THE MECHANISM OF ACTION OF POLYCHLOROSUBTILIN

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Polychlorosubtilin (PCS) inhibits the growth of *Escherichia coli*. The antibiotic affected neither respiration nor glycolysis while the synthesis of nucleic acids and proteins were feebly hindered. Formation of aminoacyl-tRNA, peptide bonds and translocation from A to P sites of ribosomes were insignificantly influenced by the drug. The antibiotic exerted its effect(s) on ribosomes by interfering with the 30 S subunits. The 23 S and 30 SP were both sensitive to the drug but the latter was more obviously affected. Changes after developing resistance to the drug by the bacteria were localized in the 30 SP, 23 S and accordingly the 30 S subunits. The principal action of PCS was to cause multisited miscoding upon the incorporation of labeled aminoacyl-tRNA, therefore, malformed protein fractions (abnormal) were synthesized. As a natural consequence such abnormal fractions would not be expected to manifest the vital metabolic activities in the normal way.

Polychlorosubtilin (PCS) was the first recorded polychloro-non-nitrogenous metabolite isolated from bacterial fermentation broths[1] and the structure of which is still to be elucidated. The antibiotic possesses substantial activity against some Gram-positive and -negative bacteria. The present paper describes studies on the mechanism of action of the antibiotic.

Materials and Methods

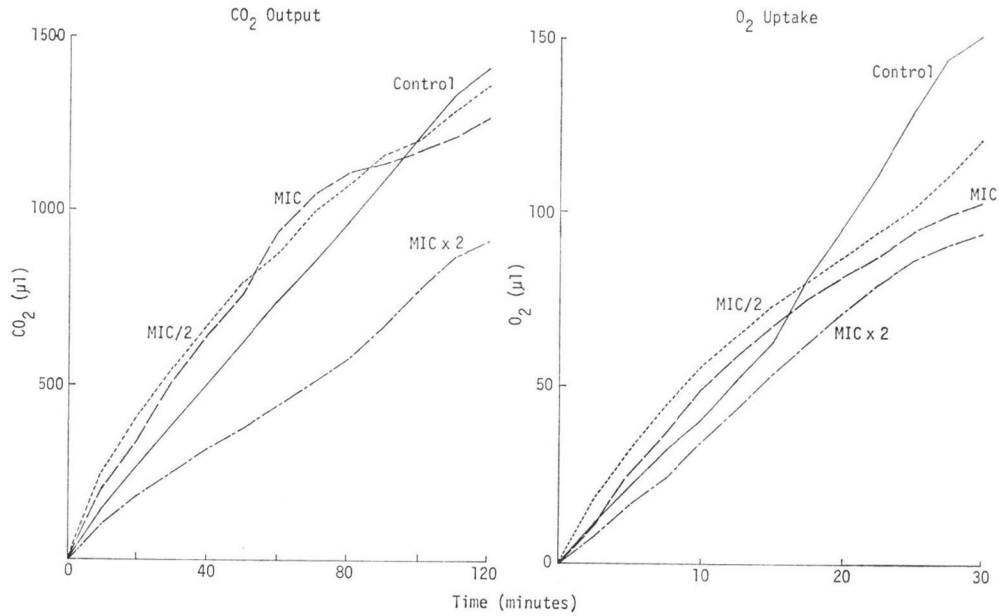
The following labeled compounds were used: (4,5-^{3}H) lysine monohydrochloride, 82 Ci/m mol; (U-^{14}C) thymidine, 517 mCi/m mol and (2-^{14}C) uridine, 465 mCi/m mol; these tagged chemicals were from the Radiochemical Centre, Amersham. The other chemicals were obtained from Sigma Chemical Co., Saint Louis, Mo., U.S.A.

Escherichia coli B was grown in a nutrient broth of the following composition (g/100 ml): peptone, 0.5; NaCl, 0.5; meat extract, 0.15 and yeast extract, 0.15. The bacterial cells were incubated at 30°C and 220 rpm. The incubation was terminated by centrifugation at the mid-logarithmic phase of growth to harvest the bacterial cells. The collected biomass was washed repeatedly with sterile saline solution before being used in the following experiments. PCS was always supplemented to the reaction media to attain the MIC level (12 μg/ml) and the optical density (OD) of the inoculated media was adjusted to 0.1 at 660 nm unless otherwise indicated.

