Biochemical Pharmacology, 1960, Vol. 3, pp. 264–271. Pergamon Press Ltd., Printed in Great Britain.

INVESTIGATION ON THE MODE OF ACTION OF *CYCLO*SERINE UPON PROTEIN SYNTHESIS OF *E.COLI* AND ANIMAL CELLS

II—ACTION OF L-*CYCLO*SERINE ON PROTEIN METABOLISM OF ALANINE AND ON ENZYMIC PREPARATIONS

P. BARBIERI, A. DI MARCO, L. FUOCO, P. JULITA, A. MIGLIACCI and A. RUSCONI

Laboratori Ricerche Farmitalia, Milano, Italy

(*Received* 9 *February* 1960)

Abstract—The action exerted by L-*cyclo*serine on protein metabolism of alanine in animal and bacterial cells has been investigated. High concentrations of L-*cyclo*serine inhibit the incorporation of alanine-^{14}C into proteins while low concentrations increase it. It has also been demonstrated that the L-isomer of *cyclo*serine, contrary to the D-isomer, causes a marked inhibition on the enzymic transamination which involves the formation of L-alanine.

INTRODUCTION

IT IS known that the L- and D-enantiomorphs of *cyclo*serine possess different antibacterial activities[1] and pharmacological properties.[2–5] In our previous biochemica experiments[6] we observed that L-*cyclo*serine, contrary to the D-isomer, increases alanine-^{14}C incorporation into proteins of *E. coli*.

The present paper deals with the influence of L-*cyclo*serine on protein metabolism of alanine and with the transaminating activity of bacterial suspensions and rat liver homogenates.

MATERIALS AND METHODS

A. *Strains, preparation of extracts, chemicals*

(a) *E. coli* 147, a thymine and phenylalanine-requiring strain, isolated in our laboratory, was grown on a Davis[7] medium containing limited amounts of thymine, and was harvested for radioactive experiments when growth was stopped as a consequence of thymine exhaustion. The Davis synthetic medium was supplemented with 25 μg/ml of Difco casein hydrolysate and 75 μg/ml of DL-phenylalanine.[6]

(b) Acetone-dried cells of *E. coli* B and 147 were prepared according to Umbarger method.[8] For enzymatic experiments, the packed cells were suspended in 10 vol of cold potassium phosphate buffer solution (0·1 M; pH 7·4) previously homogenized at 0 °C in a Potter apparatus for 5 min.

(c) Hepatoma ascites AH130 cells from albino rats were withdrawn 7 days after inoculation. The strain was kindly supplied by Professor T. Yoshida. Cells were washed with isotonic solution, suspended in two volumes of Krebs–Ringer-HCO_3 synthetic medium at pH 7·4 and incubated at 37 °C for 2 hr, for radioactive experiments.

(d) Liver, brain and hepatoma ascites AH130 cell homogenates from Wistar rats were prepared in a glass homogenizer with a Teflon pestle at 1000 rev/min at 0 °C for

1 min in 10 vol of a 0·25 M sucrose solution. The suspensions were incubated at 37 °C for 1 hr, with shaking, for radioactive experiments.

(e) Rat liver homogenates were prepared in a glass homogenizer with a Teflon pestle, using 20 ml of ice-cold potassium phosphate buffer (0·1 M; pH 7·4) per g of freshly dissected tissue, for enzymatic experiments.

(f) Supernatant fractions from liver and hepatoma ascites AH130 homogenate, according to (d), were obtained by centrifuging at 15,000 *g* for 10 min at 0°C, for radioactive experiments.

(g) A suspension of microsomes and pH 5 enzymes was prepared in medium A[9] from the liver homogenates mentioned above.

(h) The D-isomer of *cyclo*serine was isolated from a culture of *St. gariphalus* obtained in the Farmitalia Antibiotic Department of Settimo Torinese. The L-isomer was obtained by the Farmitalia Chemical Department of Settimo Torinese, after isolation from the synthetic racemate.

