grown in 90 per cent horse serum and then treated with cortisone, showed increased liver cell parenchymal outgrowth and differentiation. The susceptibility of these cultures was greatly increased so that both macrophages and differentiated liver parenchyma were destroyed.

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THE DEVELOPMENTAL SIGNIFICANCE OF ALANINE DEHYDROGENASE IN BACILLUS SUBTILIS

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Genetic functional elements (genes) can be divided into basic and developmental genes, even though these functions may sometimes overlap. Basic genes govern the formation of the basic constituents of all cells, i.e., DNA, RNA, proteins, cell membranes, etc. In contrast, developmental genes are required only for the change of one cell type into another one, that is, for the differentiation of a multicellular organism or for the change into another phase of the life cycle of a unicellular organism. Whereas mutations in basic genes are lethal (if homozygous) unless the missing compound can be fed from without, mutations in developmental genes effect only a specific type of differentiation and are not lethal for individual cells although the organism may not survive in the long run. The biochemical pathway leading toward a specific type of differentiation can be unraveled conclusively by the isolation and biochemical characterization of mutants blocked in the corresponding developmental genes.

A particularly simple system, which lends itself to such a genetic analysis of differentiation, is that of sporulation and germination in bacilli.[1] This paper analyzes in *B. subtilis* the first steps in spore germination, which are specifically initiated by L-alanine.[2, 3]

L-alanine could initiate germination for a variety of reasons: by giving rise to other compounds (D-alanine, L-glutamate, L-aspartate, L-serine, pyruvate), by its attachment to transfer RNA or to some other component in the cell, or by its incorporation into protein. The most promising explanation of the action of L-alanine was prompted by the observations that (heat-activated) spores of *B. cereus* deaminated L-alanine to pyruvate[4] by means of alanine-dehydrogenase (AlD)[5] and that deamination and germination properties were well correlated.[6] These findings suggested that AlD is necessary for the initiation of germination by L-alanine[7] in spite of another seemingly contradicting observation that the rate of germination was inversely correlated with the amount of AlD within spores.[8] We decided to settle the question of whether or not AlD is required for germination by isolating mutants that were deficient in AlD activity.

AlD catalyzes the reaction:

$$\overset{+}{{}_3}HN\text{—}\overset{CH_3}{\overset{|}{C}}\text{—}COO^- + NAD^+ + OH^- \leftrightarrow O{=}\overset{CH_3}{\overset{|}{C}}\text{—}COO^- + NADH + NH_4^+$$

The enzyme could be utilized also in the reverse direction (favored by the equilibrium constant) for the fixation of ammonia. Since most bacilli have no glutamic dehydrogenase activity,[9] it had been suggested that AlD might be necessary in the vegetative growth of such organisms, both for the formation of L-alanine[8-10] and as the major source of ammonia fixation.[9, 10] However, the low level of AlD in properly grown vegetative cells rendered this assumption unlikely.[11] Indeed, the AlD$^-$ mutants described in this paper can still grow in a minimal-glucose medium, whereas their germination can no longer be initiated by L-alanine. AlD in *B. subtilis* is therefore not a basic but a developmental enzyme.

Materials and Methods.—Bacteria: Derivatives of Spizizen's transformable strain of *B. subtilis:* 60009, prototroph; 60021, requires indole or tryptophan; 60229, requires indole, unable to produce alanine dehydrogenase; 60158, requires L-glutamate or L-aspartate, on which it grows slowly, reverts frequently.

Media: For vegetative growth: Minimal (M)- glucose (G) medium[12] plus 50 μg/ml L-tryptophan or 500 μg/ml L-glutamate when required, and 50 μg/ml L-alanine for induction of AlD. For selection of AlD$^+$ transformants or revertants: M + 1 mg/ml L-alanine + 50 μg/ml L-tryptophan; plates were incubated for three days at 37°. *Sporulation medium*[13] = S-medium.