Effect of PCS on respiration and glycolysis using the normal manometric techniques was conducted[2] and the results are given in Fig. 1 and its legend. Next, the effects of the drug on *de novo* synthesis of nucleic acids and proteins of the bacterial cells were investigated in presence of the corresponding tagged intermediates as described in legend to Fig. 2. Subsequently, action of the drug on activities of aminoacyl-tRNA synthetases was explored using a fraction of ribosome-free supernatant (S-150) of *E. coli*[3]. The effect of PCS on the binding of labeled amino acids by the tRNA synthetase was then investigated; Table 1 and legend.

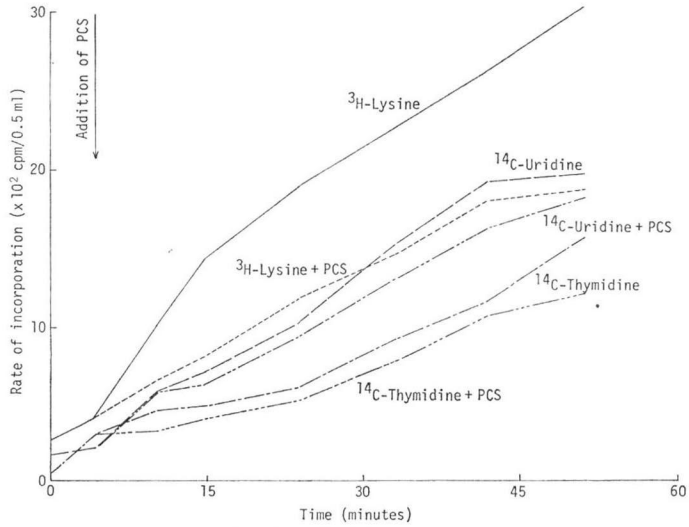
The possible action of the drug on the role played by ribosomes and their subunits was then investigated. Ribosomes and their subunits were obtained from cells sensitive to PCS and from cells that acquired resistance against the drug during successive transfers of the organism on media supplemented with increasing concentrations of the drug; from 0.6 μg/ml to 250 μg/ml of media. It is assumed that any significant difference in response to PCS between the ribosomes obtained from sensitive bacteria and

Fig. 1. Effect of the PCS on respiration and glycolysis.



E. coli were grown overnight, washed with sterile saline solution and then suspended in M/15 phosphate buffer of pH 7.0. The reaction flask contained 1.0 ml of the bacterial suspension which contained *ca.* 14 mg of cellular material and the antibiotic at the MIC level. The total volume was made up to 3.0 ml with buffer. Glucose was used as substrate at a final concentration of 0.02 mg/ml. Incubation temperature was 30°C and the gas phase was pure nitrogen. Carbon dioxide evolution was measured at 5-minute intervals.

Fig. 2. Effect of PCS on rate of incorporation of tagged intermediates into proteins, DNA and RNA of *E. coli*.



Cells of the bacterium were prepared as described in the text and were then suspended in fresh medium containing any of the following labeled compounds per 10 ml of culture media, 5.0 μCi of uridine, 5.0 μCi of thymidine and 2.5 μCi of lysine to trace their incorporation in RNA, DNA and proteins respectively. Incubation was resumed at 37°C for 5 minutes and then to half of the volume of each reaction fluid dioxane solution of PCS was added to attain the MIC level while to the second half an equal volume of dioxane without PCS was added. Incubation was resumed, samples were withdrawn at different time intervals and mixed with equal volumes of 5% ice-cold TCA. Insoluble materials were collected on millipore membrane filter discs (pore size 0.45 μ), washed twice with ice cold TCA solution, rinsed with diethyl ether and finally counted in 10 ml scintillation liquid containing 6 g PPO and 0.49 g dimethyl-POPOP/liter toluene.

those from resistant cells will indicate the site of action of the drug.

