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A Comparison between two Procedures for Extracting the Nucleotide Pool of *Escherichia coli*

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A comparison has been made between the nucleotide content of two extracts of *Escherichia coli* obtained by treating the cells with 0.6 N perchloric acid at 0° and ethanol-water at 100°, respectively.

It is found that the latter extract contained substantial amounts of purines, pyrimidines and nucleosides, whereas only small amounts of purines were found in the perchloric acid extract. Quantitative estimation of the nucleoside 5′-phosphates showed that the perchloric acid extract contained more of these substances. dAMP and dCMP were detected in both extracts.

Bacteria contain a complex pool of nucleotides, which participate in a number of important reactions in the cell. In the past, therefore, a number of papers on bacterial nucleotides have appeared. This work has recently been reviewed by Jonsen and Laland.[1] In extracting the nucleotide pool, 0.6 N perchloric acid at 0° has been the reagent most commonly employed.[2,3] The acidity of the extract might result in a rupture of certain acid labile linkages which occur in nucleotides. The use of this reagent, particular when a quantitative estimate of the pool is desired, is therefore open to some objection. In some cases a neutral reagent such as ethanol-water at 100° has been used. For instance Harris *et al.*[4] used this procedure in the case of yeast.

In an attempt to evaluate the two methods, *Escherichia coli* cells have been extracted with 0.6 N perchloric acid at 0° and with ethanol-water at 100°, respectively.

A comparison has been made between the qualitative composition of the nucleotides and nucleoside polyphosphates in the two extracts as well as a comparison between the amounts of the different nucleoside 5′-phosphates.

MATERIALS AND METHODS

The nucleotide reference substances were purchased from Nutritional Biochemical Corporation, Sigma Chemical Company, and Mann Research Laboratories. Ribose and deoxyribose were obtained from Sigma Chemical Company. Analytical grade Dowex 1 × 8, 200 to 400 mesh, anion exchange resin was provided by Dow Chemical Company.

Bacteria. A laboratory strain of *E. coli* originally isolated from human faeces was used.

Growth media. The organism was maintained on a solid medium prepared as follows: Difco beef extract, 6 g; NaCl, 6 g; $Na_2HPO_4.2H_2O$, 5.8 g and Difco peptone, 10 g were dissolved in 1000 ml of water and the pH adjusted to 8.8 by the addition of 10 % NaOH. Difco Agar, 25 g was added and the mixture autoclaved at 120° for 2 h.

The liquid medium [5] used was prepared as follows: NH_4Cl, 2.0 g: $Na_2HPO_4.2H_2O$, 7.5 g: KH_2PO_4, 3.0 g: NaCl, 3.0 g: $MgCl_2.6H_2O$, 85.6 mg and $Na_2SO_4.10H_2O$, 260 mg were dissolved in 980 ml of water and autoclaved at 120° for 2 h. 4 g of glucose was dissolved in 20 ml of water and sterilized separately. The two solutions were then mixed aseptically.

Growth and harvesting of bacteria. Before use, the organism was subcultured at least 10 times on 2 ml of the liquid medium in stationary cultures in test tubes (10 × 1 cm) for 16 h at 37°. Larger batches of bacteria were then grown in 2 l conicals containing 500 ml of the liquid medium. Each flask was inoculated with 2 ml of a stationary culture and shaken at 37° on a Gyrotory Incubator Shaker. When the culture had reached the desired density, the cells were harvested by centrifugation in a Sharpless centrifuge with precooled rotor (0°) or in a refrigerated centrifuge at 0°. The cells were washed with 3 volumes of icecold medium and reisolated by centrifugation at 0°.

The optical density of the culture was measured in 1 cm cells at 650 mμ in a Beckman DU spectrophotometer.

Extraction of cells. Two alternative methods have been used. In the first, wet cells were extracted with 3 volumes of 0.6 N of $HClO_4$ at 0° for 30 min. After centrifugation at 0°, the cell residue was reextracted in an identical manner with 1 volume of 0.6 N $HClO_4$. The combined extract was neutralized with 5 N KOH in an icebath, and the precipitate removed by centrifugation. The supernatant was concentrated to about 5 ml by freeze drying and additional precipitate removed by centrifugation.

The other method consisted of heating cells with 3 volumes of aqueous 50 % ethanol for 6 min at 100°. After centrifugation at room temperature, the cell residue was reextracted in an identical manner with 2 volumes of aqueous 50 % ethanol. The combined extract was freeze-dried before being submitted to fractionation.

Ion exchange chromatography. The bacterial extracts were fractionated on Dowex 1 × 8 column in the formate form by using the gradient technique of Hurlbert, Schmitz, Brumm and Potter.[6] 5 ml fractions were collected and the optical density of the eluate determined continuously at 260 mμ in a LKB Uvicord. Before fractionation, the pH of the extract was adjusted to 8.2 and the extract diluted to a final concentration of 20 O. U./ml*.

