at 37° to 1×10^8/ml or less, and used as the inoculum for further experiments. In most experiments trace elements (Beadle and Tatum, 1945) were added, but they did not significantly affect the AlD or LDH concentration.

The *time course of AlD induction* was measured under conditions of especially good aeration, using a gas-dispersion tube with fritted cylinder (Pyrex), in a 1-liter culture. Insufficient aeration resulted in greatly increased LDH activity; this was undesirable since LDH interferes with the AlD assay by oxidizing NADH in the presence of pyruvate. The bacteria were grown from an inoculum of 10^2/ml. When the optical density at 600 mμ of the bacterial culture reached about 0.3 (1.2×10^8 bacteria/ml), a sample was taken and L-alanine was added as AlD inducer; later actinomycin D or chloramphenicol were added as required. Since actinomycin caused foaming, resulting in the gradual loss of the antibiotic from the medium, one drop of Dow Corning Antifoam B was added. After addition of the inducer, samples were taken at different times by utilizing the aeration pressure of the growth bottle to drive out rapidly the required 40- to 50-ml aliquots. The graduate cylinders used for receiving this liquid contained 6 mg chloramphenicol and 0.2 mmole NaCN in order to stop further protein synthesis. After immediate shaking the optical density was measured and exactly 40 ml was centrifuged in a Serval swinging-bucket centrifuge at 9500 rpm for 5 minutes. The bacterial pellet formed was washed with 10 ml 0.05 M Tris plus 100 μg/ml chloramphenicol, *p*H 8, and then resuspended in 2 ml 0.05 M Tris, *p*H 8, 100 μg/ml chloramphenicol, and 10^{-3} M mercaptoethanol. After addition of one drop of chloroform the mixture was shaken and 0.1 ml lysozyme (5 mg/ml) and deoxyribonuclease (20 μg/ml) was added. The mixture was incubated at 37° until lysis was complete, about 30 minutes. The lysate was centrifuged at 15,000 rpm for 20 minutes and the supernatant was dialyzed against 0.05 M Tris, *p*H 8. (Absence or presence of 10^{-4} M EDTA or of 10^{-3} M mercaptoethanol did not significantly influence the enzymatic activity observed.) No AlD remained attached to the debris because all activity could be washed out of the pellet. The incorporation of C^{14}-valine was measured as described by Levinthal *et al.* (1962).

For the *growth at low concentrations of phosphate* the following additions were used per liter of medium: 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 7 \cdot H_2O$, 1 g Na citrate, 5 g glucose, 12.1 g Tris adjusted to *p*H 6.8 with NaOH, and trace elements. Potassium phosphate was added to give the desired phosphate concentration. In order to permit adaptation, the bacteria were transferred

[1] Abbreviations: ALD, alanine dehydrogenase; LDH, lactic dehydrogenase; NAD^+ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; $VNADP^+$ and NADPH, nicotinamide adenine dinucleotide phosphate, oxidized and reduced forms, respectively; Tris, tris(hydroxymethyl)aminomethane.