Preparation of ribosomal subunits (50S, 30S & 30SP) and their reconstitution was carried out as described in detail elsewhere[4,5,6]. Ribosomes were initially separated into 30 S and 50 S fractions by dialysis against a low Mg^{++} concentration and were then purified by sucrose density gradient (5~20%) centrifugation. The 30S subunits were dissociated further to 30 SP and 23 S core by centrifugation to equilibrium in 5.2 M CsCl solution with 0.04 M Mg^{++} and 2×10^{-4} M EDTA. The ribosomal subunits from sensitive and resistant cells were used in various combinations with poly U, poly A and poly C to trace the incorporation of labeled amino acids which were selected in such a way as to demonstrate miscoding effects. Experimental details and composition of the media are shown in legend to Table 2.

The effect of PCS on the binding of (^{14}C) phenylalanyl-tRNA to ribosomes and 30 S subunits of sensitive and resistant bacteria was subsequently investigated in the presence of poly U, poly A or poly C as described in legend to Table 3.

Further study was carried out on the effect of PCS on protein synthesis *via* possible interference with peptide bond formation between peptidyl-tRNA on the peptidyl site (P site) and the AA-tRNA on the amino-acyl site (A site) of the ribosomes. This reaction is known to be catalyzed by a peptidyl-transferase which is an integral part of the ribosomes[7~11]. To investigate such possible effect of PCS on peptide bond formation, the reaction of puromycin with N-acetyl-phenylalanyl-tRNA[12,13] was allowed to proceed in the presence of PCS, GTP and G factor which translocates N-acetyl-phenylalanyl-tRNA from the ribosomal A site to the P site. Experimental details are given in legend to Table 4.

Table 1. Effect of PCS on the activity of aminoacyl synthetase.

^{14}C-Amino acid	cpm/20 μl of reaction mixture		
	PCS 12 μg/ml	Control	% Inhibition
Alanine	2,935	3,103	5.41
Arginine	4,372	4,185	− 4.47
Aspartic acid	3,887	4,098	5.15
Cysteine	4,232	3,916	− 8.08
Glutamic acid	4,801	4,658	− 3.07
Glycine	4,074	7,867	16.68
Histidine	4,168	3,799	− 9.68
Leucine	3,950	4,282	7.76
Lysine	4,872	4,725	− 3.12
Methionine	3,432	3,071	−11.76
Phenylalanine	3,205	3,389	5.44
Serine	4,114	4,005	− 2.72
Threonine	3,103	2,794	−11.06
Tyrosine	2,233	2,158	− 3.48
Valine	4,099	4,169	1.68
Proline	4,273	4,370	2.22

The reaction mixture contained the following:

(a) ATP, 0.1 M (100 μl); CTP, 0.05 M (10 μl); phosphoenolpyruvate, 0.33 M (30 μl); Tris-HCl pH 7.6, 2 M (50 μl); mercaptoethanol, 1 M (50 μl); $MgCl_2$, 1 M (20 μl): (^{14}C) lysine or any other labeled amino acids (5 μCi) and water (130 μl).

(b) 50 μl of mixture (a) were set in ice cold bath, 1 μg/10 μl of phosphoenolpyruvate kinase was added followed by 30 μl of *E. coli*-tRNA (2mg/ml) and then 30 μl of the aminoacyl synthetase was added. The tubes were incubated at 28°C for 35 minutes when 15 μl samples were withdrawn at different time intervals and counts for the ice cold 5% TCA insoluble fraction were measured.

Finally, effect of PCS on protein contents of *E. coli* and the approximate molecular weights of the cellular proteins were determined by gel filtration as described by ANDREWS[14]. The residual bacteria-free broths were analysed for their amino acid content using two-dimensional paper chromatography and the following developing solvent systems; butan-1-ol - acetic acid - water (18: 2: 5) as the first solvent and phenol solution (80% w/v) containing 0.015% of 8-hydroxyquinoline as the second solvent. The acetic acid portion of the first solvent was sometimes replaced by ammonia solution to allow better identification of some amino acids.

Results

The results of Fig. 1 indicate that the drug did not exert a significant influence on the two metabolic channels (glycolysis or respiration) while the results of Fig. 2 demonstrate that protein synthesis was more sensitive to the drug than nucleic acid synthesis. Data of Table 1 could hardly indicate any discrete

Table 2. Effect (s) of PCS on incorporation of some amino acids guided by polyhomonucleotides.