Isolation of AlD$^-$ mutants: Bacteria 60021 were UV-irradiated to 10^{-3} survivors and filtered onto (HA) Millipore filters with a pore size of 0.45 μ. The filters, containing about 20 bacteria per square centimeter, were placed on plates containing MG + tryp + ala and incubated at 37°C for 16–20 hr. When tiny colonies had formed, the filters were transferred to plates containing 0.05 *M* Tris pH 8 + 10^{-3} *M* $MgCl_2$ and left for 3 hr in the cold, in order to "dialyze" away the substrates of various enzymes. Subsequently, the filters were transferred to assay plates containing 0.05 *M* Tris, pH 8, 0.7% agar, 0.01 *M* L-alanine, 3 μg/ml methylene blue, and 100 μg/ml 2,3,5/triphenyl tetrazolium chloride. The addition of NAD, diaphorase, or phenazine methosulfate was neither required nor effective, except that phenazine methosulfate killed some of the bacteria. After incubation in the dark at 37°C for 2 hr or longer, most colonies were dark red, a few per cent had white sectors, and about 5 in 10^4 were white. These white colonies and some white sectors were touched under the dissecting microscope by a thin platinum wire which was then streaked as a small patch onto a new Millipore filter placed on MG + tryp + ala.

After incubation for 15–20 hr, the new filters were transferred to plates containing the same buffer as above + 0.2 ml of freshly prepared 10 mg/ml lysozyme + 5×10^{-3} *M* mercaptoethanol, spread on top of the plate. These plates were incubated for 2 hr at 37°C to obtain some bacterial lysis. The filters were transferred in dim light to plates containing a thin layer of 0.05 *M* Tris, 0.7% agar, 0.01 *M* L-alanine, 0.001 *M* NAD, 200 μg/ml phenazine methosulfate (Sigma), and

300 μg/ml p-nitroblue tetrazolium (Sigma). The AlD liberated from the lysed bacterial patches caused the rapid appearance of a blue stain at 37°; this distinguished between actual AlD$^-$ mutants (not staining) and others perhaps defective in the oxidation-reduction pathway from NADH to methylene blue. The subsequent assay of AlD in liquid cultures[11] usually agreed with the result of the plate assay.

The nitroblue-tetrazolium method has also been used directly for the isolation of mutants (Millipores with colonies were exposed to lysozyme only for $^1/_2$ hr). However, about $^1/_2$ of the colonies isolated did not contain any viable bacteria. Nevertheless, this method may be useful for the isolation of mutants of dehydrogenases whose substrate cannot enter an intact cell.

Aspartase assay: Bacteria grown to an OD_{600} of 0.5 were concentrated 50-fold or more, lysed by 100 μg/ml lysozyme in buffer containing 0.1 *M* potassium phosphate, pH 7.0, 10^{-3} *M* mercaptoethanol, and 10^{-3} *M* $CaCl_2$. This buffer preserves the otherwise unstable activity.[14] After 20 min centrifugation at 17,000 rpm and gel filtration of the supernatant with coarse grade Sephadex G25, 0.9 ml of the extract plus 0.1 ml of 0.2 *M* aspartate were incubated for 1 hr at 37°C; a control was prepared without incubation. Subsequently, 0.5 ml 10% TCA was added to the reaction mixture, the precipitate removed by centrifugation, and the amount of ammonia determined by Nessler's reagent, the OD_{470} being read immediately after addition of the reagent. The specific activity is expressed in 10^{-2} mM of ammonia liberated per hour and per unit of bacterial OD_{600} measured before lysis.

Extracts for other enzyme assays in vegetative cells were prepared as described earlier.[11] The same paper has also described the method of *AlD assay.*[11]

Extracts of spores were prepared by vigorous sonication in the presence of an equal weight of glass beads, using a Mullard sonicator. The breakage of spores was established by the appearance of enzymes, like diaphorase, not further mentioned in this paper.

Transaminases were assayed spectrophotometrically by coupling the reaction to either LDH or MDH.[15] The concentrations were: Tris, pH 8, 0.05 *M;* L-aspartate or L-alanine, 0.02 *M;* α-ketoglutarate or pyruvate, 0.01 *M;* pyridoxal phosphate, 50 μg/ml; NADH, 0.15 μM/ml; LDH or MDH, 0.035 units/ml. The assay mixture and two controls (leaving out one of the two substrates) were run simultaneously by recording the decrease of OD_{340} in the Gilford model 2000 spectrophotometer. The specific activity is 1000 times the OD_{340} change per min which bacteria of $OD_{600} = 1$ would produce after lysis without any dehydrogenases contaminating the extract.

