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Studies on *Escherichia coli* Enzymes Involved in the Synthesis of Uridine Diphosphate-*N*-Acetyl-Muramyl-Pentapeptide

E. J. J. LUGTENBERG

Laboratory for Microbiology, State University, Catharijnesingel 59, Utrecht, The Netherlands

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The specific activities of L-alanine:D-alanine racemase, D-alanine:D-alanine ligase, and the L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanyl-D-alanine adding enzymes were followed during growth of *Escherichia coli*. The specific activities were nearly independent of the growth phase. D-Alanine:D-alanine ligase was inhibited by D-alanyl-D-alanine, D-cycloserine, glycine, and glycyl-glycine. L-Alanine:D-alanine racemase was found to be sensitive to D-cycloserine, glycine, and glycyl-glycine. The L-alanine adding enzyme was inhibited by glycine and glycyl-glycine.

Uridine-5′-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-*m*-diaminopimelic acid-D-Ala-D-Ala (further referred to as UDP-MurNAc-pentapeptide) are the precursors for the murein of *Escherichia coli* (7). The amino acids are added to UDP-MurNAc by specific adding enzymes. The presence of four of these enzymes, adding L-alanine, D-glutamic acid, L-lysine, and the dipeptide D-alanyl-D-alanine, respectively, to the appropriate uridine nucleotide has been demonstrated in *Staphylococcus aureus* (4-6). All adding enzymes are highly specific for both uridine nucleotides and amino acid substrates, they require Mg or Mn ions and adenosine triphosphate (ATP), and possess *p*H optima of about 8.5.

D-Alanine is probably synthesized from L-alanine by racemization. The presence of L-alanine:D-alanine racemase in *E. coli* K-12 has been demonstrated by Wijsman, who also described a temperature-sensitive lysis mutant lacking L-alanine:D-alanine racemase activity in vitro (Thesis, Amsterdam, 1970).

D-Alanine can be converted to D-alanyl-D-alanine by D-alanine:D-alanine ligase. This enzyme has been detected in *E. coli* (2), *S. aureus* (6), and *Streptococcus faecalis* (13, 15, 16).

A number of components that interfere with the alanine metabolism in murein synthesis have been described. D-Cycloserine (DCS) has a conformation similar to that of D-alanine (21) and inhibits D-alanine:D-alanine ligase and alanine racemase in *S. aureus* (20) and *S. faecalis* (14). In *E. coli*, D-alanine:D-alanine ligase appears to be the major site of inhibitory action by DCS (Chambers et al., Bacteriol. Proc. p. 119, 1963).

D-Alanyl-D-alanine inhibits the D-alanine:D-alanine ligase of *S. faecalis* in vitro (16).

There are indications that high concentrations of glycine have a specific action on the mucopeptide layer of *E. coli* (8) and cause accumulation of uridine nucleotides in *S. aureus* (19), *Vibrio fetus* (3), and *Mycobacterium smegmentis* (23). The uridine nucleotides accumulated in *S. aureus* have been identified tentatively as UDP-MurNAc, UDP-MurNAc-L-Ala-D-Glu-L-Lys, UDP-MurNAc-tripeptide, and UDP-MurNAc-pentapeptide, the latter two containing glycine instead of L-alanine (19). The authors propose that inhibition of the L-alanine adding enzyme would be a sufficient explanation of the physiological effects of glycine, although they do not exclude other effects of glycine on cell wall synthesis. Yabu (23) purified D-alanine:D-alanine ligase from *M. smegmentis* and found that the enzyme activity was inhibited for about 50% by 0.1 M glycine or glycyl-glycine. Both components gave rise to uridine nucleotide accumulation in vivo. This effect was reversed by D-alanyl-D-alanine, L-alanine, and D-alanine in this order of effectivness. On the basis of these results Yabu (23) proposed that the action of glycine and glycyl-glycine on the growth of *M. smegmentis* is due to the inhibition of the L-alanine

adding enzyme to a large extent and to the partial inhibition of the D-alanine:D-alanine ligase.

Neither the presence in *E. coli* of the enzymes enumerated above nor their inhibition by the compounds mentioned has been well described. Because our results with *E. coli* mutants in murein synthesis indicated that a number of them possessed a defective enzyme in the synthesis of murein precursors (12), we decided to study first a number of properties of these enzymes including their possible inhibition by the above-mentioned compounds. The results of this study are presented in this paper.