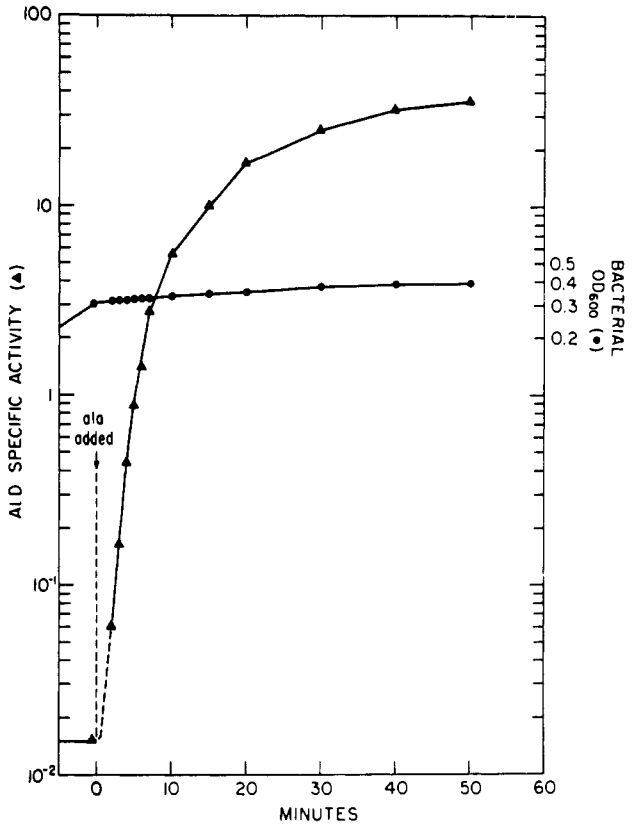

FIG. 1.—Induction of alanine dehydrogenase. Bacteria were vigorously aerated in minimal glucose medium plus trace metals and induced by addition of L-alanine (100 μg/ml) at zero time. For preparation of extracts see Methods.

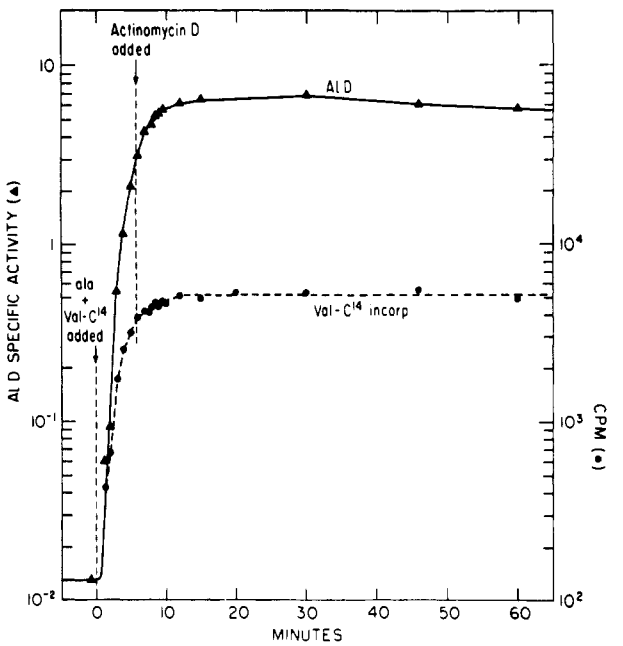

FIG. 2.—Effect of actinomycin D on induced alanine dehydrogenase and general protein synthesis. Bacteria vigorously aerated in minimal glucose medium plus trace metals to OD_{600} = 0.36. L-Alanine (100 μg/ml) and C^{14}-L-valine (10 μg/ml, 0.01 μc/ml) were added at time zero. After 5½ min actinomycin D (10 μg/ml) and one drop antifoam were added. Samples were withdrawn and extracts prepared as described in Methods.

twice in the low phosphate medium using a small inoculum. Good aeration was obtained by bubbling air through the liquid.

AlD Assay.—For the NAD assay the reaction mixture contained 0.05 M Tris, *p*H 8, 0.3 mM NAD, 0.02 M L-alanine, and enzyme. All components but alanine were mixed and the optical density at 340 mμ was recorded; it remained constant. Alanine (one-tenth of final volume) was then blown into the cuvet from a lambda pipet and the change of optical density was recorded for as long as necessary to obtain a reliable rate. For higher activities the enzyme was sufficiently diluted to permit an accurate determination of the initial rate. As a control, the reaction was permitted to go to equilibrium, 0.1 ml 1 M NH_4Cl was added, and the decrease of OD_{340} was observed; this indicated the reversal of the observed reaction. For the NADH assay the reaction mixture contained 0.05 M Tris, *p*H 8, 0.015 mM NADH, 0.01 M sodium pyruvate, 0.05 M ammonium chloride, and enzyme. When both pyruvate and ammonia were left out the measured NADH oxidase activity was very weak. When only the ammonia was omitted the measured LDH activity was usually found to be low.