Spores were obtained in S-medium,[13] 10-fold concentrated in phosphate buffer, treated with 100 μg/ml lysozyme, and then purified by differential centrifugation including 12 times washing with distilled water. The spores were stored (for less than one month) at a concentration corresponding to $OD_{625} = 10$ in distilled water in the cold

Germination: Spores were suspended in 1/15 *M* K_2H-NaH_2-PO_4 buffer at pH 7.3 (or other buffers when specially stated). Usually the suspension was kept for 30 min at 25° and then added to a cuvette in the compartment of a Gilford recording spectrophotometer. After equilibration at the experimental temperature of 37° 0.1 ml of the germinating agent was blown into the cuvette. Germination was measured by the decrease in OD_{625}.[16] The initial OD^i_{625} was 0.4–0.45. A plot of OD/OD^i against time gave a germination curve whose slope is here approximated by three quantities: (1) the *maximal rate* k_m, determined at the inflection point, if there was a visible lag, (2) a lag time, defined by the intersection between the tangent at the inflection point and the horizontal line through OD^i, and (3) the ratio OD^f/OD^i, where OD^f is the value at 60 min.

Results.—Germination of AlD$^+$ spores: During germination the refractive index of spores and consequently the extinction at 625 mμ decrease.[16] In normal AlD$^+$ spores this process was initiated by L-alanine as shown in the lower curves of Figure 1. An increase in the concentration of L-alanine did not appreciably affect either the maximal germination rate k_m or the lag (see Table 1). In addition to L-alanine some analogues had been earlier found to initiate germination, although at a lower rate, in a medium containing glucose.[3] We found the same agents also effective in phosphate buffer alone (see Table 2).

Spores of *B. cereus* require a treatment at elevated temperatures before they can efficiently respond to L-alanine.[1] In our AlD$^+$ strain of *B. subtilis* we have ob-

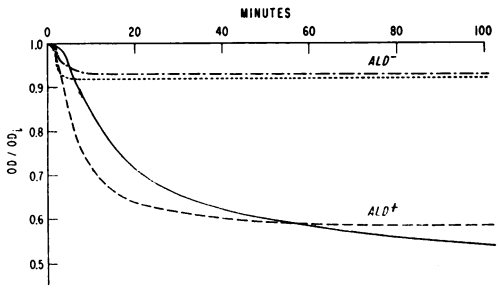

FIG. 1.—Initiation of germination by L-alanine. Germination medium: 1/15 *M* sodium phosphate, pH 7.3, 50 μg/ml L-alanine. Initial OD_{625} was about 0.45. ·—·—· = AID$^-$ spores of 60229, without heat treatment; --- = AID$^-$ spores of 60229, treated for 4 hr at 65° in 1/30 *M* sodium phosphate, and washed; —— = AID$^+$ spores of 60021, without heat treatment; —— = AID$^+$ spores of 60021, with heat treatment. Spores were heat-treated at 10 × their final concentration.

TABLE 1

DECREASE OF OD_{625} AT DIFFERENT L-ALANINE CONCENTRATIONS

L-alanine concentration in mM	AID$^+$ Spores Lag (min)	AID$^+$ Spores k_m	AID$^-$ Spores Lag (min)	AID$^-$ Spores k_m
0.56	1.0	1.9	2.0	0.93
2.24	1.2	2.1	1.2	1.2
11.2	1.2	2.0	1.5	1.0
56.0	1.4	1.9	1.8	1.1

Measured in phosphate buffer, pH 7.3. k_m = maximal rate of change of OD/OD^i per hour.

served only a small enhancement of the germination rate by treatment at 65° (see Fig. 1). Nevertheless, the spores showed the typical heat resistance: k_m decreased significantly only when the spores were treated at 70° for more than one hr (k_m was about 80% of its initial value after 5 hr at 70°). In the following experiments spores were not heat-treated except when noted.

Since germination did not require heat activation, we wondered why it did not occur immediately after the production of spores. Taking fresh and "used" sporulation medium, we measured germination by OD_{625} decrease and observed outgrowth under the phase contrast microscope. "Used" sporulation medium had supported bacterial growth until spores began to form; it had then been sterilized by filtration. Whereas good germination and outgrowth took place