MATERIALS AND METHODS

Chemicals. D-Alanyl-D-alanine was obtained from International Chemical and Nuclear Corp., City of Industry, Calif., D-cycloserine from Fluka, Buchs, Switzerland, and novobiocin from Upjohn Co., Kalamazoo, Mich.

Radiochemicals. ^{14}C-L-alanine (U), specific activity 156 mCi/mmole, ^{14}C-D-alanine (U), specific activity 40.9 mCi/mmole, and DL-glutamic acid-*1*-^{14}*C*, specific activity 19.8 mCi/mmole were obtained from the Radiochemical Centre, Amersham, England. α-ϵ-Diaminopimelic acid-*1(7)*-^{14}*C* (Dpm), specific activity 9.8 mCi/mmole was obtained from Calbiochem, Los Angeles, Calif., and ^{14}C(U)-D-alanyl-^{14}C(U)-D-alanine, specific activity 64.4 mCi/mmole was prepared from ^{14}C(U)-D-alanine as described previously (12).

Strains. *E. coli* K-12, strain KMBL-146, and its temperature-sensitive lysis mutant TKL-10 were obtained from A. Rörsch (Medical Biological Laboratory RVO-TNO, Rijswijk, The Netherlands). *E. coli* strain K-235 was obtained from McIntire (Abbott Laboratories, North Chicago, Ill.). A spontaneous streptomycin-resistant derivative of this strain was used. The genetic markers of these strains have been described previously (12).

Buffers. The buffers used were as follows: A, 0.02 M potassium-phosphate, *p*H 7.8; B, 0.02 M potassium-phosphate, *p*H 7.2; C, a solution of 0.2 M tris-(hydroxymethyl)aminomethane-hydrochloride, 0.01 M $MnCl_2$, and 0.02 M ATP, final *p*H 8.5.

Solvents. The following solvents were used: A, isobutyric acid-1 M ammonia (5:3, v/v); B, ethanol-1 M ammonium acetate, *p*H 7.2 (5:2, v/v).

Uridine nucleotides. UDP-MurNAc was accumulated by *S. aureus* strain 524/SC as described by Wishnow et al. (22). To cells growing exponentially in CGPY medium (10) supplemented with 0.5% glucose, novobiocin was added in a final concentration of 64 μg/ml. Cells were harvested after shaking at 37 C for 1 hr. UDP-MurNAc was extracted from washed cells and purified as described earlier for UDP-MurNAc-pentapeptide (11), except that an NaCl gradient of 0.0 to 0.6 M was used in the Dowex column. The accumulation and purification of UDP-MurNAc-L-Ala, UDP-MurNAc-L-Ala-D-Glu and UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm has been described previously (11, 12).

Preparation of enzyme extracts (4). All strains were grown at 37 C in CGPY medium supplemented with 0.5% glucose. An overnight culture was diluted 1:10 and incubated at 37 C under aeration. When the optical density (OD) was half of that of an overnight culture, a 500-ml culture was centrifuged at 18,000 × *g* at 4 C for 10 min. All subsequent operations were performed at 4 C. The pellet was washed twice in buffer A and resuspended in 7.5 ml of the same buffer. The concentrated cells were disrupted by ultrasonic oscillation (10). The suspension was centrifuged for 10 min at 24,000 × *g*. Three volumes of saturated ammonium sulfate, *p*H 7.4, containing 10^{-4} M ethylenediaminetetraacetic acid were added dropwise to the supernatant fluid. The precipitate was sedimented by centrifugation at 24,000 × *g* and dissolved in 3 ml of buffer B. The solution was stored at −20 C in 0.5-ml portions until used. The protein concentration, determined by the method of Lowry et al. (9), was about 8 mg/ml.

Enzyme assays (4). For the determination of adding enzymes, 10-μliter amounts of the following solutions were mixed in the cold. (i) The appropriate radioactive amino acid or dipeptide (^{14}C-L-alanine, specific activity 156 mCi/mmole; ^{14}C-DL-glutamic acid, specific activity 3.65 mCi/mmole of D-glutamic acid; ^{14}C-Dpm, specific activity 9.8 mCi/mmole; or ^{14}C-D-alanyl-^{14}C-D-alanine, specific activity 20 mCi/mmole); all solutions contained a radioactive concentration of 10 μCi/ml. (ii) A 2 mM solution of the appropriate uridine nucleotide. (iii) Buffer C. (iv) When the L-alanine adding enzyme was determined, a freshly prepared solution of DCS (0.01 M) was added to inhibit alanine racemase. When the other enzymes were determined, 10 μliters of distilled water was added. Instead of distilled water, solutions of inhibitors were sometimes added. (v) Enzyme solution in the appropriate dilution.

