Synthesis of Cyclohexylpuromycin and its Reaction with *N*-Acetylphenylalanyl-Transfer Ribonucleic Acid on Rat Liver Ribosomes

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1. Cyclohexylpuromycin, an analogue of puromycin in which a cyclohexane ring replaces the aromatic benzene ring of the *L*-phenylalanyl moiety of the nucleoside, has been synthesized and examined for its ability to release *N*-acetylphenylalanine from tRNA attached to rat liver ribosomes. 2. DL-Cyclohexylpuromycin was active in reacting with *N*-[³H]acetylphenylalanyl-tRNA on rat liver ribosomes to form *N*-[³H]acetylphenylalanylcyclohexylpuromycin. 3. The reaction product *N*-acetylphenylalanylcyclohexylpuromycin and the corresponding analogue *N*-acetylphenylalanylpuromycin were chemically synthesized for evaluation of the structure of the released *N*-acetylphenylalanyl-containing material. 4. The results obtained suggest that the model of Raacke (1971) for puromycin reactivity needs further examination with regard to the role played by the aromatic ring system of the *L*-phenylalanyl moiety of the nucleoside.

A theoretical model for the interaction of puromycin (IX) with peptidyl-tRNA on ribosomes has been proposed by Raacke (1971). The model is based on the fact that the puromycin molecule can assume a U-shaped configuration with the benzene ring stacked under the dimethyladenine ring system (Sundaralingam & Arora, 1969), and that in this configuration it can react with the 3'-terminus of tRNA (-pCpCpA-amino acid). This interaction is the result of a continuous stack of four hydrophobic

rings. The sequence of stacking (shown in Scheme 1) is dimethyladenine, adenine, *p*-methoxybenzene and cytosine. Although this is a very interesting and important hypothesis, very little work has been carried out to substantiate the idea. We therefore decided to synthesize an analogue of puromycin in which the aromatic benzene ring of the nucleoside is replaced by a puckered cyclohexane ring which is incapable of hydrogen bonding and intercalation through hydrophobic stacking, as it lacks the necessary π orbitals present in an aromatic ring system.

Chemically synthesized DL-cyclohexylpuromycin (VI) was found to be active in releasing *N*-acetylphenylalanine from its respective tRNA carrier bound to rat liver ribosomes. These results suggest that hydrophobic stacking between puromycin and the 3'-terminus of tRNA may not be necessary for puromycin reactivity as suggested by Raacke (1971).

Experimental

Chemicals and reagents

Puromycin dihydrochloride, puromycin aminonucleoside, GTP (disodium salt) and benzyloxycarbonyl chloride were obtained from the Sigma Chemical Co., St. Louis, U.S.A. Phenylalanyl-tRNA and ATP (disodium salt) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Yeast tRNA was supplied by Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Triethylamine and t.l.c. plates (silica gel 60F₂₅₄, with fluorescent indicator) were supplied by E. Merck A.G., Darmstadt, Germany. Dicyclohexylcarbodi-

Scheme 1. Possible interaction of puromycin with the CpCpA terminus of tRNA

The interaction is based upon hydrophobic stacking as evidenced by model building. A^P represents 6-dimethyladenine of the puromycin aminonucleoside.

imide and L-phenylalanine were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. 3-Cyclohexylpropionic acid was obtained from K & K Laboratories, Plainview, N.Y., U.S.A. N-Hydroxysuccinimide was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. All other reagents were of analytical grade. Solvents were dried and redistilled. Before use they were stored over pellets of the molecular-sieve compound 3A.

Methods

Thin-layer chromatography. For qualitative analysis, chromatography was carried out with small plates (2cm×8cm). Preparative chromatography was carried out with large plates (10cm×20cm). The following solvent systems were used: A, chloroform-methanol (22:3, v/v); B, chloroform-methanol (19:3, v/v). For paper chromatography on Whatman no. 1 paper, solvent C was used: butan-1-ol-acetic acid-water (78:5:17, by vol.).

