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PACTAMYCIN, AN ANTIBIOTIC THAT INHIBITS PROTEIN SYNTHESIS*

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Abstract—Pactamycin primarily inhibited protein synthesis in KB cells and *Bacillus subtilis* with much less effect on RNA or DNA synthesis. Pactamycin injected into rats at a level of 0·82 mg/kg caused about 50 per cent inhibition of both hydrocortisone- and tryptophan-induced increase in tryptophan pyrrolase in rat liver.

In the cell-free system isolated from *Escherichia coli*, polyproline synthesis was inhibited 50 per cent at 0·75 μg/ml, although polyphenylalanine synthesis was not inhibited even at 100 μg/ml. Pactamycin did not affect the formation of prolyl-sRNA. Also, in agreement with the above observations, pactamycin inhibited the incorporation into protein of prolyl-sRNA and not of phenylalanyl-sRNA. These results indicate that pactamycin inhibited some step in the transfer of amino acyl-sRNA to ribosomes. Ribosomes isolated from the liver of pactamycin-treated rats had 30 per cent of the activity of control ribosomes, whereas the activity of the 105,000 *g* supernatant was not affected. Pactamycin, unlike chloramphenicol, inhibited protein synthesis by the rabbit reticulocyte cell-free system at high poly U level.

PACTAMYCIN is an antitumor antibiotic with marked cytotoxicity against KB cells in culture[1] and marked activity against several tumors *in vivo*.[2] Its biological and chemical properties have been described.[2, 3] Pactamycin was shown to inhibit the incorporation of amino acids into protein in KB cells,[1] HeLa cells,[4] and in rabbit reticulocytes.[5] Felicetti *et al.*,[6] working with the cell-free protein synthesizing system of rabbit reticulocytes, suggested that pactamycin inhibits protein synthesis by altering a ribosomal site involved in the interaction between *m*-RNA,† *s*-RNA and ribosomes. This paper reports the inhibition of protein synthesis by pactamycin in KB cells in cell culture, *Bacillus subtilis*, rat liver, and the *Escherichia coli* and reticulocyte cell-free systems.

MATERIALS AND METHODS

Cytotoxicity of pactamycin against KB cells was determined by the method of Smith *et al.*[7] The incorporation of radioactive precursors into RNA, DNA, and protein in KB cells was determined as previously described.[8] RNA was determined by the orcinol method,[9] and DNA by Burton's modification of the diphenylamine method.[10] Cell protein was determined by the method of Oyama and Eagle.[11] Radioactive samples were counted in a Packard Tri-Carb scintillation counter.

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† Abbreviations used in this paper are: S-105, 105,000 *g* supernatant fraction from *E. coli* or liver homogenate; TCA, trichloroacetic acid; TP, tryptophan pyrrolase; CMC, carboxymethyl cellulose; *m*-RNA, messenger RNA; *s*-RNA, soluble RNA; ID_{50}, dose to cause 50 per cent inhibition; GTP, CTP, UTP, refer to the triphosphates of guanosine, cytidine and uridine respectively.

Female rats of the Wistar–Upjohn strain (140–160 g) were used in the isotope incorporation experiments. The conditions for the maintenance of the rats and the method used to determine isotope incorporation into rat liver were as described by Gray *et al.*[12] Tryptophan pyrrolase experiments were conducted with male, adrenalectomized, Sprague–Dawley rats of 140–160 g. The enzyme assay was done by the method of Gray *et al.*[12]

The reticulocyte cell-free protein synthesizing system was prepared by the method of Weisberger *et al.*[13] For this purpose male rabbits of about 2·2 kg in weight were used. To induce reticulocytosis they were injected S.C. with 1 ml of 2·5% phenylhydrazine HCl, pH 7·0, for 5 days. Blood, with a reticulocyte count between 70–95 per cent, was obtained on the seventh day by cardiac puncture.

