Biochemical Pharmacology, 1960, Vol. 3, pp 101–109. Pergamon Press Ltd., Printed in Great Britain.

INVESTIGATIONS ON THE MODE OF ACTION OF *CYCLO*-SERINE UPON PROTEIN SYNTHESIS IN *ESCHERICHIA COLI*

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(*Received* 29 *July* 1959)

Abstract—D-*cyclo*serine and L-*cyclo*serine interactions with DL-alanine 1-C^{14} incorporation into the proteins of an *E. coli* strain were investigated.

The actions of the D- and L-isomers of *cyclo*serine on protein synthesis are closely related to alanine metabolism. The incorporation of algal protein hydrolysate C^{14} into bacterial proteins does not seem to be influenced by D- and L-*cyclo*serine.

A comparison of the effects promoted by D-*cyclo*serine and by D-alanine on the incorporation of DL-alanine-1-C^{14} into bacterial proteins shows that D-*cyclo*serine behaves like a D-alanine analogue.

PREVIOUS investigations carried out in our laboratories[1] have shown a specific antagonism of D-α-alanine both on the inhibition of the bacterial growth and on protoplast formation by D-*cyclo*serine in *E. coli*.

In particular, these researches suggested that D-*cyclo*serine may interfere specifically with the bio ynthesis of a polypeptide-complex of the cell wall, containing D-alanine, considered necessary for the cell integrity.[2, 3]

The present paper deals with the action of *cyclo*serine upon protein biosynthesis by a mutant strain of *E. coli*.

In order to avoid complications resulting from parallel processes of growth, we utilized a thymine and phenyl-alanine requiring strain which synthesizes proteins under conditions of thymine starvation.

MATERIALS AND METHODS

(*a*) *Test organism. E. coli* 147, a thymine and phenylalanine requiring strain, was isolated in our laboratories.[4]

E. coli 147 protoplasts were obtained after ten hours incubation of a cell suspension in a medium containing 1250 units/ml of penicillin. We also used a cell-free supernatant prepared by centrifugation at 20,000 *g* of a protoplast suspension lysed by digitonin.[5]

(*b*) *Experimental media.* The synthetic medium of Davis, supplemented with Difco casein hydrolysate (25 μg/ml) and DL-phenylalanine (75 μg/ml), was used for cell suspensions of *E. coli* 147.

The synthetic medium of Davis, supplemented as above, and enriched with sucrose up to 20 per cent and $MgSO_4$ up to 0·2 per cent, was used for protoplast suspensions.

The density of the bacterial suspension in the incubation flasks was about 50–60 mg of cells (dry weight) per 100 ml of medium.

(*c*) *Incubation.* Suspensions were kept, without shaking, at 37 °C for 40 or 120 min according to experimental needs.

The bacterial cells were grown in a Davis medium containing limiting amounts of thymine, and were harvested when growth stopped from thymine exhaustion (48 hr).

E. coli 147 cells are able to synthesize proteins even after thymine starvation, provided that phenylalanine is present.

(*d*) *Chemicals.* The labelled protein-precursors were an algal protein hydrolysate-C^{14} and DL-alanine-1-C,^{14} supplied by the Radiochemical Centre of Amersham (England) while unlabelled natural amino-acids were from Ciba (Bale).

L-*cyclo*serine from synthesis (96 per cent pure) and D-*cyclo*serine from biosynthesis (98 per cent pure) were kindly supplied by the Farmitalia Chemical Laboratories of Settimo Torinese (Italy).

(*e*) *Protein extraction.* The protein metabolism of bacterial suspensions incubated with radioactive compounds was blocked by adding trichloracetic acid (TCA) to give a final concentration of 5 per cent. The protein fraction was purified by washing TCA-insoluble material three times with cold and twice with hot 5 per cent TCA (15 min at 95 °C), then once with ethanol and three times with hot ethanol–ether 3:1 (5 min at 65 °C).[6]

Finally the protein fraction was completely dried with ether.

(*f*) *Cell wall material.* Sometimes we used a residual cell fraction, previously extracted with hot TCA, resisting the tryptic digestion.[7] The specific activity of the residual fraction, probably containing some polypeptide compounds from the bacterial cell wall, was determined after extraction with hot ethanol–ether 3:1.

(*g*) *Radioactivity measurements.* In any case the radioactivity of the experimental samples was measured using 1 cm^2 or 2 cm^2 polyethylene planchets and thin mica-window Tracerlab G.M. tubes (TGC2) and employing, when necessary, self-absorption correction factors.

Duplicate samples were always prepared.