The *specific activity of AlD* is here defined as 10^3 times the initial increase of OD_{340}/minute which would be produced in the NAD assay by an amount of bacterial culture that gave an OD_{600} = 1 before it was lysed.

The *specific activity of LDH* is defined in the same way, except that NADH was used.

Actinomycin D was a gift of both Dr. K. Folkers at Merck Sharp and Dohme Research Laboratories, Rahway, N. J., and Dr. H. W. Bond at the Cancer Chemotherapy National Service Center, NIH, Bethesda, Md. It was dissolved in 70% acetone at a concentration of 1 mg/ml.

Cycloserine D was a gift of the Commercial Solvents Corporation, Terre Haute, Ind.

D-*alanyl*-D-*alanine*, L- *and* D-*alanine methyl ester*, and L- *and* D-*methylalanine* were prepared by the Cyclo Chemical Corporation, Los Angeles, Calif. All other compounds are commercially available.

RESULTS

Time Course of AlD Induction by L-*Alanine.*—*B. subtilis* grown on glucose minimal medium with strong aeration had a low specific activity of both AlD (0.02) lactic dehydrogenase (LDH) (<0.1). Upon addition of L-alanine the specific activity of AlD increased rapidly, as shown in Figure 1, while that of LDH remained low as long as the aeration was strong. The lag of the AlD induction was less than 1 minute, and the specific activity increased more than 1000-fold in 30 minutes. When L-alanine was added at different concentrations and the cells were harvested 30 minutes later, the specific AlD activity increased linearly with the alanine concentration up to 10 μg/ml and then remained about constant up to 1000 μg/ml L-alanine. However, increasing concentrations of L-alanine caused an increasing reduction in the bacterial growth rate. After prolonged growth in the presence of L-alanine the growth rate increased again (adaptation to alanine).

Addition of chloramphenicol (100 μg/ml) at any time after induction stopped the further increase of specific activity. When chloramphenicol was added together with alanine no AlD induction was observed.

Effect of Actinomycin D.—When Actinomycin D (2 or 10 μg/ml) was added together with the inducer L-alanine (50 μg/ml) the specific AlD activity did not increase. However, when Actinomycin D was added at some later time, the enzymatic activity continued

TABLE I

DEREPRESSION OF AlD AT LOW CONCENTRATIONS OF PHOSPHATE

Bacteria were grown in low phosphate medium, as outlined in Materials and Methods, to the OD_{600} stated in the table. Alkaline phosphatase was significantly derepressed only in the prototroph cultures containing 1×10^{-4} M phosphate and grown to an OD_{600} of 0.44 or more.

Bacteria	Phosphate (M)	Potassium (M)	Bacterial OD_{600}	Specific Activity
Prototroph	0.125	0.2	0.34	0.02
	0.125	0.2	0.36	0.03
	1×10^{-2}	1.7×10^{-2}	0.29	0.05
	2×10^{-3}	3.3×10^{-3}	0.41	0.20
	5×10^{-4}	8.3×10^{-4}	0.32	2.3
	5×10^{-4}	8.3×10^{-4}	1.33	1.7
	2×10^{-4}	3.3×10^{-4}	0.32	2.7
	2×10^{-4}	3.3×10^{-4}	0.92	2.6
	1×10^{-4}	1.7×10^{-4}	0.23	5.0
	1×10^{-4}	1.7×10^{-4}	0.44	7.3
	1×10^{-4}	0.1	0.46	6.8
Alk. phosphatase$^-$ ind$^-$	5×10^{-4}	8.3×10^{-4}	1.3	4.5
	2×10^{-4}	3.3×10^{-4}	1.4	9.2
	1×10^{-4}	1.7×10^{-4}	0.47	12.4
	5×10^{-5}	8.3×10^{-5}	0.21	19.1

to rise for a few minutes and then remained constant (see Fig. 2). Figure 2 also shows the incorporation of radioactive valine, added together with the alanine. Protein synthesis continued for some time and then stopped, the curve running parallel to that of AlD formation.