For the assay of D-alanine:D-alanine ligase, the following solutions were mixed: (i) 10 μliters of ^{14}C-D-alanine, specific activity 40.9 mCi/mmole, 20 μCi/ml; (ii) 10 μliters of buffer C; (iii) 20 μliters of distilled water or solutions of components to be tested; and (iv) 10 μliters of enzyme solution. In most experiments 10 μliters of UDP-MurNAc-tripeptide (2 mM) and 10 μliters of distilled water were added instead of 20 μliters of water.

L-Alanine:D-alanine racemase was determined in a mixture containing 10 μliters of each of the following solutions: (i) ^{14}C-L-alanine (U), specific activity 156 mCi/mmole, 10 μCi/ml; (ii) UDP-MurNac-tripeptide (2mM); (iii) pyridoxal-5-phosphate (2 mM), which was replaced by distilled water in most of the experiments because it did not stimulate the enzyme activity; (iv) buffer A; and (v) undiluted enzyme solution. The D-alanine formed by the racemase was converted to D-alanyl-D-alanine, which, in turn, was added to UDP-MurNAc-tripeptide. The latter two reactions were rather quantitative (*see below*) and catalyzed by enzymes present in the extract. After incubation, the enzymes were inactivated by heating for 2 min in a boiling-water bath.

After cooling, each mixture was applied as a 1.5-cm streak to Whatman no. 1 chromatography paper and chromatographed in solvent A for 16 hr. After autoradiography (10) for 5 days, radioactive spots were excised and counted in a liquid scintillation counter.

RESULTS

General assay procedures. The enzyme preparations of strain KMBL-146 could be stored at −20 C for at least 5 weeks without significant loss of activity of the four adding enzymes, the L-alanine:D-alanine racemase, and the D-alanine:D-alanine ligase. All enzyme activity was located in the soluble fraction. After disruption, the washed pellet contained in all cases less than 1% activity of the four adding enzymes and the D-alanine:D-alanine ligase. The absence of alanine racemase activity in the pellet could not be demonstrated because the assay method is based on the presence of D-alanine:D-alanine ligase activity, which is absent in the pellet. Although autoradiograms suggested that the radioactive uridine nucleotide products were sometimes contaminated, they were essentially pure, as shown by elution from the paper and rechromatography in solvent A for 4 days and in solvent B for 16 hr. Autoradiograms from these chromatograms showed only one spot.

When the L-alanine adding enzyme was assayed in the presence of DCS, no other radioactive product than UDP-MurNAc-L-Ala was formed. In the absence of DCS, a small amount of ^{14}C-D-Ala-D-Ala and a very small amount of pyruvic acid were formed.

Besides UDP-MurNAc-dipeptide, two radioactive compounds were formed during assay of the D-glutamic acid adding enzyme. Because they were formed in the same amounts in the absence of UDP-MurNAc-L-Ala, they probably are unrelated to the D-glutamic acid adding enzyme activity. They could be separated easily from UDP-MurNAc-dipeptide by chromatography and therefore did not interfere with the assay.

During assay of the *m*-Dpm adding enzyme and the D-alanyl-D-alanine adding enzyme, no by-products were observed.

Later in this paper it will be shown that the specific activity of the D-Ala-D-Ala adding enzyme is many times higher than that of the ligase, which, in turn, is higher than that of the racemase. We have used these phenomena in the assay of the latter two enzymes. When UDP-MurNAc-tripeptide was included in the assay mixture, D-alanyl-D-alanine was almost quantitatively converted to UDP-MurNAc-pentapeptide.

To check this new racemase assay method, we compared the specific activities of this enzyme in extracts of strain KMBL-146 and its temperature-sensitive lysis mutant TKL-10. Wijsman (Thesis, Amsterdam, 1970) found that the racemase activity in the mutant was 11% and 3% when determined by the method of Berberich et al. (1) at 28 and 42 C, respectively. With our assay procedure we found 9.0 and 9.8% at 30 C and 42 C, respectively. These results indicate that our assay procedure is useful. No efforts were made to explain the different results at 42 C. When the extracts of wild type and mutant were mixed, the product had 90% of the activity of the separately assayed extracts, suggesting that the low racemase activity was not due to the overproduction of an inhibitor for the enzyme. Therefore, this result agrees with Wijsman's explanation for the behavior of this mutant, namely a changed alanine racemase.

