STUDIES ON N-(PURIN-6-YLCARBAMOYL)THREONINE

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N-(PURIN-6-YLCARBAMOYL)THREONINE

By

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ABSTRACT:

N-[(9-β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl]-threonine, a hypermodified nucleoside found in transfer RNA represents the archetype structure of a group of synthetic ureidopurines which promote cell division in plants. L-threonine was shown to be incorporated into this nucleoside in Tobacco tissue and Rhizopogon roseolus transfer RNA. Also, this nucleoside was isolated as a free molecule in Rhizopogon roseolus growth medium.

Chromatographic analysis of an enzymic hydrolysate of 3 g of yeast transfer RNA failed to indicate the presence of a lipophilic derivative of PCT riboside. It is concluded that PCT riboside occurs in transfer RNA with a free carboxyl group.

ACKNOWLEDGEMENTS

I am particularly thankful to Dr. Hall, and to Dr. Guern from Paris who made it possible for me to come to McMaster University, within an exchange program between Canada and France.

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INTRODUCTION

N-[(9-β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl]-threonine (PCT-R, fig. 1) and its corresponding base (PCT, fig. 2) is a hypermodified nucleoside that has been isolated first from yeast transfer ribonucleic acid and characterized as such by Chheda et al. (1969) and Schweizer et al. (1969). It has been found in Escherichia coli, animal tissue and plant tissue tRNA (Dyson et al., 1970). Ishikura et al. (1969) detected it in E. coli methionyl-, lysyl-, and seryl-tRNA. Its presence as the nucleoside, among other methylated nucleosides (Chheda, 1969) in human urine is probably the result of the catabolism of human tRNA. Recently, it has been located adjacent to the 3' end of the anticodon in tRNA sequences of Torulopsis utilis isoleucyl tRNA (Takemura et al., 1969), yeast lysyl tRNA (Smith et al., 1971) and E. coli methionyl-tRNA Cory et al., 1968) (fig. 3). In this latter case it is not present in the formyl-methionyl species (Dube et al., 1968).

This suggests that PCT occurs only in those tRNA molecules that respond to codons beginning with the letter A (table 1) and specifically it is located adjacent to the 3' end of the anticodon where it appears to be essential

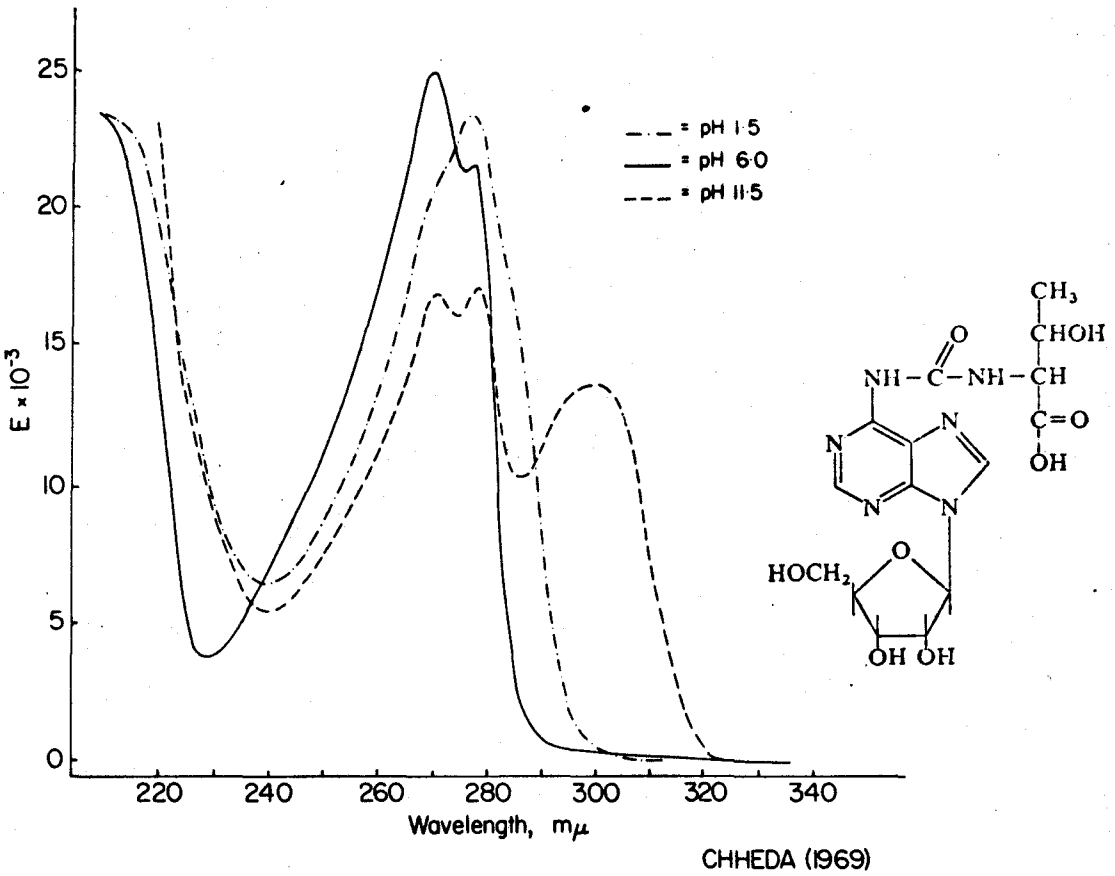


Figure 1. Ultraviolet absorption spectra and structure of N[(9-β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl]-threonine.

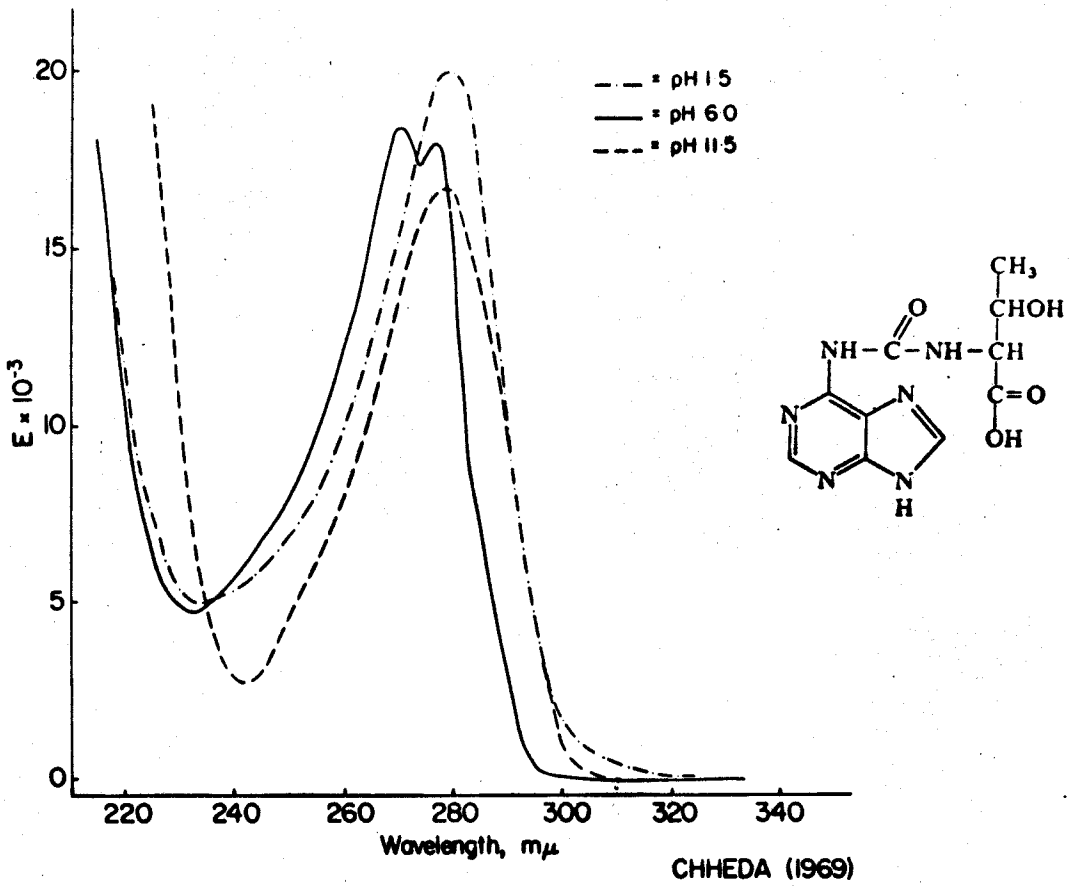
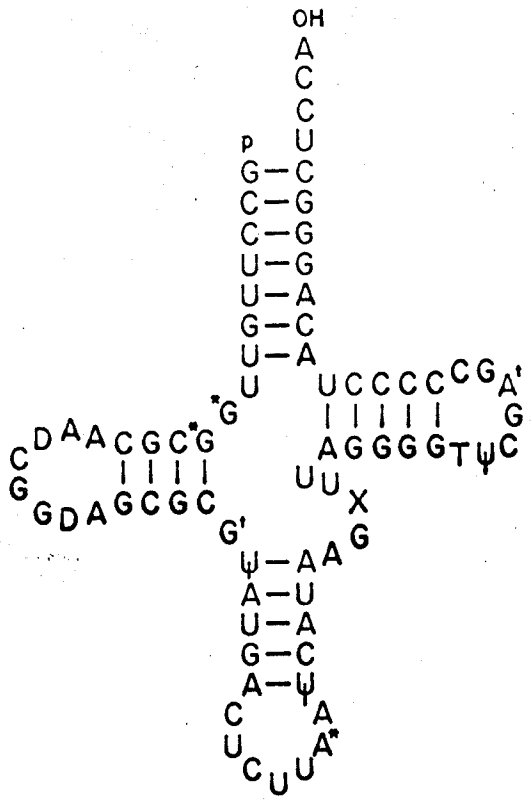
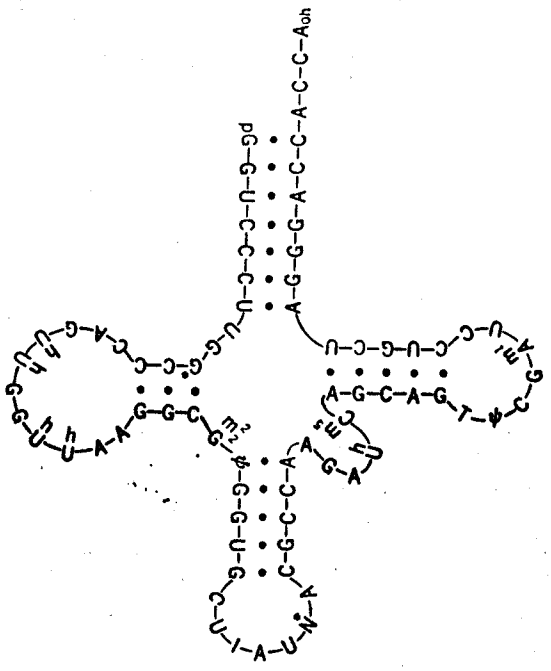


Figure 2. Ultraviolet absorption spectra and structure of N-(purin-6-ylcarbamoyl)threonine.



Yeast tRNA^{Lys}

Smith et al. (1971)



Torulopsis utilis tRNA^{Ile}

Takemura et al. (1969)

Figure 3. Primary sequence of two tRNA species showing the location of N-(purin-6-yl-carbamoyl)threonine (N* or A*) next to the 3'-end of the anticodon.

First position (5′ end)	Second position U	C	A	G	Third position (3′ end)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Term[a]	Term	A
	Leu	Ser	Term	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	GluN	Arg	A
	Leu	Pro	GluN	Arg	G
A	Ileu	Thr	AspN	Ser	U
	Ileu	Thr	AspN	Ser	C
	Ileu	Thr	Lys	Arg	A
	Meth	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

[a] Chain terminating (formerly called "nonsense").

Table 1. The genetic code

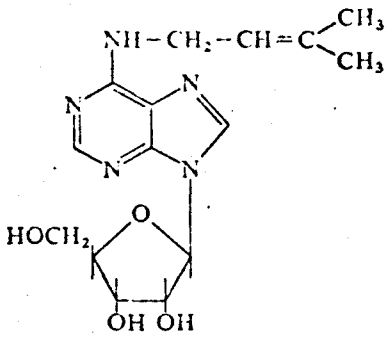
for codon-anticodon recognition (J. Miller et al., 1972).

In all these respects, PCT-R can be compared to another hypermodified nucleoside, N^6-(Δ^2-isopentenyl)-adenosine which occurs in the tRNA adjacent to the 3' end of the anticodon, and only in those tRNA molecules that respond to codons beginning with the letter U (review by Hall, 1970).

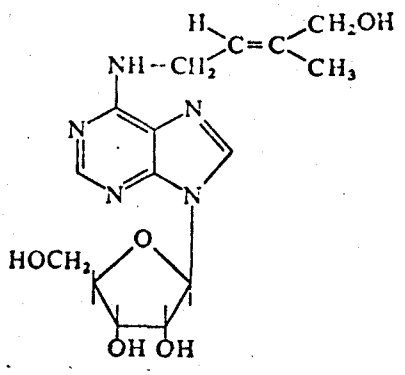
Moreover, i^6-Ado and its derivative zeatin-riboside (fig. 4) are naturally occuring cytokinins in plants. Cytokinins are plant hormones that promote cell division, induce differentiation in plant tissue cultures, release lateral buds from apical dominance and stimulate the rate of germination of seeds. They also exert a strong antisenescent effect in excised leaves (see review by Fox, 1969).

Although PCT itself has no cytokinin activity synthetic derivatives with a lipophilic side-chain (without the hydroxyl and carboxyl groups) promote cell division in plant tissues (Dyson et al., 1970; McDonald et al., 1971; Dyson et al., 1972). The parallelism between PCT and i^6-Ado (specific location in tRNA and potential cytokinin activity) has lead Dyson et al. (1970) to present the concept of PCT as a physiologically active molecule.

