Studies in Amino Acid 'Biogenesis: the Synthesis of Alanine from Pyruvate and Ammonia

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SUMMARY: Washed suspensions of certain bacteria can synthesize alanine from pyruvate and ammonia. This has been studied in detail in *Bacillus subtilis*. Several other amino acids can be formed from the corresponding keto-acids, but synthesis was most rapid in the case of alanine. Notably, glutamic acid is formed from keto-glutarate relatively slowly, whilst oxalacetate fails to give aspartate. Pyruvate can accept an amino group from a number of amino acids by transamination (but aspartic and glutamic acids are less effective than ammonia itself). It is possible that the reductive amination of pyruvate may proceed directly rather than through transamination from some other primary amino-acceptor.

The last step in the synthesis of an amino acid is often the exchange of the carbonyl group of the appropriate keto acid for an amino group. When the ultimate source of the latter is ammonia we can refer to the reaction as 'reductive amination':

This reaction has been most studied in the case of pyruvic acid and alanine. It was first demonstrated in animals by Embden & Schmidt (1912) but was not investigated in detail until Kritzman (1947) examined the reaction in liver and kidney slices. Since then it has been demonstrated in washed suspensions of *Clostridium* sp. (Nisman, Rayneaud & Cohen, 1947) and in *Aerobacter aerogenes* (Paretsky, 1948). Holden, Wildman & Snell (1951) have also shown that *Lactobacillus* will grow on media in which certain essential amino acids have been replaced by the corresponding keto acids. Extracellular production of alanine has been demonstrated in *Corynebacterium diphtheriae* growing on casein hydrolysate medium (Linggood & Woiwod, 1949), while Goodlow, Mika & Braun (1950), Braun, Goodlow, Kraft, Altenbern & Mead (1951), and Altenbern & Housewright (1951) showed that extracellular alanine production in *Brucella abortus* had an interesting significance as a determining factor in the rough-smooth transformation. Most of their work was concerned with the pyruvate-asparagine transamination, but they also showed that alanine could be slowly formed from ammonia and pyruvate.

The first suggestion as to the mechanism of the reaction came from Kritzman's (1947) work with liver slices (cf. Kritzman & Melik-Sarkisyan, 1948):

- (1) Pyruvate + CO₂ = oxalacetate,
- (2) Oxalacetate + NH₃ = aspartate,
- (3) Aspartate + pyruvate = oxalacetate + alanine.

He supported this scheme by showing that carbon dioxide or one of the dicarboxylic acids was required and that (in the presence of carbon dioxide),

aspartate appeared diminished and alanine was the sole final product. Nisman *et al.* (1947) found an initial production of aspartate and glutamate when using washed suspensions of *Clostridium*, and Konikova, Kritzman & Jakobson (1948) and Konikova, Kritzman, Jakobson & Samarina (1949) reported similar results with *Escherichia coli*. (See also Kritzman, Jakobson & Konikova (1948); Vishepan & Mogilevsky (1948); and Jakobson, Konikova, Kritzman & Melik-Sarkisyan (1949). In most cases only summaries of the Russian papers have been available to us.) Paretsky (1948), however, found no carbon dioxide requirement for formation of alanine from pyruvate and ammonia in cell-free extracts of *Aerobacter aerogenes*.

The object of the present work was to investigate the characteristics of the system and the mechanism of the reaction in *Bacillus subtilis*.

METHODS

A strain of *Bacillus subtilis* (strain 4 in our list) was used throughout except for the comparative experiments cited in Table 8. The medium used was Nutrient Broth No. 1 (Oxo, Ltd., London), supplied in the form of dry granules. It contained Lab-Lemco, 0.1%; yeast extract, 0.2%; peptone, 0.5%; NaCl, 0.5%. Towards the end of this work we found that Nutrient Broth No. 2 (Oxo, Ltd.; Lab-Lemco, 1.0%; peptone, 1.0%; NaCl, 0.5%) gave more active suspensions and this medium was used for the experiments in Table 8.