Preparation of subcellular fractions. Rat liver homogenates were prepared in medium A, containing (final concentrations) 0.25M-sucrose, 5mM-MgCl₂, 25mM-KCl and 50mM-Tris-HCl buffer (pH 7.6). Rat liver polyribosomes were prepared according to the method of Wettstein *et al.* (1963) with slight modification in that sodium deoxycholate was replaced by a mixture of sodium deoxycholate (final concentration 0.5%, w/v) and Triton X-100 (final concentration 0.5%, w/v). The polyribosomes were finally suspended in medium A and stored frozen at -15°C.

Washed polyribosomes. These were prepared by a modification of the method described by Siler & Moldave (1969) as follows. Polyribosomes were incubated in a buffer containing 50mM-Tris-HCl (pH 7.6), 25mM-KCl, 1mM-dithiothreitol, 5mM-MgCl₂, 80mM-NH₄Cl and 1mM-puromycin for 30min at 37°C. Thereafter, 10ml portions of the ribosomal suspensions were layered over 25ml of 0.5M-sucrose in medium A buffer containing 0.5M-NH₄Cl and centrifuged at 99000g for 4h in the Spinco no. 30 rotor. The polyribosome pellets were taken up in medium A, cleared of debris by low-speed centrifugation, and stored frozen at -15°C.

pH5 enzyme. This was prepared from a sample of rat liver cell sap as described by Hawtre & Nourse (1966). It was taken up in medium A and kept at -15°C.

[³H]Phenylalanyl-tRNA. This was prepared as previously described by Herrington & Hawtre (1971) with the exception that small amounts of yeast tRNA and purified yeast phenylalanyl-tRNA were present in the original incubation mixture. The final preparation of tRNA had an extinction (E_{260}/E_{280}) ratio of 2.1 and gave 32.5×10^4 c.p.m./100μg of tRNA.

[³H]Acetylphenylalanyl-tRNA. This was prepared by a modification of the method of Pellegrini *et al.* (1972). The reaction medium for acetylation con-

tained: N-hydroxysuccinimide ester of acetic acid (50mg, 0.32mmol) in 1.0ml of dimethyl sulfoxide; [³H]phenylalanyl-tRNA (2.8mg) and 0.2ml of 0.5M-potassium phosphate buffer, pH 6.8 (final concentration 0.03M). The final reaction volume was 3.2ml. Incubation was carried out for 2.5h at 37°C. The reaction mixture was cooled in ice, and 0.3ml of 20% (w/v) potassium acetate added, followed by 2.5vol. of cold ethanol. After standing overnight, the tRNA was recovered by centrifugation and washed twice by suspension in ethanol-dimethylformamide (3:1, v/v) followed by centrifugation. The acetylated product was dissolved in water and exhaustively dialysed against water and then frozen at -15°C. It gave an extinction (E_{260}/E_{280}) ratio of 1.80 and 38×10^4 c.p.m./100μg of tRNA. Alkaline hydrolysis of a sample of N-[³H]acetylphenylalanyl-tRNA followed by paper chromatography in solvent C gave one radioactive spot corresponding to N-acetylphenylalanine.

Translocation (G factor). This was prepared from a sample of pH5 supernatant (adjusted to pH 7.6 with 2M-KOH) according to the method described by Felicetti & Lipmann (1968). Purification was taken as far as the calcium phosphate-gel stage. The partially purified enzyme was finally dialysed against a buffer consisting of 0.25M-sucrose, 0.05M-Tris-HCl (pH 7.6), 1mM-MgCl₂ and 1mM-mercaptoethanol, and then frozen in small portions at -15°C.

Measurement of radioactivity. In experiments concerned with the binding of N-[³H]acetylphenylalanyl-tRNA to washed polyribosomes, samples were transferred to Millipore filters and washed with Nirenberg buffer, 0.1M Tris-acetate (pH 7.2), 20mM-magnesium acetate and 50mM-KCl (Nirenberg & Leder, 1964). Filters were counted for radioactivity in toluene containing 0.5% (w/v) of 2,5-diphenyloxazole and 0.03% (w/v) of 1,4-bis-(5-phenyloxazol-2-yl)benzene. In the ethyl acetate extractions, the samples were finally counted for radioactivity in Bray's (1960) solution.