EXPERIMENTAL RESULTS

(*a*) *Action of* D-cyclo*serine on the incorporation of algal protein hydrolysate* $-C^{14}$ *in bacterial proteins.* D-*cyclo*serine, in concentrations up to 40 μg/ml does not seem to influence the incorporation of algal protein hydrolysate-C^{14} in *E. coli* 147 proteins. On the other hand chloramphenicol, under the same experimental conditions, shows the known inhibiting action even at 25 μg/ml (Table 1).

TABLE 1. ACTION OF D-*cyclo*SERINE AND CHLORAMPHENICOL (CHL) ON THE INCORPORATION OF ALGAL PROTEIN HYDROLYSATE-C^{14} INTO PROTEINS OF *E. coli* 147

Experiment	Control	+ D-*cyclo*serine			+ CHL
		10 μg/ml	20 μg/ml	40 μg/ml	25 μg/ml
1	5,486	—	5,558	—	3,089
2	21,000	22,000	22,800	21,500	—

Values are given as cpm/mg of protein

Also in a cell-free system (supernatant at 20,000 $\times$ *g* of *E. coli* 147 protoplasts lysed by digitonin), D-*cyclo*serine up to 50 μg/ml does not cause variations in algal hydrolysate incorporation into proteins (Table 2).

TABLE 2. ACTION OF D-*cyclo*SERINE ON THE INCORPORATION OF ALGAL PROTEIN HYDROLYSATE-C^{14} INTO PROTEINS OF AN *E. coli* CELL-FREE SYSTEM

Control	+ D-*cyclo*serine		
	12 μg/ml	25 μg/ml	50 μg/ml
574	551	545	553

Values are given as cpm/mg of protein

(*b*) *Action of* D-cyclo*serine on the incorporation of* DL-*alanine*-1-C^{14} *into bacterial proteins.* D-*cyclo*serine (20μg/ml) reduces remarkably DL-alanine-1-C^{14} incorporation into total proteins as well as into the cell wall fraction of *E. coli* 147, where the decrease is more evident (Table 3).

TABLE 3. ACTION OF D-*cyclo*SERINE ON THE INCORPORATION OF DL-ALANINE-1-C^{14} INTO PROTEINS AND CELL WALL FRACTION OF *E. coli* 147

Experiment No.	Total proteins		Cell-wall fraction	
	Control	D-*cyclo*serine (20 μg/ml)	Control	D-*cyclo*serine (20 μg/ml)
1	10,200	4,750	16,000	6,000
2	1,775	725	4,680	710

Values are given as cpm/mg of protein

However it is improbable that the primitive site of action of D-*cyclo*serine be situated in the bacterial cell wall; in fact a decrease in DL-alanine-1-C^{14} incorporation was demonstrated also in protoplasts (Table 4).

TABLE 4. ACTION OF D-*cyclo*SERINE ON THE INCORPORATION OF DL-ALANINE-1-C^{14} INTO PROTEINS OF PROTOPLASTS OF *E. coli* 147

Control	+ D-*cyclo*serine (25 μg/ml)
6,983	2,112

Values are given as cpm/mg of protein

However, DL-alanine-1-C^{14} incorporation into proteins does not decrease in proportion to the increase of antibiotic concentration. In particular insignificant variations in protein specific activity were observed when the antibiotic concentration ranged from 1·2 to 2·4 μM/ml. The data reported in Table 5 demonstrate the similar influence of equimolecular concentrations of unlabelled D-alanine, D-*cyclo*serine and unlabelled D-alanine + D-*cyclo*serine on the incorporation of DL-alanine-1-C^{14} into proteins.

TABLE 5. EFFECT OF D-*cyclo*SERINE AND UNLABELLED D-ALANINE ON THE INCORPORATION OF DL-ALANINE-1-C^{14} INTO PROTEINS OF *E. coli* 147

D-*cyclo*serine		D-alanine		D-*cyclo*serine + D-alanine	
μM/ml	cpm/mg	μM/ml	cpm/mg	μM/ml	cpm/mg
0	2,331	0	2,331	0	2,331
0·08	1,901	0·08	1,823	0·04 + 0·04	1,895
1·20	972	1·20	1,286	0·6 + 0·6	1,260
2·40	855	2·40	1,106	1·2 + 1·2	954

Values are given as cpm/mg of protein

(*c*) *Action of* L-cyclo*serine on the incorporation of algal protein hydrolysate-*C^{14} *and* DL-*alanine-*1-C^{14} *into proteins of E. coli* 147. L-*cyclo*serine at concentrations of 10·2 μg/ml does not cause variations in algal protein hydrolysate-C^{14} incorporation into proteins of *E. coli* 147 cells. On the other hand, at the same concentration, it considerably increases the total protein specific activity either in the whole cells or in protoplasts of *E. coli* 147, when DL-alanine-1-C^{14} is used as protein precursor (Table 6).