The *E. coli* cell-free protein synthesizing system was prepared by the method of Nirenberg and Matthaei.[14] For this purpose *E. coli* B cells harvested in middle log phase were obtained from General Biochemical Inc., Chagrin Falls, Ohio.

^{14}C-phenylalanyl-*s*-RNA and ^{14}C-prolyl-*s*-RNA were prepared by the method described by von Ehrenstein and Lipman.[15] For charging with amino acids, the reaction mixture contained (per ml): 100 μmole of Tris–HCl, pH 7·2; 5 μmole of magnesium acetate; 3 μmole ATP; 20 μmole phosphoenolpyruvate; 40 μg pyruvate kinase; 25 mμmole of each of 20 L-amino acids minus the ^{14}C-amino acid; 10 mg *E. coli s*-RNA stripped of any attached amino acids; 200 mμmole L-^{14}C-phenylalanine or L-^{14}C-proline (5×10^6 cpm); and 2 mg (as protein) of dialyzed 105,000 *g* supernatant from alumina-ground *E. coli*. The reaction was conducted for 10 min at 37° and the charged *s*-RNA was isolated as described by von Ehrenstein and Lipman.[15] The ^{14}C-phenylalanyl-*s*-RNA and ^{14}C-prolyl-*s*-RNA had specific activities of 7700 cpm/mg *s*-RNA and 17,000 cpm/mg *s*-RNA, respectively.

To assay for radioactivity incorporated into protein in the cell-free systems, the reaction was stopped with 5 ml of 10% TCA plus bovine serum albumin (0·5 mg/ml). The precipitated protein was washed twice with 10% TCA, heated for 20 min at 70° with 0·5 N perchloric acid, washed twice more with 10% TCA, once with ethanol–ether (3:1), and finally with ether. The dry material was dissolved in formic acid and counted in the scintillation counter.

E. coli B *s*-RNA, stripped of any attached amino acids, was obtained from General Biochemicals Inc. Poly U and poly C were obtained from Miles Laboratory. All radioactive precursors were bought from New England Nuclear Corp. Pactamycin was produced in The Upjohn Company and has the empirical formula $C_{28}H_{40}N_4O_8$ (mol. wt., 560). It was dissolved in water or any other vehicle by adjusting the pH to 2 with 0·1 N HCl. After the antibiotic had dissolved, the pH was raised slowly to 5·5–6·0 with 0·1 N NaOH.

RESULTS

Effect on cell growth

The inhibition of KB cell growth by increasing concentrations of pactamycin is shown in Fig. 1. Cell growth was maximally inhibited during the first 24 hr, after which the cells grew at a rate similar to that of the untreated cells. Pactamycin was inactivated when incubated in Eagle's complete medium for 24 hr at 37°, which allowed the surviving cells to grow at a rapid rate after the first day. Addition of pactamycin to cells which had already been exposed to the antibiotic for 42 hr, caused inhibition of