TABLE 2

EFFECT OF DIFFERENT AGENTS ON THE OD_{625} DECREASE

Compound	Concentration (mg/ml)	AID$^+$ Spores Lag (min)	AID$^+$ Spores k_m	AID$^+$ Spores OD^f/OD^i (in %)	AID$^-$ Spores Lag (min)	AID$^-$ Spores k_m	AID$^-$ Spores OD^f/OD^i (in %)
L-alanine	0.25	1.2	1.9	56	1.2	1.2	90
L-alanine	5	1.4	1.9	53	1.8	1.07	91
L-α-aminobutyrate	0.25	1.7	1.3	51	0.8	1.09	90
D-α-aminobutyrate	1		0.06	88		0.07	94
β-alanine	2.5	2.0	1.1	53	0.7	1.4	89
L-cysteine	5	1.7	1.1	52	1.2	1.1	91
D-cysteine	5		0.06	87		0.04	97
L-isoleucine	4	1.3	1.3	52	1.1	1.4	90
L-leucine	10		0.19	79		0.24	90
L-methionine	6		0.23	78		0.29	92
L-norvaline	4	2.0	1.3	49	1.0	1.4	89
L-serine	5		0.18	82		0.13	97
L-valine	4	1.9	1.3	55	0.5	1.2	89
D-valine	4					0.06	98

Measured in phosphate buffer, pH 7.3. OD^f_{625} = value at 60 min. The compounds were mostly added at three times the concentration required by Woese *et al.*[3] for 50% of maximal germination.

The rate k_m of the maximal OD change per hour was less than 0.03 when any one of the following compounds (in brackets: concentration in mg/ml) were added either to AID$^+$ or AID$^-$ spore suspensions: adenine (0.2), adenosine (0.5), D-alanine (0.5), p-aminobenzoic acid (0.05), ascorbic acid (0.001), L-aspartate (5), L-arginine (0.2), biotin (0.001), cytosine (0.1), dipicolinate (0.5), folic acid (0.001), glucose (0.25), gluconate (0.4), L-glutamate (0.2), glycine (5), guanine (0.1), D-histidine (0.25), inosine (0.5), i-inositol (0.001), D-isoleucine (4), L-lysine (0.2), nicotinic acid (0.001), pantothenate-Ca (0.001), L-phenylalanine (0.05), L-proline (5), pyridoxine (0.001), rioflavin (0.001), ribose (0.3), ribose-5-phosphate (0.46), sarcosine (5), thiamine HCl (0.001), L-threonine (0.25), thymine (0.1), L-tryptophan (0.25), L-tyrosine (0.25), uracil (0.1).

The small effect of some D-amino acids might be caused by contaminating L-amino acids in the preparation.

in the fresh medium (rich in amino acids), no such effect could be observed for at least $2^1/_2$ hr in the "used" medium except when L-alanine (50 μg/ml) had been added. No other one of the usual 20 amino acids, vitamines, purines, or pyridines (at 50 μg/ml) stimulated germination. Addition of yeast extract (which contains about 4% alanine) was as efficient as that of L-alanine alone.

Germination defect in AlD$^-$ spores: AlD$^-$ mutants were isolated by a plate assay described under *Materials and Methods*. The specific activity of AlD in vegetative cells grown in MG + tryp + ala, was less than 0.1 (DPN assay) for mutant 60229, whereas it was 40 for the parent strain 60021. In a spore extract the specific activity of AlD (NADH assay, per OD_{625} of a spore suspension) was 30 for AlD$^+$ and at least 20 times smaller for AlD$^-$ spores. The AlD$^-$ strain could be transformed to AlD$^+$ by prototroph DNA (for a plating medium which selects AlD$^+$ transformants, see *Materials*). The reversion frequency of the AlD$^-$ strain was less than 10^{-7}. It sporulated as well as the AlD$^+$ parent strain.

With respect to germination the AlD$^-$ strain behaved abnormally. After addition of L-alanine, the extinction of AlD$^-$ spores decreased only slightly (Fig. 1) whereas the marked decrease observed for AlD$^+$ spores was blocked, obviously because it depended on the enzymatic activity of AlD. The extinction of more highly purified refractile AlD$^-$ spores continued to decrease slowly.