Effect of Aeration.—The oxygen tension in the growth medium had a strong effect upon the level of AlD. Whereas for strong aeration (glass frit) the specific activity of AlD was 0.02, growth in a rotary shaker, which provided less air to the medium, resulted in a higher specific activity: about 0.5–1 when 200 ml medium was rapidly shaken in a 500-ml Erlenmeyer flask and 0.2–0.4 when 50 ml medium was shaken in a 250-ml Erlenmeyer flask. The specific activity of AlD did not change very much throughout the bacterial growth.

Derepression of AlD at Low Concentrations of Phosphate.—When *B. subtilis* was grown with strong aeration in minimal-glucose medium containing low concentrations of potassium phosphate (see Materials and Methods) the specific activity of AlD increased with decreasing phosphate concentration, as shown in Table I. This effect occurred at phosphate concentrations at which phosphate was not growth limiting; in contrast, alkaline phosphatase was appreciably derepressed only when phosphate became limiting; e.g., growth could not progress beyond $OD_{600} = 0.4$ in 1×10^{-4} M phosphate or beyond $OD_{600} = 0.2$ in 5×10^{-5} M phosphate. A mutant (P8) unable to produce alkaline phosphatase also showed the increase in AlD activity when grown in low phosphate concentrations. When the concentration of phosphate was decreased, that of potassium was usually also reduced; but the increase of AlD activity is clearly the result of the low phosphate concentration, because the same result was observed when a low-phosphate medium contained 0.1 M KCl (see Table I).

AlD Induction by Other Compounds.—The induction of AlD by various compounds was determined in two ways. In the first method the bacteria were grown to an OD_{600} of about 0.3, the compound was added, and the culture was shaken for another hour. In this way induction could be observed even if the compound inhibited bacterial growth. Results are given in Table II.

TABLE II

INDUCTION OF AlD ONE HOUR AFTER ADDITION OF COMPOUND

Bacteria were shaken or aerated to $OD_{600} \sim 0.3$, and aliquots were rapidly distributed into flasks (50 ml into 250-ml Erlenmeyer flasks) containing the compounds and immediately shaken (rotary) at 37°. After 1 hour the bacteria were harvested and extracts prepared (see Methods). The AlD specific activity in the control varied according to the air supply but was never higher than 0.5. All values in the table are at least a factor two above the corresponding control. The following compounds produced less than a factor two or no increase: adenine, D-alanyl-D-alanine, alanine amine, DL-alacreatine, L-α-aminobutyric acid, L-α-aminobutyric acid + L-glutamic acid, DL-2-amino-1-propanol, 3-amino-1-propanol, L-arginine, L-aspartic acid, betaine, L-citrulline, creatine, L- or D-cycloserine, L- or D-glutamic acid, glycine, glycylcyanine, isobutyric acid, D-isoleucine, D-leucine, D-methionine, β-methoxyethylamine, *N*-methyl-L- or D-alanine, nicotinamide, L-ornithine, D-pantethine, D-panthenol, pantoyltaurine, pantothenate, propionic acid, pyruvate, riboflavin, sarcosine, thiamine.

Addition to Minimal Glucose Medium	Concentration (μg/ml)	Specific Activity
None	—	0.2–0.5
L-Alanine	20 or 50	25–45
D-Alanine	20 or 50	15–30
L-Alanine methyl ester	50	38
D-Alanine methyl ester	50	37
D-α-Aminobutyric acid	50	30–40
D-Aspartic acid	50	8–13
L-Cysteine	50	2.2
D-Cysteine	50 or 200	23–30
L-Cysteine sulfinic acid	50	6.4
DL-2,3-Diamino propionic acid	50	1.0
DL-Homocysteine	50	7.6
L-Homocysteine thiolactone	50	5.0
D-Homocysteine thiolactone	50	3.0
L-Isoleucine	50	7.5
L-Leucine	50	1.8
L-Methionine	50	6–8
L-Norvaline	50	17
D-Norvaline	50	14
L-Serine	50	9.2
D-Serine	50	8–9
L-Threonine	50	6.5
D-Threonine	50	10.6
L-Valine	50	3.5
D-Valine	50	12