(i) Pyridoxal-phosphate was supplied by La Roche; ^{14}C-amino acids were obtained from the Radiochemical Centre of Amersham (England).

B. *Biochemical methods*

(1) The preliminary estimation of the protein content of the subcellular fractions (d), (f) and (g) was performed according to Kunitz.[10]

(2) *Protein-*^{14}C *extractions.* Proteins were extracted according to Rabinovitz method.[11] Protein metabolism of the samples incubated with radioactive aminoacids and *cyclo*serine, was blocked by addition of trichloracetic acid (TCA) to give a 5 per cent final concentration. The protein fraction was purified by washing TCA insoluble material twice with cold, twice with hot, (at 95°C for 15 min) and again twice with cold 5 per cent TCA; then once with ethanol and three times with hot ethanol–ether 3 : 1 (at 65 °C for 5 min). Finally the protein fraction was completely dried with ether.

(3) *Radioactivity measurements.* When the amount of material was scarce, it was suspended in chloroform and transferred into polyethylene planchets, then the solvent was evaporated. In any case the radioactivity of the experimental samples was measured using 1 cm^2 polyethylene planchets and thin mica window Tracerlab G.–M. tubes (TGC2) and employing, when necessary, a self-absorption correction factor.

(4) *Determination of transaminase activity.* The transaminase activity of liver homogenates and *E. coli* cells suspensions (strain B and strain 147), in the presence and absence of L-*cyclo*serine, was tested according to the following reactions:

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{L-glutamate} \qquad (1)$$

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxalacetate} + \text{L-glutamate} \qquad (2)$$

Pyruvate was analysed as 2 : 4-dinitrophenyl-hydrazone while oxalacetate was previously converted to pyruvate.[12] Rat liver homogenates were prepared in potassium phosphate buffer according to (e). *E. coli* cell homogenates were prepared in the buffer solution according to (b). The *E. coli* buffered suspension was not diluted further when used in testing L-alanine reaction (equation (1)) but it was diluted when used to test the L-aspartate reaction (equation (2)). A five fold dilution was employed in the experiments with strain 147 and an eight fold dilution with strain B. The sample was prepared as follows: to 0·1 ml of enzymic preparation were added 0·9 ml of potassium

phosphate buffer (0·1 M; pH 7·4) containing 0·05 mM of L-aminoacid, 0·02 mM of ketoacid, 100 μg of pyridoxal phosphate, and some of either one or the other of the two isomers of *cyclo*serine when this substance was used. The final volume of the sample was 1 ml. The enzymic processes were blocked by addition of 10 % TCA. Rat liver homogenates were incubated at 37 °C for 10 min. For *E. coli* dried cells of the two *E. coli* strains, which exhibit a weak transaminating action towards L-alanine and a strong one towards L-aspartate, we had to use two different concentrations, as mentioned. In addition, for alanine reaction the *E. coli* 147 cell suspensions

TABLE 1. ACTION OF LCS ON THE INCORPORATION OF ALGAL PROTEIN HYDROLYSATE-^{14}C AND ALANINE-^{14}C INTO PROTEINS OF *E. coli* 147 AND RAT HEPATOMA ASCITES AH130

Labelled precursor	Cell system*	Controls	LCS 0·1 μM/ml	LCS 1 μM/ml
Algal protein hydrolysate	*E. coli* 147	7622	7297	—
Algal protein hydrolysate	AH 130	570	563	473
DL-Alanine C14	*E. coli* 147	3417	5865	2770
L-Alanine C14	AH 130	575	2370	1867

Values are given as counts/min per mg of protein.

* The final volume of each sample of bacterial cell suspension was 10 ml or 20 ml according to the experiment; in any case the bacterial suspension showed an optical density of 1000 at 700 mμ. Samples were incubated for 40 min without shaking.

The final volume of each sample of hepatoma ascites cell suspension was 2 ml and samples contained 1μC of either L-alanine-^{14}C or algal protein-^{14}C hydrolysate. They were incubated for 2 hr with shaking.