In spite of this block in germination, the AlD$^-$ spores did react with L-alanine, as revealed by a limited decrease in extinction (Fig. 1, Table 1). This decrease occurred also after addition of alanine analogues that initiated germination of AlD$^+$ spores (see Table 2). However, even in phosphate, carbonate, or Tris buffer (0.1 *M*, pH 7.3) alone, the extinction slowly decreased ($k_m \leq 0.2$), leveling out at $OD^f/OD^i \approx 90$ per cent, after 3 hr at 37° (or a week at 4°); subsequently, AlD$^-$ spores no longer responded to L-alanine, ($k_m < 0.1$), whereas AlD$^+$ spores still reacted normally (but with a smaller lag). Repeated washing and storage in water did not recover the initial reaction. A limited OD decrease was also found with Ca-dipicolinate (0.04 *M*) ($k_m = 0.8$) without germinating AlD$^-$ or AlD$^+$ spores; after an OD decrease of 10 per cent the reaction stopped, even when followed up to 5 hr at 37°. When L-alanine was added to these spores, the AlD$^-$ spores were unreactive whereas the OD of AlD$^+$ spores decreased much more rapidly ($k_m = 5.1$) and with a smaller lag (40 sec) than without the pretreatment. The release mechanism evidenced by these reactions will be analyzed in detail elsewhere.

Whereas AlD$^+$ spores swelled and grew out in an L-alanine containing minimal medium, no such effect was observed for the AlD$^-$ spores. Even in the rich S-medium, which contains 2 mg/ml yeast extract and glucose, only 30 per cent of the AlD$^-$ spores were swollen after 7 hr shaking at 37°, and further outgrowth proceeded slowly. AlD$^+$ spores, in contrast, exhibited 14 per cent swelling and 35 per cent outgrowth after one hr, and 10 per cent swelling, 45 per cent outgrowth, and 25 per cent long threads after two hr in this medium. Eventually, both AlD$^-$ and AlD$^+$ spores gave rise to colonies on plates with rich medium. On the selective plates with M + ala + tryp, however, only the AlD$^+$ spores produced colonies.

The assimilation of ammonia: AlD$^-$ mutants grew in MG + tryp at the same rate as their parent strain (doubling time of 65 min at $OD_{600} = 0.4$). The deficiency of alanine dehydrogenase therefore does not prevent the uptake of ammonia. Since neither of these strains showed glutamic dehydrogenase activity, some other enzyme must be responsible for the assimilation of inorganic ammonia.

TABLE 3

SPECIFIC ACTIVITY OF L-ASPARTASE

Strain	Growth Medium: MG L-asp	MG D-asp	MG + 500 μg/ml L-glutamate L-asp	Tryptone broth L-asp	Tryptone broth D-asp
60009 = prototroph	3.6	0.09	2.5	4.3	0.06
	6.2		2.2	7.7	
60158 = L-glutamate⁻	—	—	0.20	0.10	0.10
			0.37	0.20	

Indeed, we have observed a high specific activity of *aspartase* which reversibly catalyzes the conversion of fumarate and ammonia into L-aspartate (see Table 3). The conclusion that aspartase is necessary for the assimilation of ammonia is supported by the following observation. One of our mutants (60158) requiring L-glutamate or L-aspartate for growth showed a very low aspartase activity (see Table 3) but had normal AlD activity. The low amount of aspartase apparently forced the mutant to utilize (by transamination) glutamate or aspartate as an amino source.

It may be surprising that AlD is not used for the assimilation of ammonia, at least in aspartase⁻ mutants. This may be partially due to the low activity of AlD in cells grown in minimal medium with good aeration.[11] The major reason, however, is apparently the low transaminase activity between L-alanine and either L-glutamate (spec. activity ≤ 1) or L-aspartate (spec. activity ≤ 3). Similar results have been obtained by Thorne *et al.* who did not find any L-alanine-L-aspartate transaminase.[17] Ammonia present in the form of L-alanine can therefore not be readily converted into the much needed ammonia source L-glutamate. In contrast, the interconversion between L-glutamate and L-aspartate can be readily achieved since the corresponding transaminase activity is high (specific activity 60–80). (The transaminases were measured on extract of prototroph bacteria, 60009, grown in MG; about the same specific activities were observed after growth in MG plus L-alanine, L-glutamate, or L-aspartate). Transamination between L-alanine and several other amino acids is actually so rare that L-alanine itself can barely serve as amino source, although it can undoubtedly enter the cell as evidenced by the induction of AlD.[11] This lack of amino group transfer from L-alanine has been observed most clearly when bacteria were grown in a minimal medium lacking NH_4^+. Table 4 shows that in contrast to L-alanine several other L-amino acids served as a good source of amino groups.