Washed suspensions. Roux bottles containing 150 ml. medium were inoculated with a few drops of a young broth culture and incubated at 37° for 15–17 hr., i.e. until the culture was near the end of the logarithmic phase of growth. This gave maximum enzyme yield though earlier harvesting would have given greater specific activity. Old cultures were less active and gave much greater blanks for amino-N in the absence of substrate. The organisms were collected by centrifugation in glass tubes. When plastic tubes were used the suspensions were almost inactive; the reason for this is not known. After washing twice on the centrifuge with distilled water the organisms were suspended in a suitable volume of 0.2M phosphate buffer pH 7.0, to give a concentration of 15–30 mg. dry wt./ml. Repeated washing in water or saline did not impair activity (cf. Konikova *et al.* 1948). The density of the suspensions was determined turbidimetrically using the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex), which was calibrated by dry-weight determinations. In the experiment on age-of-culture effect (Fig. 4) each suspension was standardized by dry-weight determination to avoid errors arising from variation of the turbidity factor with age. Suspensions suffered no appreciable loss in activity on storage at 0° for up to 48 hr.

Reagents. During the earlier stages of this work sodium and ammonium pyruvate were prepared in solution as required, by neutralizing redistilled pyruvic acid with sodium or ammonium hydroxide at 0°. Later commercial sodium pyruvate became available and pyruvic acid was no longer used; 'ammonium pyruvate' was obtained by using a solution of sodium pyruvate and ammonium sulphate. Phenylpyruvic acid was prepared according to

Herbst & Shemin (1939). We gratefully acknowledge gifts of sodium α -keto-butyrate and α -ketoisovalerate from Dr C. Long (Aberdeen); sodium fluoracetate from Dr B. D. Saunders (Cambridge); sodium α -ketoisocaproate from our colleague Dr W. Lijinsky.

Standard experimental technique. All washed suspension experiments—including determinations of Q_{Alanine} —were performed under the following standard conditions, except where otherwise stated. The buffered bacterial suspension (40–60 mg. dry wt. of organisms), the substrate (ammonium pyruvate, final concentration 0.12M), and any special reagents (inhibitors, etc.) were placed in a 15 ml. conical glass centrifuge tube, making a total volume of 6 ml. with a final strength of buffer (phosphate, pH 7) 0.1M. The tube was placed in a 37° water bath for 3 hr. Occasionally the organisms tended to settle on the bottom of the tube and periodic mixing was necessary. The reaction was stopped by addition of 0.3 ml. 2N-HCl. In a few experiments (referred to below) the reaction was stopped by addition of trichloroacetic acid, and in the transamination experiments by heating to 100° for 5 min. This avoided the introduction of unwanted anions which might have interfered with the subsequent ion-exchange treatment. In all cases the organisms were centrifuged and discarded and the supernatant fluid stored at 0° till required. Blanks were performed by incubating substrate and suspension separately and mixing only at the end of the incubation period. The blank amino-N was usually negligible (Table 1).

Paper chromatography. Orthodox techniques were used; descending single-dimensional chromatograms were run for 24–48 hr. with a solvent mixture consisting of butanol (480 ml.), acetic acid (95 ml.), butyl acetate (35 ml.) and water (195 ml.). This system readily resolved alanine from all other amino acids normally found in proteins. In some cases results were confirmed by using the phenol + water system.

Determination of amino acids. In most experiments paper chromatography showed that only one amino acid was present (usually alanine). This amino acid could then be estimated by the nitrous acid method of Van Slyke (1929). Ammonia was present in most experiments and interfered with this method. It was removed by adding NaOH till the solution was alkaline to thymol blue, and boiling gently for a further 0.5 min. after the smell of ammonia had disappeared. Control runs showed that this removed the ammonia without loss of amino acid. In the transamination experiments where aspartic acid or glutamic acid were present as well as alanine, the latter was separated by an ion-exchange method described below.

Results are expressed in most cases in terms of Q_{Alanine} , defined as $\mu\text{l.}$ of alanine produced per mg. (dry wt.) of organism per hour. Densities of suspensions of *Bacillus subtilis* 4 were determined turbidimetrically, the instrument being calibrated by dry-weight determinations; but the results in Table 8 are based on nitrogen determinations and are expressed as $q_{\text{Alanine}}^{\text{N}}$.

RESULTS

Characteristics of the system

Variance between replicates. Washed suspensions were prepared from 18 identical cultures of *Bacillus subtilis* 4 and the Q_{Alanine} value for each was determined under standard conditions. The coefficient of variation of an individual determination was 5.2%.