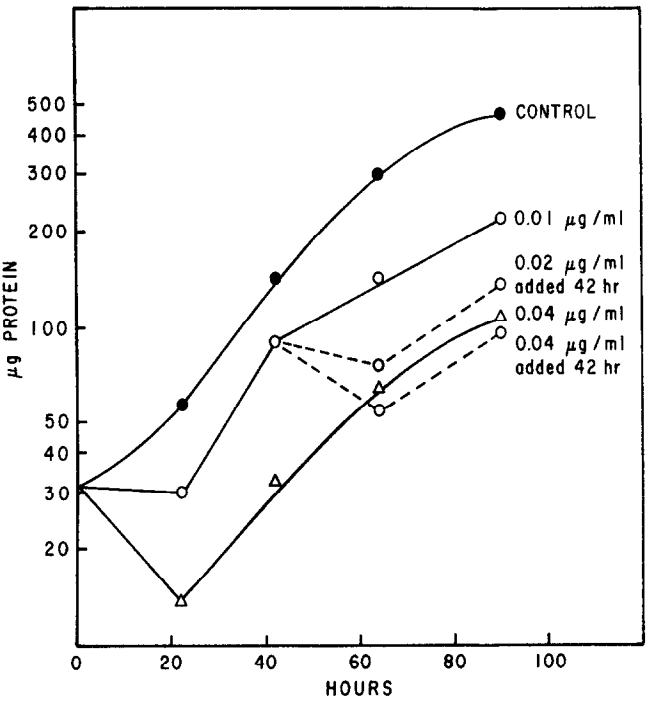

FIG. 1. Growth inhibition of KB cell monolayers by pactamycin. Dotted lines indicate growth inhibition when pactamycin was added at 42 hr to cells previously exposed to 0·01 μg/ml. In all other experiments pactamycin was added at 0 hr.

cell growth. This indicated that the cells which had grown in the presence of the agent had not developed resistance to it. In this system ID_{50} of pactamycin was 0·01 μg/ml. Inhibition of cell growth by pactamycin was not reversed by the addition of mixtures of purines or pyrimidine bases, nucleosides and nucleotides, or amino acids or vitamins.[1]

Macromolecular synthesis by mammalian cells exposed to pactamycin

Pactamycin inhibited the incorporation of valine into protein more than the incorporation of precursors into DNA or RNA of KB cells as shown in Table 1. Thus

TABLE 1. EFFECT OF PACTAMYCIN ON INCORPORATION OF LABELED PRECURSORS INTO DNA, RNA AND PROTEIN OF KB CELLS*

Pactamycin (μg/ml)	Valine→ Protein (% inhibition)	Thymidine→ DNA (% inhibition)	Uridine→ RNA (% inhibition)
0·01	38	28	0
0·02	69		10
0·04	81	65	4

* The cells were incubated for 1 hr in the presence of the drug and radioactive precursor. The labeled precursors were added at the following levels: DL-1-^{14}C-valine (0·1 μc/23 μmole valine/ml medium); ^{3}H-uridine and ^{3}H-thymidine (0·2 μc/8 μg substrate/ml medium). The controls (no pactamycin) incorporated label to give $1·8 \times 10^4$ cpm/mg protein, 3×10^4 cpm/mg DNA, and $2·8 \times 10^4$ cpm/mg RNA.

valine incorporation into protein was inhibited 81 per cent by 0·04 μg/ml ($7·2 \times 10^{-8}$ M) pactamycin compared to 65 per cent and 4 per cent inhibition of precursor incorporation into DNA and RNA, respectively. These results were confirmed with several other precursors as shown in Table 2.

TABLE 2. EFFECT OF PACTAMYCIN ON INCORPORATION OF VARIOUS PRECURSORS INTO DNA, RNA AND PROTEIN OF KB CELLS*

Labeled precursor	Pactamycin (μg/ml)	Protein /(% inhibition)	DNA (% inhibition)	RNA (% inhibition)
Lysine	0·01	30		
Lysine	0·02	75		
Proline	0·01	26		
Proline	0·02	76		
Phenylalanine	0·01	30		
Phenylalanine	0·02	74		
Cytidine	0·08		95	31
Hypoxanthine	0·08		87	38

* The cells (2×10^6/ml medium) were incubated for 1 hr in the presence of antibiotic and labeled precursor. The amino acids were added at levels similar to those shown in Table 1. ^{3}H-cytidine and ^{14}C-hypoxanthine were added at 0·2 μc/4 μg substrate/ml medium.

The sequence in which incorporation of labeled precursors into protein, RNA, and DNA was inhibited is shown in Fig. 2. Incorporation of valine into protein was inhibited first to be followed by inhibition of labeling of DNA and RNA. These results suggest that pactamycin primarily inhibits amino acid incorporation into protein.