When L-alanine:D-alanine racemase was assayed, a low amount of alanyl-alanine and an extremely low amount of pyruvic acid were formed besides UDP-MurNAc-pentapeptide. The addition of pyridoxal-5-phosphate to the assay mixture did not stimulate the alanine racemase activity. The reason may be that this cofactor is not necessary or, more likely, that it is bound to the enzyme, as has been found for the corresponding enzyme from *Pseudomonas putida* (18).

During assay of D-alanine:D-alanine ligase in the presence of UDP-MurNAc-tripeptide, UDP-MurNAc-pentapeptide and a small amount of alanyl-alanine were formed. When fresh enzyme preparations were used, some labeling into pyruvic acid was observed, which decreased when the enzyme had been stored at −20 C for a few weeks (10). Pyruvic acid was synthesized when D-alanine was the radioactive substrate, in agreement with the results of Wijsman (Thesis, Amsterdam, 1970), who showed that pyruvic acid was formed via D-alanine when L-alanine was the only carbon source. D-Alanine:D-alanine ligase was preferentially assayed in the presence of UDP-MurNAc-tripeptide to achieve a better separation between the product and alanine. Moreover, we observed that the yield was higher in that case, suggesting that the enzyme was inhibited by its product.

Effects of manganese ions and ATP on activity of the enzymes. The omission of manganese ions or ATP from the assay mixtures of all adding enzymes and D-alanine:D-alanine ligase resulted in no measurable enzyme activities. The radioactivities of the

products of the assayed enzymes were even lower than those of the control. Because the assay of alanine racemase was dependent on two other enzymes, the influence of lack of manganese and ATP on this enzyme was not determined. In the alanine racemase assays of Rosso et al. (18), Berberich et al. (1), and Wijsman (Thesis, Amsterdam, 1970), divalent cations and ATP were absent during the assay, suggesting that alanine racemase does not require these components for activity. The requirements of the *E. coli* enzymes for ATP and Mn or Mg ions are similar to those of the corresponding enzymes of *S. aureus* (4).

The formation of pyruvic acid in the D-alanine:D-alanine ligase assay was stimulated threefold by the absence of manganese ions.

Enzyme activities at different temperatures. Because we expected adding enzymes with a temperature-sensitive character in a number of temperature-sensitive lysis mutants (12), we wanted to know if the enzymes could be determined at 30 C as well as at 42 C. The reaction was followed for 2 hr at 22, 30, 37, and 42 C. The maximum temperature at which the curve was linear was 42 C for the L-alanine and D-glutamic acid adding enzymes, 37 C for the D-alanyl-D-alanine adding enzyme and the D-alanine:D-alanine ligase (tested in the presence of UDP-MurNAc-tripeptide), and 30 C for the *m*-Dpm adding enzyme and the L-alanine:D-alanine racemase (assayed in the presence of UDP-MurNAc-tripeptide). The amounts of substrates were not limiting during these assays. The relative activities after 60 min of incubation at different temperatures are given in Table 1. The D-glutamic acid adding enzyme is very active even at 45 C.

Another criterion for thermostability was obtained by preincubation of enzyme in buffer B for 10 min at different temperatures, followed by enzyme assay at 30 C. The results are given in Table 2. Although the L-alanine adding enzyme gave a linear curve at 42 C, a considerable amount of activity was lost during incubation of the enzyme for 10 min at this temperature, probably because cofactors or substrates or both, which may protect the enzyme, were lacking.

TABLE 1. *Enzyme activity as a function of temperature*[a]

Enzyme	Relative activity[b] (%) at			
	22 C	37 C	42 C	45 C
L-Ala adding enzyme	66	69	40	
D-Glu adding enzyme	73	152	182	231
m-Dpm adding enzyme	71	65	20	
D-Ala-D-Ala adding enzyme	67	82	68	
D-Ala:D-Ala ligase	40	98	78	
L-Ala:D-Ala racemase	57	104	111	

[a] All incubations were carried out for 1 hr. The racemase and synthetase were determined in the presence of UDP-MurNAc-tripeptide.

[b] Relative to activity at 30 C.