TABLE 4

AMINO ACIDS AS AMMONIA SOURCE

Growth Medium MG − $(NH_4)_2SO_4$ + 0.1 *M* conc. of:	Doubling Time: 60009 Prototroph	60021 Indole⁻
Nothing	>1200	>1200
L-alanine	342 → 120	480 → 115
L-aspartate	77	62
L-glutamate	128 → 103	130 → 107
L-arginine	74	65
L-ornithine	75	60
L-proline	130 → 75	126 → 82
NH_4Cl	87	74

The bacteria were grown in MG (+ 50 μg/ml L-tryp for 60021) to 1 × 10^8/ml, washed twice, and inoculated at 5 × 10^7/ml in the indicated media (containing tryp in case of 60021). Doubling times were measured during exponential growth. The arrow indicates slow adaptation from one doubling time to a lower one over an extended range of OD_{600}, the last value being that observed at OD_{600} of about 0.2.

Discussion.—Germination which is initiated by L-alanine clearly requires an active AlD since it does not occur in AlD$^-$ spores. The deamination is normally preceded by two additional reactions, the release of some compound(s) and a trigger reaction. Each of the three reactions can be activated by L-alanine.

The release is indicated by a gradual 10 per cent decrease of extinction observed when AlD$^-$ spores are exposed to L-alanine, its analogues, Ca-dipicolinate, or more slowly to buffer alone. Whereas the L-alanine-induced release of Ca^{++}[18] and dipicolinate,[19] a peptide,[20] and amino acids[18] has been demonstrated for normal spores, that of AlD$^-$ spores will have to be investigated.

The release reaction apparently reduces dormancy. Whereas freshly prepared AlD$^+$ spores germinate in L-alanine sluggishly,[21] they respond much faster after pretreatment by buffers or, most effectively, by Ca-dipicolinate. The latter has been reported to induce germination,[22] as long as the temperature is below 30°[23] and to release Ca^{45}.[23] Under our conditions (37°) only the release mechanism seems to be initiated by Ca-dipicolinate, since even in AlD$^+$ spores only the 10 per cent decrease in OD_{625} occurs.

L-alanine and its analogues must trigger germination by a mechanism different from the release reaction, since Ca-dipicolinate or buffers alone did not cause complete germination. The effect of L-alanine itself could be attributed to the deaminating activity of AlD, yielding the readily utilizable carbon source pyruvate. However, this substrate mechanism does not seem to explain the germinating effect of analogues like L-cysteine or β-alanine, which are at most very weak substrates of AlD,[24] or of good substrates that are deaminated to inefficient carbon sources. A better explanation is suggested by the observation in *B. cereus*[4] that a few minutes after the addition of L-alanine to normal (AlD$^+$) spores, NH_4^+ and pyruvate can be derived from endogenous sources. It would therefore seem most likely that L-alanine or its effective analogues trigger the liberation and deamination of *endogenous* alanine, without being required as substrates in germination. This trigger mechanism explains, e.g., the initiating effect of L-cysteine more plausibly, at least in *B. subtilis*, than an earlier proposal[6] which assumed that pyruvate is produced from L-cysteine by means of a cysteine desulfhydrase. If the desulfhydrase were involved, our AlD$^-$ spores should still germinate in the presence of cysteine; instead they undergo only the limited decrease in extinction, just as with L-alanine. The initiating alanine analogues all have an affinity toward AlD[24] suggesting that AlD itself might be involved in the trigger mechanism. For example, Ca^{++} or Mn^{++} could aggregate AlD into an inactive complex[24] in the spore; the addition of L-alanine or certain analogues might then change the folding of AlD, release the metal ions, activate AlD, and thus initiate the chain of further reactions. This aggregation would explain also the inhibition of germination by mercuric ions[21] which complex and inactivate AlD extremely efficiently.[24]

Whereas AlD is required for germination, it is not needed for the growth of vegetative bacteria, for AlD$^-$ mutants grow at the same rate as AlD$^+$ strains in minimal glucose medium. (Abnormal growth conditions can be found in which the enzyme is required for vegetative growth: if glucose is replaced by L-alanine, AlD$^+$ strains, but not AlD$^-$ strains, do grow at a slow rate.) Since GDH is also missing in our strains, ammonia seems to be mainly assimilated by L-aspartase, as was also shown by a special mutant. The amino group of aspartate is then transferred by