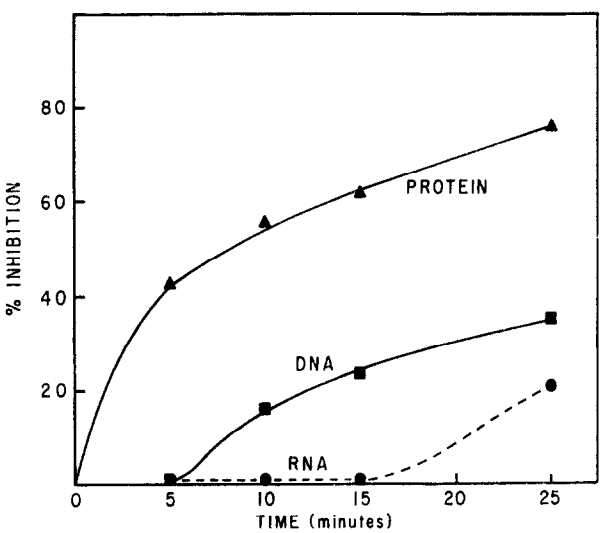

FIG. 2. Pactamycin inhibition of uptake of labeled precursors into RNA, DNA and protein of KB cells suspension. KB cells (2×10^6/ml medium) in suspension were incubated for 30 min in the presence of radioactive precursor and pactamycin. The labeled precursors were added at the following levels: ^{3}H-cytidine and ^{3}H-thymidine (0·7 μc/3 μg substrate/ml medium) and DL-1-^{14}C-valine (0·7 μc/23 μg valine/ml medium). Samples were immediately chilled in the presence of large excess of unlabeled precursor, cells were centrifuged and then resuspended in 10% TCA. Control experiments indicated that this procedure stopped uptake of labeled precursor immediately. Protein, RNA and DNA fractions were obtained as described in Methods.

Pactamycin also inhibited the incorporation of valine into liver protein, when the antibiotic was injected i.p. or i.v. into rats (Table 3). The inhibition obtained at these antibiotic doses is similar to the inhibition seen when TP synthesis was studied (see Table 5).

TABLE 3. INHIBITION BY PACTAMYCIN OF ^{14}C-VALINE INCORPORATION INTO RAT LIVER PROTEIN*

Injection route	Pactamycin (mg/kg)	Valine → Protein (% inhibition)
Intravenous	0·2	36
Intravenous	1·0	72
Intraperitoneal	0·2	42
Intraperitoneal	1·0	85

* The rats were exposed to pactamycin and ^{14}C-valine for 1 hr. Four rats (140–160 g) were used per experiment and each rat received 30 μc of DL-1-^{14}C-valine of 1800 μc/μmole. Liver protein in the control rats had a specific activity of 2×10^3 cpm/mg protein.

Inhibition of macromolecular synthesis in B. subtilis

Since pactamycin is active preferentially against gram-positive organisms, its effect on macromolecular synthesis by *B. subtilis* was investigated. The results (Table 4) indicate that pactamycin inhibited total protein synthesis without affecting the synthesis of DNA or RNA. Similar results were also obtained when incorporation of labeled precursors was studied.

TABLE 4. EFFECT OF PACTAMYCIN ON THE TOTAL CONTENT OF PROTEIN, RNA AND DNA IN GROWING *B. subtilis* CELLS*

Pactamycin (μg/ml)	Cell protein (mg/ml)	Cell DNA (mg/ml)	Cell RNA (mg/ml)
0 (control)	1·8	0·257	0·581
0·05	1·36	0·262	0·634

* *B. subtilis* cells growing in Difco Pen-assay medium were exposed to pactamycin for 30 min followed by determination of total protein, DNA and RNA in the control and treated cells. At zero time the cells /ml medium contained 1·2 mg protein, 0·16 mg DNA, and 0·39 mg RNA.