50S	30S	23S	30SP	Mix.	Incorporation with poly U				Incorporation with poly A				Incorporation with poly C			
					Phenylalanine		Leucine		Lysine		Glutamic		Proline		Alanine	
					cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
S	S			C	7,400	100	144	100	4,785	100	98	100	3,556	100	104	100
				C+A	858	12	705	490	182	3	881	899	373	11	707	680
S	R			C	6,889	100	212	100	4,723	100	104	100	3,842	100	104	100
				C+A	6,136	89	208	98	4,496	95	99	95	3,406	89	98	94
R	S			C	6,078	100	179	100	3,946	100	90	100	4,115	100	97	100
				C+A	1,045	17	617	345	146	4	1,154	1,282	232	6	488	503
R	R			C	7,093	100	283	100	4,574	100	84	100	4,503	100	138	100
				C+A	6,490	92	289	102	4,287	94	91	108	4,302	96	129	94
S		S	S	C	6,582	100	115	100	4,351	100	102	100	4,371	100	103	100
				C+A	704	11	803	968	418	9	1,463	1,434	199	5	1,574	1,528
S		S	R	C	5,775	100	132	100	4,506	100	123	100	4,547	100	139	100
				C+A	2,889	50	374	283	907	20	538	437	379	8	421	303
S		R	S	C	7,243	100	147	100	4,434	100	117	100	4,765	100	110	100
				C+A	1,600	22	591	402	1,327	30	985	842	186		742	67
S		R	R	C	6,451	100	126	100	3,859	100	78	100	4,103	100	91	100
				C+A	5,883	91	104	83	4,032	105	72	92	3,907	95	94	103
R		S	S	C	7,058	100	129	100	3,948	100	103	100	1,223	100	135	100
				C+A	642	9	786	609	178	5	1,607	1,560	141	12	1,176	1,019
R		S	R	C	5,500	100	202	100	4,636	100	148	100	4,778	100	104	100
				C+A	4,461	81	316	156	1,025	22	589	398	320	7	317	305
R		R	S	C	6,125	100	192	100	4,068	100	105	100	4,447	100	97	100
				C+A	1,237	20	514	268	1,001	25	1,237	1,178	354	8	401	413
R		R	R	C	7,040	100	188	100	4,595	100	78	100	4,641	100	83	100
				C+A	6,378	91	156	83	4,498	98	80	103	4,357	94	78	94

C : Control reaction mixtures.
C+A: Control reaction mixtures+PCS 12 μg/ml of total volume.
S : *E. coli* sensitive to PCS.
R : *E. coli* resistant to PCS.
% : Percent of control.

The reaction mixture as indicated in the Table contained in 0.5 ml the following components: 50 S, 200 μg; 30 S, 120 μg; 23 S core, 70 μg; 30 SP, 60 μg; *E. coli* 105,000$\times g$ supernatant 150 μg, *E. coli* B tRNA, 150 μg; poly U, poly A or poly C, 20 μg; ATP, 1 μ mole; GTP, 0.05 μ moles; creatine phosphate, 2 μ moles; creatine phosphokinase, 50 μg; ^{14}C-lysine (305 mCi/mM) 0.1 μl; ^{14}C-glutamic acid (265 mCi/mM) 0.1 μl; ^{14}C-proline (255 mCi/mM) 0.1 μl; ^{14}C-alanine (162 mCi/mM) 0.1 μl; $MgCl_2$, 10 mM; NH_4Cl, 50 mM; 2-mercaptoethanol, 6 mM and Tris-HCl, 10 mM at pH 7.4. Incubation was at 37°C for 30 minutes. Assessment of the rate of reactions was carried out as described in legend to Fig. 2.

effect of PCS on the formation of aminoacyl-tRNA. An interesting finding that is exhibited by results of Table 2 is that resistance to PCS was principally localized in the ribosomes, 30 S subunit, 23 S core and 30 SP. An obvious miscoding influence was exerted by the drug on the incorporation of amino acids

Table 3. Effects of PCS on binding of ^{14}C-phenylalanyl-tRNA to ribosomes and 30 S subunits guided by polyhomonucleotides.