TABLE 2. ACTION OF LCS ON THE INCORPORATION OF L-ALANINE-^{14}C-G INTO PROTEINS OF MAMMALIAN SUBCELLULAR SYSTEM

Labelled precursor: L-alanine-^{14}C-G (μc/ml)	System*	Controls	LCS 0·1 μM/ml	LCS 1 μM/ml
1·9	Rat liver homogenate	2·34	3·60	2·53
1·9	Rat liver homogenate + α-ketoglutarate	5·42	3·98	4·19
3·5	Rat brain homogenate	4·34	4·13	3·19
1·9	Supernatant 15,000 *g* of rat hepatoma ascites AH 130	2·57	2·00	3·40
1·9	Supernatant 15,000 *g* of rat liver	4·94	3·98	3·93
1·2	Microsomes + EpH5 of rat liver	10·34	11·59	13·83

Values are given as counts/min per mg of protein.

* Each sample of liver and brain homogenate contained in a final volume of 2 ml : 1 ml of homogenate preparation in 0·25 M sucrose; 0·2 ml of a 0·25 M sucrose, 0·016 M ATP, 0·2 M α-ketoglutarate,[13] and 0·05 M $MgCl_2$ solution; 0·5 ml of potassium phosphate buffer pH 7·4 (0·1 M); 0·3 ml of a 0·25 M sucrose solution containing L-alanine-^{14}C (specific activity 5 μc/μM).

Each sample of 15,000 *g* supernatant fraction contained, in a final volume of 2 ml of medium A: 20 μM of HDP, 2 μM of ATP, 20 mg of supernatant fraction proteins, 3·8 μc of L-alanine-^{14}C.

Each sample of microsomes + E pH 5 suspension contained, in a final volume of 1 ml of medium A: 1 μM of ATP, 0·5 μM of GTP, 10 μM of PEP, 20 μg of PEP-kinase, 1·2 μc of L-alanine-^{14}C, 10 mg of microsomal proteins and 2·5 mg of E-pH 5 proteins.

All samples were incubated for 1 hr at 37 °C with shaking.

were incubated for 4–5 hr and the *E. coli* B cell suspension for 60–90 min. All samples were incubated for 10 min when the reaction contained L-aspartate.

EXPERIMENTAL

Effect of L-cyclo*serine on alanine-*^{14}C *incorporation into proteins*

When whole cells of *E. coli* 147 and rat hepatoma ascites AH130 are employed and when the protein precursor is α-alanine-^{14}C, L-*cyclo*serine, at the concentration of 0·1 μM/ml, causes an increase in the incorporation of labelled aminoacids into cell proteins. If α-alanine-^{14}C is replaced by a mixture of ^{14}C-amino acids from an algal protein hydrolysate, there is no significant change.

On the other hand, when the incorporating system consists of rat subcellular fractions, the stimulating action of L-*cyclo*serine is doubtful. (Table 2).

TABLE 3. PERCENTAGE VARIATIONS OF THE SPECIFIC ACTIVITY OF CELL PROTEINS OF *E. coli* 147 INCUBATED WITH DL-ALANINE-^{14}C IN THE PRESENCE OF DCS, LCS, PLP AND A MIXTURE OF LCS AND PLP

Experiment no. 1		Experiment no. 2	
Additives (μM/ml)	% Variations of protein specific activity as compared to the control	Additives (μM/ml)	% Variation of protein specific activity as compared to the control
LCS 0·04	+ 7·8	LCS 0·04	+ 33·2
—		LCS 0·10	+ 47·5
—		LCS 0·20	+ 47·0
PLP 0·04	− 23·6	PLP 0·04	− 14·2
PLP 0·12	− 30·0	—	
PLP 0·32	− 24·7	—	
LCS 0·04 PLP 0·04	− 7·5	LCS 0·04 PLP 0·04	+ 21·2
LCS 0·04 PLP 0·12	− 6·5	— —	
LCS 0·04 PLP 0·32	− 11·7	— —	
— —		LCS 0·10 PLP 0·04	+ 33·2
— —		LCS 0·20 PLP 0·04	+ 33·4
DCS 0·20	− 27·0		
DCS 0·20 PLP 0·04	− 47·5		
DCS 0·20 PLP 0·12	− 49·5		
DCS 0·20 PLP 0·32	− 51·0		

* PLP = Pyridoxalphosphate.

