ISOLATION OF CYTOKININS FROM tRNA.

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SUMMARY. A method is described for the isolation of cytokinins from tRNA. The cytokinin active ribosides are recovered from enzymatic hydrolysates of tRNA by extraction with ethyl acetate. The extracts are fractionated by chromatography on Sephadex LH-20 columns in 35% ethanol. The method appears to be quantitative and resolves individual cytokinins such as the ribosides 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (2iPA) and 6-(3-methyl--2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (ms2iPA).

Partition chromatography on Celite columns has been used for the isolation of cytokinin ribosides from tRNA hydrolysates (1,2,3). However, we found (4,5) that the Celite columns failed to separate individual cytokinins, and the cytokinin active fraction contained impurities from the Celite. The high mobility of cytokinin ribosides on these columns suggested that solvent extraction of tRNA hydrolysates would be effective in their purification. The procedure combined with adsorption chromatography on Sephadex columns gives a simple, rapid method for isolating cytokinin ribosides.

MATERIALS AND METHODS

Hydrolysis and fractionation of tRNA. E. coli B tRNA, partially degraded (Schwarz Biochemicals) and Saccharomyces lactis tRNA (obtained from R.M. Bock, U. of Wisconsin) were used. The tRNA was hydrolyzed with enzymes to ribosides (4,5,6). The dried tRNA hydrolysates were extracted with 1 ml of the upper phase of ethyl acetate-water (5:1, v/v) per ca. 220 A_{260} O.D. units of tRNA. Usually the solvent was decanted after each extraction, but if suspended material was present the extracts were centrifuged and then decanted. The extracts were dried at room temperature and redissolved in

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the appropriate solvent for chromatography or for bioassay. Sephadex LH-20 and G-10 (Pharmacia), swollen and washed in the appropriate solvents, were used for column fractionations. Details are given in the legends to the figures. The tobacco bioassay (4,7) was used to determine cytokinin activity.

Table 1

Cytokinin activity from Saccharomyces lactis tRNA.

Extract number	tRNA concentration A_{260} units/100 ml medium 1.76	8.8	44.0	220.0	1100.0
	Avg. fresh weight (g/flask)				
1,2	3.5	6.0	6.6	6.0	4.4
3,4	0.7	0.8	1.4	3.5	5.9
5,6	0.6	0.6	1.2	1.0	1.0
7,8	0.8	0.5	0.9	0.7	0.7
9,10	1.0	0.6	0.6	0.6	0.9
Residue	0.8	1.0	0.4	0.2	0.1
Kinetin control (µg/l)	0	1	3	5	10
Avg. fresh wt. (g/flask)	0.8	1.8	3.3	4.9	6.8

The lyophilized enzymic hydrolysate (2,200 A_{260} units) was extracted with water-saturated ethyl acetate. The ten extracts were pooled as indicated in column 1 and divided into two equal parts. One part was fractionated on Sephadex LH-20 columns (see Fig. 1), and the other part was bioassayed. The concentrations refer to the A_{260} units of yeast tRNA extracted and not absorption of the extracts.

Table 2

Cytokinin activity from E. coli tRNA.

Extract number	tRNA concentration A_{260} units/100 ml medium 1.76	8.8	44.0	220.0	1100.0
	Avg. fresh weight (g/flask)				
1	0.4	1.5	3.8	5.4	7.0
2	0.4	0.4	0.4	2.6	6.4
3	0.3	0.5	0.3	0.6	3.6
4,5,6	0.5	0.6	0.5	0.6	0.7
7,8,9	0.6	0.6	0.5	0.5	0.5
Residue	0.6	0.3	0.4	0.2	0.1
Kinetin control (µg/l)	0	1	3	5	10
Avg. fresh wt. (g/flask)	0.5	1.8	3.3	3.8	4.9

The lyophilized enzymic hydrolysate (2,200 A_{260} units) was extracted with water-saturated ethyl acetate. The ten extracts were pooled as indicated in column 1 and divided into two equal parts. One part was fractionated on Sephadex LH-20 columns (see Fig. 2), and the other part was bioassayed. The concentrations refer to the A_{260} units of E. coli tRNA extracted and not the absorption of the extracts.