		bound ^{14}C phenylalanyl-tRNA (cpm)		% of control cpm	
		*R	*S	*R	*S
Basic reaction mixture I	+poly U (control)	1,205	1,318	100	100
	+poly U+PCS	1,081	1,371	90	104
	+poly C	64	56	100	100
	+poly C+PCS	162	1,117	253	1,995
	+poly A	63	81	100	100
	+poly A+PCS	137	938	217	1,158
Basic reaction mixture II	+poly U (control)	883	794	100	100
	+poly U+PCS	734	213	83	27
	+poly C	68	76	100	100
	+poly C+PCS	117	735	172	967
	+poly A	59	47	100	100
	+poly A+PCS	66	509	112	1,083

Basic reaction mixture I: 10 μ moles Tris-HCl of pH 7.4; 10 μ moles KCl; 3 μ moles magnesium acetate; 1.2 μ moles B-mercaptoethanol; 0.2 mg ribosomes; 60 μg ^{14}C-phenylalanyl-tRNA (13,908 cpm); 10 μg of poly U, A or C and 2.5 μg in a final volume of 0.2 ml.

Basic reaction mixture II: As in mixture I, except that ribosomes were replaced by 30 S subunits. Incubation was carried out at 30°C for 30 minutes and then binding of the labeled supplement to ribosomes or 30 S subunits was assessed by the millipore filter technique.

*R: ribosomes or 30S subunits obtained from the bacteria resistant to PCS.

*S: ribosomes or 30S subunits obtained from the bacteria sensitive to PCS.

Table 4. Effect of PCS on the reaction of puromycin with N-acetyl ^{3}H-Phe-tRNA.

Reaction mixtures	N-acetyl-Phe-puromycin formed (cpm)		% of control	
	*R	*S	*R	*S
Basic reaction mixture (control)	1,202	1,123	100	100
−Puromycin	316	287	25.8	25.6
−GTP+105 S	984	895	81.9	79.7
+PCS (12 μg/ml)	1,194	1,202	99.3	107
+PCS (50 μg/ml)	1,231	1,181	102.4	105.2
+PCS (100 μg/ml)	1,098	1,141	91.3	101.6
+Fusidic acid 10^{-4}M	947	882	78.8	78.5

Basic reaction mixture contained/0.2 ml: 12.5 μ moles Tris-HCl buffer of pH 7.4; 2.5 μ moles magnesium acetate; 12.5 μ moles KCl; 1.5 μ moles B-mercaptoethanol; 10 μg poly U; 0.4 mg ribosomes; 25 μ moles GTP and 48 μg N-acetyl ^{3}H-Phe-tRNA (4,072 cpm).

Each reaction mixture was incubated with 100 μg puromycin in 0.04 ml of 2.5% dimethyl formamide at 30°C for 6 minutes and with each of the following antibiotics: 100 μg fusidic acid; 12, 50 and 100 μg PCS.

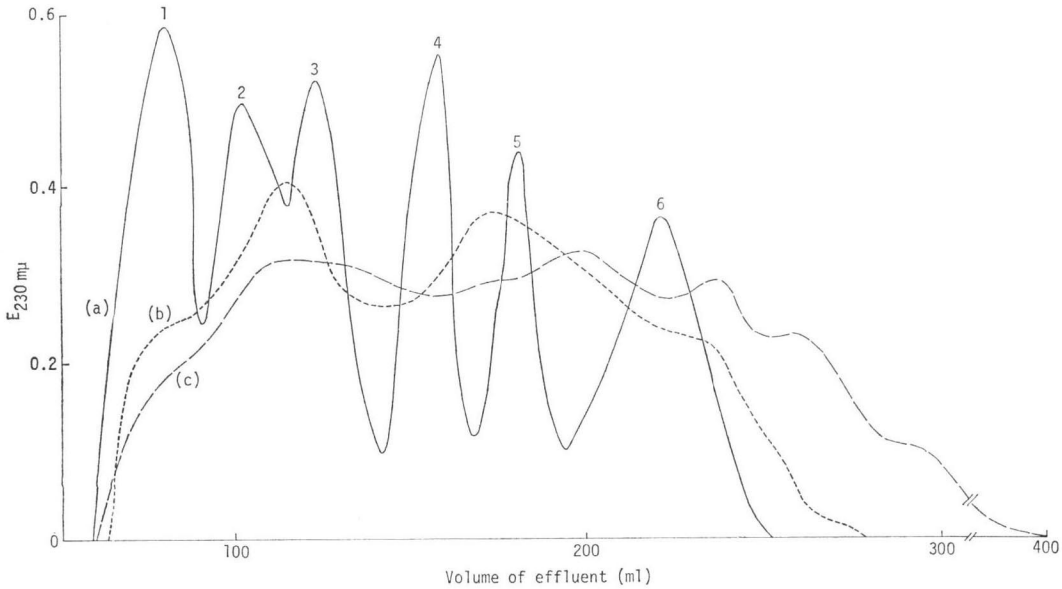
Reactions were stopped by dropping the pH to 5.5 by acetate buffer and 1.5 ml ethyl acetate was then added. One ml of the ethyl acetate was placed on a planchet, evaporated and radioactivity was then determined.