We were surprised to find that the specific activity of the L-alanine adding enzyme of *E. coli* strain K-235 was much higher than that of the corresponding enzyme in two *E. coli* K-12 strains tested. The L-alanine adding enzyme activity of strain K-235 was about equal at 30 and 42 C, whereas the K-12 enzyme at 42 C possessed only 40% of the activity found at 30 C. The specific activity of the L-alanine adding enzyme of strain K-235 was 4 and 10 times higher than in K-12 strains, when assayed at 30 and 42 C, respectively, whereas the other enzymes had activities comparable to those of strain KMBL-146. These results show that the K-12 enzyme is temperature-sensitive compared with the K-235 enzyme. This difference in specific activity may also exist in vivo. It is difficult to say whether the relatively high amounts of precursors in strain K-235 (11) are caused by a more active L-alanine adding enzyme than that of strain K-12.

Dependence of specific activities of the enzymes on the growth phase. An overnight culture of *E. coli* strain KMBL-146 in CGPY medium containing 0.5% glucose was diluted 1:10 in the same medium and vigorously aerated at 37 C. The OD increased exponentially without lag with a generation time of 45 min. Samples of the overnight culture and of the growing culture were taken at times indicated in Fig. 1A. Extracts were prepared as described above. After protein assay, the enzyme samples were diluted to the appropriate protein concentrations. Enzyme assays were performed at 30 C as described above. Alanine racemase and D-alanine:D-alanine ligase were assayed in the presence of UDP-MurNAc-tripeptide. The calculated specific activities are plotted in Fig. 1B and C. For all enzymes, the lowest specific activity was found in the overnight culture. The specific activities of D-alanyl-D-alanine adding enzyme and of L-alanine adding enzyme increased and reached a maximum in the early logarithmic phase, in contrast to the other activities measured. The specific activities of the adding enzymes increased more than those of the racemase and the ligase. The time at which the highest activity was found differed from enzyme to enzyme.

TABLE 2. *Influence of preincubation on enzyme activity*[a]

Preincubation temp	Relative activity (% of control)					
	L-Ala adding enzyme	D-Glu adding enzyme	*m*-Dpm adding enzyme	D-Ala-D-Ala adding enzyme	D-Ala:D-Ala ligase[b]	L-Ala:D-Ala racemase
42	68	102	74 88	101	72	91
50	0	67	97 119	0	30	82[c]
55			145			
60	0	2	13	0	0	0[d]

[a] After preincubation for 10 min in buffer B, the enzyme solution of strain KMBL-146 was cooled and part of it waŝ used for enzyme assay at 30 C for 1 hr. The ligase and recemase were assayed in the presence of UDP-MurNAc-tripeptide. This table presents the remaining activity, relative to the control (100%) which was not preincubated.

[b] No excess of D-alanyl-D-alanine observed.

[c] D-Alanyl-D-alanine was present in excess in twice the amount of the control.

[d] D-Alanyl-D-alanine was present in excess in four times the amount of the control.

Ito and Strominger have followed the specific activities of the adding enzymes during growth of *S. aureus*. They found that the specific activity of the D-alanyl-D-alanine adding enzyme decreased dramatically in the early stationary phase and was almost zero in older cultures (6). This phenomenon was not observed in *E. coli*. The very high specific activity of the D-Ala-D-Ala adding enzyme relative to the other three adding enzymes, also observed in *S. aureus* (6), was striking. As described above, this phenomenon was used in the assays of the D-alanine:D-alanine ligase and the L-alanine:D-alanine racemase.

Inhibition of D-alanine:D-alanine ligase by D-alanyl-D-alanine. Our initial assays of D-alanine:D-alanine ligase were carried out in the absence of UDP-MurNAc-tripeptide. The assay has the disadvantage that separation between alanine (R_F 0.56) and alanyl-alanine (R_F 0.65) is not always complete. Therefore, the reaction product was converted to UDP-MurNAc-pentapeptide (R_F 0.10) by the addition of UDP-MurNAc-tripeptide to the assay mixture. Comparison between mixtures with and without UDP-MurNAc-tripeptide showed that the radioactivity of the products increased by the addition of UDP-MurNAc-tripeptide. The results obtained by monitoring the course of the reaction indicated that a constant rate was only obtained when UDP-Mur-NAc-tripeptide was added (Fig. 2), suggesting an inhibition of D-alanine:D-alanine ligase by its product. This hypothesis was tested by the addition of increasing amounts of unlabeled D-alanyl-D-alanine to the assay mixture (without UDP-Mur-NAc-tripeptide). The results (Table 3) showed that severe inhibition of D-alanine:D-alanine ligase by D-alanyl-D-alanine occurred.