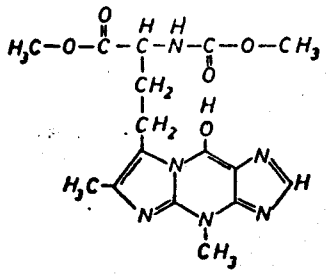
It should be mentioned that cell-division promotion activity of coconut-milk had been attributed to diphenyl-urea by Shantz et al. (1955), and subsequently hundreds of derivatives of diphenylurea were tested for biological



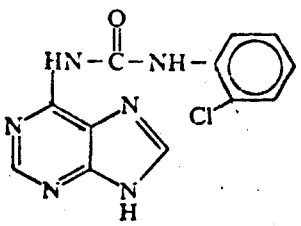
N^6-(Δ^2-isopentenyl)adenosine



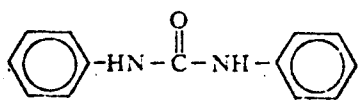
trans-zeatin riboside



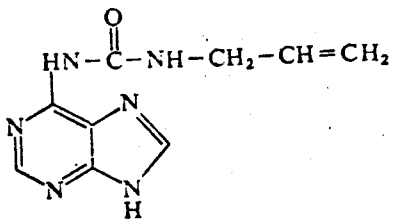
Y^+



o-chlorophenylureidopurine



1-3-diphenylurea



Allylureidopurine

Figure 4. Structures of some of the molecules which have been reported to promote cell division in plant tissue cultures.

activity (Kefford et al., 1966). Diphenylurea is not known to occur naturally and perhaps its physiological activity can be attributed to the structural resemblance to the N^6-ureidopurines.

The report by Hecht et al. (1970) of cytokinin activity associated with the fluorescent base Y^+ (Thiebe et al., 1971) found adjacent next to the anticodon in yeast, liver and wheat germ phenylalanyl-tRNA supports also the hypothesis of a relation between cytokinin activity and the presence of these molecules at a key position in tRNA molecules.

The purpose of this project was to study the in vivo incorporation of L-threonine in the side-chain of PCT. The incorporation of a radioactive precursor of the side-chain, more specific than the labelling of the purin ring could be used as a tool for the detection of PCT as a free molecule in a plant system and the isolation of a modified form of PCT as reported in the original isolation by Chheda et al. (1969).

MATERIALS AND METHODS

Adenine [8-^{14}C], specific activity 50 mc/mmole, adenine [8-^{3}H] specific activity 14 C/mmole and L-threonine-^{14}C, uniformly labeled with a specific activity of 210 mc/mmole were purchased from Schwarz/Mann. D.L-threonine-^{3}H, generally labeled, specific activity 80 mc/mmole, was purchased from Amersham/Searle.

A. RADIOACTIVITY MEASUREMENTS

Small aliquots (50 µl to 100 µl) of the tRNA preparations were dissolved overnight in 20 ml vials with 100 µl of NCS (Nuclear Chicago solubilizer); then 10 ml of Bray's solution (Bray, 1960) was added. For fractions from the Sephadex LH-20 columns, 1 ml aliquots were counted in 10 ml of Bray's solution. The radioactivity in the chromatograms was measured as follows: when dry, each strip of paper (about 3 cm wide) was cut in pieces of 1 or 2 cm wide, parallel to the origin. As the efficiency for tritium is very low on paper, each piece was put separately in 20 ml vials and eluted for 30 min with 1 ml of hot 35% ethanol, then 10 ml of Bray's solution was added.

The radioactivity was measured in a Nuclear Chicago scintillation counter Mark I, using the external standardization to determine the efficiencies for both tritium and carbon 14. With 1 ml of 35% ethanol in 10 ml of Bray's solution, the efficiencies were respectively 18% for tritium and 45% for ^{14}C, with 13.5% of the ^{14}C disintegrations going into the tritium channel.

B. CHROMATOGRAPHIC TECHNIQUES

1. Paper and thin layer chromatography.

Paper chromatography was performed in the descending manner using Whatman No. 1 paper (when not stated) or Whatman 3 MM paper. Solvent systems and Rf values are listed in table 2. All solvents used were reagent grade and redistilled in an all-glass apparatus.

When the fluorescent nucleoside Y was purified, MN-silica gel N-HR (Macherey, Nagel & Co.) plates, 0.5 mm thick were prepared. For rapid analytical purposes, EASTMAN CHROMATOGRAM sheets 6060 Silica Gel with fluorescent indicator were used in an ascending manner. The Rf values observed were very similar to those obtained by paper chromatography.

Ultraviolet absorbing compounds were visualized under U.V. light, and threonine was localized on chromatograms with a ninhydrin test.

COMPOUND	SOLVENT SYSTEMS A	B	C	E
PCT	0.00	0.47	0.43	0.00
PCT, Ribosyl	0.00	0.38	0.40	0.00
Product 1	0.53	0.82	--	0.61
Product 2	0.23	0.70	--	0.30
Adenine	0.30	0.60	0.28	--
Adenosine	0.30	0.57	--	0.22
Threonine	--	0.53	0.58	--
Zeatin	0.72	--	0.62	--
N^6-(Δ^2-isopentenyl)-adenosine	0.83	0.85		0.85
N^6-N^6-dimethyladenosine	0.65	0.75		0.65
N^6-methyladenosine	0.45	0.90		0.45
2'-O methyladenosine	0.47	0.75		0.45
2-thio-5-carboxymethyl-uridine methyl ester	0.10	0.66		0.64
5 methyluridine	0.20	0.65		0.25
2'-O-methyluridine	0.20	0.65		0.43

A 1-butanol-water-concentrated ammonium hydroxide (86:14:5)
B 2-propanol-1% aqueous ammonium sulfate (2:1)
C 2-propanol-concentrated hydrochloric acid-water (680:170:144)
D 2-propanol, water, concentrated ammonium hydroxide (7:2:1)
E Ethylacetate-1-propanol-water (4:1:2) upper phase

Table 2. Paper chromatography - Rf values

2. Column partition chromatography with diatomaceous earth.

Celite-545 (Johns-Manville's trademark of diatomaceous earth) is used as described by Hall (1971 - p.210). Two parts of Celite-545 are mixed with one part of solvent (aqueous phase). This "dry mixture" is packed into a precision-bore heavy-wall glass column with a tight-fit plunger in such a way that the compacted layers are equal in height to the diameter of the column. Then the sample, dissolved in a small volume of aqueous phase (one-seventh of the volume of solvent used to prepare the column) is mixed with 2 parts of Celite-545. This mixture is packed on the top of the column as described above. Solvent systems E (ethylacetate, 1 propanol, water:4:1:2) and F (ethylacetate, 2-ethoxyethanol, water: 4:1:2) were used.

3. Sephadex LH-20 column chromatography

Sephadex LH-20 is prepared by hydroxypropylation of Sephadex G-25, this treatment confers lipophilic properties to the dextran gel which allow the use of organic non-polar solvents. Its exclusion limit is at about MW=1500-2000, compared to 5000 for the parent G-25. Therefore the separation obtained is not based on gel filtration, but rather on some kind of interactions between the gel and the solutes, as illustrated by the different

elution volume of PCT with 35% aqueous ethanol at normal pH (5.8) and with 35% aqueous ethanol at pH 2.5 (fig. 19 and fig. 20). At pH 2.5, the repellent effect between the few carboxyl groups on the gel and the carboxyl group on PCT is suppressed and PCT with its nondissociated carboxyl group is retarded.

A Sephadex column, type K25/45 (Pharmacia) was packed with 50 g of Sephadex LH20 gel swollen in 35% aqueous ethanol. This column could be used either with 35% aqueous ethanol at normal pH or with 35% aqueous ethanol, pH 2.5, provided that it was prealably equilibrated overnight with the appropriate solvent. The relative ultraviolet absorbancy of the eluent was always monitored at 254 nm or 280 nm with an ultraviolet analyzer, Isco, model UA-2.

4. Dowex-50 cation exchange chromatography

Analytical grade Dowex 50W-X8 resin (H^+, 200-400 mesh) was obtained from Bio-Rad.

The resin can be bulk prepared and equilibrated, instead of equilibrating each column. Enough resin is stirred several times with 3 volumes of distilled water until the supernatant is colorless. The resin is poured in a column (2.5 cm x 40 cm) where it is washed for 6 hrs with 2 N HCl and rinsed with distilled water until the pH of the eluent is 5. This prepared and ready to use resin is stored in distilled water at 4°C.

C. OTHER TECHNIQUES

1. All ultraviolet absorption spectra were recorded on a Cary model 14 spectrophotometer.

2. Mass spectra were obtained with a high resolution CEC model 21-110B mass spectrometer in the Department of Chemistry, McMaster University. The sample was introduced by a direct inlet system.

3. Nuclear magnetic resonance spectra were recorded with a Varian Associates HA-100 spectrometer in the Department of Chemistry, McMaster University.

4. Quantitative ion-exchange chromatography of L-threonine was performed on an Amino-acid analyzer Beckman model 120C, according to Spackman et al. (1968).

5. Gas chromatography analysis of N^6-(Δ^2-isopentenyl)adenosine was performed by W.H. Dyson on a Hewlett-Packard model 402 gas chromatograph, according to the method of Babcock et al. (1970).

D. TISSUE CULTURES

1. Medium. Fox's medium (J.E. Fox, 1963) was used for all the cultures. The composition is as follows (mg/ml): NH_4NO_3:1000, KNO_3:1000, $Ca(NO_3)_2$-$4H_2O$:500, KH_2PO_4:250, H_3BO_3:5, KI:0.8, Mg SO_4-$7H_2O$:300, KCl:50, $ZnSO_4$-$7H_2O$:7.5, $MnSO_4$-H_2O:5, thiamine-HCl:0.1, nicotinic acid:0.5, pyridoxine-HCl:0.5, glycine:2, Na_2Fe-EDTA:35, i-inositol:100, sucrose:30,000, and when required α-naphthaleneacetic acid:1 mg/l, and kinetin:0.5 mg/l.

The pH is adjusted to 5.8-6 with 1 N sodium hydroxide. When necessary, media are solidified with 0.7% Difco Bacto-agar.

2. The strain of Tobacco tissue (0-1) used does not require any auxin or cytokinin and was derived from normal pith callus of Nicotiana tobaccum, variety Wisconsin 38 by J.E. Fox (1963). It came to our laboratory via W.D. Dyson.

3. Soybean tissue requires both an auxin and a cytokinin for growth. This cytokinin requirement is absolute and therefore the cytokinin-dependent growth of this particular strain of tissue constitutes the most sensitive biological test for cytokinins. Pieces of tissue (2 mg) are placed, three to a 25 ml flask, on 10 ml of solid medium with an auxin plus an aliquot of the solutions to be tested. The flasks are previously autoclaved at 18 lb/square inch, at 121°C for 5 min. After incubation in the dark at 24°C for 4 weeks, the pieces are weighed and their fresh weights are compared to controls grown on basal medium devoid of cytokinin.

The tissue was derived from the cotyledon of glycine max (LL) variety Merril Acme, in C.O. Miller's laboratory and came to this laboratory via J.E. Fox and W.H. Dyson.

4. Rhizopogon roseolus, a mycorrhizal fungus (Basidiomycete) has been shown to be a good producer of zeatin-riboside in culture (C.O. Miller, 1967). It came to our laboratory via G. Miura from C.O. Miller's laboratory. It is grown in liquid suspension in the basal medium described above, without any auxins or cytokinins. Cultures are inoculated with mycelial suspensions that have been ground for 5 seconds in a sterile Waring blender with the same fresh medium. They are incubated with aeration on a gyrotory shaker at 27°C in the dark.

E. EXTRACTION OF TRANSFER RNA

Transfer RNA was extracted according to the method of Holley et al. (1961) adapted to the nature of the biological material.

1. Tobacco tissue tRNA

The procedure (Dyson et al. 1969) is described in terms of 300 g fresh weight of tissue. The tissue is homogenized in a Waring blender for 30 seconds with 1.5 liters of cold (-5°C) 95% ethanol. The homogenate is allowed to settle for 10 min and is then filtered through a Büchner funnel. The precipitate is washed with cold 95% ethanol until the efluent is colorless. It is rinsed three times with 100 ml of acetone. After drying in a desiccator in vacuo, the residue is homogenized with 200 ml of distilled phenol, 200 ml of 0.1 M Tris-HCl buffer (pH 6, containing KCl 0.06 M, $MgCl_2$ 0.01 M) and

with 30 ml of sodium dodecyl sulfate. After centrifugation (16000 g for 10 min) the aqueous phase recovered is extracted twice with 150 ml of buffer-saturated phenol. Then the residual phenol is extracted from the last aqueous phase with ether in a separatory funnel.

After evaporation in vacuo of the residual ether, 0.1 V of sodium acetate 20%, pH 5.4 is added to the aqueous phase. The nucleic acid is precipitated according to the method of Bellamy and Ralph (1968) by addition of cetyltrimethylammonium bromide (1% solution). The precipitate is recovered by centrifugation and resuspended in 70% ethanol containing 0.1 M sodium acetate, centrifugated again and washed twice with 95% ethanol.

The crude nucleic acid preparation is dissolved in 3 ml of Tris-HCl buffer, 0.1 M, pH 7.5 containing 0.1 M NaCl and placed on top of a column (2 cm x 20 cm) of DEAE cellulose (Cl^-) previously equilibrated with the same buffer. The column is washed with 0.3 M NaCl in Tris-HCl 0.1 M, pH 7.5 until the eluent is free of any UV absorbing material (the UV absorbancy at 254 nm is monitored with a Isco UV analyzer, model UA_2). The tRNA is eluted with a solution of 1 N NaCl. The yield is about 1 A_{260} unit per g of tissue (fresh weight). The tRNA solution is then diluted with 2.5 volumes of cold ethanol to precipitate the tRNA.

2. Rhizopogon tRNA

All the glassware used is baked at 180°C to free it of any ribonuclease activity. The mycelium (1 g to 3 g, fresh weight) is stirred for 5 hours at least in a 100 ml beaker with 10 ml of distilled phenol and 10 ml of Tris-HCl buffer, 0.1 M, pH 7.5. The aqueous phase is separated by centrifugation (10000 g, 10 min) and the phenol phase is re-extracted with 10 ml of buffer. The aqueous phases are pooled and re-extracted two times with 10 ml of distilled phenol each time. After washing the aqueous phase with ether to extract any residual phenol, the traces of ether are removed in vacuo.

The procedure described above is followed except that the tRNA fraction is purified on a smaller DEAE cellulose column (0.8 cm x 10 cm). The yield is about 6 A_{260} units per g of mycelium (fresh weight). Fifty mg of yeast tRNA are added as carrier to the tRNA solution before precipitation with 2.5 volumes of ethanol.

F. HYDROCHLORIC ACID HYDROLYSIS OF tRNA (CHHEDA ET AL., 1969)

The tRNA is dissolved in 1 ml of distilled water in a 10 ml erlenmeyer flask. This solution is heated to 100°C over a steam-bath, then hot 2 N hydrochloric acid (1 ml) is added. The flask is stoppered and left for 10 more minutes on the steam-bath, then the flask is rapidly

cooled in ice water. The solution is evaporated to dryness in vacuo and the residue is repeatedly dissolved in water (1 ml) and evaporated to dryness until the pH is approximately 5. Finally, the hydrolysate is dissolved in 2 ml of water.