RESULTS AND DISCUSSION

Ethyl acetate extraction of tRNA hydrolysates. Five to six extractions with water-saturated ethyl acetate removed all cytokinin activity from hydrolyzed yeast tRNA (Table 1) and E. coli (Table 2), and three extractions removed all but traces of the activity. The tRNA residues (Table 1) had no cytokinin activity, but at the highest tested concentrations they inhibited growth. The results gave no indication of inhibitors in the ethyl acetate extracts.

Adsorption chromatography on Sephadex columns. Separation of cytokinin containing ribosides from other ribosides present in ethyl acetate extracts was achieved with Sephadex LH-20 columns in 35% ethanol (Figs. 1 and 2). The cytokinins 2iPA and ms2iPA were separated from the early, large peak of ultraviolet absorption and from each other. The nature of the early peak, which had no cytokinin activity, has not been investigated.

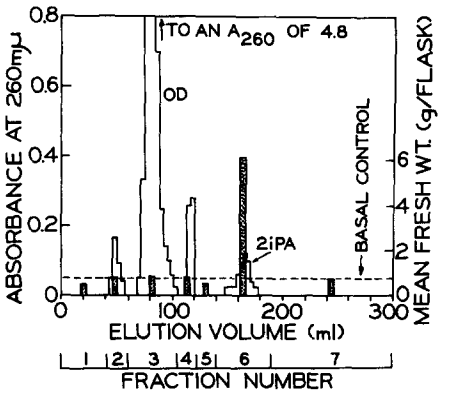

Figure 1. Distribution of cytokinin activity in the Sephadex LH-20 fractionation of an ethyl acetate extract of S. lactis tRNA hydrolysate. A sample equivalent to 1100 A_{260} units of tRNA was taken from the combined first and second ethyl acetate extracts in Table 1, redissolved in 1 ml of 35% ethanol and chromatographed in 35% ethanol on a Sephadex LH-20 column (2.4 x 20 cm, 25 g). Fractions of 3.5 ml were collected. The fractions were pooled as indicated, reduced to dryness, dissolved in 5.0 ml distilled water, and bioassayed.

The mobility of cytokinin ribosides on LH-20 columns was altered by adjusting the ethanol concentration. The order of their elution remained the

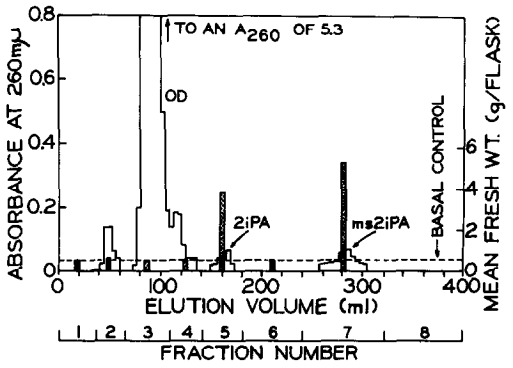

Figure 2. Distribution of cytokinin activity in the Sephadex LH-20 fractionation of an ethyl acetate extract of E. coli tRNA hydrolysate. A sample (equivalent to 1100 A_{260} units of tRNA) was taken from the first ethyl acetate extract in Table 2. Procedure same as in Fig. 1. Fractions of 4 ml were collected.