Table 3 reports the percentage variations occurring in the specific activity of *E. coli* 147 proteins when bacterial suspensions are incubated, in the presence of DL-alanine-^{14}C, either with L-*cyclo*serine, or pyridoxalphosphate or a mixture of the two.

Influence of L-cyclo*serine and* D-cyclo*serine on the transamination by enzymic systems*

Rat liver homogenates. When the transaminating activity of rat liver homogenate is tested in the reaction L-alanine + α-ketoglutarate ⇌ pyruvate + glutamate, D-*cyclo*serine does not show any inhibiting action up to a concentration of 1 μM/ml while the inhibiting action of L-*cyclo*serine is already strong at concentrations of 0·02 μM/ml and complete at concentrations of 0·20 μM/ml.

TABLE 4. ACTION OF DCS AND LCS ON THE FORMATION OF PYRUVATE FROM L-ALANINE + α-KETOGLUTARATE IN THE PRESENCE OF RAT LIVER HOMOGENATE

DCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of pyruvate formation	LCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of pyruvate formation
1·00	0·04	2·25	0·02	0·04	54·00
1·00	0·40	7·00	0·02	0·40	57·50
2·00	0·04	3·40	0·20	0·04	98·00
2·00	0·40	2·00	0·20	0·40	98·50

When the reaction L-aspartate + α-ketoglutarate ⇌ oxalacetate + glutamate is used, both D- and L-*cyclo*serine, in concentrations ranging from 0·02 μM/ml to 0·2 μM/ml, show only a slight inhibiting action.

TABLE 5. ACTION OF DCS AND LCS ON OXALACETATE FORMATION FROM L-ASPARTATE + α-KETOGLUTARATE IN THE PRESENCE OF RAT LIVER HOMOGENATE

DCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxyalacetate formation	LCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxalacetate formation
0·02	0·04	2·50	0·02	0·04	2·00
0·02	0·40	11·50	0·02	0·40	14·00
0·20	0·04	10·00	0·20	0·04	9·30
0·20	0·40	16·50	0·20	0·40	16·00
2·00	0·04	43·40			

E. coli cells. When the transaminating activity of a suspension of *E. coli* dried cell is tested on the reaction L-alanine + α-ketoglutarate ⇌ pyruvate + glutamate, D-*cyclo*serine in concentrations up to 0·2 μM/ml has scarcely any effect on the formation of pyruvate, while L-*cyclo*serine displays an inhibiting action even stronger than that observed with liver homogenate.

The enzymic preparations from *E. coli*, incubated at 37 °C for a prolonged time, form pyruvate even in the absence of amino or ketoacids. The pyruvate formed in this case is not affected by addition of 1 μM/ml of D- or L-*cyclo*serine and therefore is ascribed to metabolic pathways not involving alanine transamination. The inhibition of bacterial alanine transamination by L-*cyclo*serine was determined by subtracting

the amount of pyruvate produced in the "blank" from that produced in the samples incubated with alanine. The percentage inhibition of the transamination L-alanine $\rightleftharpoons$ glutamate exerted by *cyclo*serine on the enzymic preparation of *E. coli* 147 and B, respectively, are reported in Tables 6 and 7.