Chemical syntheses

DL-2-Bromo-3-cyclohexylpropionic acid. This was prepared by a standard procedure, from 3-cyclohexylpropionic acid with bromine and phosphorus trichloride. After distillation under vacuum, the product crystallized on standing. It was recrystallized from light petroleum (b.p. 100-120°C), m.p. 58-59°C; *m/e* 234, 236 (*M*⁺).

DL-2-Amino-3-cyclohexylpropionic acid (I). DL-2-Bromo-3-cyclohexylpropionic acid (2.12g, 9mmol) was treated with 10ml of aq. 25% (w/v) NH₃ at 50°C. After 1h the product began to crystallize. The reaction was then allowed to proceed for 24h. The resulting crystalline product was washed successively with cold water, methanol and ether. It was recrystallized from hot water, m.p. 246-247°C (Found:

C, 60.3; H, 9.8, N, 7.7; $C_9H_{18}O_2N$ requires C, 60.0; H, 10.1; N, 7.8%). $\nu_{\max}$. (KBr) 2550, 2100 (amino acid peaks), 3100 ($-\text{NH}_3^+$), 1590 (amino acid II), 2900 (satd. C-H) cm^{-1} .

DL-N-Benzylloxycarbonyl-2-amino-3-cyclohexylpropionic acid (II). *DL*-2-Amino-3-cyclohexylpropionic acid (600mg, 3.5mmol) was dissolved in 4M-NaOH (0.88ml, 3.5mmol) at 0°C. The solution was stirred magnetically and to it was added benzyloxycarbonyl chloride (655mg, 3.85mmol) and 4M-NaOH (1.05ml) in alternate portions (five separate additions). After stirring the solution at room temperature for 15 min it was extracted with ether to remove unchanged benzyloxycarbonyl oxychloride. The reaction mixture was then extracted with chloroform. The chloroform extract was washed with water and then evaporated to dryness *in vacuo* at 20°C. The crude product was recrystallized from ethyl acetate and light petroleum (b.p. 60–80°C), m.p. 114–116°C (Found: C, 65.8; H, 7.7; $C_{17}H_{33}NO_4$ requires C, 66.9; H, 7.6%; m/e 305 (M^+)).

N-Hydroxysuccinimide ester of N-benzylloxycarbonyl-2-amino-3-cyclohexylpropionic acid (III). *N*-Hydroxysuccinimide (63mg, 0.55mmol), *N*-benzyloxycarbonyl-2-amino-3-cyclohexylpropionic acid (164mg, 0.54mmol) and dicyclohexylcarbodi-imide (111mg, 0.55mmol) were dissolved in 2.5ml of dioxan and allowed to stand at room temperature for 15h. Dicyclohexylurea was removed by filtration and the filtrate evaporated to dryness under reduced pressure. The product was recrystallized from ethyl acetate and light petroleum (b.p. 60–80°C) to give large clusters of fine needles (150mg, 60%). m.p. 131–132°C; m/e 402 (M^+)).