Identification of ultraviolet absorbing substances. Appropriate fractions obtained from the ion exchange column were pooled, freeze-dried and when necessary, the ammonium formate removed by sublimation in vacuum. The residues were dissolved in a small amount of water and chromatographed on Whatman No. 1 paper in some of the following solvent systems. Solvent 1, butan-1-ol-acetic acid-water (4:1:5 v/v): solvent 2, isobutyric acid- 0.1 N EDTA-conc. aqueous NH_3 (sp.g. 0.910) (50:0.8:30 v/v); solvent 3, 96 % ethanol-conc.aqueous NH_3-water (80:5:15 v/v); solvent 4, butan-1-ol-water (86:14): solvent 5, 96 % ethanol-1 M-aqueous ammonium acetate (pH 7.6) (75:30 v/v).

For the identification of purines and pyrimidines, paper chromatography in solvent 6, propan-2-ol-conc. aqueous HCl-water (68:17.6:14.4 v/v) was used.

All the spots were located by photography in the ultraviolet light. The identification of substances by their chromatographic properties was verified by measuring their ultraviolet absorption spectrum in 0.1 N HCl and 0.1 N NaOH, respectively.

The presence of 5′-substituted nucleotides was verified by a periodate spray [7] and purine linked deoxyribose detected by the diphenylamine spray.[8]

Hydrolysis of nucleotides. For the liberation of purines and pyrimidines respectively, N HCl for 1 h at 100° and conc. formic acid for 2 h at 175° in sealed tubes were used.

Quantitative estimation of nucleotides. Spots on paper chromatograms located by ultraviolet photography and appropriate blanks were cut out and eluted with 0.1 M HCl at room temperature for 2 h. The optical density at the wavelength of maximum absorption was determined. The amount of nucleotide was calculated from the optical density at the wavelength of maximum absorption using the molecular extinction coefficient

* O.U. = optical density at 260 mμ of the solution × volume (in ml).

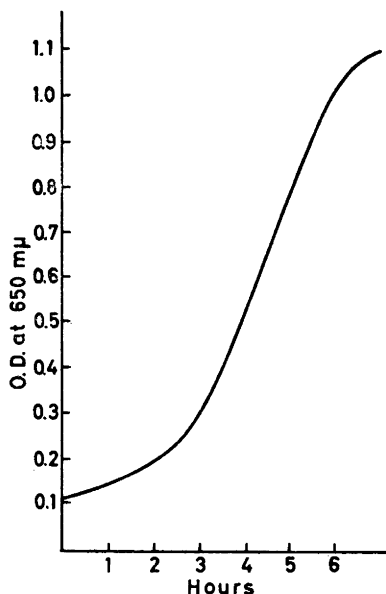

Fig. 1. Growth curve of *E. coli* cells used for preparing extracts of the nucleotide pool.

reported in the literature.[9] Identification and estimation of DPN was identified and estimated by the cyanide method of Colowick. Kaplan and Ciotti.[10]

Estimation of pentose. Deoxyribose was determined by the Burton diphenylamine test.[11] Pyrimidine linked deoxyribose was estimated after reduction with sodium amalgam.[12]

RESULTS

Cells were harvested when the culture had reached an optical density of 0.80 (growth curve see Fig. 1). One half of the cells was extracted with ethanol-water and the other half with perchloric acid (see methods). The extracted material contained 1250 and 410 O. U. respectively. It is therefore obvious that the ethanol-water procedure is far more efficient in extracting ultraviolet absorbing material than perchloric acid. This was confirmed by the following experiment:

The cell residue remaining after the acid extraction was boiled for 6 min with ethanol-water and yielded 720 O.U. The sediment remaining after the ethanol-water extraction was extracted at 0° by perchloric acid. This extract, however, contained only 8 O. U.

The two extracts (containing 1250 and 410 O. U. respectively) were fractionated on identical columns. The elution diagrams are given in Figs. 2 and 3, respectively. It is seen by comparing the figures that the amount of ultraviolet absorbing material which is eluted by water, is much greater in Fig. 2 than in Fig. 3. Thus, the amount of such material extracted by the ethanol-water procedure exceeds by far the amount of material extracted by the perchloric acid procedure. In the case of the ethanol-water extract, total optical units of the material eluted with water was 46, whereas it was only 3.6 in the case of the perchloric acid extract.

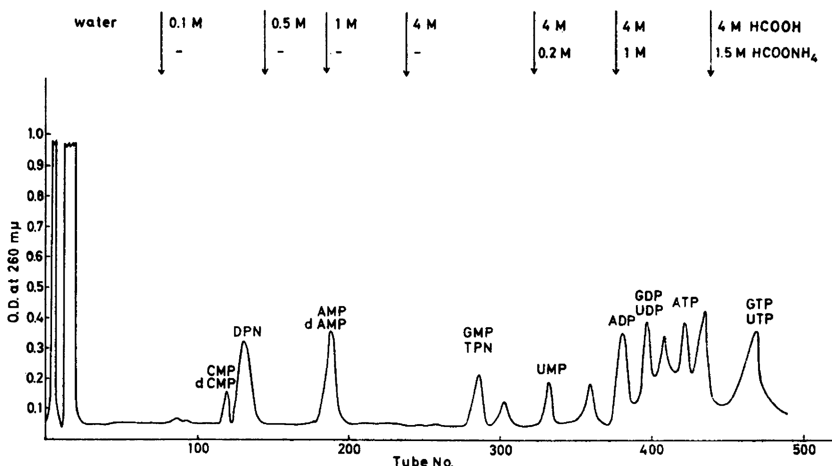

Fig. 2. The gradient elution diagram on a Dowex 1 × 8 formate column (1.2 × 18 cm) of material extracted with ethanol-water from 6 g of wet *E. coli* cells. Volume of mixing flask 1000 ml. The unmarked peaks are not identified.