In the second method the compound was added together with a small bacterial inoculum and the flask shaken until the titer was about 2×10^8/ml. In this case a strong growth inhibition by the added compound had to be counteracted. Such strong inhibitors are L-α-aminobutyric acid, DL-2,3-diaminopropionic acid, D-cysteine, L-cysteinesulfinic acid, L- or D-homocysteine, D-leucine, L- or D-norvaline, L- or D-serine, L- or D-threonine, and D-valine. The inhibition by both strong and weak growth inhibitors could be competitively counteracted by L-glutamate and other donors of amino groups. Details will be published elsewhere. Since L-glutamate neither induced AlD nor prevented the induction by any other compound, it could be used to facilitate the growth of cultures in the presence of a growth-inhibiting inducer (see Table III).

Lack of NH_4^+ did not cause an increase of AlD activity. Bacteria were aerated in minimal glucose plus trace metals to $OD_{600} = 0.42$, centrifuged, resuspended in minimal glucose medium lacking $(NH_4)_2SO_4$, and shaken for 75 minutes. The specific activity was 1.3 (bacterial $OD_{600} = 0.30$) as compared to a control

TABLE III

INDUCTION OF AlD BY GROWTH IN THE PRESENCE OF VARIOUS COMPOUNDS

Bacteria, grown in minimal glucose medium, were inoculated at a titer of about 1×10^6 into 200 ml minimal glucose medium plus the indicated additions. The cultures were shaken at 37° until the titer was about 2×10^8. They were then harvested and extracts were prepared (see Materials and Methods). The AlD specific activity was less than 1 for: L-aspartic acid, L- or D-glutamic acid, glycine, α-ketoglutaric acid, DL-lactic acid, pyruvic acid; or for L-glutamic acid plus any one of the following: L-α-aminobutyric acid, pyruvic acid, mercaptoethanol.

Addition to Minimal Glucose Medium	Concentration (μg/ml)	Specific Activity
None	–	0.5–1
L-Alanine	50–100	20–50
D-Alanine	50	12
L-Glutamate plus	50	
DL-α-Aminobutyric acid	50	6.1
L-Cysteine	50	20
DL-Cysteine	50	12
L-Cysteine sulfinic acid	50	6.1
L-Homocysteine thiolactone	50	35
D-Homocysteine thiolactone	50	26
DL-Homocysteine	50	35
L-Methionine	50	7.0
L-Norvaline	50	20
L-Serine	50	2.2
D-Serine	50	12

culture resuspended in minimal glucose, which had a specific activity of 1.5 (bacterial OD_{600} = 0.58). Another control containing no ammonia but 200 μg/ml L-alanine gave a specific activity of 68 (bacterial OD_{600} = 0.38).

The Effect of D-Alanyl-D-alanine.—Both L- and D-alanine were effective as inducer of AlD, whereas D-alanyl-D-alanine neither induced nor prevented the induction by L- or D-alanine (Table IV). To find out whether D-alanyl-D-alanine can enter the bacteria, we employed D-cycloserine, which is known to inhibit the formation of both D-alanine and D-alanyl-D-alanine (Strominger, 1962). When D-cycloserine (300 μg/ml) was added to minimal glucose together with 1×10^6/-ml bacteria no turbidity was observed even after 2 days. However, growth occurred when either D-alanine or D-alanyl-D-alanine were also added (5–200 μg/ml). The D-alanyl-D-alanine was only slightly less effective than D-alanine. Cultures with 20 μg/ml of D-alanyl-D-alanine or more grew up to full turbidity before they lysed. On the other hand, cultures with 5 μg/ml of D-alanine grew up fully, whereas with the same amount of D-alanyl-D-alanine they lysed after the

TABLE IV

EFFECT OF D-ALANYL-D-ALANINE ON THE INDUCTION OF AlD

Bacteria, grown in minimal glucose medium plus trace metals to OD_{600} = 0.24, were distributed (50 ml into 250-ml Erlenmeyer flasks) containing the indicated compounds and shaken for one hour at 37°. Extracts were prepared as stated in Materials and Methods.