TABLE 6. ACTION OF L-*cyclo*SERINE ON PROTEIN SYNTHESIS OF *E. coli* 147

Precursor	System	L-*cyclo*serine		
		0	10·2 μg/ml	25 μg/ml
Protein hydrolysate-C^{14}	Cells	7,622	7,297	—
DL-alanine-1-C^{14}	Cells	3,417	5,865	—
DL-alanine-1-C^{14}	Protoplasts	6,983	—	17,194

Values are given as cpm/mg of protein

(*d*) *Comparison of the effects of the enantiomorphs of* cyclo*serine and alanine on the incorporation of* DL-*alanine-*1-C^{14} *into the protein of E. coli* 147. To make clear the quantitative relationships of the observed antagonism between the enantiomorphs of *cyclo*serine and those of alanine, we compared the effects induced on DL-alanine-1-C^{14} incorporation into bacterial proteins by equimolecular amounts of D- and L-alanine and of D- and L-*cyclo*serine. DL-Alanine-1-C^{14} was used as a protein precursor and was added to the Davis medium enriched with 25 μg/ml of Difco casein hydrolysate and 75 μg/ml of DL-phenylalanine. The incubation time was generally reduced to 40 min.

The incorporation of a labelled precursor into a given compound is proportional, *ceteris paribus*, to the concentration of this precursor in its pool. In our case, any variation of DL-alanine specific activity in its pool, promoted by adding unlabelled L- or D-alanine as a diluent will occur according to the law of isotopic dilution and will be reflected by a corresponding variation of the protein specific activity.

Now if A is the activity of the amount b of a given amino acid in the pool, the specific activity is

$$a = \frac{A}{b}.$$

Adding a given amount x of the same amino acid unlabelled, the specific activity becomes

$$y = \frac{A}{b + x};$$

we can write the following proportion:

$$a : y = \frac{A}{b} : \frac{A}{b + x}$$

from which:

$$y = \frac{a}{1 + \frac{x}{b}} \tag{1}$$

Whenever the addition of an unlabelled amino acid induces no significant variations of protein synthesis or of the precursor pool size, the variations of protein specific activity, due to this addition, are expressed by equation (1), where y means the observed protein specific activity (as cpm/mg), x the amount of the diluent amino acid in the medium (as μM/ml), and a the protein specific activity of the control (made equal to 100).

Our experimental data concerning L-alanine dilution are in agreement with equation (1) (Fig. 1).

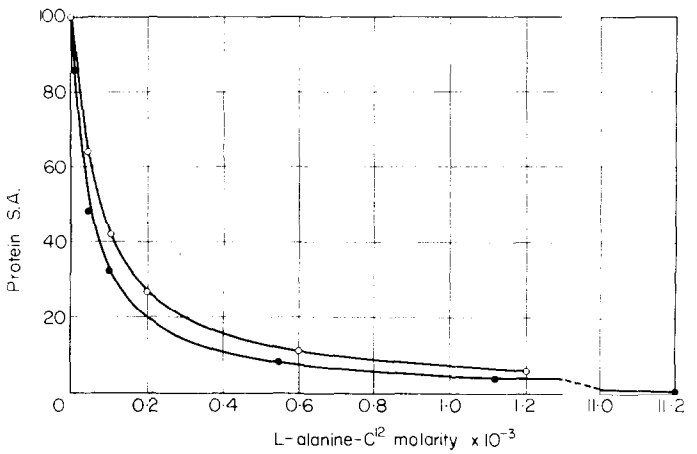

FIG. 1. Effect of exogenous L-alanine-C^{12} on the incorporation of DL-alanine-1-C^{14} into proteins Expt. No. 1 ○—○: 40 min incubation; b = 0·0747. Expt. No. 2 ●—●: 120 min incubation; b = 0·0498.

Considering $a = 100$, b can be determined according to:

$$b = x\left(\frac{y}{a - y}\right)$$

From Fig. 1 it is apparent that for high values of x, y tends to zero. Therefore it may be deduced that L-alanine is easily converted into its D-isomer, presumably without any variation in the L-alanine/D-alanine ratio in the pool of the precursors.

Molar concentrations of L-*cyclo*serine identical to those used for L-alanine did not induce similar effects (Figs. 1 and 2). The curve in Fig. 2 shows an increase in specific activity for the lowest doses of L-*cyclo*serine and a decrease for the highest ones.