RESULTS

SECTION I BIOSYNTHESIS OF N-(PURIN-6-YLCARBAMOYL)THREONINE

A. BIOSYNTHESIS OF PCT IN TOBACCO TISSUE tRNA

1. Incorporation of [^{14}C]-L-threonine into PCT of Tobacco tissue tRNA

Three weeks old Tobacco 0-1 tissue (each 250 ml flask contained approximately 10 to 12 g of tissue, and 10 flasks were used) was incubated with 20 µc of [^{14}C]-L-threonine (specific activity 210 µc/µmole). The radioactive solution was made up to 1 ml with distilled water and autoclaved for 5 min at 121°C, and 0.1 ml of this solution was applied on the top of each culture.

After 8 days of incubation, the tissue (325 g fresh weight) was extracted and yielded 280 A_{260} units of tRNA after elution from the DEAE cellulose column. The total incorporation was 1800 cpm (counting efficiency 75%) into tRNA. This fraction was acid hydrolysed (see procedure) and the hydrolysate, after removal of the hydrochloric acid, was applied to a column (1 cm x 10 cm) of previously prepared Dowex 50W-X8 (H^+) (200-400 mesh). The column was washed with 50 ml of 0.001 N HCl and eluted with a 500 ml hydrochloric acid gradient (0.2 N to 0.8 N) (fig. 5). Each fraction was evaporated to dryness in vacuo to free it of hydrogen chloride. Fraction IV was identified

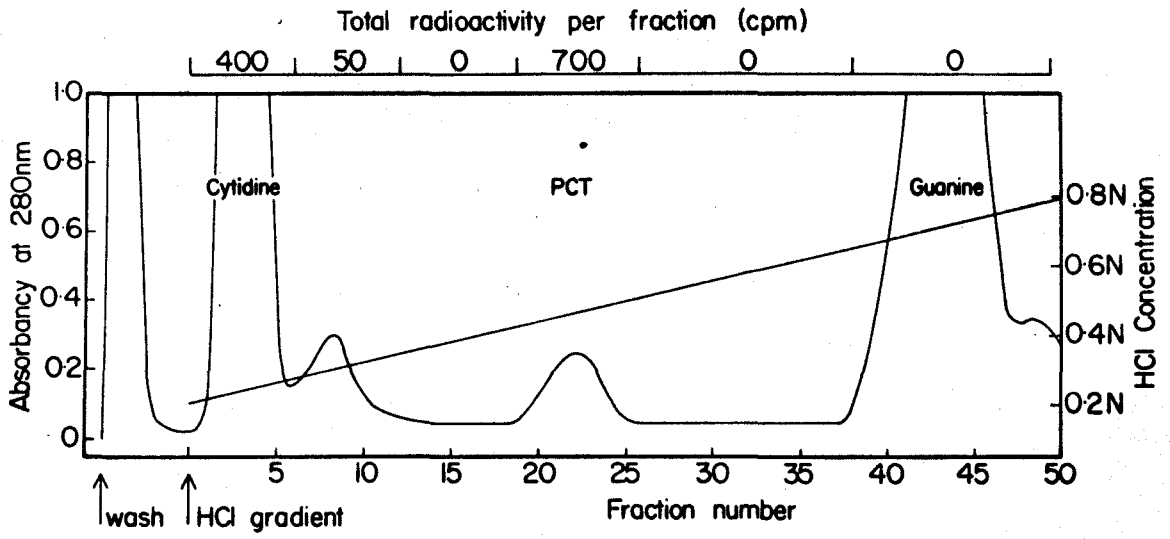


Figure 5. Isolation of N-(purin-6-ylcarbamoyl)-threonine from an hydrochloric acid hydrolysate of tobacco tissue on a 1 cm x 10 cm column of Dowex 50W-X8 (H^+, 200-400 mesh). The column was eluted with a linear gradient of hydrochloric acid (0.2 N - 0.8 N, total volume 500 ml).

by its UV spectra as PCT. A one-fifth aliquot of each fraction was counted, the total radioactivity associated with the PCT fraction was 700 cpm. In solvent systems B and C, this radioactivity cochromatographed as a discrete spot with PCT.

2. Study of the turn-over of ^{14}C-L-threonine labeled PCT in Tobacco tissue tRNA

In order to detect a possible turn-over of the tRNA molecules another experiment was carried out. Twenty flasks at the same stage as in the first experiment were labeled over a period of 4 days (6 µc each) and subsequently incubated for 6 days for half of the cultures, and 12 days for the remaining half. The first set (6 days incubation) yielded 330 A_{260} units of tRNA with a total incorporation of 3300 cpm. After hydrochloric acid hydrolysis of this tRNA and Dowex-50-column chromatography, 600 cpm cochromatographed with the PCT fraction. This fraction was hydrolysed for 2 hr in one ml of 0.1 N sodium hydroxide. Paper chromatography in solvent C showed that the ^{14}C radioactivity moved with L-threonine. The second set (12 days incubation) of cultures yielded 300 A_{260} units of tRNA with a total incorporation of 700 cpm in the PCT fraction after Dowex-50-column chromatography.

3. Attempt to isolate a soluble form of PCT in Tobacco tissue

The 2 liters ethanolic extract of the first

experiment which contained 15% of the original radioactivity was reduced to 50 ml under vacuo. In order to remove the pigments present, the solution was extracted twice with 50 ml of petroleum ether. After removal of the traces of ether and addition of 500 µg of cold synthetic PCT riboside, the solution was made 0.1 N with respect to HCl and heated for 15 min at 100°C on a steam-bath. The flask was rapidly cooled in ice-water. The solution was evaporated under vacuo, but the removal of the hydrogen chloride appeared impossible due to the formation of a very thick and gummy residue. This residue was dissolved in 15 ml of distilled water and the pH was adjusted to 5 with 3 ml of 1 N sodium hydroxide.

This solution was absorbed on a Dowex-50-column (2 cm x 30 cm). The column was washed with one l of 0.001 N hydrochloric acid in order to remove UV absorbing impurities and the column was eluted with one l of a hydrochloric acid linear gradient (0.2 N to 1 N). The cold marker PCT could not be recovered on the elution profile. Radioactivity measurements showed the presence of a major radioactive product which chromatographed with L-threonine in several solvent systems (approximately 10^4 cpm/ml for the first 10 ml fractions down to a level of 100 cpm/ml at the end of the gradient). For this reason, it appeared impossible to separate a soluble form of PCT from the bulk of the radioactivity.

4. Conclusion

[^{14}C]-L-threonine is incorporated in the side-chain of PCT in the tRNA of Tobacco tissue, but no evidence could be found of the presence of a soluble form of PCT in this system. However, the level of incorporation of [^{14}C]-threonine in PCT in situ in the tRNA is very low, and is at the limits of the working conditions. Therefore, the fungus, Rhizopogon roseolus, which had been shown to produce free zeatin-R in remarkable amounts (C.O. Miller, 1967) was examined for the presence of PCT.

B. BIOSYNTHESIS OF PCT IN RHIZOPOGON ROSEOLUS

1. PCT in Rhizopogon tRNA

First, the presence of PCT in Rhizopogon roseolus tRNA had to be established.

Two days after inoculation, a 400 ml culture was incubated with 30 µc of [^{14}C]-L-threonine, and 25 µc of [8-^{3}H]-adenine for 3 days (growth is exponential between day 2 and day 5). Then, the culture was filtered through a Büchner funnel and the mycelium was washed twice with 20 ml of fresh medium.

After extraction and purification of the tRNA, the yield was 18 A_{260} units. The total incorporation was 400 000 dpm for ^{3}H and 78 000 dpm for ^{14}C. After acid hydrolysis of the tRNA (with 50 mg of yeast tRNA as carrier) and separation on Dowex-50 (fig. 6) the PCT

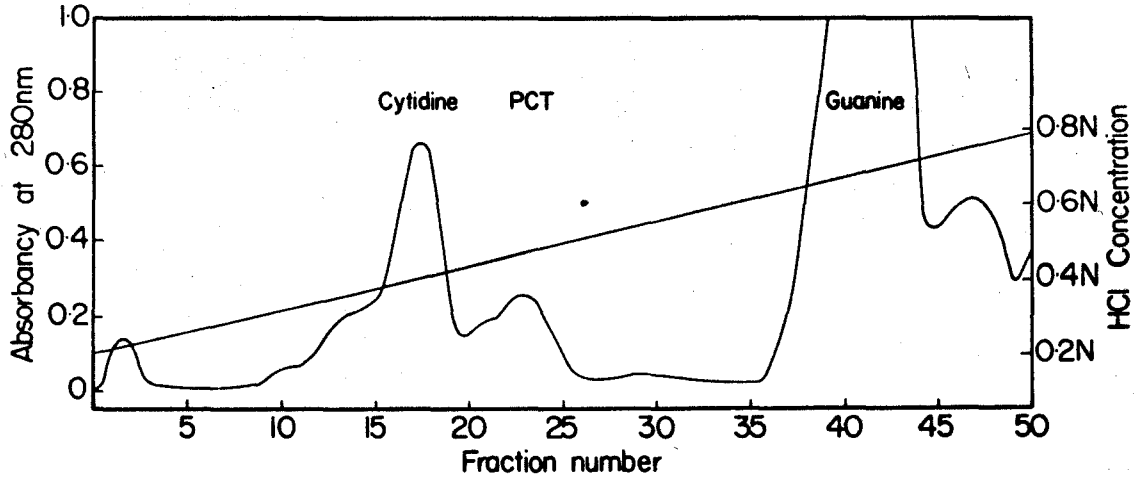


Figure 6. Isolation of N-(purin-6-ylcarbamoyl)-threonine from an HCl digest of Rhizopogon tRNA (plus 50 mg of yeast tRNA as carrier) as in fig. 5.

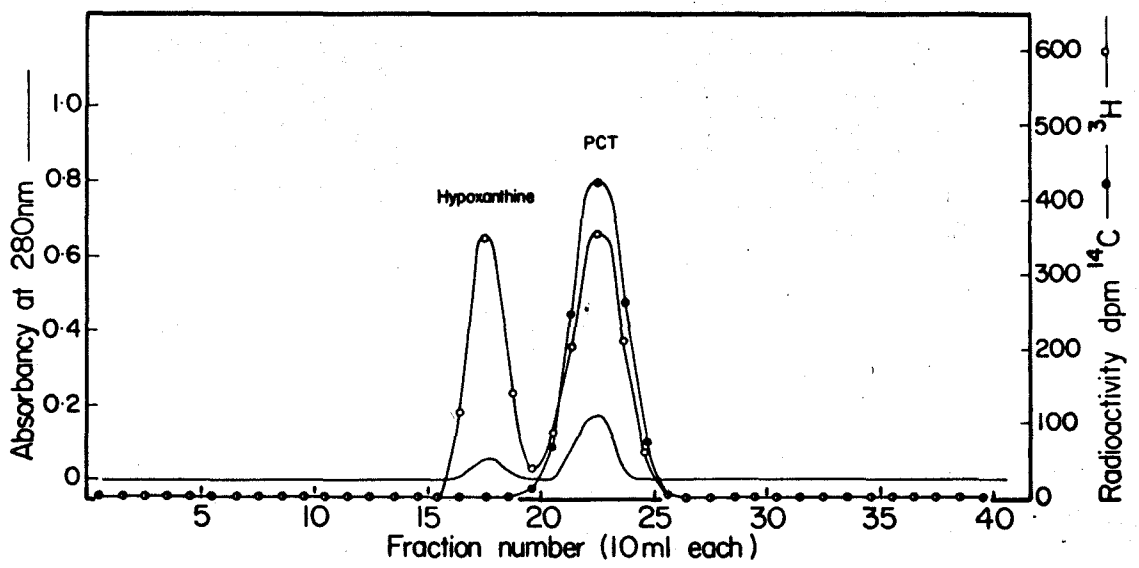


Figure 7. Purification of N-(purin-6-ylcarbamoyl)-threonine after Dowex-50W-X8 column chromatography on a Sephadex LH-20 column (50 g, 2.5 cm x 40 cm). The column was eluted with 35% aqueous ethanol, pH 2.5. Radioactivity was measured on 1 ml aliquot of each fraction.

fraction was purified on a LH-20 column. The PCT fraction appeared to be double labeled with ^{14}C and ^{3}H (fig. 7) and was well separated from tritiated material identified as hypoxanthine by its UV spectra.

For further characterization, the PCT fraction (1000 dpm for ^{14}C and 900 dpm for ^{3}H) was chromatographed in solvent systems B and D. In those systems, the double radioactivity moved coincidentally with the UV quenching spot of PCT, and no other residual radioactivity was observed on the chromatogram.

In another experiment, using the same incubation conditions but with 140 µc of [^{3}H]-DL-threonine and 20 µc of adenosine-[8-^{14}C] the incorporation was 1300 dpm for ^{14}C and 2000 dpm for ^{3}H in the whole PCT fraction after LH-20 purification. After sodium hydroxide hydrolysis, (as in section IA.2, page 22) paper chromatography in solvent C showed that the ^{14}C radioactivity moved with adenine and that the ^{3}H radioactivity coincided with threonine.

2. PCT released in the medium

A 50 ml culture (2 days old) was incubated for 4 days[1] with 30 µc of [^{14}C]-L-threonine and 200 µc of [8-^{3}H]-adenine in presence of cold PCT-R (10^{-4} M, final concentration). The culture was filtered through a Büchner funnel and the

[1]footnote: After 4 days of incubation the pH of the medium is 2.5

medium was collected and separated into two fractions of 45 ml and 5 ml respectively. The second fraction (one-tenth of the total volume) was used to measure the amount of zeatin produced.

Nine-tenths of the medium was reduced to 10 ml with a flash evaporator and made 0.1 N with respect to hydrochloric acid. After heating for 20 min at 100°C, the solution was partially neutralized with 2 N sodium hydroxide until the pH was 5, and the solution was absorbed on a Dowex-50-column (2 cm x 20 cm). The column was washed with 800 ml of distilled water and eluted with 300 ml of 1 N ammonium hydroxide. The ammonia eluate was dried in vacuo, redissolved in 5 ml of distilled water and then passed through a smaller Dowex-50-column (1 cm x 15 cm) and the products were eluted as previously described (fig. 8). After purification on an LH-20 column, the PCT fraction appeared to be double labeled and well separated from the bulk of the radioactivity (fig. 9). The total incorporation was 14 000 dpm for ^{14}C (threonine moiety) and 84 000 dpm for ^{3}H (adenine moiety), (ratio of Adenine/threonine, 6).