Inhibition of tryptophan pyrrolase synthesis in rat liver

Pactamycin inhibited both the hydrocortisone- and tryptophan-induced increase in TP as shown in Table 5. The toxicity of tryptophan plus pactamycin limited the antibiotic level which could be used when substrate induction of TP was studied. The results with hydrocortisone indicate that pactamycin at high doses also lowered the basal level of TP synthesis.

TABLE 5. INHIBITION OF TRYPTOPHAN PYRROLASE (TP) SYNTHESIS IN RAT LIVER BY PACTAMYCIN*

Inducing agent	Pactamycin (mg/kg)	TP activity†	Inhibition (%)
CMC‡ (control)		1·2 ± 0·29	
CMC plus hydrocortisone		4·12 ± 0·17	0
CMC plus hydrocortisone	3·3	0·65 ± 0·22	>100
CMC plus hydrocortisone	1·65	1·295 ± 0·29	97
CMC plus hydrocortisone	0·82	2·5 ± 0·33	55
CMC plus tryptophan		2·78 ± 0·08	0
CMC plus tryptophan	0·82	1·99 ± 0·3	50

* Five rats were used per experiment and received pactamycin ip. simultaneously with the inducing agent. The rats were harvested 4 hr later. Each value represents the average of 5 rats with the mean deviation indicated.
† The TP activity is expressed as μmole kynurenine/ml homogenate/hr.
‡ CMC = carboxymethyl cellulose.

Inhibition of protein synthesis in different cell-free systems

The effect of different levels of pactamycin on protein synthesis by the rabbit reticulocyte cell-free system is shown in Fig. 3. Fifty per cent inhibition was obtained at 0·5 μg/ml of pactamycin in agreement with the results obtained by Colombo *et al.*[5] with the same system. Protein synthesis in this system was not inhibited by chloramphenicol (100 μg/ml) at the level of poly U (25 μg/ml) used.

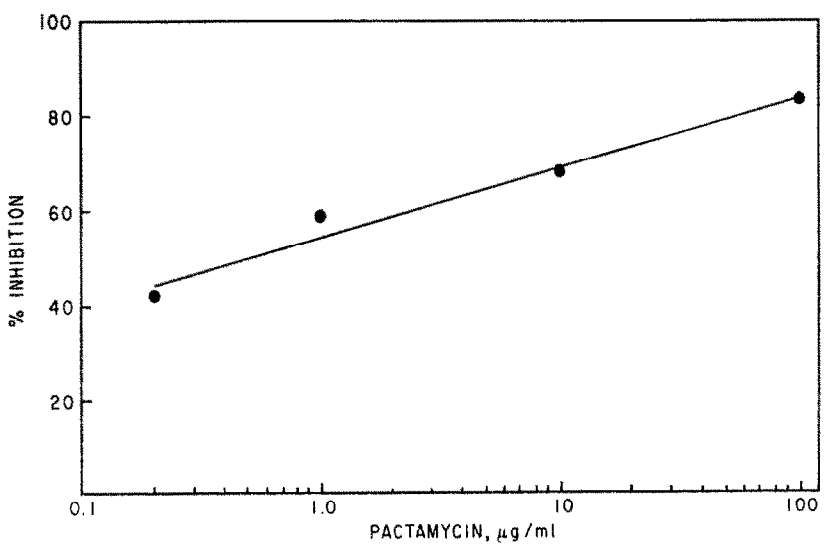

FIG. 3. Inhibition of protein synthesis in the reticulocyte cell-free system. The reaction mixtures contained the following in μmole/ml. 100 of Tris-HCl pH 7·8; 10 of magnesium acetate; 50 of KCl; 6 of β-mercaptoethanol; 0·05 each of 20 L-amino acids minus L-phenylalanine; 0·025 each of GTP, CTP, UTP, 5 of sodium phosphoenol pyruvate; 1 of ATP; 20 μg pyruvate kinase; 0·025 of L-^{14}C-phenylalanine (1·6 × 10^5 cpm); 0·4 mg of ribosomal protein and 0·2 mg of pH-5 protein. The total volume of the reaction mixture was 1 ml. The control tube (no antibiotic) incorporated 1000 cpm/mg protein.