Table 1. *Synthesis of alanine*

Reagents and suspension were incubated together at 37°, the reaction stopped by addition of 0.06 ml. of 2N-HCl, the organisms removed by centrifugation and the amino-N determined in the supernatant.

<i>B. subtilis</i> (50 mg./ml.) (ml.)	2	2	2	2
Sodium pyruvate (0.5M) (ml.)	3	3	3	—
Ammonium sulphate (0.25M) (ml.)	3	3	—	3
Water (ml.)	—	—	3	3
Incubation time (hr.)	3	0	3	3
Alanine produced (mg. N)	5.02	0.05	0.00	0.08

Overall reaction. Table 1 shows that pyruvate and ammonia were required. In this experiment the concentrations of both organism and substrate used were a little higher than in the standard technique, to facilitate detection of any alanine formed in the absence of substrate or suspension. Although the reaction involves reduction no separate reducing agent was required, the pyruvate itself acting as hydrogen donor and the addition of a separate H-donor, e.g. formate did not accelerate the reaction. Up to 25% of the pyruvate metabolized was converted to alanine; the remainder underwent dismutation. The fate of the pyruvate was discussed in a preliminary note by Sewell & King (1954). The ammonia is used exclusively for alanine formation (Table 2).

Table 2. *Nitrogen balance in alanine synthesis*

A suspension of *Bacillus subtilis* (4 ml., 100 mg. total dry wt.) was incubated with ammonium sulphate (1 ml. of 0.25M) and sodium pyruvate (6 ml. of 0.5M), for 3 hr. at 37°. 3 ml. of 10% trichloroacetic acid was added and the material centrifuged. Total N was determined in the deposit. The supernatant was assayed for ammonia and alanine. In the control, trichloroacetic acid was added *before* incubation.

	Expt. (mg. N)	Control (mg. N)
'Protein'	5.88	5.86
Alanine	3.89	0.00
Ammonia	3.15	7.17
Total	12.92	13.03

Product of reaction. Alanine was demonstrated by paper chromatography and by isolation and chemical characterization; it proved to be the DL-mixture (Fairhurst, 1952).

Time of incubation. A 3 hr. incubation period at 37° was normally used; alanine synthesis was almost linear over this period but further incubation produced an appreciable falling-off in the rate of synthesis.

pH value of optimum activity. The fairly sharp optimum at pH 6.8–7.0 (Fig. 1) was lower than the figure of pH 8.5 given by Kritzman *et al.* (1948) for liver.

Concentration of NH_4^+ . The Q_{Alanine} value was determined in a series of experiments in which the ammonium sulphate concentration was varied from 0.5 to 0.03 M. The pyruvate concentration was constant at 0.25 M. Thin suspensions (2 mg./ml.) were used to ensure that, even at the lowest NH_4^+ concentration, 80 % of the ammonia would remain unchanged. Otherwise, the experiments were performed under standard conditions. Fig. 2 shows that even the lowest ammonium sulphate concentration (0.03 M) saturated the system.

Concentration of pyruvate. Q_{Alanine} was determined in a series of experiments in which the pyruvate concentration was varied. The ammonium sulphate concentration was constant at 0.12 M. Again thin suspensions were used, and incubation also was shortened to 2 hr. This ensured that the pyruvate concentration did not fall significantly during the experiment even at the lowest concentrations used (0.015 M). Pyruvate determinations were performed at the end of the experiment and showed that in no case had more than one-third of the pyruvate been utilized. The Michaelis constant, K_s , was determined by the reciprocal plot method of Lineweaver & Burk (1934); Fig. 3 gives the plots for experiments with two different suspensions, giving K_s values of 0.12 and 0.13 M respectively. It is not known, however, whether these surprisingly high values represent a true affinity constant for the system or whether other factors are involved such as competition for pyruvate by other metabolic pathways.

Presence of oxygen. The majority of experiments were performed in test tubes with no special precautions to ensure either aeration or anaerobiosis. No increased activity was found when nitrogen-filled Thunberg tubes were used, but vigorous aeration had a marked adverse effect. This accords with the findings of Altenbern & Housewright (1951) for alanine synthesis in *Brucella abortus*; see also Fowler & Werkman (1955). We found the alanine yield decreased by about half on shaking in air-filled Warburg manometers; more thorough aeration reduced the yield to zero; activity was fully restored when aeration was stopped. Whether this was a true inhibition of the enzyme or whether it arose from the diversion of the pyruvate to other metabolic channels is not known. The organism itself cannot grow under strict anaerobiosis.