L-Alanine (μg/ml)	D-Alanine (μg/ml)	D-Alanyl-D-alanine (μg/ml)	Specific Activity
20	—	—	42
20	—	50	47
—	20	—	21
—	20	50	22
—	—	50	0.4
—	—	—	0.4

TABLE V

COMPETITION EXPERIMENTS FOR INDUCTION OF AlD BY L-ALANINE

The compounds were added 10 minutes before alanine and the cultures grown for 1 hour after the alanine addition.

Compound	Concentration (μg/ml)	L-Alanine (μg/ml)	Specific Activity[a]
L-Arginine	200	10	1.0
L-Aspartate	200	10	1.0
Citrate	5000	20	1.1
D-Glyceraldehyde	100	40	1.1
D-Glycerate	100	40	1.3
Glycerol	100	40	1.2
Glycine	200	40	1.1
α-Ketobutyrate	100	40	1.8
α-Ketoglutarate	200	40	0.95
Lactate	200	40	0.91
Oxaloacetate	100	40	1.4
Pantothenate	10	50	0.95
Pyruvate	100	5	1.0
Pyruvate	500	5	0.92
Pyruvate	2000	5	0.95
L-Serine	200	40	1.8

[a] Relative to culture to which only alanine was added.

OD_{600} had reached 0.5. The D-alanyl-D-alanine, being quite pure, thus seems to be able to enter the cell. We have not excluded the alternative that some of the D-alanyl-D-alanine was hydrolyzed outside or at the surface of the cell and enough D-alanine was formed to support growth but not to induce AlD. We therefore conclude only tentatively that D-alanyl-D-alanine is neither an inducer nor a repressor of AlD.

Attempt to Find a Compound that Competes with AlD Induction by L-Alanine.—Various compounds were added 10 minutes before the addition of the inducer L-alanine, and the bacteria were shaken for 1 hour or more. The AlD specific activity, determined in the extracts, showed no significant reduction of L-alanine induction for any of the compounds tested, as shown in Table V.

Absence of Glutamic Dehydrogenase.—No glutamic dehydrogenase activity was found, irrespective of the growth conditions (with or without L-alanine or L-glutamate and with much or little aeration) and irrespective of the assay conditions (with NAD or NADH, $NADP^+$ or NADPH).

DISCUSSION

The rapid increase of AlD specific activity observed after addition of alanine stops immediately when chloramphenicol is added. This shows that the induction of AlD requires *de novo* protein synthesis. Actinomycin D also stops any AlD induction when added together with alanine, but when it is added later the specific activity of AlD continues to increase for a few minutes and then stops, the curve running parallel to that of general protein increase. Actinomycin D seems predominantly to inhibit DNA-directed RNA synthesis (Hurwirtz *et al.*, 1962; Levinthal *et al.*, 1962). Our result indicates therefore that alanine induces the formation of a specific messenger RNA that initiates the production of AlD. This *specific* RNA remains active for about as long as does the *general* messenger RNA.

The specific activity of AlD was much higher in bacteria grown at low than at high concentrations of phosphate. This suggests the existence of an internal repressor which at low concentrations of phosphate is not produced in sufficient quantity to repress the

synthesis of AlD. The rapid induction by alanine would then indicate that the inducer can rapidly inactivate or compete with the repressor and does not first have to be metabolically altered. As an alternative but less likely explanation, the increase of AlD could be caused by an internal inducer which is formed when the concentration of phosphate is low.