6-Dimethylamino-9-[1'-[3'-(N-benzylloxycarbonyl-2'-amino-3'-cyclohexylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (V). (a) To a solution of puromycin aminonucleoside (45mg, 0.15mmol) and *N*-benzyloxycarbonyl-2-amino-3-cyclohexylpropionic acid (76.3mg, 0.25mmol) in dimethylformamide (1.5ml) was added dicyclohexylcarbodi-imide (37mg, 0.18mmol) in 0.3ml of dimethylformamide. After 48h at room temperature, dicyclohexylurea was removed by filtration. The remaining solution was evaporated to dryness under reduced pressure at 37°C. The residue was extracted with light petroleum (b.p. 69–80°C) and ether to remove unchanged dicyclohexylcarbodi-imide and *N*-benzyloxycarbonyl-2-amino acid. The crude product was taken up in solvent A and chromatographed on silica gel G (60F₂₅₄) plates (10cm $\times$ 20cm) with solvent A. The appropriate band (R_F 0.87) was eluted with chloroform-methanol (1:1, v/v). After removal of the solvent the product was recrystallized from ethanol to give white rosettes (47.6mg, 53%), m.p. 179–181°C (Found: C, 59.9; H, 6.9; $C_{29}H_{39}N_7O_6$ requires C, 59.9; H, 6.8%). $\nu_{\max}$. (KBr) 1690 (urethane), 1660 (C=O) amide I), 1600 (heterocyclic C=N) cm^{-1} .

(b) The alternative approach involved prior activation of the cyclohexylamino acid (II) with *N*-hydroxysuccinimide to give compound (III), which was then allowed to react with puromycin aminonucleoside (IV) to give the required compound (V). Puromycin aminonucleoside (10mg, 0.034mmol) and *DL*-*N*-benzyloxycarbonyl-2-amino-3-cyclohexylpropionic ester of *N*-hydroxysuccinimide (14.1mg, 0.035mmol) were dissolved in 0.5ml of pyridine-water (7:3, v/v). After 4h at room temperature the solvent was removed under reduced pressure, the residue dissolved in solvent A and then subjected to t.l.c. as described in method (a). Recrystallization from ethanol gave rosettes (10mg, 51%), m.p. 179–181°C. The ultraviolet and infrared spectra were identical with those of the sample prepared by method (a). A mixed-melting-point determination with sample (a) gave no depression.

6 - Dimethylamino - 9 - [1'[3' - (2'' - amino - 3'' - cyclohexylpropionamido) - 3' - deoxy - β - D - ribofuranosyl]]purine (VI). To a solution of compound (V) (42mg, 0.08mmol) in methylCellosolve (2.2ml) was added 8mg of 10% palladium-charcoal catalyst. Hydrogenation was continued until the evolution of CO_2 was complete (20min). The catalyst was removed by filtration through a bed of Celite, and the filtrate evaporated to dryness under reduced pressure at 37°C. The product was recrystallized from ethanol (22.5mg, 70%), m.p. 193–195°C; m/e 447 (M^+). $\nu_{\max}$. (KBr) 2800, 2700 ($-\text{CH}_2-$ stretching), 1640 (C=O amide I) cm^{-1} .

N-Hydroxysuccinimide ester of N-acetyl-L-phenylalanine (VII). To a solution of *N*-acetyl-L-phenylalanine (272mg, 1.32mmol) and *N*-hydroxysuccinimide (156mg, 1.35mmol) in dioxan (3.5ml) was added a cold (10°C) solution of dicyclohexylcarbodi-imide (276mg, 1.35mmol) in 0.3ml of dioxan. After 16h at room temperature, dicyclohexylurea was removed by filtration, and the filtrate evaporated to dryness under reduced pressure at room temperature. The product was recrystallized from propan-2-ol (264mg, 66%), m.p. 143–145°C; m/e 207 (*N*-acetyl-L-phenylalanine), 115 (*N*-hydroxysuccinimide).

N - Acetyl - L - phenylalanyl cyclohexylpuromycin (VIII). A solution containing *DL*-cyclohexylpuromycin (5mg, 0.011mmol) and the *N*-hydroxysuccinimide derivative of *N*-acetyl-L-phenylalanine (7.5mg, 0.024mmol) in 0.25ml of pyridine-water (7:3, v/v) was allowed to stand at room temperature for 3 days. The solvent was removed under reduced pressure and the residue subjected to t.l.c. (silica gel 60F₂₅₄) with solvent A. Extraction of the appropriate band gave compound (VIII), which was finally recrystallized from ethanol; m/e 636 (M^+), 618 ($M^+ - 18$).