Nature of keto acid. Various keto acids (neutralized to pH 7 with NaOH) were incubated with ammonium sulphate under standard conditions, save that the final concentration of keto-acid was 0.25 M, and the reaction was arrested by addition of trichloroacetic acid (final concentration, 2.5 %). Parallel experiments with pyruvate were set up in each case. The cells were removed by centrifugation and the supernatants examined by paper chromatography and assayed for amino-N. In each case only one amino acid was found. The relative rates of formation were (alanine=100), valine from α -keto-*iso*-valeric acid, 65; leucine from α -keto-*iso*-caproic acid, 50; α -aminobutyric acid from α -ketobutyric acid, 40; glutamic acid from α -ketoglutaric acid, 10. No reductive amination of phenylpyruvic acid was observed. Oxalacetic acid gave

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 1. Effect of pH on rate of alanine synthesis. Q_{Alanine} was determined under standard conditions, the reagents being buffered with 0.1 M-acetate (pH 4.5–5.5), 0.1 M-phosphate (pH 6–8) or 0.1 M-borate (pH 8.5–9.5).

Fig. 2. Relation between ammonium salt concentration and rate of alanine synthesis. Q_{Alanine} was determined under standard conditions save that the final concentration of the organism was 2 mg./ml. dry wt. Sodium pyruvate concentration, 0.12 M; final concentrations of ammonium sulphate indicated on abscissa.

Fig. 3. Relation between pyruvate concentration and rate of alanine synthesis: reciprocal plots of two experiments. (Method of Lineweaver & Burk, 1934.) Q_{Alanine} was determined under standard conditions, except that thin suspensions were used (final concentration of organism, 1 mg./ml. (upper line) and 0.5 mg./ml. (lower line) and the incubation time was reduced to 2 hr. This ensured that the pyruvate concentration was not significantly reduced during the experiment, even at the lowest concentration employed. Pyruvate estimations at the end of the experiment confirmed this. Ammonium sulphate concentration, 0.12 M throughout. Reciprocal of Q_{Alanine} ($1/V$) plotted against reciprocal of molar pyruvate concentration ($1/S$).

considerable amounts of alanine but no aspartic acid; presumably it was decarboxylated to pyruvate, which then formed alanine. The negative results with oxalacetic acid and the slow reaction of α -ketoglutaric acid were important as they might be regarded as excluding these substances as intermediates in the formation of alanine. Two possibilities must be considered: (1) Failure of the keto acids to penetrate the cell membrane. This was investigated by freeing a suspension of the organism from endogenous substrates (as far as possible) by aerating for $1\frac{1}{2}$ hr. at 37° , and examining its capacity for dehydrogenating various substrates on incubation with methylene blue in Thunberg tubes (Table 3). Since both oxalacetate and α -ketoglutarate were more rapidly oxidized than formate or pyruvate, it seems reasonable to suggest that they could enter the cell readily. (2) It had been assumed that oxalacetate and α -ketoglutarate (like pyruvate) need no separate H-donor for reductive amination, and their ready reduction of methylene blue would support this view. However, suspensions of the organism were incubated with the keto acids and ammonia in the presence of formate, shown to be an active H-donor (Table 3); no increase in reductive amination was observed.

Table 3. *Reduction of methylene-blue by various substrates in the presence of organism*

The body of each Thunberg tube contained 1 ml. substrate (0.1 M), 2 ml. of 0.2 M-phosphate buffer, pH 7, and 1 ml. of 0.0002 % methylene blue. The hollow stoppers contained 1 ml. (9.5 mg. dry wt.) of washed suspension of *Bacillus subtilis*. The tubes were filled with oxygen-free nitrogen and placed in a water bath at 37° ; the contents were mixed after 12 min. and the time required for complete reduction recorded.