In addition to alanine, several analogs induce an increase in the specific activity of AlD. It appears that both the amino and the carboxyl groups of alanine are required for induction. The only compounds which still induce, although these groups have been altered, are L- or D-alanine methylester; this induction may be due to alanine liberated by an esterase. The α-carbon group is also required for induction, since glycine does not induce. When one or two hydrogen atoms of the methyl group of alanine are substituted some inductive capacity is retained. The resulting D-amino acid is often a better inducer than the L-amino acid, as long as one hydrogen on the $-CH_3$ is replaced by only a small group. When the replacements become bulkier, the L-amino acids become better inducers. In some of those cases in which both the L- and the D-compound is active, one of them might be converted into the active compound by an L-D-amino acid racemase.

Most of the inducers inhibit growth. But there is no strict correlation between the two properties, since for example L-α-aminobutyric acid inhibits but does not induce, whereas D-α-aminobutyric acid and D-aspartic acid induce but do not inhibit.

The complexing properties of alanine analogs with AlD have been determined by O'Connor and Halvorson (1961) for a partially purified preparation of AlD of *B. cereus;* it may be reasonable to assume that the AlD of *B. subtilis* has similar properties. Our results show first of all that many D-amino acids are inducers but not substrates, and some substrates, such as L-α-aminobutyric acid, are not inducers. More generally, a compound that complexes with the enzyme (substrate or inhibitor) is not necessarily an inducer since compounds such as glycine and sarcosine, lacking the α-carbon, inhibit but do not induce AlD. But conversely, most inducers seem to complex with AlD. Detailed measurements will have to be made of the complexing properties of all inducers with the AlD of *B. subtilis* before it can be decided whether some inducers do not complex the enzyme, as was found in the case of β-galactosidase (Jacob and Monod, 1961).

The germination of spores of bacilli is initiated by L-alanine (Hills, 1949) and certain of its L-amino acid analogs (Woese *et al.*, 1958; O'Connor and Halvorson, 1961b). Most of these initiators are substrates of AlD (O'Connor and Halvorson, 1961a,b), whereas D-amino acids, which reduce the rate of L-alanine-initiated germination, are inhibitors of AlD. O'Connor and Halvorson (1961b) therefore suggested that AlD activity is essential for germination initiated by L-alanine. If this is correct, L-alanine may be particularly effective in germination because it also induces the formation of AlD. An induction of AlD by exposure of spores of *B. cereus* to L-alanine has recently been reported (Halvorson, 1963). Other inducers of AlD might also initiate germination if they are added to spores together with a noninitiating amount of L-alanine, or, better, L-α-aminobutyric acid, which does not induce AlD. One experiment of this kind has already been reported for D-cysteine, which is a strong inhibitor of AlD and yet initiates germination under the above conditions (Krask, 1961; O'Connor and Halvorson, 1961b). Krask has proposed that this initiating effect is caused by the inhibition of alanine racemase by D-cysteine. An alternative possibility is that D-cysteine initiates by inducing the formation of AlD. The two possibilities could be distinguished experimentally by the use of D-cycloserine, since this compound also inhibits the alanine racemase but does not induce AlD.

The reaction constants of AlD greatly favor the formation of L-alanine from pyruvate. If all three substrates of the reaction, pyruvate, NH_4^+, and NADH, were present in sufficient concentration inside the cell, AlD would presumably increase autocatalytically, because the alanine produced would induce the formation of more enzyme, which in turn would produce an increased amount of L-alanine, and so on. Actually, the cells have a small specific activity of AlD even when they are grown in minimal medium containing 0.5% pyruvate in place of glucose and the usual amount of NH_4^+. Hence the factor restricting the excessive formation of L-alanine seems to be the concentration of NADH. This assumption would explain why the AlD concentration is very low when the aeration is excellent, because most NADH is then converted to NAD, and why the AlD concentration increases when the oxygen tension in the medium decreases.

The internal repression of AlD appears useful to the cell for two reasons. First, it prevents the excessive drainage of pyruvate into non-energy-producing pathways. Second, it avoids the accumulation of L-alanine which, for some unknown reason, inhibits growth. The specific activity of AlD is actually so low, when the aeration is strong and no inducer is present, that in these cells most ammonia is probably assimilated by a reaction different from the amination of pyruvate.

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