The double radioactivity moved with PCT on paper chromatography in three different solvents (B,C and D) with a constant ratio between ^{14}C and ^{3}H for each strip of paper counted. No significant radioactivity was found elsewhere on the chromatograms (fig. 10).

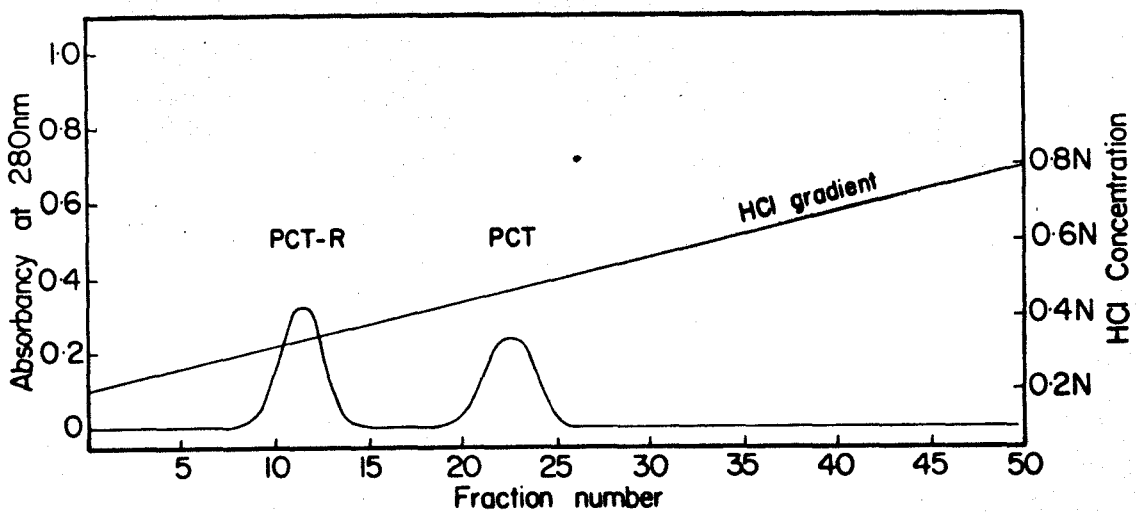


Figure 8. Separation of a mixture of PCT-R and PCT added to 50 ml of Rhizopogon medium by Dowex-50 column chromatography (1 cm x 15 cm). The column is washed with 100 ml of 0.001 N HCl and eluted as in figure 5.

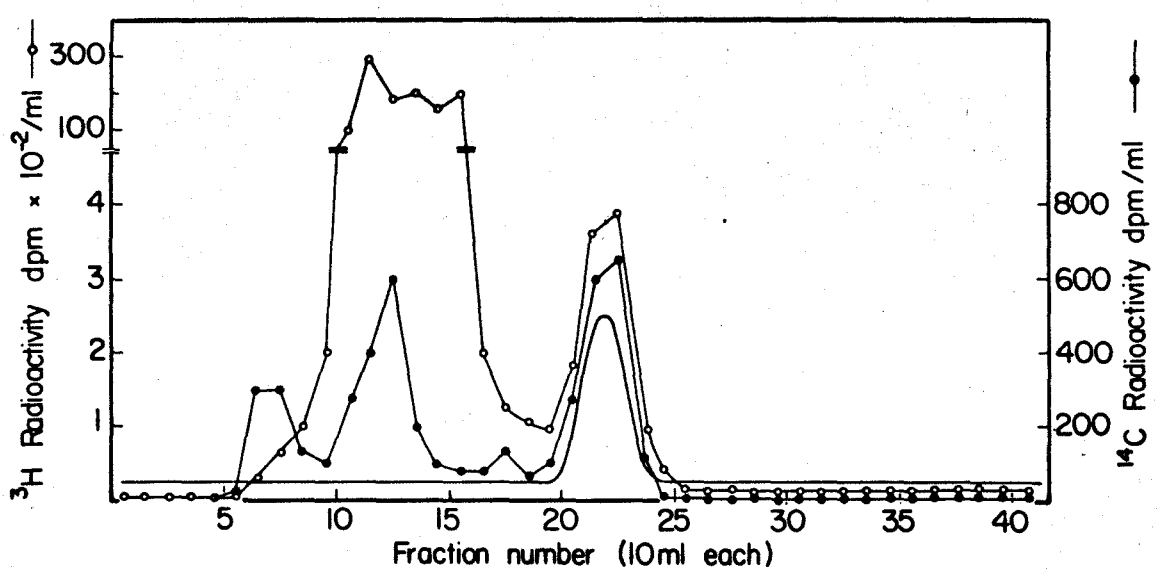


Figure 9. Purification of PCT from Rhizopogon medium after isolation by Dowex-50 column chromatography on a Sephadex LH-20 column (as in figure 7).

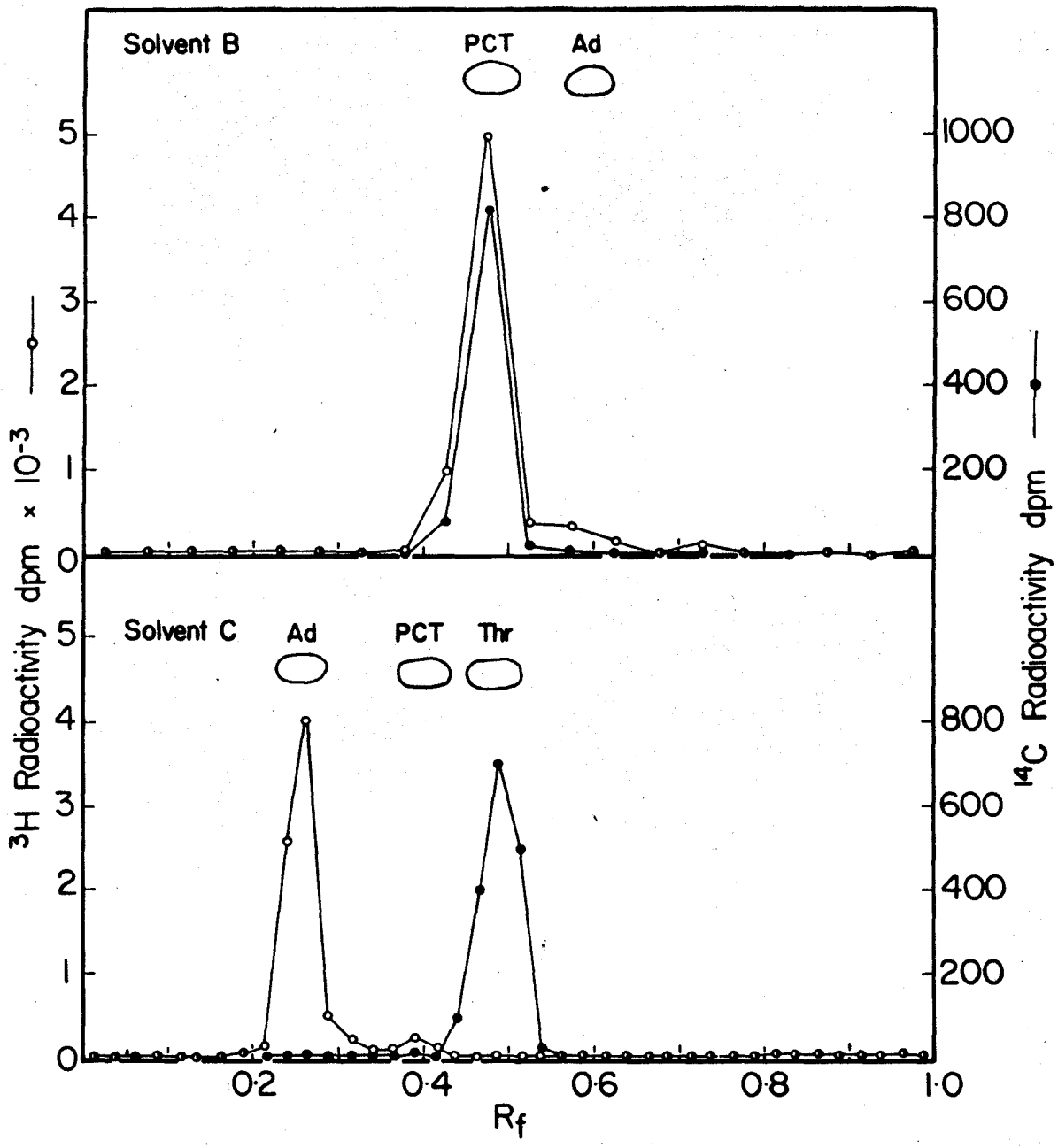


Figure 10. Paper chromatography of the double labeled PCT isolated from Rhizopogon roseolus medium (fig. 9). After sodium hydroxide degradation (bottom), the double radioactivity separates between adenine (^{3}H) and L-threonine (^{14}C) respectively.

After sodium hydroxide hydrolysis (as in Section I.A.2, page 22) of the double-labeled product, paper chromatography in solvent system C (fig. 10) showed that the ^{14}C radioactivity moved with threonine and the ^{3}H radioactivity moved with adenine.

tRNA was extracted from the mycelium grown in this experiment. The PCT fraction isolated from this tRNA (as in section I.B.1 page 24) contained: [^{3}H]-Adenine, 2,550 dpm, [^{14}C]-L-threonine, 990 dpm (ratio of adenine/threonine, 2.6).

3. Amount of zeatin released under the same conditions

I wished to relate the amount of PCT released in the medium to the amount of zeatin produced. One-tenth of the medium (pH 2.5) plus 50 µg of cold zeatin as a marker was absorbed on a Dowex-50W-X8 column (1 cm x 15 cm). The column was washed with 200 ml of 1 N hydrochloric acid, rinsed with 100 ml of water and eluted with 100 ml of 1 N ammonium hydroxide. The ammonia eluate was dried in vacuo and the residue dissolved in 0.5 ml of 35% ethanol which was applied to a Sephadex LH-20 column (fig. 11), equilibrated and eluted with 35% ethanol. The fractions containing the marker zeatin were combined and zeatin itself was separated from adenine and another labeled compound by paper chromatography (fig. 12) in solvent

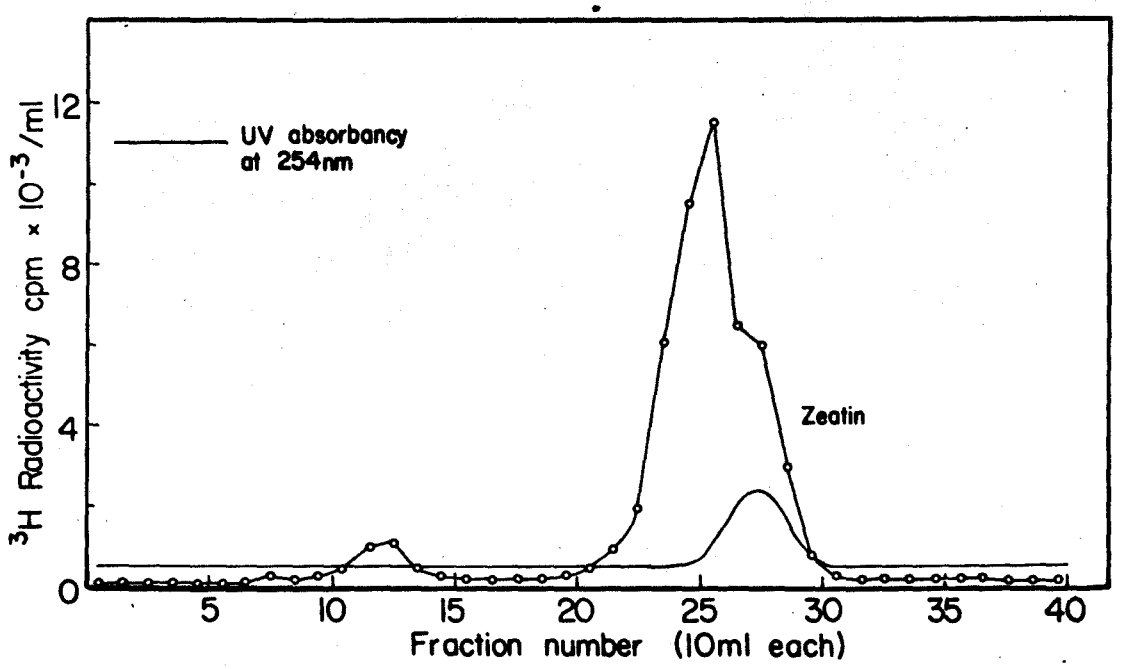


Figure 11. Purification of zeatin from Rhizopogon roseolus medium after ion-exchange chromatography (Dowex-50W-X8, H^+) on a Sephadex LH-20 column eluted with 35% aqueous ethanol.

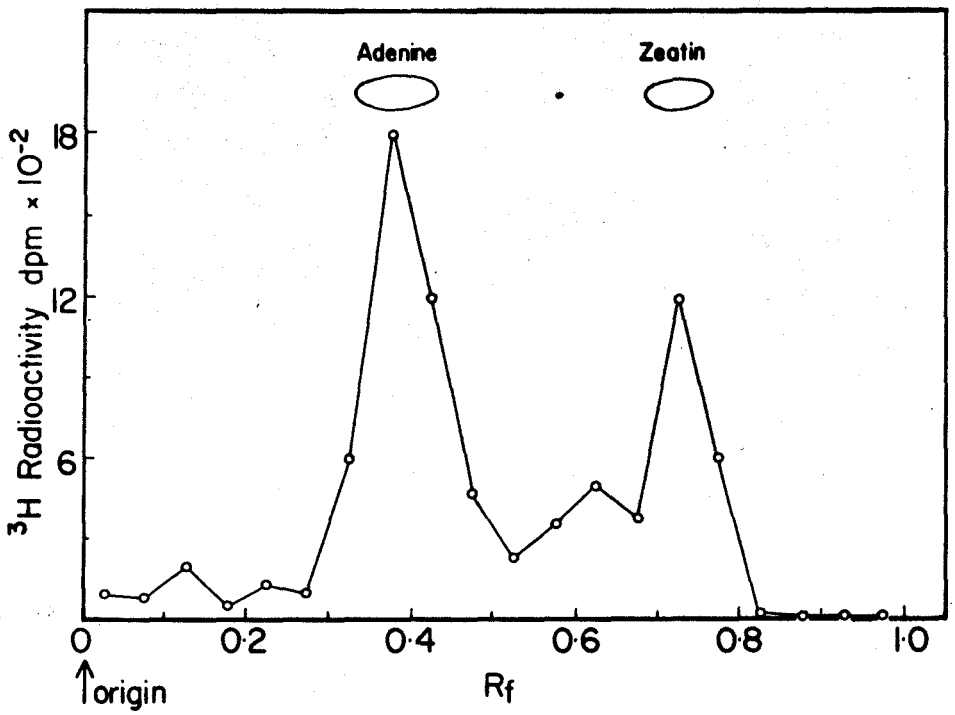


Figure 12. Paper chromatography purification of the zeatin fraction obtained after Sephadex LH-20 chromatography (fig. 11). The chromatogram was cut into 20 pieces and each piece was counted in a liquid scintillation counter.

systems A and C. In both systems, the total incorporation in the zeatin fraction was about 1.7×10^6 dpm. This 0.4% incorporation of [^{3}H]-adenine into zeatin agrees with previous results (G.A. Miura et al., 1969) and is 20 times the incorporation into the PCT fraction.