Cell-free protein synthesis was further studied by using the readily available *E. coli* 105,000 *g* supernatant and ribosomes. The inhibition of poly C-directed ^{14}C-proline incorporation is shown in Fig. 4. Polyproline synthesis was inhibited 50 per cent at 0·75 μg/ml. In contrast to inhibition of polyproline synthesis, polyphenylalanine synthesis directed by poly U was only marginally inhibited, even at 100 μg/ml of pactamycin (Table 6). In this system puromycin (100 μg/ml) almost completely inhibited polyphenylalanine synthesis.

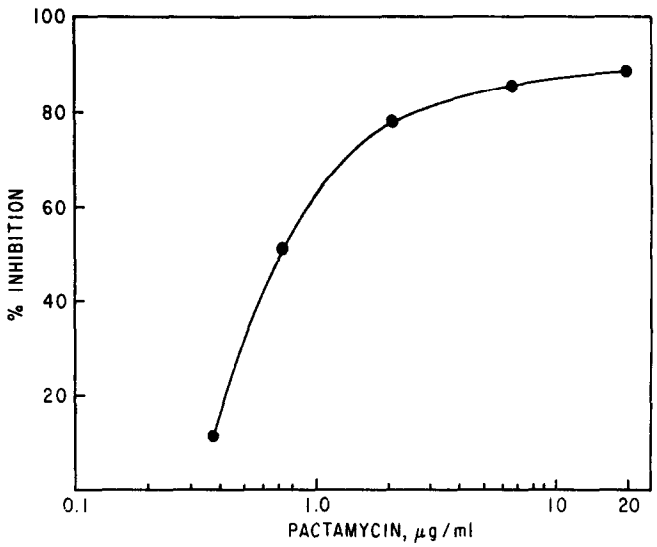

FIG. 4 Inhibition of protein synthesis in the *E. coli* cell-free system. The reaction mixture contained the following in μmole/ml: 100 of Tris-HCl, pH 7·8; 50 of KCl; 1 of ATP; 6 of β-mercaptoethanol; 5 of sodium phosphoenol pyruvate; 20 μg of pyruvate kinase; 0·05 of each of 20 L-amino acids minus L-proline; 0·03 of GTP, CTP, UTP each; 0·1 of L-^{14}C-proline (68 × 10^4 cpm); 50 μg of poly C; 1·6 mg (protein) *E. coli* ribosome; 1·6 mg (protein) of *E. coli* S-105. Total volume of the reaction mixture was 0·5 ml. Tubes were incubated for 20 min at 37°, after which they were deproteinized with 10% TCA and the precipitates washed and counted as described under Methods.

TABLE 6. LACK OF INHIBITION OF POLYPHENYLALANINE SYNTHESIS BY PACTAMYCIN*

Addition	Protein (cpm/mg)	Inhibition (%)
Complete, zero time	178	
Complete, 30 min	7400	
Complete, 30 min + pactamycin	7250	2
Complete, 30 min + puromycin	510	95·5

* The reaction mixture was the same as that for Fig. 4 except that L-^{14}C-phenylalanine and poly U replaced L-^{14}C-proline and poly C. Pactamycin and puromycin were present at 100 μg/ml.

Absence of pactamycin effect on the transfer of proline to s-RNA

The transfer reaction was studied by determining the radioactivity bound to RNA after incubation of *s*-RNA with *E. coli* S-105 fraction and ^{14}C-proline. In the absence of ribosomes the protein fraction does not become labeled. The results shown in Table 7 indicate that pactamycin did not affect the transfer of ^{14}C-proline to *s*-RNA.