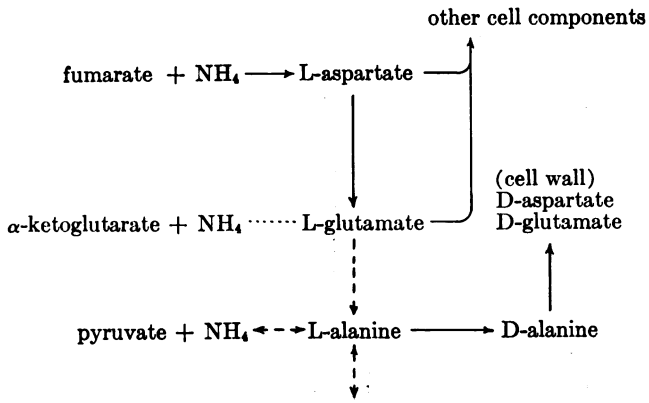

FIG. 2.—Pathway of amino groups in *B. subtilis*. The arrows indicate the major use of the enzymes when *B. subtilis* is grown in minimal medium. All reactions are readily reversible and will be used in the opposite direction when the corresponding substrate is added. —— = strong activity observed; --- = weak activity observed; . . . = no activity observed. Vertical connections are made by transaminases, horizontal connections by other enzymes.

transamination to L-glutamate and from there to other cell components (see Fig. 2). L-alanine (and the D-amino acids derived from it) may receive its amino group by transamination from L-glutamate or some other amino acid (L-serine), or by direct amination of pyruvate if the AlD activity is sufficiently high. Aspartase, which has no cofactor requirement, may be the major enzyme catalyzing ammonia assimilation in most bacilli, since GDH activity has been observed only in very few strains.[9]

Summary.—Mutants deficient in alanine dehydrogenase (AlD) activity have been isolated by a plate assay method. Whereas they sporulated normally, germination could no longer be initiated by L-alanine or its analogues. Nevertheless, the addition of these compounds, or of Ca-dipicolinate, or buffer alone, to AlD$^-$ spores did cause a partial decrease of OD_{625}, indicating the release of some compounds (s). This release seems to reduce dormancy. In addition, L-alanine or its analogues specifically trigger some other reaction required for germination. Further studies on the AlD$^-$ strain and another mutant revealed that ammonia is assimilated by aspartase rather than AlD. AlD is therefore a developmental rather than a basic enzyme.

We thank Dr. H. O. Halvorson for stimulating discussions.

Abbreviations: Tris, trishydroxymethylaminomethane; NAD, NADH, nicotinamide diphosphopyridine nucleotide, oxidized and reduced form, respectively; AlD, alanine dehydrogenase; LDH, lactic dehydrogenase; MDH, malic dehydrogenase.

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*THE FORMATION OF λ BACTERIOPHAGE BY λ DNA IN DISRUPTED CELL PREPARATIONS**

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DNA, prepared from coliphage λ, will give rise to the formation of intact λ phage in spheroplasts from a number of *E. coli* strains although incapable of infecting intact bacterial cells.[1, 2]

The present report is concerned with experiments involving λ DNA and the *E. coli* mutant 3350/λ, a strain not infected by either λ bacteriophage or λ DNA. However, formation of λ bacteriophage from *E. coli* 3350/λ can be demonstrated after the addition of λ DNA if the bacterial cells are first subjected to a variety of procedures which damage or disrupt them, but do not involve spheroplast formation. Such bacterial preparations do not show increased virus titer when intact λ bacteriophage is added.

Three disruptive processes have been studied: (1) freezing and thawing cultures of *E. coli* 3350/λ; (2) freezing the cells and disrupting them in a Hughes press;[3] and (3) mixing the cells with an equal weight of powdered Pyrex glass, freezing, and disrupting in a Hughes press.

Experimental Procedure.—Disrupted cell preparations and virus assay: *E. coli* 3350/λ, grown in Fraser medium[4] to late log phase (*ca.* 2×10^9 cells per ml) was used for preparation of disrupted cells and spheroplasts. *E. coli* W-1485 was used in the assay plating of λ bacteriophage.[5]

Cells were centrifuged at 6,000 × *g* for 20 min, washed once by resuspension in 10 ml of Fraser medium, and centrifuged at 12,000 × *g* for 10 min. The cell paste, in approximately 20-mg aliquots, was applied with the tip of a microspatula to a chilled steel block which had previously been sterilized by rinsing with 95% ethanol and chilled to −35°C with solid CO_2. The frozen