4. PCT is released as PCT-riboside

Until to this point, PCT had always been isolated after hydrochloric acid hydrolysis of tRNA. I questioned whether acid hydrolysis partially degrades the product. For example, the carboxyl group of PCT in the tRNA could be esterified (see for example Chheda et al., 1969).

The experiment was repeated, using the same incubation conditions (as in Section I.B.2, page 26), except PCT-R was absent from the culture medium. After a 4 days incubation time (30 µc of [^{14}C]-threonine, and 200 µc of [^{3}H]-adenine) the medium plus 200 µg of cold PCT-R was passed through a Dowex-50-column (1 cm x 15 cm) which was, after washing with 500 ml of distilled water, eluted with a hydrochloric acid gradient. The PCT riboside marker eluted earlier on the elution profile (fig. 8) and was definitely identified as the riboside by its UV spectra. Fractions containing PCT-R were pooled and heated at 100°C for 5 minutes (in 0.4 N HCl) in order to obtain the base which is better separated from the bulk of the radioactivity on LH-20. Under these conditions, the total incorporation into PCT was 23 000 dpm for ^{14}C

and 145 000 dpm for ^{3}H.

5. Is there a lipophilic form of PCT?

The possibility of the presence of a lipophilic form of PCT (i.e. without a free carboxyl group) was investigated in a third experiment (conditions identical to those described in section I.B.2 page 26). The medium of the 50 ml culture which contained 60 millions dpm for ^{3}H and 3.75 millions dpm for ^{14}C, was sequentially extracted with petroleum ether, water-saturated-ethylacetate and water-saturated-1-butanol. For each solvent, the aqueous phase was extracted twice with 50 ml of solvent in a separatory funnel.

The first extract contained 3.2×10^{6} dpm for ^{3}H but no significant ^{14}C radioactivity. Therefore this fraction was not investigated.

The total ethylacetate extract contained 12.5 millions dpm for ^{3}H and 20 000 dpm for ^{14}C and the 1-butanol extract contained 44 millions dpm for ^{3}H and 140 000 dpm for ^{14}C. In each case the radioactivity was measured on a one-tenth aliquot. After evaporation of the solvent the ethylacetate extract and the 1-butanol extract were dissolved in 0.8 ml of 35% ethanol and applied to the top of a Sephadex LH-20 column which was eluted with the same solvent. One hundred fractions of 10 ml each were collected. Measurement of the radioactivity showed

that in both cases, all the ^{14}C radioactivity was localized to an early fraction (tubes 12 to 15). Paper chromatography analysis of this fraction could not establish a coincidence between the ^{14}C and ^{3}H radioactivity.

The aqueous phase remaining after the 1-butanol extraction was investigated for the presence of PCT-R as in Section I.B.4, page 33 . Double-labeled PCT was characterized by its chromatographic properties and by its sodium hydroxide hydrolysis products. The total incorporation was 16 000 dpm for ^{14}C and 105 000 dpm for ^{3}H. This result, similar to those obtained above (Sections I.B.2 and I.B.4) shows clearly that PCT is not released into Rhizopogon medium in a lipophilic form but as the nucleoside.

SECTION II CONCERNING THE NATURAL FORM OF PCT IN YEAST tRNA

A. INTRODUCTION

When PCT was first isolated from the acid hydrolysate of 30 g of yeast tRNA, Chheda et al. (1969) reported the presence of a small amount (about 10%) of N-(purin-6-ylcarbamoyl)threonine which did not have a free carboxyl group. Its paper chromatography properties are of special interest: in solvent E (ethylacetate, 1-propanol, H_2O=4:1:2, upper phase) its Rf is 0.68 and can be compared to those of i^6-Ado (0.84), zeatin-riboside (0.54), Y riboside (0.54) Y^+ and Y^{++} (0.75 and 0.95 respectively according to R. Thiebe et al., 1968).

If PCT itself has no cytokinin effect - the negative charge of the carboxyl group preventing PCT from penetrating the cells - one can speculate that this lipophilic derivative might be active and could be the native form of PCT in transfer RNA. Therefore yeast tRNA was hydrolysed under milder conditions and the hydrolysate was examined for the presence of this derivative.

B. ENZYMIC HYDROLYSIS OF YEAST tRNA

Three grams of yeast tRNA were hydrolysed

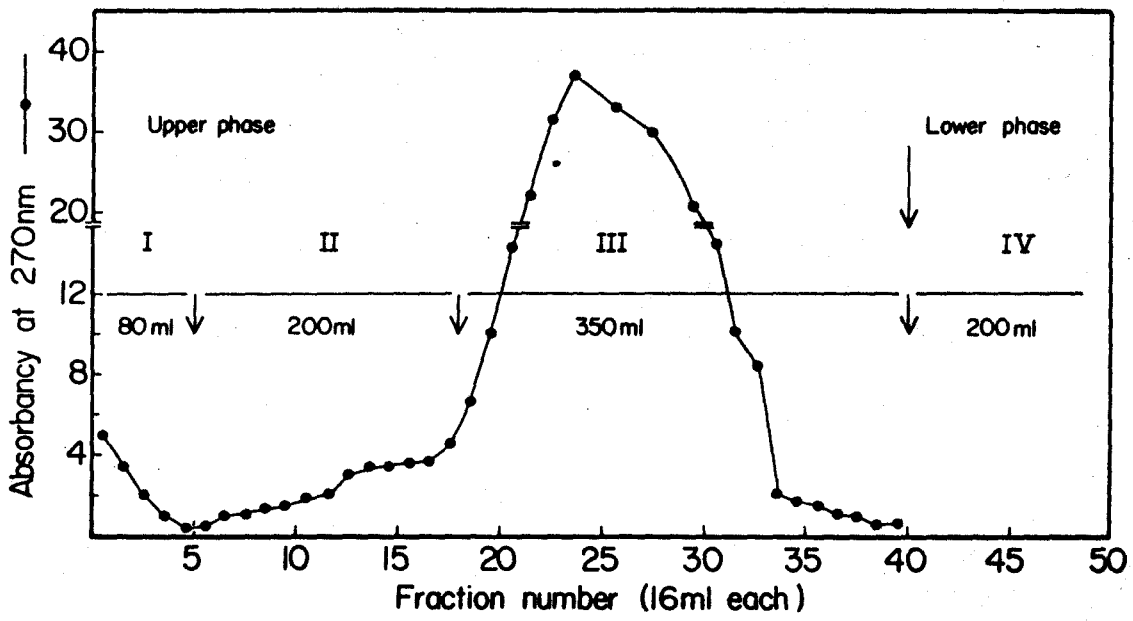


Figure 13. Celite-545 partition chromatography of an enzymic digest of 1 g of yeast tRNA, solvent E (column 2.5 cm in diameter, containing 120 g of Celite-545).

enzymically according to the procedure described by Hall (1971, p. 212) tRNA was dissolved in 300 ml of 0.005 M magnesium chloride solution, then 500 mg of lyophilized Crotalus adamanteus venom and 10 mg of bacterial alkaline phosphatase were added. A few drops of toluene were added to inhibit bacterial contamination and the mixture was incubated at 37°C after adjustment of the pH to 8.6. This pH was adjusted with 1 ml of 1 N sodium hydroxide each hour for 6 hours. Then 5 mg of bacterial alkaline phosphatase and 150 mg of snake venom were added. Incubation was continued until the uptake of NaOH had ceased. After a total of 14 hours the reaction was stopped by heating at 60°C for 30 min. The solution was kept at 4°C overnight and centrifuged at 15,000 x g for 1 hr. The supernatant was dried down and dissolved in 30 ml of solvent E (lower phase).

C. FRACTIONATION OF THE HYDROLYSATE

The first step used was column partition chromatography, as described by Hall (1971, p. 210). One-third of the above solution was mixed with 20 g of Celite-545. This mixture was then packed onto the top of a previously prepared glass column containing 120 g of Celite-545 mixed with 60 ml of solvent E (lower phase). The column was eluted with the upper phase of solvent E, as shown in

fig. 13. This procedure was repeated 3 times to fractionate the total hydrolysate.

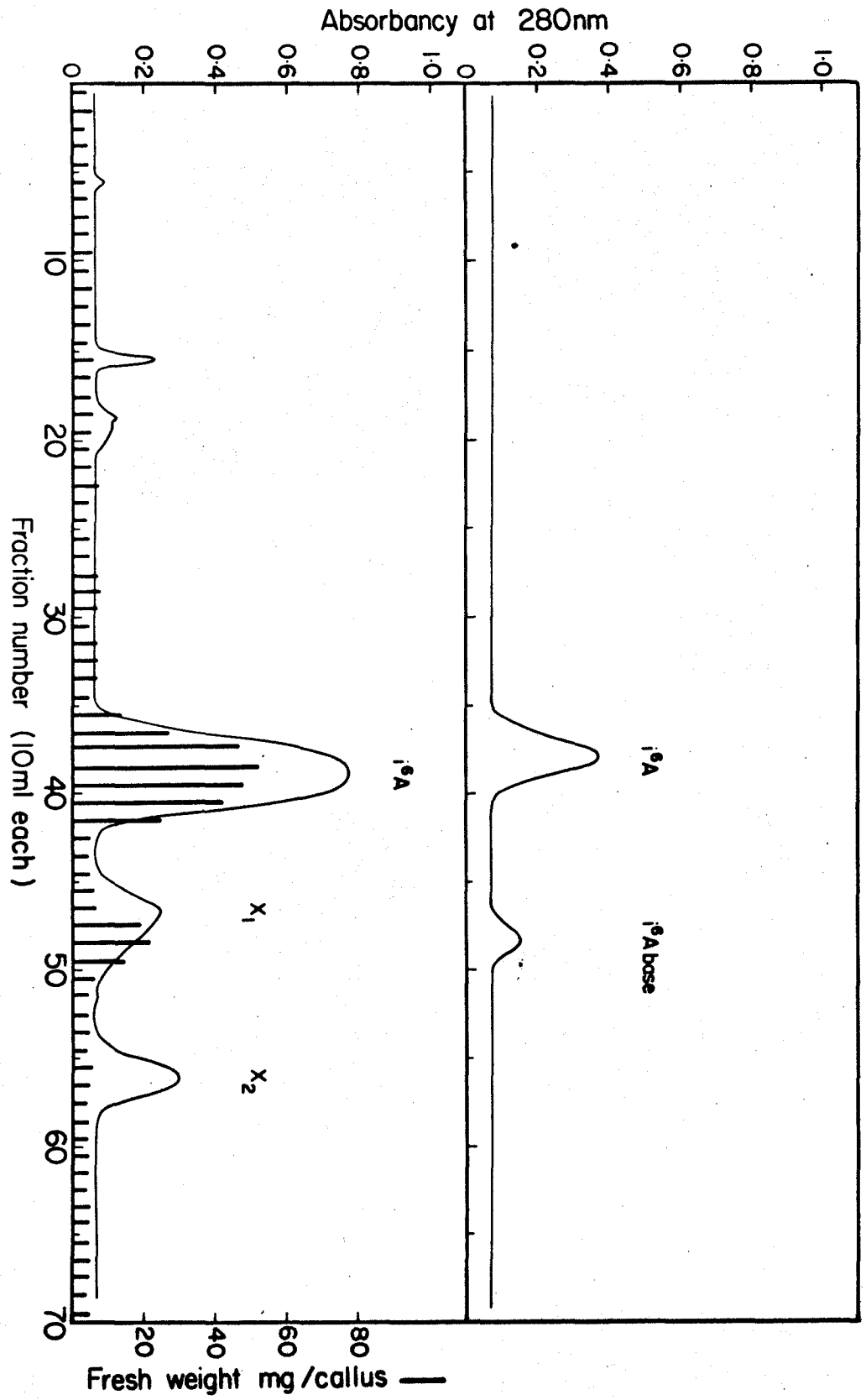
1. Fraction I

Fraction I from the 3 columns (80 ml each) were combined, evaporated to dryness under vacuo and redissolved in 2.5 ml of 35% ethanol. An oily precipitate was removed by centrifugation in a 15 ml bench top centrifuge tube. The supernatant was then applied to a 35% ethanol Sephadex LH-20 column. The elution profile is shown in fig. 14. N^6-(Δ^2-Isopentenyl)adenosine (120 A_{269} units) was identified by means of its UV absorption spectra and its gas chromatography retention time. This represents a 0.06 moles % moles nucleosides frequency for i^6-Ado which is close to the figure reported by Robins et al. (1967).

The two fractions following i^6-Ado, designated X_1 and X_2 for convenience presented unknown and very similar UV absorption spectra (fig. 15) and identical paper chromatography Rf (0.90 solvent E, 0.36 solvent B). As these compounds were observed in a different experiment which had only the Celite-545 (solvent E) partition chromatography and the Sephadex LH-20 chromatography steps in common, it was obvious that they were contaminants washed off the Celite-545. This was verified by eluting a blank Celite-545 column (50 g) with the same solvent. Prior to this finding, cytokinin activity of this fraction was

Figure 14 a. Elution volume of N^6-(Δ^2-isopentenyl)-adenosine (i^6Ado) and N^6-(Δ^2-isopentenyl)adenine (i^6A base) from a Sephadex LH-20 column (50 g) eluted with 35% aqueous ethanol.

b. Fractionation by Sephadex LH-20 column chromatography of Celite I fraction (fig. 13). Biological activity (soybean assay) was tested on a 50 µl aliquot of each fraction.