N-Acetyl-L-phenylalanylpuromycin (X). This was prepared in a manner identical with that for the cyclohexylpuromycin derivative (VIII) via *N*-hydroxysuccinimide activation. The product was

Scheme 2. Synthesis of DL-cyclohexylpuromycin (VI)

B, 6-Dimethyladenine; Cbz, benzyloxycarbonyl.

recrystallized from ethanol (9.0mg, 76%), m.p. 200–202°C; m/e ($M^+ - 18$). Compound (X) was also prepared through *p*-nitrophenyl activation of *N*-acetylphenylalanine and by direct coupling in the presence of dicyclohexylcarbodi-imide.

Results and Discussion

For the synthesis of DL-cyclohexylpuromycin (VI, Scheme 2), we employed a simple approach by which the cyclohexylalanine fragment of the molecule was made and then attached to puromycin aminonucleoside (IV). DL-3-Cyclohexyl-2-aminopropionic acid (I) (DL-cyclohexylalanine) was prepared from cyclohexylpropionic acid through bromination, followed by treatment with aqueous ammonia (Rudman *et al.*, 1952). In order to be certain that the intermediate monobromo acid had its bromine atom in the correct α - or 2-position, it was subjected to nuclear-magnetic-resonance analysis. This clearly indicated bromine substitution in the correct position.

Treatment of DL-cyclohexylalanine (I) with benzyl-oxycarbonyl chloride gave the *N*-benzyloxycarbonyl derivative (II), which was then attached to puromycin aminonucleoside (IV) by two methods (Scheme 2). The first method involved coupling by means of

dicyclohexylcarbodi-imide to give DL-*N*-benzyloxycarbonylcyclohexylpuromycin (V). Hydrogenation of the latter gave crystalline DL-cyclohexylpuromycin (VI). The second method involved activation of DL-cyclohexylalanine with *N*-hydroxysuccinimide and reaction of the activated intermediate (III) with puromycin aminonucleoside (IV) to give the same product (V) as obtained by the dicyclohexylcarbodi-imide coupling procedure. These reactions are shown in Scheme 2.

It was of considerable importance to determine whether *N*-acetylphenylalanylcyclohexylpuromycin (VIII) was formed in the reaction between cyclohexylpuromycin and *N*-acetylphenylalanyl-tRNA on ribosomes or not. Compound (VIII) was therefore synthesized by bringing the *N*-hydroxysuccinimide ester of *N*-acetylphenylalanine (VII) into reaction with DL-cyclohexylpuromycin. For comparative purposes *N*-acetylphenylalanylpuromycin (X) was synthesized by the same method (Scheme 3).

In the biochemical experiments *N*-[^3H]acetylphenylalanyl-tRNA was bound to washed rat liver ribosomes (previously treated with puromycin and NH_4Cl as described in the Experimental section) at a high concentration of Mg^{2+} , equivalent to 20mM.

Scheme 3. Synthesis of *N*-acetylphenylalanylcyclohexylpuromycin (VIII) and *N*-acetylphenylalanylpuromycin (X)

B, 6-Dimethyladenine.

Fig. 1. Effect of concentration of DL-cyclohexylpuromycin and puromycin on the release of N -[^3H]acetylphenylalanine bound to rat liver ribosomes

Each reaction tube contained, in a final volume of 0.5 ml: Tris-HCl buffer, pH 7.6 (50 mM); KCl (60 mM); MgCl_2 (20 mM); NH_4Cl (20 mM); mercaptoethanol (1 mM); GTP (potassium salt), pH 8.0 (0.5 mM); poly(U) (50 μg); N -[^3H]acetylphenylalanyl-tRNA (28 μg , 10.7×10^4 c.p.m.); G factor (0.4 mg of protein); washed ribosomes (0.9 mg). Incubations were carried out for 30 min at 37°C . Each reaction tube received 1.0 ml of 0.1 M-Tris-HCl (pH 7.6) and 3 ml of ethyl acetate. After mixing and centrifugation, portions (2 ml) of the upper phase were taken for radioactivity counting in Bray's (1960) solution. Each sample was corrected for radioactivity extracted by a blank determination. (a) Puromycin; (b) DL-cyclohexylpuromycin.