TABLE 6. ACTION OF DCS AND LCS ON PYRUVATE FORMATION FROM L-ALANINE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* 147 SUSPENSION

DCS + PLP (μM/ml)		% Inhibition of pyruvate formation	LCS + PLP (μM/ml)		% Inhibition of pyruvate formation
0·20	0·04	21·00	0·02	0·04	88·00
0·20	0·40	12·00	0·02	0·40	97·50
1·00	0·04	58·00	0·20	0·04	96·50
1·00	0·40	41·00	0·20	0·40	100·00
			1·00	0·04	90·00
			1·00	0·40	100·00

TABLE 7. ACTION OF DCS AND LCS ON PYRUVATE FORMATION FROM L-ALANINE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* B SUSPENSION

DCS + PLP (μM/ml)		% Inhibition of pyruvate formation	LCS + PLP (μM/ml)		% Inhibition of pyruvate formation
0·20	0·04	10·00	0·02	0·04	63·00
0·20	0·40	39·00	0·02	0·40	91·50
			0·20	0·04	93·20
			0·20	0·40	93·20

When the test reaction is L-aspartate + α-ketoglutarate $\rightleftharpoons$ oxalacetate + glutamate, neither D- nor L-*cyclo*serine show any inhibiting action in concentrations up to 0·20 μM/ml. In concentrations of 1 μM/ml their inhibition values are almost similar. The percentage inhibition values of L-*cyclo*serine on oxalacetate formation by enzymic preparations of *E. coli* 147 and B, respectively, are reported in Tables 8 and 9.

TABLE 8. ACTION OF DCS AND LCS ON OXALACETATE FORMATION FROM ASPARTATE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* 147 SUSPENSION

DCS + PLP (μM/ml)		% Inhibition of oxalacetate formation	LCS + PLP (μM/ml)		% Inhibition of oxalacetate formation
0·02	0·04	0	0·02	0·04	0
0·02	0·40	0	0·02	0·40	0
0·20	0·04	0	0·20	0·04	0
0·20	0·40	0	0·20	0·40	0
1·00	0·04	30·00	1·00	0·04	46·00
1·00	0·40	30·00	1·00	0·40	44·00

TABLE 9. ACTION OF DCS AND LCS ON THE OXALACETATE FORMATION FROM ASPARTATE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* B SUSPENSION

DCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxalacetate formation	LCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxalacetate formation
0·02	10	0	0·02	0·04	2·50
0·02	100	0	0·02	0·40	0
0·20	10	3·50	0·20	0·04	0
0·20	100	0	0·20	0·40	0
1·00	10	20·00	1·00	0·04	32·00
1·00	100	5·00	1·00	0·40	13·00

DISCUSSION

The L-isomer of *cyclo*serine, as well as the D-isomer, does not seem to affect the incorporation into cell proteins of ^{14}C amino acids from algal protein hydrolysate. On the other hand, if we use alanine-^{14}C instead of an amino acid-^{14}C mixture *cyclo*serine exerts a remarkable action on the incorporation of this amino acid into cell proteins. In fact, low concentrations of L-*cyclo*serine increase the incorporation of this amino acid into protein while high concentrations decrease it.

Experiments carried out with L-*cyclo*serine and pyridoxalphosphate showed that these substances promote opposite effects on the incorporation of DL-alanine-^{14}C into protein of *E. coli* 147.

The depressing activity of pyridoxalphosphate, upon DL-alanine-^{14}C incorporation, may be ascribed to the property of this coenzyme to accelerate the biosynthesis of alanine, as it is better observed in vitamin B_6 requiring micro-organisms.[14]

Considering the antibacterial properties of L-*cyclo*serine as well as its inhibiting action on alanine $\rightleftharpoons$ ketoglutarate transamination, the increase in protein radioactivity induced by L-*cyclo*serine could be considered as the effect of the inhibition exerted by this drug on the synthesis of alanine.

From the radioactive data (Table 3) we wonder whether a possible active opposition does occur between pyridoxalphosphate and L-*cyclo*serine towards some enzymic steps of alanine metabolism, even if the results of the first experiment of Table 3 would support this possiblity.