Inhibitory action of DCS on enzymes of E. coli. Enzymes in extracts of strain KMBL-146 were assayed in the absence and presence of DCS. The L-alanine adding enzyme and the D-Ala-D-Ala adding enzyme were completely resistant to 0.4 μmole of DCS per ml. D-Alanine:D-alanine ligase and L-alanine:D-alanine racemase were inhibited by DCS (Fig. 3). Both enzymes were assayed in the presence of UDP-MurNAc-tripeptide. L-Alanine:D-alanine racemase was assayed with the help of the DCS-sensitive D-alanine:D-alanine ligase and the insensitive D-alanyl-D-alanine adding enzyme. The ligase activity was at least 15 times higher than the racemase activity (Fig. 1). If the racemase were insensitive to DCS, inhibition of racemase activity would be only expected at DCS concentrations which inactivate such a large part of the ligase that the remaining activity is too low to convert all synthesized D-alanine. However, if the racemase were sensitive to DCS, inhibition would be expected at lower DCS concentrations. Experiments indicated that the racemase was even more sensitive to DCS than the ligase. DCS concentrations which gave 50% inhibition of racemase and ligase were 1.5 and 12 nmoles/ml, respectively (Fig. 3).

Autoradiograms showed that during the ligase assay with fresh enzyme in the presence of a DCS concentration which inhibited the enzyme by at least 80%, pyruvate disappeared and a new spot with R_F 0.35 (in solvent A) appeared. The new spot was almost absent when the extracts had been stored at −20 C for 2 weeks. No significant ligase activity was lost during that period. The new component was also synthesized from L-alanine, although much less efficiently than from D-alanine. For reasons summarized above, we suppose that it was synthesized by an enzyme which is not

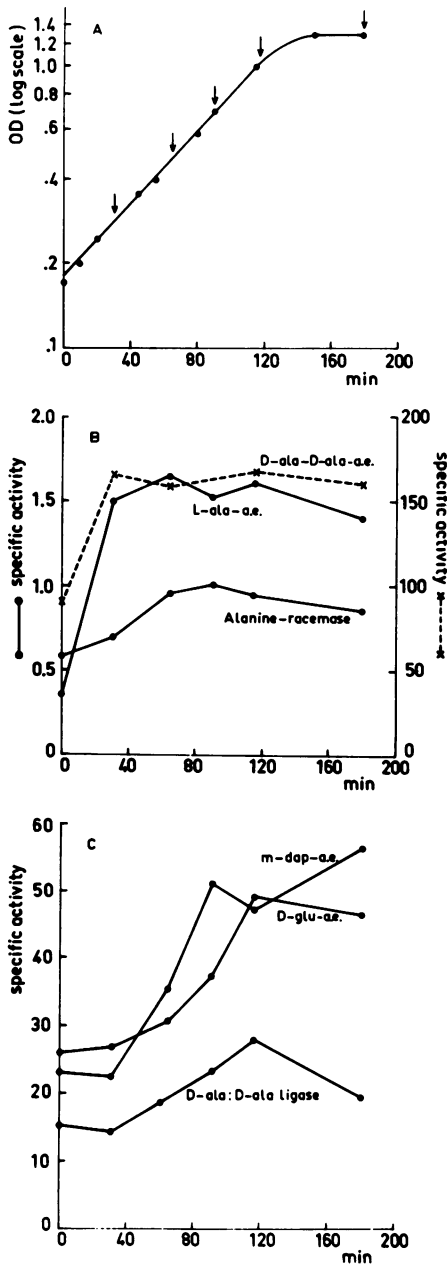

FIG. 1. *Relationship of specific activity to the growth phase of E. coli strain KMBL-146. A, Optical density of the culture at 37 C. Arrows indicate times at which samples of bacteria were isolated for enzyme assays. First sample (zero-time) was the overnight culture. B and C, Specific activities of various enzymes, expressed in nanomoles of product per milligram of protein after incubation for 1 hr at 30 C. Note the difference in scale in B between the D-alanyl-D-alanine adding enzymes and the other two enzymes.*

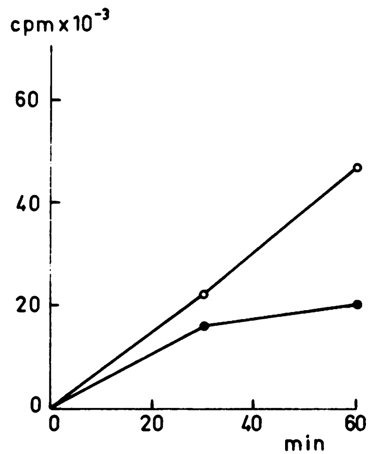

FIG. 2. *Influence of UDP-MurNAc-tripeptide on the activity of the D-alanine: D-alanine ligase of strain KMBL-146. Incubations were carried out at 30 C in the absence (closed circles) and presence (open circles) of UDP-MurNAc-tripeptide (0.4 mM).*