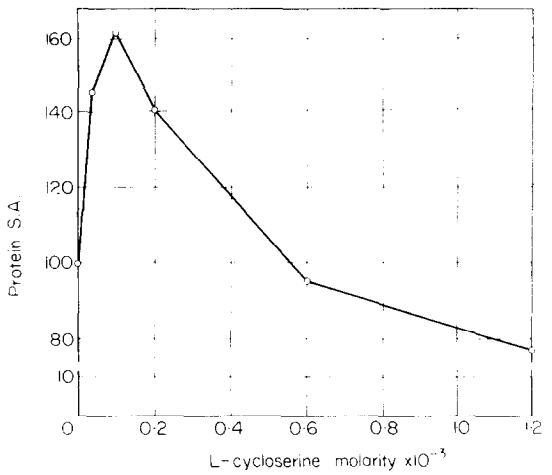

FIG. 2. Effect of L-*cyclo*serine on the incorporation of DL-alanine-1-C^{14} into proteins (40 min incubation).

When unlabelled D-alanine is added as diluent, the radioactivity incorporated into bacterial proteins from DL-alanine-1-C^{14} does not tend to zero as the amino acid concentration increases, but reaches a minimum value which thereafter remains constant. This value varies remarkably from one experiment to the other and ranges from 45 to 15 per cent of the activity incorporated into the control.

Supposing that under our conditions D-alanine is not converted into L-alanine, the minimum constant radioactivity which appears in proteins in presence of high levels of unlabelled D-alanine should represent only the incorporation of L-alanine-1-C^{14}.

Therefore the dilution process should be represented by:

$$y = C + \frac{a - C}{1 + \frac{x}{b}} \tag{2}$$

where C is the minimum constant value of the protein specific activity, due to the presence of L-alanine-1-C^{14}.

Considering $a = 100$, b can be determined according to:

$$b = x \frac{y - C}{a - y}$$

D-alanine dilution curves appear to fit quite well with equation (2), where y tends to C when x tends to high values (Fig. 3).

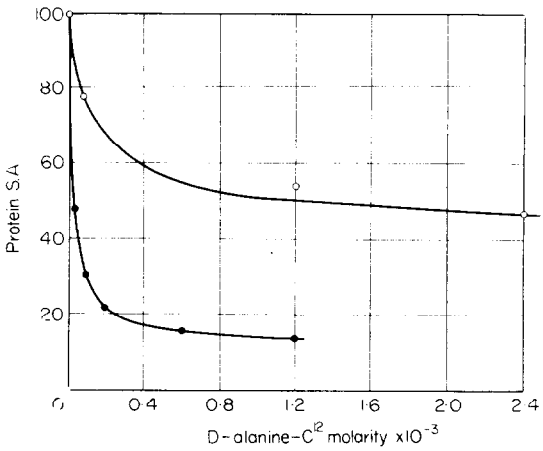

FIG. 3. Effect of exogenous D-alanine-C^{12} on the incorporation of DL-alanine-1-C^{14} into proteins. Expt. No. 1 ○—○: 40 min incubation; $b = 0{\cdot}14$, $c = 45$. Expt. No. 2 ●—●: 40 min incubation; $b = 0{\cdot}023$, $c = 13$.

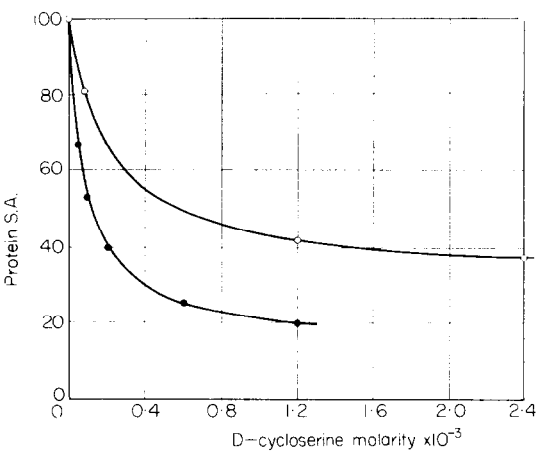

FIG 4. Effect of D-cycloserine on the incorporation of DL-alanine-1-C^{14} into proteins. Expt. No. 1 ○—○: 40 min incubation; $b = 0{\cdot}20$, $c = 32$. Expt. No. 2 ●—●: 40 min incubation; $b = 0{\cdot}08$ $c = 15$.

The curves represented by the same symbol belong to the same experiment.

Also the curves of protein specific activity plotted against molar concentrations of D-*cyclo*serine, in the same range as those used for D-alanine (Fig. 3 and Fig. 4), appear to be in agreement with equation (2).