Fig. 2. Rate of release of N -[^3H]acetylphenylalanine from ribosomes by DL-cyclohexylpuromycin and puromycin

Reaction conditions were identical with those of Fig. 1. Portions (2 ml) of the upper ethyl acetate phase were counted for radioactivity directly in Bray's solution. ●, Puromycin (1 mM); ○, DL-cyclohexylpuromycin (2 mM); ○, blank extraction.

This is due to the fact that N -acetylphenylalanyl-tRNA will not bind to rat liver ribosomes at 5 mM- MgCl_2 even in the presence of T factor (transferase I) and GTP (Siler & Moldave, 1969). In separate

experiments we have confirmed these observations. Conditions for optimum binding of N -acetylphenylalanyl-tRNA were determined with respect to poly(U), tRNA and ribosome concentrations.

Results presented in Fig. 1 illustrate the effect of increasing concentrations of DL-cyclohexylpuromycin (b) and puromycin (a) on the release of N -[^3H]acetylphenylalanine from ribosomes. It is seen that DL-cyclohexylpuromycin catalyses a definite release of N -acetylphenylalanine as measured by the ethyl acetate extraction procedure (Leder & Bursztyn, 1966; Siler & Moldave, 1969). Maximum activity with puromycin was obtained at a concentration of approx. 0.5 mM. Determination of the Michaelis-Menten constant from these results (see insert to Fig. 1a) gave a value of 2.3×10^{-4} M, similar to that obtained by Fahnestock *et al.* (1970) for the reaction of puromycin with N -formylmethionine in the *Escherichia coli* fragment reaction. DL-Cyclohexylpuromycin showed optimum activity at an approximate concentration of 1.3 mM. Determination of the Michaelis-Menten constant from these results gave a value of 7.7×10^{-4} M (see insert to Fig. 1b). If we assume that the DL compound contains 50% L-isomer, the K_m value would presumably be of the order of approximately 4×10^{-4} M. These results indicate that replacement of the aromatic benzene ring portion of the puromycin molecule with a cyclohexane ring lowers activity by

Fig. 3. Reaction of DL-cyclohexylpuromycin with N - $[^3\text{H}]$ -acetylphenylalanine bound to ribosomes and an examination of the reaction products by t.l.c.

Reaction mixtures were as described for Fig. 1 and were incubated for 30 min at 37°C . Each reaction tube then received 1 ml of 0.1 M-Tris-HCl (pH 7.6) and 3 ml of ethyl acetate. After mixing of the contents, the phases were separated by centrifugation at 2000g for 5 min. The upper buffer-saturated ethyl acetate phase was removed and 2 ml counted for radioactivity directly in Bray's solution. A further 0.5 ml portion was evaporated to dryness *in vacuo* at room temperature. The minute residue remaining was taken up in 0.4 ml of 96% ethanol at 40°C and then subjected to t.l.c. (10 cm $\times$ 20 cm plates) with solvent A. Plates were dried, and 0.5 cm-wide strips cut out with a scalpel. Each strip was extracted with 3 ml of hot ethyl acetate (boiled for 10 s). The extracts were cleared by low-speed centrifugation and 2 ml portions counted for radioactivity in Bray's solution. Before removal of the strips, plates were examined under u.v. light. (VI) Cyclohexylpuromycin; (VIII) N -acetylphenylalanyl cyclohexylpuromycin. N -Acetylphenylalanine (VII) was detected by charring with perchloric acid.

approx. 50% and the K_m value by half. Nevertheless, the cyclohexyl derivative is active in releasing N -acetylphenylalanine from its tRNA carrier on ribosomes. The results presented in Fig. 2 illustrate the rate of release of N - $[^3\text{H}]$ acetylphenylalanine from ribosomes by DL-cyclohexylpuromycin and puromycin. They indicate a fairly linear rate of release up to 30 min reaction time.