TABLE 3. *Influence of the D-alanyl-D-alanine concentration on the activity of D-alanine: D-alanine ligase*[a]

Addition to assay mixture	Products (counts/min)		Percentage of radioactivity in products
	UDP-MurNAc-pentapeptide	D-Alanyl-D-alanine	
UDP-MurNAc-tripeptide 0.4 mM	35,500	1,130[b]	100
None		16,100[c]	44
D-Alanyl-D-alanine			
0.4 μM		14,050	38
4 μM		10,200	28
40 μM		2,660	7

[a] Enzyme from an extract of strain KMBL-146 was tested. The assay mixture without unlabeled D-alanyl-D-alanine but containing UDP-MurNAc-tripeptide was considered as the least inhibited mixture. Incubation was carried out for 60 min at 30 C.

[b] This amount of D-alanyl-D-alanine was probably present during the whole incubation and represents a concentration of 0.31 μM.

[c] The D-alanyl-D-alanine concentration at the end of the assay was 4.45 μM.

related to the ligase or racemase. No attempts have been made to identify this component.

Inhibitory action of glycine and glycylglycine. Three enzymes were sensitive to glycine and glycyl-glycine, namely L-alanine:D-alanine racemase, D-alanine:D-alanine ligase, and the L-alanine adding enzyme (Fig. 4). The

inhibitor concentrations required for 50% inhibition are given in Table 4. The D-glutamic acid adding enzyme and the D-alanyl-D-alanine adding enzyme were completely resistant to final concentrations of 125 mM glycine or glycyl-glycine.

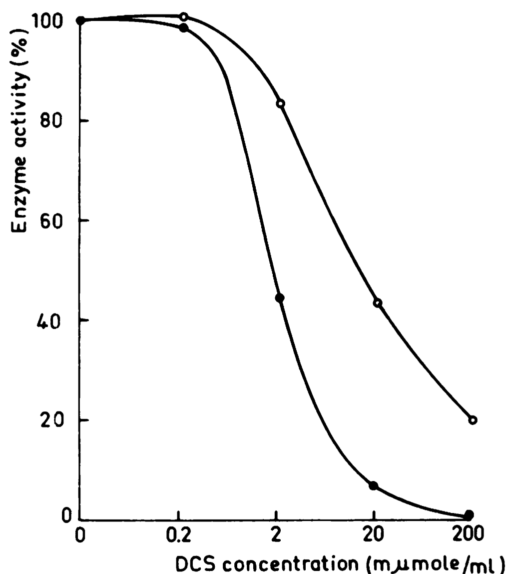

FIG. 3. *Inhibition of L-alanine: D-alanine racemase and D-alanine: D-alanine ligase by D-cycloserine (DCS). Incubations were carried out at 30 C for 1 hr as described in text. UDP-MurNAc-tripeptide (0.4 mM) was present in all mixtures. The activity in the UDP-MurNAc-pentapeptide spot was counted. This radioactivity, expressed as percentage of the activity of a mixture without DCS, was plotted against the logarithm of the DCS concentration. Open circles: D-alanine: D-alanine ligase; closed circles: L-alanine: D-alanine racemase.*

Strominger and Birge (19) have identified the uridine nucleotides that are accumulated in *S. aureus* by glycine. They found two nucleotides in which glycine was incorporated instead of L-alanine. This suggests that the action of glycine on the L-alanine adding enzyme can be partly explained by utilization of glycine instead of L-alanine. We have not checked if UDP-MurNAc-Gly was synthesized by the *E. coli* L-alanine adding enzyme.

Yabu (23) found that purified D-alanine:D-alanine ligase of *M. smegmentis* was inhibited 50% by 0.1 M glycine or glycyl-glycine. The concentrations required to inhibit the corresponding *E. coli* enzyme are considerably lower, and glycyl-glycine was less effective than glycine (Table 4). The latter compound was also more effective against the L-alanine adding enzyme. The sensitivity of the racemase to glycine and glycyl-glycine was about equal.