Absorbancy at 280nm
0
0·2
0·4
0·6
0·8
1·0
Fraction number (10ml each)
10
20
30
40
50
60
70
i⁶A
i⁶A base
X1
X2
20
40
60
80
Fresh weight mg/callus

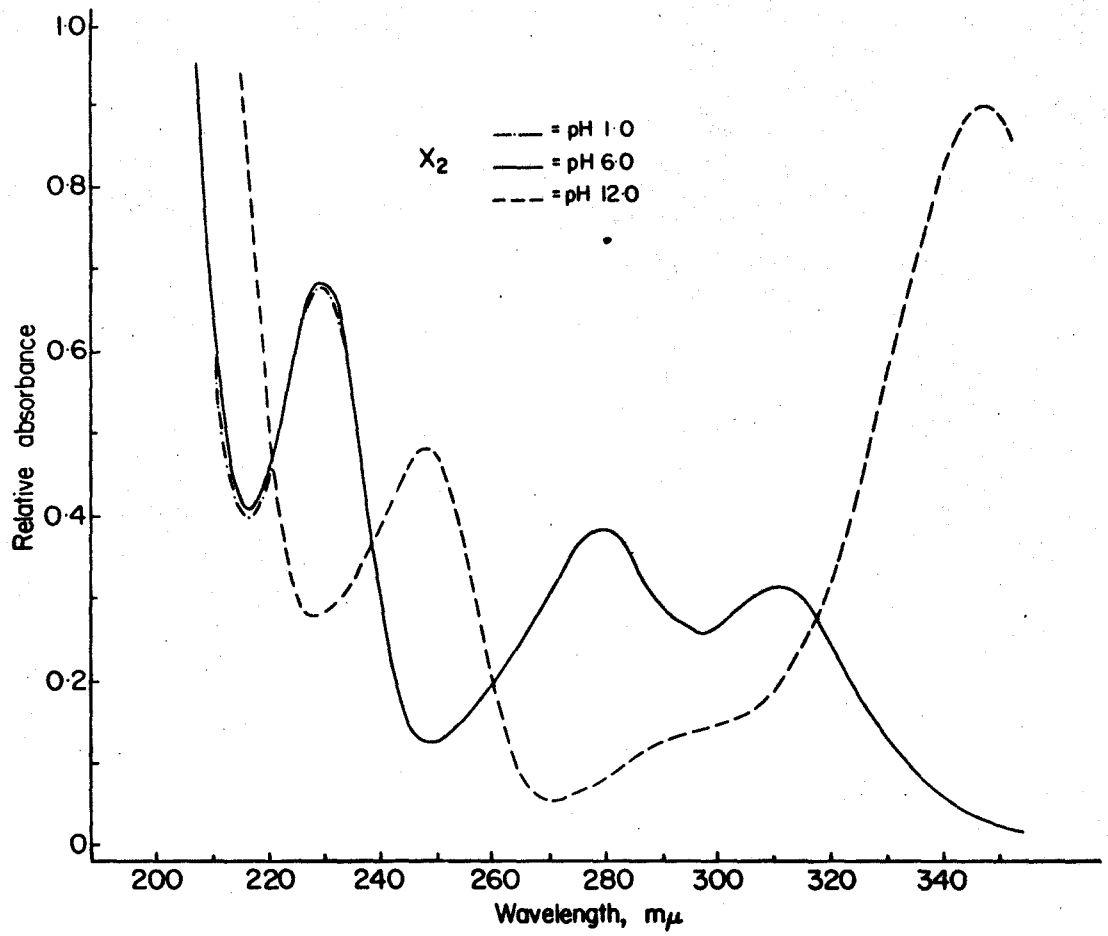


Figure 15. Ultraviolet absorption spectra of compound X_2 (fig. 14b), a contaminant extracted from Celite-545.

tested. The weak activity present in tubes 48 to 50 was probably due to a small amount of N^6-(Δ^2-isopentenyl)adenine (fig. 14).

2. Fraction II

Fraction II was evaporated in vacuo and the residue was applied in 1.5 ml of 35% ethanol to the same Sephadex LH-20 column (fig. 16a). This step did not prove very useful, except for the separation of an early sub fraction (IIa) (tubes 18 to 20) which gave a very bright fluorescent spot under UV light at Rf 0.50 after paper chromatography with solvent E. As some UV quenching material was present at Rf 0.45-0.50, the total fraction dissolved in 5 ml of distilled water was extracted twice with the same volume of chloroform. This extract was further purified by silica gel TLC (MN-silica gel N-HR) with solvent E. Elution of a fluorescent band of Rf 0.50-0.55 yielded 3 A_{235} units of a material presenting the same UV spectra as the hypermodified nucleoside Y (Thiebe et al., 1968). On the basis of this evidence and on the basis of its paper chromatography properties, the isolated fluorescent material was identified as Y riboside. Its biological activity was tested with the soybean tissue assay. Y riboside was either added to the cooling medium by millipore filtration or added prior autoclaving for 7 min at 121°C. No biological activity could be detected in the range of

concentration tested (10^{-6} M to 10^{-9} M, final concentration in the medium, based on E_{239} = 30 000 (Thiebe et al., 1968).

The sub-fraction IIb (tubes 21 to 29) was analysed by paper chromatography (Whatman 3MM) with solvent E. Three bands of UV quenching material were obtained with Rf of 0.22, 0.45-0.50, and 0.66 respectively. After elution with distilled water from the chromatogram, the materials obtained were chromatographed again on Whatman No. 1 and silica gel sheets (Eastman 6060).

Band Rf 0.22 contained only one product identified as adenosine by its UV absorption spectra and its Rf values.

Material of band Rf 0.45-0.50 rechromatographed on paper in solvent system B gave a heavy band at Rf 0.90 identified as N^6-methyladenosine by means of its UV absorption spectra and its Rf values on silica gel sheets TLC, a light band at Rf 0.75 which chromatographed like 2'-O methyladenosine on silica gel sheets TLC in solvent systems A and D, and another light band at Rf 0.60 which chromatographed as 2-methyladenosine on TLC in the same solvents A and D.

Material from band Rf 0.66 chromatographed on paper in system solvent B yielded a broad band of UV quenching material between Rf 0.65 and Rf 0.75, but in solvent system A good separation was obtained with a light band at Rf 0.65 and a band at Rf 0.70. Material of Rf 0.65 is very likely N^6,N^6-dimethyladenosine on the basis

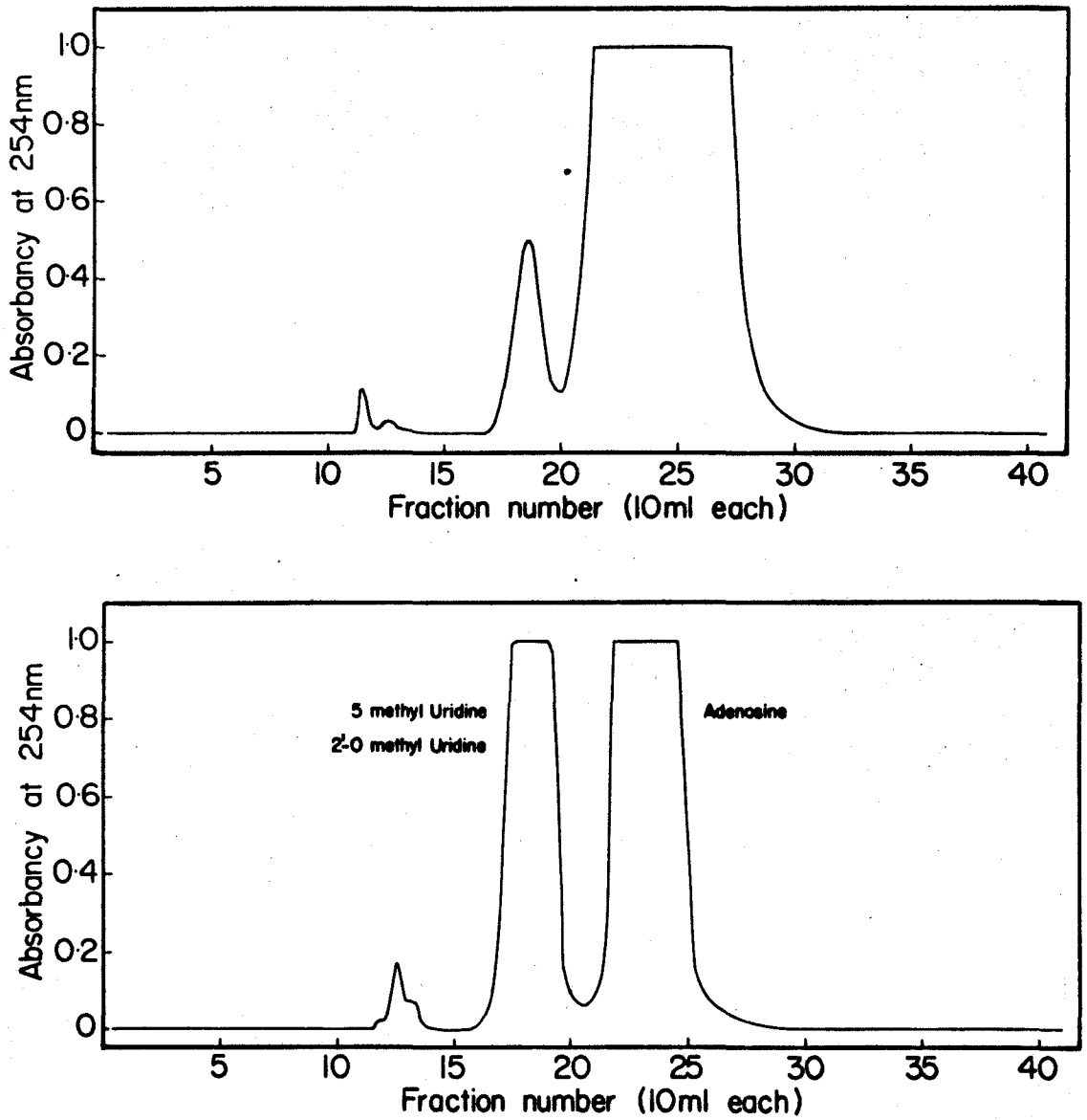


Figure 16. Fractionation by Sephadex LH-20 column chromatography of Celite II (top) and Celite III (bottom) fractions (fig. 13).

of its first Rf in solvent system E and its modified adenosine like UV absorption spectra. Material at Rf 0.10 was eluted from the paper chromatogram and purified on silica gel (MF-254) TLC with solvent E. Material at Rf 0.70-0.80 was eluted from the gel with 50% aqueous ethanol. Its UV absorption spectra were identical to those of 2-thio-5-carboxymethyl uridine methyl ester (Baczynskyj et al., 1968). The yield was 4.5 A_{242} units at pH 7 or 0.2 µmoles.

3. Fraction III

A one-tenth aliquot of combined fraction III was fractionated first by Sephadex LH-20 chromatography (fig. 16b). Material of the first peak (tubes 17 to 20) had UV absorption spectra similar to those of 5 methyluridine. Paper chromatography in solvent system E confirmed its presence at Rf 0.25 and indicated the presence of another nucleoside at Rf 0.43. After elution from the chromatogram, this material chromatographed as 2'-O-methyluridine on silica gel sheets TLC and had its UV absorption spectra.

The next peak (tubes 22 to 27) contained only one material as indicated by silica gel sheets TLC with Rf values of adenosine.

4. Fraction IV

This fraction was analysed by ion-exchange chromatography in order to recover PCT riboside from the hydrolysate. After evaporation of the solvent the residue was dissolved in 75 ml of water and the pH was adjusted to

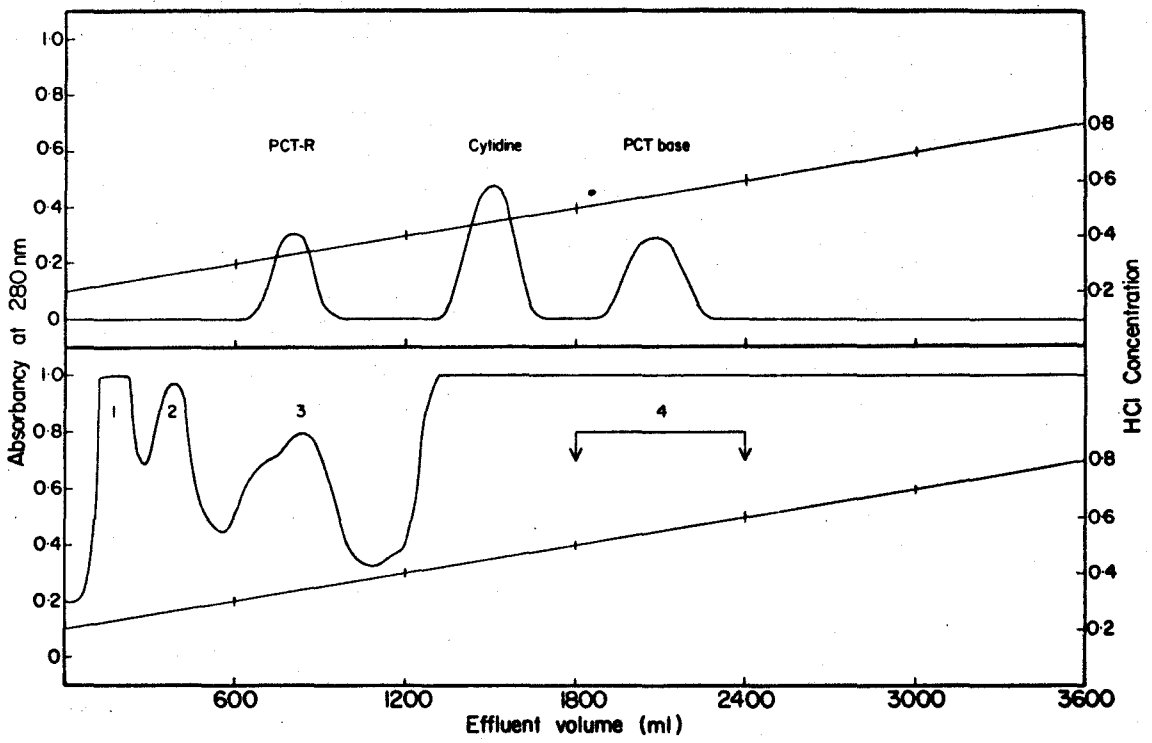


Figure 17 a. Elution pattern of PCT.R, cytidine and PCT from a 2 cm x 30 cm column of Dowex-50W-X8 (H^+, 200-400 mesh) eluted with a linear gradient of hydrochloric acid (0.2 N - 0.8 N, total volume 3.6 l).

b. Elution of the material in fraction IV (fig. 13) from an identical Dowex-50 column.

4.5 with 1 N HCl. This solution was absorbed on a previously prepared and standardized Dowex 50W-X8 (H^+, 200-400 mesh) column (2 cm x 30 cm). The column was washed with 500 ml of 0.001 N HCl and then eluted with a linear gradient of hydrochloric acid (0.3 N - 0.8 N, total volume 3.6 l) (fig. 17).