*R: refers to ribosomes obtained from *E. coli* resistant to PCS.

*S: refers to ribosomes obtained from *E. coli* sensitive to PCS.

Fig. 3. Elution diagrams for separation of reference proteins and proteins of *E. coli* treated and untreated with PCS on Sephadex G-100.

(a) Mixture of 1. human γ globulin 0.96 mg, 2. bovine serum albumin 1.92 mg, 3. ovalbumin 1.83 mg, 4. chymotrypsinogen 1.71 mg, 5. cytochrome C 1.22 mg, and 6. glucagon 1.08 mg.
(b) Protein extracted from *E. coli* untreated with PCS.
(c) Protein extracted from *E. coli* treated with PCS.



Column (2.5 × 50 cm) was equilibrated with 0.05 M Tris- HCl buffer at pH 7.5 containing 0.1 M KCl. Bacterial cells were harvested by centrifugation and then ruptured by grinding with very fine sand particles in Tris-HCl buffer at pH 7.5 and 0.1 M KCl. Residual solids were harvested by low speed centrifugation at 7,500 rpm and the supernatant fluid was applied to the Sephadex G-100 column. Details of preparation and use of gel columns are as described by ANDREWS[14].

guided by the polyhomonucleotides whenever the ribosomes and their fractions were derived from sensitive bacteria. The results of Table 3 are in line with those of Table 2; both demonstrate clearly that PCS exerted a multisited miscoding influence on amino acid incorporation and that the ribosomes and 30 S subunits of resistant bacteria were markedly less sensitive to the drug.

Data of Table 4 demonstrate that PCS did not inhibit the formation of N-acetyl-phenylalanyl-puromycin in the presence of GTP and the 105 S supernatant indicating that the drug failed to restrict peptide bond formation and the translocation step which is rather sensitive to fusidic acid[15]. This excludes the peptidyltransferase as a possible site of action of the antibiotic.

Finally the analytical data of Fig. 3 demonstrate greater abundance of low molecular weight cellular protein fractions in cells treated with PCS when compared with cells in control cultures. Lysine, proline, arginine, phenylalanine, cystine and four unidentified ninhydrin-positive spots could only be detected in cultures treated with PCS. The broth of untreated cultures contained traces of lysine, arginine and valine.

Discussion

The scope of action of PCS on the bacterial test organisms did not include glycolysis or respiration as indicated by the results shown in Fig. 1. The effects of PCS on the rate of incorporation of tagged

intermediates in *de novo* synthesis of proteins, DNA and RNA were rather feeble. Nevertheless, protein synthesis was the most sensitive to the drug and therefore exploration of a possible interference in some metabolic reactions that are involved in protein synthesis was carried out.

The results of Table 1 concerning the effects of PCS on formation on aminoacyl-tRNA revealed feeble influences which are considered insufficient to show definite action of the drug on the activity of aminoacyl synthetases. Thereafter, the action of the drug on amino acid incorporation guided by polyhomonucleotides and ribosomes or their subunits obtained from sensitive and resistant bacteria was thoroughly examined (Table 2). Whenever ribosomes and their subunits other than the 50 S were collected from bacteria sensitive to PCS considerable miscoding influence was exerted by the drug. Such effects were demonstrated as an appreciable inhibition by PCS of the incorporation of the amino acid coded by the polyhomonucleotide (right amino acid) and as stimulatory influences for incorporation of the wrong amino acids; those which were not coded for. The effect of PCS on 30 SP subunits of sensitive bacteria was more pronounced than on the 23 S core of the same origin. Furthermore, the miscoding effect of PCS on the incorporation of ^{14}C-phenylalanyl-tRNA guided by poly A, poly U, and poly C was examined (Table 3). The results indicate that the drug was only effective as a miscoding agent when the ribosomes and 30 S subunits were obtained from sensitive bacteria. The antibiotic gave marked stimulation to incorporation of the labeled phenylalanyl-tRNA guided by the wrong polyhomonucleotides; poly A and poly C; whereas inhibition was observed when poly U was involved in the reaction.