DISCUSSION

The specific activities of the six enzymes studied differed considerably (Fig. 1). We have used these differences to assay the L-alanine: D-alanine racemase with the help of D-alanine: D-alanine ligase and the D-alanyl-D-alanine adding enzyme, which have specific activities about 20 and 150 times higher, respectively.

When the Dpm adding enzyme was preincubated at different temperatures, followed by assay at 30 C, the highest activity was found when the preincubation was performed at 55 C. In this case the resulting activity was even higher than when the preparation had not been preincubated (Table 2). This result can be explained by assuming that the enzyme contains

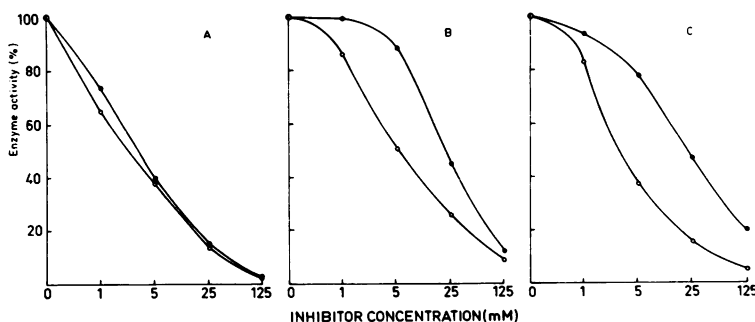

FIG. 4. *Inhibition of L-alanine: D-alanine racemase (A), D-alanine: D-alanine ligase (B), and L-alanine adding enzyme (C) by glycine (open circles) and glycyl-glycine (closed circles). Incubations were carried out for 60 min at 30 C. Assay mixtures for racemase and ligase contained UDP-MurNAc-tripeptide (0.4 mM). The radioactivity in the uridine nucleotide product was counted. This activity, relative to that of the product of an uninhibited enzyme, was plotted against the logarithm of the inhibitor concentration.*

TABLE 4. *Glycine and glycyl-glycine concentrations required to inhibit enzymes from E. coli strain KMBL-146 by 50%*[a]

Enzyme	Inhibitor (mM)	
	Glycine	Glycyl-glycine
L-Ala : D-Ala racemase	2	2.5
D-Ala : D-Ala ligase	5	20
L-Ala adding enzyme	2.5	20

[a] Assays with crude enzyme of strain KMBL-146 were performed as described in the text. The final protein concentrations were 2 mg/ml in the assays of the racemase and the L-alanine adding enzyme and 0.7 mg/ml in the assay of the ligase. Racemase and ligase were both determined in the presence of UDP-MurNAc-tripeptide. Incubations were performed at 30 C for 60 min.

subunits which have a higher activity than the complete enzyme. It is also possible that an inhibitor for the enzyme is present, which only partly inhibits the enzyme activity. The results then can be explained by assuming that the inhibitor is more sensitive to thermoinactivation than the enzyme.

D-Alanine : D-alanine ligase was strongly inhibited by its product D-alanyl-D-alanine (Table 3). This phenomenon was found by Neuhaus et al. (15) for the corresponding enzyme of *S. faecalis*. These authors assume that product inhibition prevents accumulation of D-alanyl-D-alanine in vivo and therefore also the resulting depletion of the L-alanine branch. When *E. coli* was incubated in a wall medium (10) in the presence of ^{14}C-L-alanine, a large amount of ^{14}C-alanyl-alanine accumulated and was excreted into the medium. When *E. coli* strain KMBL-146 was incubated at 37 C in minimal medium, supplemented with ^{14}C-L-alanine, the precursors were labeled, but only a small amount of ^{14}C-alanyl-alanine was excreted into the medium. No labeled alanyl-alanine was found in the cellular fraction (*unpublished data*). These results are therefore not compatible with the regulatory function of D-alanyl-D-alanine as assumed by Neuhaus et al. (15).

DCS is known to cause inhibition of murein synthesis in sensitive bacteria, accompanied by accumulation of UDP-MurNAc-tripeptide (17). In *S. faecalis* (14, 16) and *S. aureus* (20), alanine racemase and D-alanine : D-alanine ligase were sensitive to this antibiotic. Chambers et al. (Bacteriol. Proc., 1963, p. 119) concluded that D-alanine : D-alanine ligase was the major site of the in vivo action of DCS in *E. coli*. Our in vitro experiments show that both enzymes of *E. coli* are sensitive to DCS, but that the racemase is the most sensitive enzyme (Fig. 3).

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