The products in fractions 1 and 2 were not positively identified; the product in 1 had UV absorption spectra, characteristic of methylated cytidines (it is probably 2'-O-methyl cytidine).

Half of fraction 3 was evaporated to dryness in vacuo repeatedly to free it of hydrogen chloride by adding small amounts of water. This converts PCT-riboside to PCT base. The residue was dissolved in 4 ml of solvent F (lower phase) and fractionated on a partition column of Celite-545 (50 g, 2 cm x 40 cm) according to Hall's procedure (1971, p. 210) and Chheda et al. (1969). The column was eluted with one l of solvent F (upper phase) (fig. 18). PCT was separated from hypoxanthine but was still contaminated with another nucleoside. It was further purified by Sephadex LH-20 chromatography (ethanol 35%, pH 2.5). As shown on the elution profile (fig. 19) PCT was well separated from 1-methylhypoxanthine (15 A_{250} units, pH 7). PCT appeared free of ultraviolet absorbing contaminants by silica gel sheets TLC in solvents systems A,B. and C. The yield was

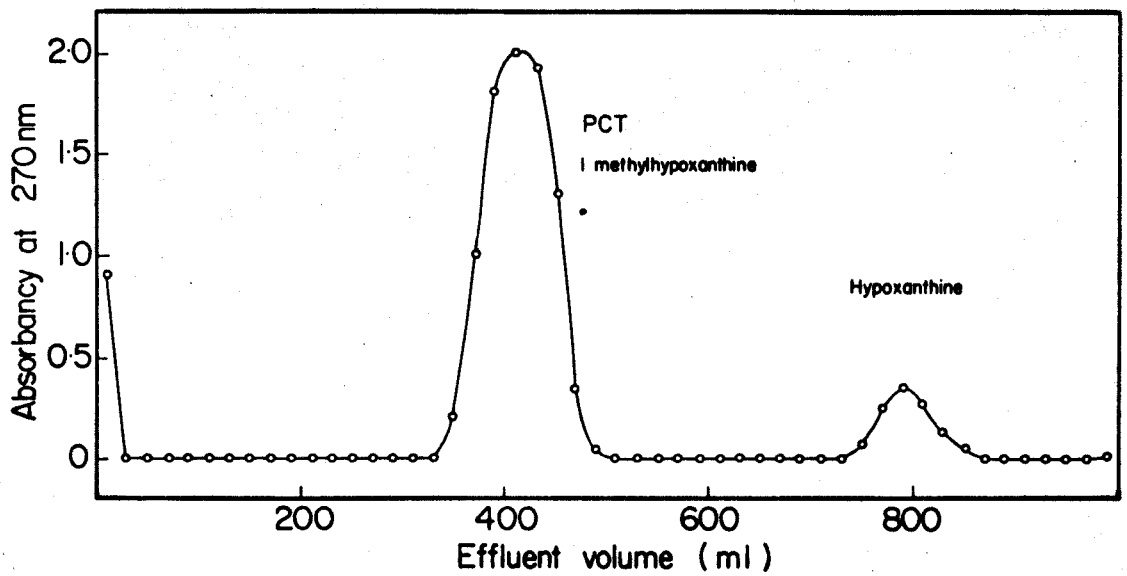


Figure 18. Fractionation of the material of fraction 3 (fig. 17) on a Celite-545 column (2 cm x 40 cm) with solvent F.

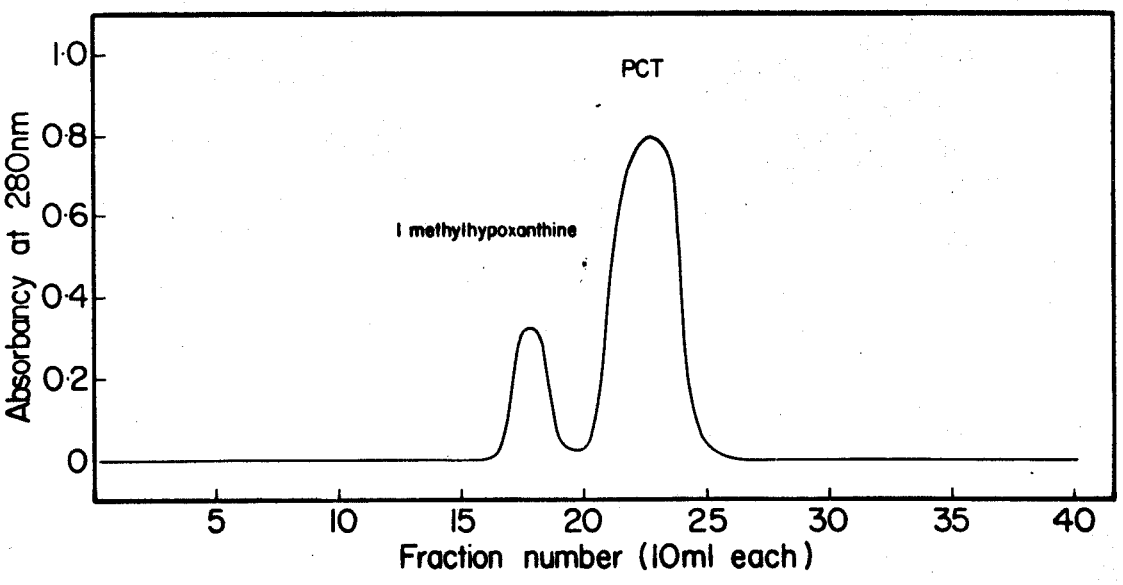


Figure 19. Separation of PCT from 1-methylhypoxanthine on a standard Sephadex LH-20 column, eluted with 35% aqueous ethanol, pH 2.5.

130 A_{270} units, pH 7, which represents 10.4 µmoles or 4.3 mg of the riboside for the total fraction.

In order to clearly demonstrate that PCT-riboside was obtained, the remaining half of fraction 3 from the Dowex column was slowly neutralized with 5 N NaOH and 1 N NaOH to pH 6 in an ice-water bath. The saline solution was evaporated to dryness in vacuo and the salt obtained was washed twice with 50 ml of 95% ethanol. Very little sodium chloride dissolved in the ethanol but the efficiency of the extraction was greater than 95% for the UV absorbing material. This procedure was repeated twice with 5 ml portions of 95% ethanol. An aliquot of this ethanolic solution was submitted to paper electrophoresis in 0.05 M citrate, pH 4, (1 hr, in a Savant flat-bed apparatus at 35 v/cm) as described by Chheda et al. (1969). A single band of UV absorbing material moved towards the anode (+ 7 cm, like PCT-R, compared to + 10 cm for PCT base). The material in this band was eluted from the electrophoregram with distilled water and the UV absorption spectra were taken. They were identical to those of PCT-R (fig. 1).

The fraction eluting from 1800 ml to 2400 ml of the gradient (fraction 4) was also investigated for the possible presence of PCT base. On a column of Dowex-50 of this size PCT-riboside is converted to some extent to PCT base. After evaporation of the hydrochloric acid, an aliquot was

submitted to paper electrophoresis as above. A single UV absorbing band moved towards the anode with the same mobility as PCT base. After recovery from the electrophoregram, this material had the same UV absorption spectra and paper chromatographic properties as PCT base. The total amount of PCT base in fraction 4 was estimated to be 44 A_{270} units at pH 7, or 2.2 µmoles.

A 0.2 µmole aliquot of the materials obtained from fractions 3 and 4 (fig. 17b) was hydrolysed in 1 ml of 0.1 N sodium hydroxide in a sealed tube for 3 hrs at 100°C. The amounts of adenine determined by its UV absorption spectra, and of threonine measured by quantitative ion-exchange chromatography (Spackman et al., 1958) in the hydrolysates were stochiometric and close to 0.2 µmoles in both cases. Thus the total amount of PCT recovered was 12.6 µmoles. Based on the reported frequency (Chheda et al., 1969) of 0.28 moles % moles nucleosides for PCT in yeast tRNA, 3 g of yeast tRNA contain 28 µmoles of PCT. Therefore using this isolation procedure, the yield is about 45%.

But this does not answer the question of PCT without a free carboxyl group. The compound reported by Chheda et al. (1969) should have been detected if it were present in the hydrolysate. The analysis procedure was sensitive enough to allow the detection of a very rare modified nucleoside such as 2-thio-5-carboxymethyluridine

methyl ester (4.5 A_{242} units at pH 7), and furthermore all the relatively "lipophilic" nucleosides could be identified.

C. ACID-CATALYZED ESTERIFICATION OF PCT WITH METHANOL

1. Introduction

At this point, I investigated the possibility that these derivatives might have formed during the extraction procedure described by Chheda et al. (1969). These authors repeatedly dissolved an acidic residue (containing PCT) in methanol and concentrated it to dryness to free it of hydrogen chloride. Under these conditions, an acid-catalyzed esterification of the free carboxyl group of PCT could occur, on the same basis as the acid-catalyzed reaction of PCT with ethanol (Schweizer et al., 1969) which yielded a product having similar Rf values to those of the PCT derivative isolated.

2. Reaction

10 mg of PCT base were dissolved in 2 ml of absolute methanol and 0.3 ml of 2 N hydrochloric acid were added. The solution was evaporated to dryness in vacuo in order to obtain an acidic residue. The residue was redissolved in 2 ml of absolute methanol, the flask was stoppered and left at room temperature (24°C). The reaction was followed by silica gel TLC (Eastman 6060) in solvent system E.

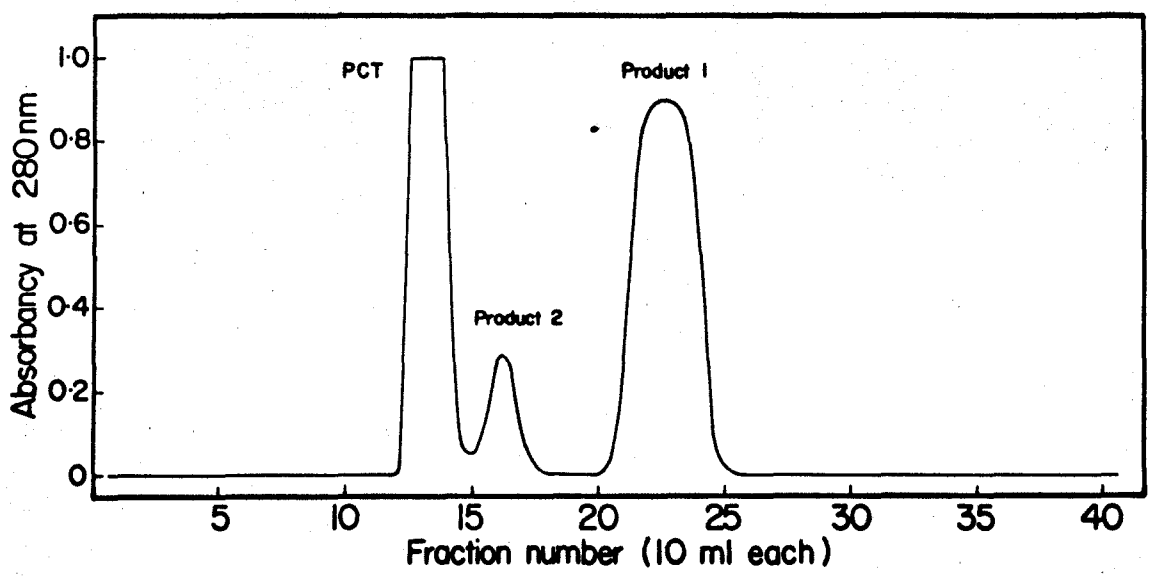


Figure 20. Separation of the 2 products of an acid-catalyzed reaction of PCT with methanol by Sephadex LH-20 chromatography. The standard column is eluted with 35% aqueous ethanol, normal pH 5.8.

PCT remained at the origin while two products moved at Rf 0.31 and 0.60 respectively. After 36 hours, the methanol was evaporated under vacuo and the residue dissolved in 5 ml of water. This solution was neutralized with 0.5 N sodium hydroxide until pH 6. After evaporation in vacuo, the residue was dissolved in 1.5 ml of 35% ethanol and applied on top of a standard Sephadex LH-20 column. The column was eluted with 35% ethanol (fig. 20). PCT eluted first, followed by two products designated for convenience as product 2 and product 1 (yields 5% and 55% respectively). It is interesting to note that product 1 has the same elution volume on Sephadex LH-20 - ethanol 35% - as PCT on the same column at pH 2.5. This already indicates a modification of the carboxyl group.

3. Product 1

Its ultraviolet abosrption spectra are identical to those of PCT and its chromatographic properties on paper are similar to those of the modified PCT reported by Chheda et al. (1969) (table 2). On paper electrophoresis, performed as above, this product moved toward the cathode (-3 cm) while PCT moved in the opposite direction (+10 cm) at pH 4.

High-resolution mass spectrum of 1: The fragmentation pattern is identical to that of PCT (Schweizer et al. 1969) except that the greatest m/e value observed is 258, instead of 244 for PCT (fig. 21). This value of 244

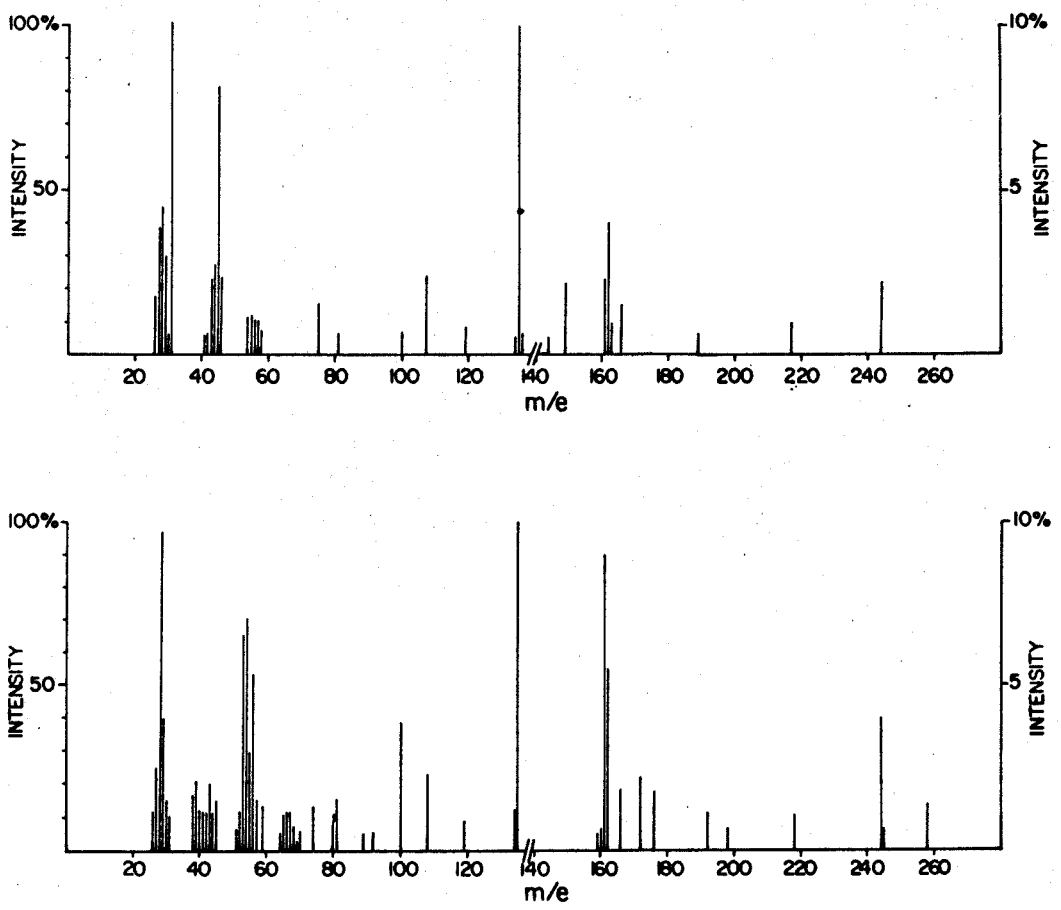


Figure 21. High resolution mass spectra of N-(purin-6-ylcarbamoyl)threonine (top) and product 1 (bottom).