Substrate	Reduction time (min.)
None	7.5
Sodium pyruvate	2.0
Sodium α -ketoglutarate	1.1
Sodium oxalacetate	1.5
Sodium formate	2.5

Search for intermediate products. Washed suspensions were incubated with pyruvate and ammonia under standard conditions and the products examined by paper chromatography at 20 min. intervals up to 2 hr. A steady production of alanine was found, without trace of any other amino acid.

Carbon dioxide not required. The formation of carbon dioxide by dismutation of pyruvate (Sewell, 1954) makes establishment of 'CO₂-free' conditions difficult. An experiment (Table 4) was performed, however, in which we endeavoured to remove the carbon dioxide as fast as it was formed by working at pH 6.5 and shaking in Warburg manometers with KOH in the centre cups. At pH 6.5 reductive amination is still fairly rapid but carbon dioxide is readily expelled from the solution. Table 4 shows that the 'CO₂-free' system (Expt. 2) was as active as the control (Expt. 1) in which KOH was not present in the manometer. Addition of oxalacetate or α -ketoglutarate did not accelerate the reaction (Expts. 3 and 4).

Transamination. If the reaction proceeds through a transamination mechanism involving the dicarboxylic acids these should be at least as effective NH_2 -donors as ammonia itself. This was investigated experimentally. The organism was incubated with the reagents shown in Table 5. The reaction was stopped by heating ($100^\circ/5$ min.) and the cells removed by centrifugation. The supernatant was passed down a column (18×1.2 cm.) of the cation-exchanger Zeokarb 225 (Permutit Co., London), treated with HCl and washed. This retained the amino acids but passed through pyruvic acid and other anions. The

Table 4. *Effect of carbon dioxide and dicarboxylic acids*

Suspension previously shaken in air for $1\frac{1}{2}$ hr./ 37° to oxidize endogenous metabolites. All reagents (except KOH) buffered at pH 6.5 with 0.1M-phosphate. Manometers filled with oxygen-free nitrogen and shaken for 1 hr./ 37° before tipping. Shaking continued for further 3 hr. Manometers dismantled and pH determined; no change detected. 0.9 ml. 10% trichloroacetic acid added. Organisms spun down and supernatants assayed for amino-N. Controls were identical with experimental series but side-bulb tipped at end of experiment.

		Expt. ...	1	2	3	4
Main flask	Suspension (40 mg. dry wt.) (ml.)	2	2	2	2	
	Buffer, pH 6.5 (ml.)	0.4	0.1	—	—	
Centre cup	KOH, 10 % (ml.)	—	0.3	0.3	0.3	
Side bulb	Ammonium pyruvate, 0.1 M (ml.)	0.6	0.6	0.6	0.6	
	Oxalacetate, 0.05 M (ml.)	—	—	0.1	—	
	α -ketoglutarate, 0.05 M (ml.)	—	—	—	0.1	
	Q_{Alanine}	20.6	20.6	17.0	18.3	

Table 5. *Formation of alanine by transamination*

Final concentrations of reagents are given; total volume, 30 ml. in each experiment. Organism and reagents (buffered to pH 7), incubated 3 hr. at 37° . Reaction stopped by heating to 100° for 5 min. Alanine separated and determined as described in text.

Organism (50 mg.)	+	+	+	+
Pyruvate, 0.25M	+	+	+	+
Ammonium sulphate, 0.025M	—	+	—	—
Glutamate, 0.05M	—	—	+	—
Aspartate, 0.05M	—	—	—	+
Alanine formed (mg. N)	0.12	2.88	0.66	0.60

column was washed with water and then eluted by perfusion with 0.5N-ammonia. The effluent was collected by means of a fraction-collector and the fractions giving a positive ninhydrin reaction were pooled. No separation of the amino acids was attempted at this stage. Most of the surplus ammonia was removed by boiling; the solution was then adjusted to pH 4 (HCl) and passed down a column (18×1.2 cm.) of the anion-exchanger Deacidite FF (Permutit Co., London), previously treated with 0.2M-sodium acetate. The column (which retained aspartic and glutamic acids, but not alanine) was washed with water, the washings added to the effluent, and the alanine estimated after confirmation by paper chromatography that no other amino acid was present. Artificial mixtures of alanine, glutamate, and ammonium pyruvate similar to the experimental supernatants were subjected to the ion-exchange separation and gave quantitative recovery of alanine.