The effects promoted by L- and D-alanine and D-*cyclo*serine on the incorporation of DL-alanine-1-C^{14} in the cell wall fraction were also investigated. The results were in agreement with those obtained for incorporation into total proteins. In fact, the decrease of the specific activity promoted by D-alanine and D-*cyclo*serine was even more evident in the cell wall fraction than in total proteins (Table 7).

TABLE 7. EFFECT OF L-ALANINE, D-ALANINE AND D-*cyclo*SERINE ON THE DL-ALANINE-1-C^{14} INCORPORATION INTO TOTAL PROTEINS (TP) AND CELL WALL FRACTION (CWF) OF *E. coli* 147

Experiment No.	Control		L-alanine (9 μg/ml)		D-alanine (9 μg/ml)		D-*cyclo*serine (25 μg/ml)		D-*cyclo*serine (25 μg/ml) L-alanine (9 μg/ml)		D-*cyclo*serine (25 μg/ml) D-alanine (9 μg/ml)	
	TP	CWF	TP	CWF	TP	CWF	TP	CWF	TP	CWF	TP	CWF
1	3924	—	1387	—	1832	—	1186	—	700	—	1300	—
2	711	1300	—	—	—	—	382	500	298	450	335	480
3	1738	2220	1052	1372	1542	1492	994	963	632	678	960	893

DISCUSSION

Our results showed that the action of D-*cyclo*serine is closely related to the utilisation of D-alanine in the protein biosynthesis of *cyclo*serine-sensitive bacterial cells and protoplasts.

Buogo *et al.*[1] made evident a competitive antagonism of D-*cyclo*serine with D-alanine which can be quantitatively studied by analysing the DL-alanine-1-C^{14} incorporation into bacterial proteins in the presence of variable amounts of unlabelled D- or L-alanine and D- or L-*cyclo*serine. By addition of increasing amounts of unlabelled L-alanine, the radioactivity incorporation into proteins tends to zero as if the amino acid could dilute both the L- and D- isomers of the labelled amino acid racemate. This is in agreement with the hypothesis of the enzymatic conversion of L-alanine into D-alanine.

On the contrary, the DL-alanine-1-C^{14} incorporation decreases after addition of unlabelled D-alanine but instead of tending to zero, reaches a minimum constant value which is probably accounted for by the fact that the L-fraction of the alanine-1-C^{14} racemate does not undergo any dilution effect and therefore it should be concluded that there is no apparent conversion of D-alanine into L-alanine under our experimental conditions, in spite of our knowledge on the behaviour of the bacterial alanine-racaemase[8].

The decrease of DL-alanine-1-C^{14} incorporation into proteins caused by increasing amounts of unlabelled D-*cyclo*serine is quantitatively similar to that observed after addition of unlabelled D-alanine, and here again the radioactivity does not decrease below a given minimum value when further amounts of this antibiotic are added. It has been observed that at the lowest concentrations used in our experiments D-alanine and D-*cyclo*serine showed additional effects (Table 5).

Buogo *et al.*,[1] observing that D-*cyclo*serine acts on sensitive micro-organisms requiring thymine and phenylalanine, provided that phenylalanine be present, that is, when conditions for protein biosynthesis obtain, were led to the conclusion that the D-*cyclo*serine action is closely related to the protein synthesis.

The observations described in the present paper show that this action is limited to the part played by alanine in protein synthesis.

Our results support the hypothesis that D-*cyclo*serine behaves as a D-alanine analogue during bacterial protein synthesis. It, or one of its derivatives, might replace the natural amino acid in protein formation.

The inhibiting action of D-*cyclo*serine on the protein incorporation of DL-alanine-1-C^{14} is more evident in the cell wall fraction than in total cellular protein.

As a significant decrease in DL-alanine-1-C^{14} incorporation is observed also in the protein of intact protoplasts, it is likely that the site of action of *cyclo*serine is located in the protoplasm, where the synthesis of the peptide-complex of the wall, containing D-alanine, may occur.

The observation that the D-*cyclo*serine inhibiting action appears to be greater on the synthesis of cell wall constituents may be explained by the larger amounts of D-alanine occurring in the cell wall than in the intracellular proteins.

The action of the L-isomer of *cyclo*serine appears to be quite different from that of D-isomer. In our experiments, L-*cyclo*serine at low concentrations enhanced DL-alanine-1-C^{14} incorporation into both bacterial cells and protoplast proteins. Only higher concentrations induced inhibiting effects.

In any case we cannot exclude that the effects shown by L-*cyclo*serine could be due to some traces of impurities present in the batch of this compound which we used.

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