The analytical data of Fig. 3 concerning gel filtration of extracts of cellular proteins of *E. coli* treated with PCS indicate a greater abundance of low molecular weight fractions. Besides, more amino acids and ninhydrin-positive metabolites were detected in culture broths treated with PCS. Such abnormal protein constituents in cells treated with PCS might be expected to be lethal to the organism.

From the above results it is concluded that the 30 S subunit is the site of action of PCS on the ribosomes. Both 30 SP and 23 S subfractions were found sensitive to the antibiotic but the former was more sensitive. Since the binding of AA-tRNA to the 30 S subunit involves codon-anticodon interaction, it is concluded that PCS acts upon the 30 S subunit in such a way that disturb the coding mechanism. This anticipated influence would result in abnormal protein fractions which fail to offer a normal metabolic melee for normal bacterial growth.

References

1) SHIMI, I. R. & M. W. KELADA: Polychlorosubtilin, an antibiotic newly isolated from metabolites of *Bacillus subtilis*. J. Antibiotics, Ser. A 18: 56~59, 1965
2) UMBREIT, W. W.; R. H. BURRIS & J. F. STAUFFER: Manometric technique. 3 rd. Ed., p. 149, 1959
3) KAJI, K.: Technique for measuring specific sRNA binding to *E. coli* ribosomes. cited in Methods in Enzymology Vol. 12, p. 692, New York, Academic press, 1968
4) TRAUB, P.; S. MIZUSHIMA, C. V. LOWRY & M. NOMURA: Reconstitution of ribosomes from subribosomal components. Methods in Enzymol. 20: 391~407, 1971
5) HOSOKAWA, K.; R. K. PUJIMURE & M. NOMURA: Reconstitution of functionally active ribosomes from inactive subparticles and proteins. Proc. Nat. Acad. Sci., U.S.A. 55: 198~204, 1966
6) STAEHELIN, T. & M. MESELSON: *In vitro* recovery of ribosomes and of synthetic activity from synthetically inactive ribosomal subunits. J. Mol. Biol. 16: 245~249, 1966
7) MONRO, R. E.: Catalysis of peptide bond formation by 50S ribosomal subunits from *E. coli*. J. Mol. Biol. 26: 147~151, 1967
8) GOTTESMAN, M. E.: Reaction of ribosome bound peptidyltransfer ribonucleic acid with aminoacyl transfer ribonucleic acid on puromycin. J. Biol. Chem. 242: 5564~5571, 1967
9) RYCHLIK, I.: Release of lysins peptide by puromycin from polylysyltransfer ribonucleic acid in the presence of ribosomes. Biochem. Biophys. Acta 114: 425~427, 1966
10) MADEN, B. E. H.; R. R. TRAUT & R. E. MONRO: Ribosome catalysed peptidyl transfer the poly phenylalanine system. J. Mol. Biol. 35: 333~345, 1968
11) MADEN, B. E. H. & R. E. MONRO: Ribosome catalysed peptidyl transfer effects of cations and pH value. Eur. J. Biochem. 6: 309~316, 1968
12) HERBERT, E. & J. SMITH: Effect of limiting concentrations of T, ribonuclease on the release of aminoacyl-oligonucleotide from unfractionated yeast acceptor RNA. J. Mol. Biol. 28: 281~294, 1967

13) WEISSBACH, H.; B. REDFIELD & N. BROT: Studies on the reaction of N-acetyl-phenylalanyl-tRNA with puromycin. Arch. Biochem. Biophys. 172: 705~710, 1968
14) ANDREWS, P.: Estimation of the molecular weight of protein by Sephadex Gel filtration. Biochem. J. 91: 595~606, 1964
15) TANAKA, N.; T. KINOSHITA & H. MASUKAWA: Mechanism of protein synthesis, inhibition by fusidic acid and related antibiotics. Biochem. Biophys. Res. Commun. 30: 278~283, 1968