The ability of other amino acids to transaminate to pyruvate was investigated qualitatively. Suspensions of the organism were incubated with pyruvate and the appropriate amino acid, and the products examined by paper chromatography. The degree of transamination was judged by the strength of the alanine spot obtained on spraying with ninhydrin. Leucine, tyrosine and arginine were about as active as ammonia itself; valine, phenylalanine, histidine, methionine, less active; tryptophan, serine and threonine, weakly active; proline, lysine, cysteine and glycine gave no appreciable transamination.

Table 6. *Action of inhibitors on alanine synthesis*

Washed suspensions of *Bacillus subtilis* 4 were incubated for 30 min. at 37° with the inhibitor at pH 7. Substrate (ammonium pyruvate) was then added and incubation continued for 3 hr. under standard conditions. The reaction was stopped by addition of HCl. The organisms were removed by centrifugation and the amino-N determined in the supernatants. Final concentrations of inhibitors are recorded in the table.

The following showed no inhibition: sodium cyanide, semicarbazide hydrochloride, hydroxylamine hydrochloride, hydrazine hydrochloride, sodium fluoracetate (all 0.002M); penicillin, streptomycin, chloromycetin, sulphathiazole (all 0.2%).

Inhibitor		Q_{Alanine}	Inhibition %
None	—	29.0	—
Iodoacetate	0.004M	5.2	82
	0.0004M	17.7	39
Sodium fluoride	0.004M	24.3	16
	0.0004M	28.4	10
2:4-Dinitrophenol	0.002M	17.6	39
	0.0002M	28.5	10
Sodium azide	0.002M	21.7	25
	0.0002M	27.8	10
Sodium arsenite	0.002M	3.5	88
	0.0002M	13.2	54
8-Hydroxyquinoline	0.001M	22.3	23
	0.0001M	28.5	10
None	—	30.5	—
8-Hydroxyquinoline	0.004M	5.5	82
	0.002M	10.5	64
None	—	31.8	—
Aureomycin	0.2%	17.8	44
	0.02%	26.5	17

Inhibitors. The effect of various inhibitors is given in Table 6. The organism was incubated with the inhibitor for 30 min. before adding the substrate (ammonium pyruvate). It is not possible, however, to distinguish between the inhibition of the alanine synthesis itself or the accompanying dissimilation of pyruvate which may be necessary for the reductive amination process.

Attempts to prepare isolated enzyme extracts. Study of the mechanism of reductive amination would be much easier if the reaction could be demonstrated in cell-free extracts. But though the system was quite stable in the washed suspensions any interference with the integrity of the cells resulted in severe loss. Thus even lyophilization destroyed 60–80% of the activity. Attempts at preparing active extracts have so far not been successful.

Factors determining activity of system

Age of culture. Activity was greatest in growing cultures and declined rapidly when growth ceased (Fig. 4). This raised considerable difficulties when investigating the influence of other cultural conditions, since it was necessary to determine the growth curve in each case in order to harvest at the same stage of growth. The poorer activity of older cultures may be due in part to exhaustion of the medium. Thus, if the organisms are removed from a 24 hr. broth culture and the medium resterilized and re-inoculated, good growth is obtained but the washed suspensions show poor activity. The nature of the substances removed during growth has not been determined. Sewell & King (1955), however, have shown that although the organism will grow on a simple synthetic medium, the reductive amination system does not develop its full activity unless certain amino acids are added to the medium.

Fig. 4. Relation between age of culture at harvesting and activity of washed suspensions. Organisms grown in Roux bottles on nutrient broth no. 1, harvested after incubation at 37° for time specified, washed suspensions prepared and incubated at 37° for 4 hr. with 0.1M ammonium pyruvate. Density of each suspension determined by dry-weight determinations.

pH of growth medium. Three sources of error had to be avoided: (1) Growth was much slower at the extreme pH values. Hence if all cultures were harvested at, say, 24 hr., it was found that those from acid or alkaline media were most active. This was due to the age-of-culture of effect, the neutral cultures being more mature and therefore less active. If, however, growth curves were prepared for each pH value and the organisms harvested towards the end of the logarithmic phase of growth, optimal activity was obtained over the neutral pH range most favourable for growth (Table 7). (2) A considerable pH shift may occur during growth in nutrient broth. Heavy buffering was undesirable in view of the risk of toxic effects with high salt concentrations, so some change in pH had to be tolerated. The buffer concentrations used and the extent of the pH shift in each case are recorded in Table 7. (3) It is known that

in some cases the pH optimum of an enzyme is dependent on the pH of the growth medium. This was not found to occur in our system.