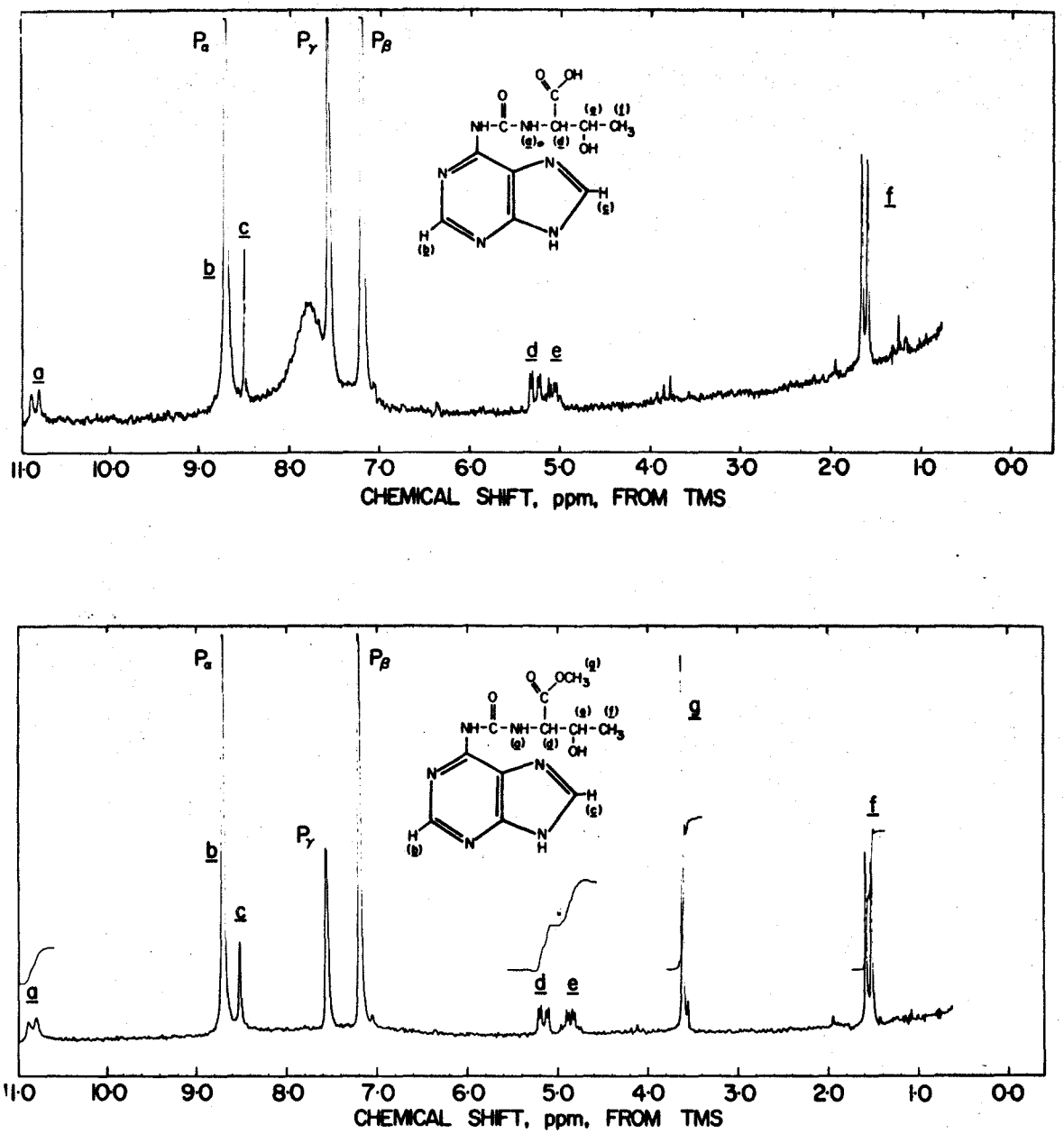


Figure 22. 100 MH_z nuclear magnetic resonance spectra of N-(purin-6-ylcarbamoyl)threonine (top) (structure after Schweizer et al., 1969) and product 1 (bottom) in pyridine-d_5.

indicates an ion with composition C_{10} H_8 N_6 O_2 corresponding to PCT after loss of two molecules of water, the value of 258 corresponds to an ion of composition C_{11} H_{10} N_6 O_2, indicating the substitution of an hydrogen by a methyl group.

Nuclear magnetic resonance spectra of PCT and product 1: Proton magnetic resonance measurements at 100 MH_Z were made on PCT and product 1 in pyridine-d_5 in order to compare their structures. The spectra obtained were qualitatively identical (fig. 22) except for a 3 protons singlet at δ 3.64 readily assignable to a methyl ester on the carboxyl group for product 1.

4. Product 2

The ultraviolet absorption spectrum at pH 7 does not present the double peak, characteristic of N^6-ureidopurines (fig. 1). Its chromatographic properties (table 2), especially in solvent E (Rf 0.30, compared to 0.20 for adenine) indicate that the carboxyl group is no longer free. This is also supported by its electrophoretic mobility (-3 cm at pH 4).

A high resolution mass spectrum of product 2 did not yield the parent ion. The strongest line in the spectrum corresponded to adenine (m/e 135) and significant but weak ions were found at m/e values of 81, 108 and 162, 172, 186, 192 and 244. Lines 162 and 244 were present

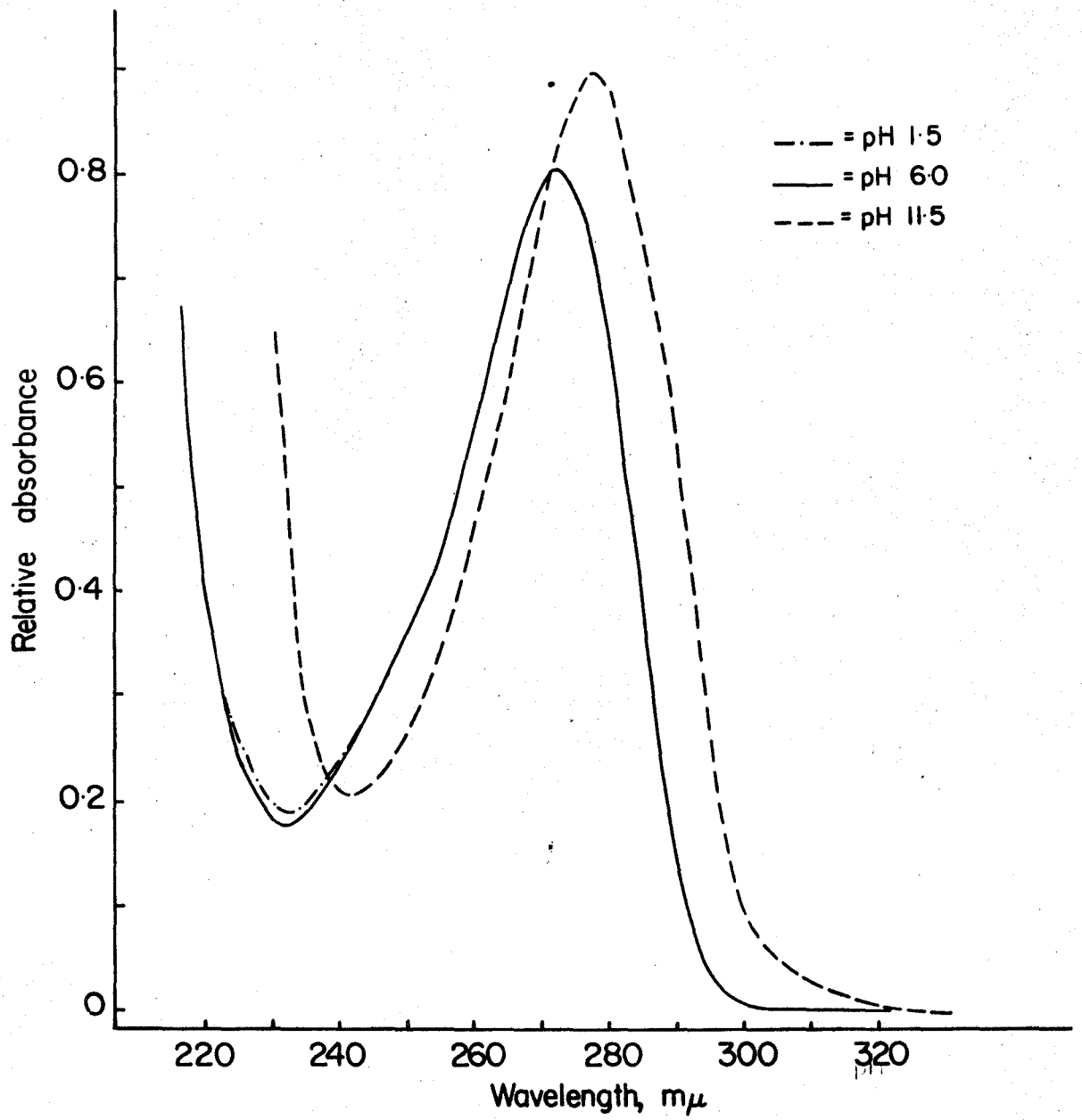


Figure 23. Ultraviolet absorption spectra of product 2 (fig. 20).

in the spectra of PCT and PCT-CH_3, lines 172 and 192 in the spectrum of PCT-CH_3 only, and line 186 was present in the spectrum of product 2 only. On base hydrolysis (0.2 N NaOH for 2 hrs) and on acid hydrolysis (0.2 N for 15 min) at 100°C the product yielded adenine, identified as such by its UV absorption spectra and its chromatographic properties on TLC sheets in several solvent systems.

All these data, however, are not sufficient to assign a structure to product 2.

DISCUSSION

The data presented here shows that L-threonine is a precursor of N-(purin-6-ylcarbamoyl)threonine of Tobacco tissue and Rhizopogon roseolus tRNA. This confirms the results of Powers et al. (1972) and Chheda et al. (1972) who established that L-threonine is a precursor of the side chain of PCT in the tRNA of Escherichia coli and rat liver respectively. [^{14}C]-L-Threonine also serves as a precursor for N-[9-β-D-ribofuranosyl-9H-purin-6-yl)carbamoyl]-threonine which is isolated from the growth medium of Rhizopogon roseolus. This finding is of special interest since Rhizopogon roseolus also excretes ribosyl zeatin, a very potent and naturally occuring cytokinin in plants.

PCT riboside was proposed by Dyson et al. (1972) as the archetype molecule for the series of biologically active synthetic N^6-alkylureidopurines and N^6-phenyl-ureidopurines. Therefore, PCT-riboside could play a role in the symbiotic relationship between Rhizopogon roseolus and its hosts.

After four days of incubation, there is 14 times more [^{14}C]-L-threonine-labeled PCT-riboside in the medium than in the tRNA molecules (33 times more on the basis

of [^{3}H]-adenine incorporation). Thus, unless there is unusually rapid turn-over of the tRNA, this result suggests that Rhizopogon roseolus is capable of synthesizing PCT-riboside independently of the tRNA. This is also supported by the different [^{3}H]-adenine/[^{14}C]-L-threonine ratios found in PCT in tRNA (2,6) and in the medium (6).

Precedence exists for the esterification of modified nucleosides which contain carboxyl groups, such as 2-thio-5-carboxymethyluridine methyl ester (Baczynskyj et al., 1968) and compound Y (fig. 4) (Thiebe et al., 1971). Concerning the possibility that PCT may also exist in tRNA as a methyl ester, the analysis of an enzymic hydrolysate of 3 g of yeast tRNA did not show any indication of the presence of such a derivative. Furthermore, facile esterification of PCT with methanol, under acidic conditions, indicates that the derivative observed originally by Chheda et al. (1969) was an artefact of isolation. This finding does not exclude that the tRNA component could be a non-lipophilic derivative that could be converted to PCT-riboside under acidic conditions. However, Chheda et al. (1972) isolated [^{14}C]-L-threonine labeled PCT riboside from an enzymic hydrolysate of rat liver tRNA by means of paper chromatography only. Therefore, all these data support the hypothesis that PCT occurs in tRNA with a free carboxyl group.

The growth-promoting activity of the methyl ester of PCT obtained was tested (soybean assay) and no activity could be detected. Also, the growth-promoting activity of kinetin (10^{-6} M) or zeatin-riboside (10^{-6} M) was not affected by PCT-CH_3 or PCT-riboside in a range of concentration of 5×10^{-5} M to 10^{-8} M. Therefore, the question that N^6-ureidopurines represent a separate class of growth promoting agents in plants remains open. It is still possible that the alkyl-type N^6-ureidopurines exert their activity through a similarity in structure to the cytokinin, N^6-(Δ^2-isopentenyl)adenine. As this cannot be tested in vivo, the study of the binding properties of the alkyl-type N^6-ureidopurines with a hypothetical receptor for N^6-(Δ^2-isopentenyl)adenine and/or the isolation of a specific receptor for N^6-ureidopurines should bring an answer to this question.

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