TABLE 7. LACK OF EFFECT OF PACTAMYCIN ON FORMATION OF ^{14}C-PROLYL-*s*-RNA*

Pactamycin (μg/ml)	Radioactivity soluble in hot perchloric acid (cpm)
0 (control)	980
200	1025
20	955

* The reaction mixture was based on that reported by Goldberg *et al.*[16] and contained in μmole/ml: 100 of Tris-HCl, pH 7·2; 10 of $MgCl_2$; 4 of β-mercaptoethanol; 4 of ATP; 10 of sodium phosphoenolpyruvate; 40 μg phosphoenolpyruvate kinase; 100 μg *E. coli* B, stripped *s*-RNA; 100 mμ mole of L-^{14}C-proline (1 μc); and 2 mg of *E. coli* S-105 protein. After incubation for 10 min at 37°, 5 ml of 10% TCA was added, the precipitate washed 3 times with 10 per cent cold TCA and the radioactivity solubilized by heating at 70° for 20 min in 0·5 N perchloric acid. Over 95 per cent of the radioactivity was thus solubilized.

*Effect of pactamycin on polyphenylalanine and polyproline synthesis from phenylalanyl-*s*-RNA and prolyl-*s*-RNA*

The results given in Table 8 indicate that pactamycin inhibited polyproline synthesis but not polyphenylalanine synthesis. Puromycin, however, inhibited synthesis of both polyphenylalanine and polyproline.

TABLE 8. EFFECT OF PACTAMYCIN ON INCORPORATION OF PHENYLALANYL-*s*-RNA AND PROLYL-*s*-RNA INTO PROTEIN

Label	Addition	cpm	Inhibition (%)
^{4}C-prolyl-*s*-RNA	Complete	659	
^{14}C-prolyl-*s*-RNA	Complete + pactamycin	178	73
^{14}C-proyl-*s*-RNA	Complete + puromycin	116	82
^{14}C-phenylalanyl-*s*-RNA	Complete	1457	
^{14}C-phenylalanyl-*s*-RNA	Complete + pactamycin	1700	−13
^{14}C-phenylalanyl-*s*-RNA	Complete + puromycin	0	100

* The reaction mixture was based on that reported by Vazquez[17] and contained in μmole/ml: 60 of Tris-HCl, pH 7·4; 100 of ammonium acetate; 10 of magnesium acetate; 5 of β-mercaptoethanol; 4 of sodium phosphoenolpyruvate; 50 μg pyruvate kinase; 0·2 of GTP; 20 μg poly U or 60 μg poly C; 2 mg 30,000 *g E. coli* supernatant protein; 1·2 mg ^{14}C-phenylalanyl-*s*-RNA (3400 cpm) or 2 mg ^{14}C-prolyl-*s*-RNA (21,000 cpm). Total volume incubated per tube was 0·5 ml. Pactamycin or puromycin was added to give 200 μg/ml. The reaction was stopped by adding 10% TCA and the mixture was treated as described under Methods.

Inhibition by pactamycin of ribosomal activity

Ribosomes and S-105 fractions were isolated from livers of untreated and pactamycin-treated rats. The ribosomes and S-105 were combined in various ways to determine their protein synthesizing ability in a cell-free system. The results given in Table 9 indicate that ribosomes from pactamycin-treated rats were defective, since they had only 33–42 per cent of the protein synthesizing ability of control ribosomes.

TABLE 9. LOWERED PROTEIN SYNTHESIZING ABILITY OF LIVER RICOSOMES ISOLATED FROM PACTAMYCIN TREATED RATS*

Ribosome source	S-105 source	Protein (cpm/mg)	(%)
Control rat	Control rat	525	100
Control rat	pactamycin rat	555	106
Pactamycin rat	Control rat	175	33
Pactamycin rat	pactamycin rat	220	42