Access to oxygen. The organism cannot grow in the anaerobic jar, so the effect of anaerobiosis could not be studied. Active suspensions could be obtained, however, from cultures grown under forced aeration.

Temperature. Here also age-of-culture effects had to be considered. Growth curves were constructed for the organism at both 27 and 37°, and it was found that 39 and 18 hr. respectively represented corresponding physiological ages. Q_{Alanine} values of 32 and 30 were obtained for the 18 hr./37° culture and 45 and 40 for the 39 hr./27° culture. The optimum temperature for enzyme formation may be a little below that for growth but the difference is not marked.

Table 7. *Effect of pH of growth medium on activity on suspensions*

Nutrient broth no. 1 was buffered as indicated ('Initial' pH) and inoculated with the organism. After harvesting at the specified time the pH of the medium was determined and recorded ('final' pH). Washed suspensions were prepared and incubated with ammonium pyruvate for 3 hr. at 37° under the standard conditions. The reaction was terminated by addition of HCl, the organisms centrifuged down, and the amino-N determined in the supernatants.

pH of nutrient broth		Buffer	Age at harvesting (hr.)	Q_{Alanine}
Initial	Final			
5.4	5.8	Phthalate, 0.05 M	66	3.2
6.0	6.3	Phosphate, 0.1 M	15	25.5
7.1	7.5	Phosphate, 0.02 M	15	25.2
8.2	8.3	Phosphate, 0.1 M	39	12.5
9.1	8.6	Borate, 0.2 M	63	4.3

Strain of organism. Growth curves were determined for the organisms listed in Table 8, using nutrient broth no. 2. Washed suspensions were prepared from cultures harvested towards the end of the logarithmic phase and incubated with ammonium pyruvate under standard conditions. In all cases alanine was the only amino acid detected both before and after acid hydrolysis. This was established by paper chromatography in the two solvent systems. It seems that reductive amination is widely but somewhat irregularly distributed. Two out of six strains of *Bacillus* and the four Gram-negative organisms showed little activity under the standard conditions of test, known to be the most suitable for *Bacillus subtilis* 4. These conditions may not necessarily have been the best for some of the other organisms investigated, and the activities given in Table 8 must be interpreted with this in mind.

Formation of alanine in growing cultures. The results described so far were obtained under the somewhat artificial conditions of non-proliferating suspensions. A few confirmatory experiments were made with growing cultures. *Bacillus subtilis* 4 was grown in a mineral medium (no. VII, Stephenson, 1949) with ammonium pyruvate (2%), ammonium lactate (2%), or glucose (2%) as sole organic nutrient. Paper chromatography of the culture filtrates (butanol/acetic acid/butyl acetate system) indicated plentiful alanine production on the pyruvate medium, somewhat less on lactate. On glucose, alanine appeared in

small amounts accompanied by some aspartic acid. The organisms listed in Table 8 (except *Pseudomonas aeruginosa* and *Bacillus megaterium* 7581) were also grown on the pyruvate medium, a very heavy broth inoculum being used to ensure good growth of the nutritionally exacting types. Control experiments showed that this did not introduce detectable amounts of free amino acids into the system. The culture filtrates were examined after several days' growth and all showed plentiful alanine production except *Aerobacter aerogenes*. It will be noted that several organisms which were poor alanine producers in the washed suspensions gave good yield in the growing cultures. In no case was any amino acid other than alanine found in appreciable amount. Certain of these organisms were also grown in the lactate and glucose media. Most gave alanine readily in the lactate medium. With glucose, alanine production was detectable in most cases; moreover (except in *Aerobacter aerogenes*), it was the only amino acid detectable save in very old cultures undergoing autolysis.

Table 8. *Comparative rates of alanine synthesis*

Organisms with NCTC numbers were obtained from the National Collection of Type Cultures; others were stock strains and the numbers refer to our own list. They were grown on nutrient broth no. 2 and harvested at the specified times. These had been determined in each case to be near the end of the phase of active growth. The washed suspensions were incubated with ammonium pyruvate under the standard conditions cited in the text. The reaction was terminated by addition of HCl, the organisms removed by centrifugation and the amino-N-estimated in the supernatants.