From this experiment it is apparent that the actions of pyridoxal phosphate and the D-isomer of *cyclo*serine do not exclude each other. In any case, assays performed on the influence of *cyclo*serine upon the transaminating activity of enzymic preparations show clearly that L-*cyclo*serine, more than the D-isomer, inhibits the transaminating reactions and displays a characteristic specificity towards the substrates of enzymic reactions. Therefore it does not affect the formation of oxalacetate from L-aspartate and α-ketoglutarate even when it is used at the same concentrations which inhibit strongly the formation of pyruvate from L-alanine and α-ketoglutarate. On the other hand, D-*cyclo*serine inhibits only partially the transaminating system when used in concentrations fifty times higher than those required for a complete inhibition by L-*cyclo*serine. It is worth noticing that L-*cyclo*serine causes the same inhibition when supplemented either with 10 μg/ml or 100 μg/ml of pyridoxalphosphate. This seems to exclude the occurrence of a competitive antagonism between L-*cyclo*serine and pyridoxalphosphate for the same transaminating enzymes.

The stereospecificity of the inhibiting action of L-*cyclo*serine (only L-*cyclo*serine inhibits the transaminating reaction of L-alanine) would rather suggest the presence of a competition, between L-*cyclo*serine and L-alanine, for the same transaminating enzyme, though previous works showed that the inhibiting action of L-*cyclo*serine on *E. coli* growth is antagonized, but not competitively, by L-alanine.[1]

In any case it is clear that the enantiomorphs of *cyclo*serine, even if possessing a common antibacterial activity, behave in a quite different way.

The D-isomer, indeed, interferes selectively with the mechanism involved in D-alanine incorporation into macromolecular complexes of the bacterial cell wall, and slightly affects the biosynthetic pathway of alanine formation, such as glutamate $\rightleftharpoons$ pyruvate transamination. Moreover, D-*cyclo*serine does not exhibit any action on L-alanine-^{14}C incorporation into proteins of mammalian cells. On the other hand, L-isomer specifically inhibits a transaminating reaction which forms L-alanine either in bacterial or in mammalian cells. This is also significant in regard to the pharmacological properties of L-*cyclo*serine.

It is doubtful whether the data concerning the effect of L-*cyclo*serine on L-alanine-^{14}C incorporation into proteins of subcellular fractions are significative, because of the too weak extent of L-alanine-^{14}C incorporation into protein, observed in our experiments (Table 2).

REFERENCES

1. A. Buogo, A. Di Marco, M. Ghione, A. Migliacci and A. Sanfilippo, *Giorn. Microbiol.* **6**, 130 (1959).
2. M. Serembe, *Boll. Soc. Ital. Biol. Sper.* **33**, 474 (1957).
3. B. Monaco, *Boll. Soc. Ital. Biol. Sper.* **34**, 1464 (1958).
4. C. Bertazzoli, A. Buogo and M. Ghione, *Farmaco, Ed. Sci.* **13**, 447 (1958).
5. J. Smrt, J. Beranek, J. Sicher, J. Skoda, V. F. Hess and F. Sorm, *Experientia* **13**, 291 (1957).
6. P. Barbieri, A. Di Marco, L. Fuoco and A. Rusconi, *Biochem. Pharmacol.* In press.
7. B. D. Davis and E. S. Mingioli, *J. Bact.* **60**, 7 (1950).
8. H. E. Umbarger and B. Magasanik, *J. Amer. Chem. Soc.* **74**, 4256 (1952).
9. E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.* **221**, 45 (1956).
10. M. Kunitz, *J. Gen. Physiol.* **35**, 423 (1952).
11. M. Rabinovitz, M. E. Olson and D. M. Greenberg, *J. Biol. Chem.* **210**, 837 (1954).
12. N. E. Tonhazy, N. G. White and W. W. Umbreit, *Arch. Biochem.* **28**, 36 (1950).
13. P. Siekevitz, *J. Biol. Chem.* **195**, 549 (1952).
14. E. E. Snell, N. S. Radin and M. Ikawa, *J. Biol. Chem.* **217**, 803 (1955).