* The reaction mixture, similar to that reported by Weinstein *et al.*,[18] contained in μmole/ml: 6 of Tris-HCl, pH 7·8; 8 of $MgCl_2$; 50 of KCl; 4·2 of β-mercaptoethanol; 1 of ATP; 0·05 of GTP, CTP and UTP; 0·04 of each of 20 amino acids minus L-phenylalanine; 5 mμmole of L-^{14}C-phenylalanine ($1·1 \times 10^5$ cpm); 4 of phosphoenolpyruvate; 50 μg of pyruvate kinase; 100 μg of poly U; 1 mg each of ribosome and S-105 protein. The reaction was stopped after 20 min at 37° by adding 5 ml cold 10%. TCA. The precipitate was washed and counted as described under Methods. Control rats received saline i.p.; treated rats received 0·7 mg pactamycin/kg twice daily for 3 days.

DISCUSSION

The evidence presented in this paper (and enumerated below) suggests that pactamycin primarily inhibits protein synthesis: (1) in *B. subtilis*, pactamycin inhibited total protein synthesis with no inhibition of RNA or DNA synthesis; (2) in KB cells incorporation of amino acid into protein was inhibited to a greater extent, and earlier than, the incorporation of labeled precursors into DNA or RNA; (3) pactamycin, like puromycin, inhibited the increase in TP synthesis induced by both hydrocortisone and tryptophan. Tryptophan stimulates the enzyme by inhibiting enzyme degradation,[19] whereas enzyme induction by hydrocortisone depends upon an increase in *m*-RNA synthesis with a concomitant increase in protein synthesis.[20] Since both mechanisms require continued enzyme protein synthesis, puromycin blocks enzyme induction by both tryptophan and hydrocortisone.[21] Actinomycin D, an inhibitor of *m*-RNA synthesis, blocks only induction by hydrocortisone.[22] These results, when considered together with the lack of inhibition of RNA synthesis in *B. subtilis*, and the fact that pactamycin does not bind to DNA (unpublished observation), indicate that pactamycin does not inhibit protein synthesis by inhibition of *m*-RNA synthesis.

Studies with the *E. coli* cell-free system indicated that inhibition of protein synthesis occurred at some step in the transfer of aminoacyl-*s*-RNA to ribosomes. Thus, pactamycin and puromycin at 100 μg/ml caused 73 per cent and 82 per cent inhibition, respectively, of the incorporation of prolyl-*s*-RNA into protein. Pactamycin appeared to alter a ribosomal site essential to protein synthesis. Liver ribosomes isolated from pactamycin-treated rats had 30 per cent of the activity of control ribosomes, but the activity of the S-105 fraction was unaffected by pactamycin treatment. Similar results were also obtained by Felicetti *et al.*[6] when they compared ribosomes from pactamycin-treated reticulocytes to those from untreated reticulocytes. Colombo *et al.*[5] also obtained complete breakdown of polysomes of reticulocytes to smaller aggregates within 30 min by pactamycin. Polysome breakdown in the presence of pactamycin allowed peptide chains to be released into the supernatant. These results indicate that pactamycin acts differently from cycloheximide and puromycin in inhibiting protein synthesis. Cycloheximide does not affect the polysome profile, whereas puromycin causes release of incomplete peptide chains with little modification of the polysome pattern in short-term incubation.[5]

Our studies indicated that polyproline synthesis directed by poly C was more sensitive to pactamycin than polyphenylalanine synthesis directed by poly U in the *E. coli* cell-free system. Similar differential sensitivity has also been observed by Kucan and Lipman[23] with chloramphenicol and by Vazquez[17] with chloramphenicol, erythromycin, and several other antibiotics. However, pactamycin, unlike chloramphenicol, inhibits protein synthesis in cell-free systems derived from reticulocytes at high poly U level. Polyphenylalanine synthesis in the reticulocyte cell-free system was markedly inhibited by pactamycin in contrast to the lack of inhibition in the *E. coli* cell-free system. This might indicate that there is some essential difference in the ribosomal sites involved in protein synthesis in the two systems.

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