Organism	Age at harvesting (hr.)	Gram	q_{Alanine}^N	
<i>Bacillus subtilis</i> 4	16	+	1070	1040
<i>Bacillus subtilis</i> NCTC 3610	16	+	210	140
<i>Bacillus megaterium</i> NCTC 2605	45	+	220	190
<i>Bacillus megaterium</i> NCTC 7581	48	+	40	40
<i>Bacillus mycoides</i> 5	16	+	670	640
<i>Bacillus pumilus</i> NCTC 7576	24	+	30	50
<i>Sarcina lutea</i> 7	45	+	330	240
<i>Sarcina lutea</i> 21	72	+	200	160
<i>Staphylococcus aureus</i> 23	24	+	190	110
<i>Streptococcus lactis</i> NCTC 662	18	+	230	180
<i>Streptococcus faecalis</i> 9	45	+	10	
<i>Mycobacterium phlei</i> NCTC 525	72	(Acid-fast)	190	130
<i>Proteus vulgaris</i> 10	16	—	10	10
<i>Escherichia coli</i> 106	16	—	30	10
<i>Aerobacter aerogenes</i> NCTC 418	18	—	10	10
<i>Pseudomonas aeruginosa</i> 15	16	—	30	10

DISCUSSION

Two types of mechanism may be considered for this reaction: (1) Indirect, by primary incorporation of the amino group into a dicarboxylic acid either through the mechanism shown to operate in liver slices by Kritzman (1947) or through the reversible glutamic dehydrogenase demonstrated in *Escherichia coli* by Adler, Hellström, Gunther & Von Euler (1938). The amino group would then be transferred to pyruvate by transamination. (2) Direct, not involving any intermediate dicarboxylic acid.

We have not found intermediate formation of aspartic or glutamic acid, as did Kritzman (1947) in liver slices or Nisman *et al.* (1947) in clostridial suspensions. Neither could we demonstrate a requirement for carbon dioxide or dicarboxylic acids. We recognize the difficulty of eliminating these substances when using intact cells. But if *Bacillus subtilis* has a carbon dioxide requirement comparable with that of Kritzman's or Nisman's systems, we could have reasonably expected to detect it. While recognizing that negative results can never be regarded as conclusive, we consider our findings not to be without significance. The observations that alanine was more rapidly formed when ammonia itself rather than aspartate or glutamate was the amino donor, and that neither oxalacetate nor α -ketoglutarate were as rapidly aminated as pyruvate also introduce the possibility that conversion of pyruvate to alanine might perhaps proceed directly rather than through transamination. We are fully aware, however, that in experiments with intact cells negative results may arise from failure of the reagents to penetrate the cell and that a transamination mechanism might be catalysed by traces of aspartate or glutamate still retained within the washed cells. It is interesting, however, that Shah (1956, unpublished observations) reports that the poor activity of cells grown on a synthetic medium is not enhanced by addition of 0.5% glutamate to the medium. We recognize that our experiments do not exclude the possibility of a transamination mechanism, and that establishment of the mechanism of the reaction may be impossible unless active cell-free extracts can be obtained.

If, however, amination can occur directly this raises the question of the alanine-pyruvate system as a path of entry for the amino group. Rowsell (1951) showed that in animal tissues alanine can participate—without intervention of glutamate—in a wide range of transaminations. It is significant that a wide range of organisms, when grown on glucose, liberate into the medium alanine but no other amino acid. Pyruvate is normally only a transitory intermediate in metabolism but may accumulate under certain conditions and even be liberated into the external medium (Dagley, Dawes & Morrison, 1951).

Since completion of this work, Wiame & Pierard (1955) have presented spectroscopic evidence for a reversible and specific DPN-coupled L-alanine dehydrogenase in a mutant of *Bacillus subtilis*. They show that the oxidative deamination of aspartate, glutamate and glycine proceeds indirectly by transamination with pyruvate followed by oxidative deamination of the alanine thus formed. Since this enzyme is reversible, it provides a potential means of direct alanine synthesis.

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