It was of considerable interest and importance to determine the mechanism of N -acetylphenylalanine release catalysed by DL-cyclohexylpuromycin. If one assumes that the reaction is analogous to that of the puromycin reaction, it would be expected that the product of the release would be N -acetylphenylalanyl cyclohexylpuromycin (VIII). To clarify this point, ethyl acetate extracts of reaction mixtures were subjected to t.l.c. with chemically synthesized N -acetylphenylalanyl cyclohexylpuromycin as a marker.

Results in Fig. 3 show that the fastest-moving radioactive peak was coincident with N -acetylphenylalanyl cyclohexylpuromycin (VIII), thus constituting fairly good evidence that this is a major reaction product. Of considerable interest was the finding of further radioactive peaks on the thin-layer chromatogram moving with lower R_F values. These have been consistently observed in all experiments. This material is not free N - $[^3\text{H}]$ acetylphenylalanine and does not appear to be small peptides, as a number of di- and tri-peptides do not migrate in the solvent system used. It is possible that they represent cyclohexylpuromycin derivatives with N -acetylphenylalanine attached to either position 2 or 5 of the ribose ring or alternatively an ON -disubstituted molecule. Detailed studies are in progress aimed at determining the structures of these unknown substances.

Similar ethyl acetate extracts from puromycin-containing reaction mixtures were subjected to t.l.c., the results of which are shown in Fig. 4. A radioactive peak is found in the position of the N -acetylphenylalanylpuromycin marker as well as two radioactive peaks of unknown structure with lower R_F values. The results are therefore analogous to those obtained with DL-cyclohexylpuromycin. Studies are in progress to determine the structure of the unknown substances.

From the results obtained in the present work it would appear that hydrophobic stacking of the aromatic benzene ring portion of the puromycin molecule is not absolutely essential for activity, as

Fig. 4. Reaction of puromycin with N - $[^3\text{H}]$ acetylphenylalanine bound to ribosomes and an examination of the reaction products by t.l.c.

Conditions for the incubations were as described for Fig. 1. The extraction procedure and t.l.c. are the same as given for Fig. 3. In earlier experiments, solvent B was used. However, in later experiments it was found that better resolution was obtained with solvent A. (VII) N -Acetyl-L-phenylalanine; (X) N -acetylphenylalanylpuromycin.

postulated by Raacke (1971), as we have shown that replacement of the benzene ring with a cyclohexane ring still enables the molecule to be fairly reactive in peptide chain release. The structural requirements for puromycin activity appear to be rather complicated if one looks at previous work in the literature. Nathans & Neidle (1963) showed that L-tyrosylpuromycin was active in an *E. coli* system, whereas L-tryptophan, L-leucine and glycine substitution gave inactive puromycin derivatives. These results suggested a requirement for an aromatic amino acid in the puromycin molecule. Symons and his co-workers (1969) looked at the supposedly rigid requirements for aromatic amino acids in the inhibition of protein synthesis by puromycin analogues, obtaining similar results to Nathans & Neidle (1963). However, they found that *S*-benzyl-L-cysteine was 78% as active as puromycin at a concentration of 0.3 mM, again suggesting aromatic ring involvement. They also showed that 5'-*O*-cytidylglycylpuromycin was 94% as active as puromycin at a concentration of 0.2 mM, suggesting that the aromatic ring system of puromycin may be occupying the binding site normally occupied by the terminal cytosine ring at the 3'-end of the tRNA molecule. Alternatively, the binding of the aromatic rings may simply be fortuitous. Various other workers have studied the problem by a similar approach (Takanami, 1964; Rychlik *et al.*, 1967; Harris *et al.* 1971; Hengesh & Morris, 1973).

Our own results suggest that further detailed work is necessary before a satisfactory explanation of the action of puromycin is found.

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