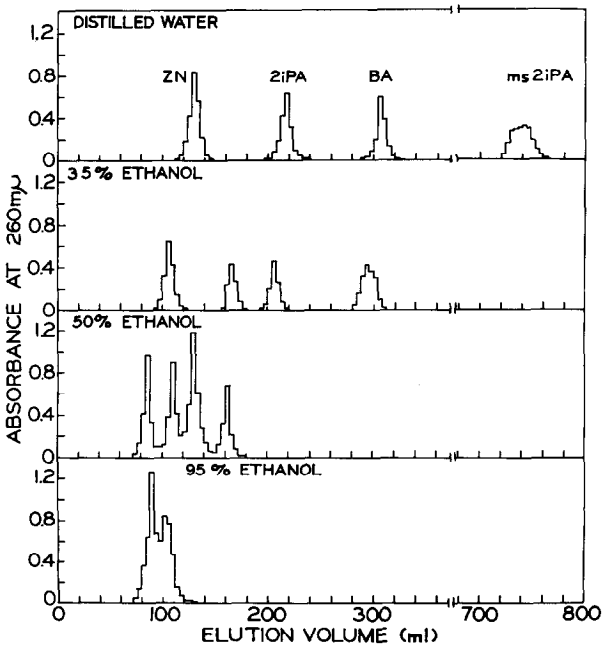

Figure 3. Effect of ethanol concentration on the elution of cytokinin ribosides from Sephadex LH-20 columns. One ml samples containing a mixture of the four cytokinin ribosides 2iPA, ms2iPA, N^6-benzyladenosine (BA), and trans-zeatin riboside (ZN) were fractionated on Sephadex LH-20 columns (upper row 2.4 x 15 cm., 20 g; others 2.4 x 20 cm., 25 g). Fractions of 4 ml were collected.

same (Fig. 3). With 50% or higher ethanol concentrations resolution of the ribosides was poor; with 95% ethanol all were eluted near the front. A final wash with 95% ethanol, therefore, assurred that all ribosides were eluted from the column. In fractionations of the ethyl acetate extracts on Sephadex LH-20 columns the polar zeatin riboside would not be separated from the early large absorption peak. Further purification of the zeatin riboside was achieved by rechromatography on Sephadex G-10 in distilled water followed by paper chromatography.

The relative mobilities of cytokinins chromatographed on Sephadex LH-20 in 35% ethanol are compared in Table 3. The free bases eluted later than the corresponding ribosides and in the same order.

The chromatographic behavior of a number of bases, ribosides, and ribotides on Sephadex G-10 columns has been described (8). Although greater resolution can be achieved on Sephadex G-10 and G-25 columns in distilled water, the solids recovered from ethyl acetate extracts are more soluble in

Table 3

Relative mobilities of cytokinins chromatographed on Sephadex LH-20 columns in 35% ethanol.

Cytokinin	Relative Mobility*
6-(trans-4-hydroxy-3-methyl-2-butenylamino)purine riboside (trans-zeatin riboside)**	1.1
6-(trans-4-hydroxy-3-methyl-2-butenylamino)purine (trans-zeatin)**	1.4
6-(3-methyl-2-butenylamino)purine riboside (2iPA)**	1.7
6-furfurylaminopurine riboside	1.8
6-benzylaminopurine riboside	2.1
6-(3-methyl-2-butenylamino)purine (2iP)**	2.1
6-furfurylaminopurine	2.2
6-benzylaminopurine	2.7
6-(3-methyl-2-butenylamino)-2-methylthiopurine riboside (ms2iPA)**	2.9
6-phenylaminopurine	3.4
6-(3-methyl-2-butenylamino)-2-methylthiopurine (ms2iP)**	5.3

*The value 1.0 represents an elution volume equivalent to one column volume. The values given are compiled from several different columns.

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ethanol solutions, and the ethanol concentration can be adjusted to shorten fractionation times on the LH-20 columns. This is convenient and important because some bases and ribosides tend to spread when large volumes of solvent are needed for their elution.

The methods described are readily scaled-up for the isolation of milligram quantities of cytokinin ribosides. The columns are simple to prepare, use of buffers and salt solutions is avoided, and all solvents are readily removed by evaporation in vacuo at low temperatures.

This procedure has been used for the isolations of 2iPA from purified yeast cysteine tRNA (9) and from crude E. coli tRNA (4).

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