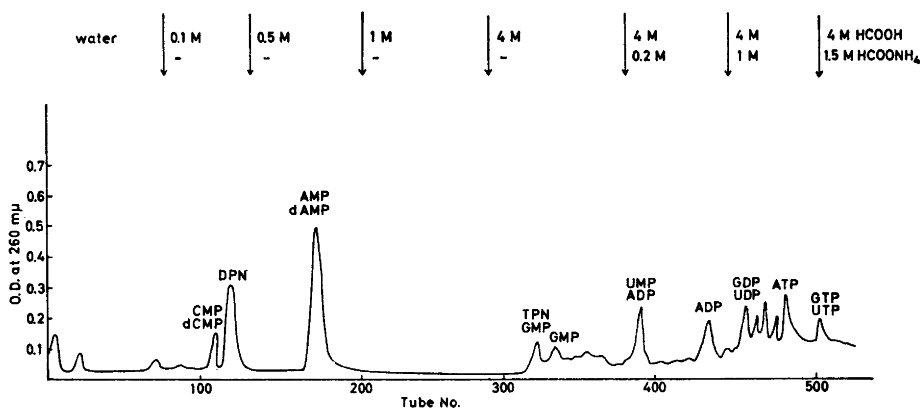

Fig. 3. The gradient elution diagram on a Dowex 1 × 8 formate column (1.2 × 18 cm) of material extracted with perchloric acid from 6 g of wet *E. coli* cells. Volume of mixing flask 1000 ml. The unmarked peaks are not identified.

As expected, the material eluted with water in the case of the ethanol-water extract contained purines, pyrimidines and nucleosides. The substances identified were adenine, guanine, hypoxanthine, uracil, adenosine, guanosine and uridine. However, in the case of the perchloric acid extract only adenine and guanine were found. This mixture of purines, pyrimidines and nucleosides was separated by chromatography in solvents 2, 4, and 6.

The following nucleotides were identified by paper chromatography and their ultraviolet absorbtion spectra in the appropriate fractions eluted from

the columns: CMP, dCMP, DPN, AMP, dAMP, TPN, GMP, UMP, ADP, UDP, GDP, ATP. The exact location of the nucleotides in each elution-diagram is indicated in Figs. 2 and 3.

The fractions were also examined for the presence of deoxyribotides. dCMP and dAMP were detected and estimated in the following way: The peak containing CMP obtained from the perchloric acid extract, was chromatographed in solvent 2. Apart from CMP, another ultraviolet absorbing substance was present which had R_F value and ultraviolet absorption spectrum identical to that of dCMP. It did not react with periodate. Upon reduction with sodiumamalgam in water, the solution gave a positive diphenylamine reaction for deoxyribose. The amount of deoxyribose corresponded to 90 % of the value which could be calculated from the ultraviolet absorption of the nucleotide.

The fraction containing AMP was chromatographed in solvent 2 and yielded beside AMP a spot with R_F value and ultraviolet absorption spectrum similar to that of dAMP, and which gave on the chromatogram a positive diphenylamine reaction for deoxyribose. The spot was not oxidized by periodate.

The amount of dCMP and dAMP in the perchloric acid and the ethanol-water extracts are seen in Table 1. In the case of dAMP only traces could be detected in the ethanol-water extract. Is is seen that DPN is present in equal quantities in the two extracts.

The amount of AMP, GMP, CMP, and UMP, respectively, was determined after purifying the appropriate fractions by paper chromatography. The CMP containing fraction was purified by chromatography in solvents 2 and 4, the three other fractions in solvent 2. The spots corresponding to the four nucleotides all reacted with the periodate spray. It is apparent from Table 1 that the content of nucleoside 5'-phosphates is higher in the acid extract than in the ethanol-water extract.

DISCUSSION

It appears from the present work that when treating *E.coli* cells with perchloric acid, small amounts of purines are extracted together with the nucleo-

Table 1. Amounts of nucleoside monophosphates in perchloric acid and ethanol-water extracts of *E. coli.*

	μmoles/6 g wet* cells in perchloric acid extract	μmoles/6 g wet* cells in ethanol-water extract
AMP	1.85	1.10
GMP	0.70	0.60
UMP	1.32	0.96
CMP	0.19	traces
dCMP	0.34	0.20
dAMP	0.06	traces
DPN	1.3	1.3

* washed cells weighed after centrifugation.

tides. However, when using hot ethanol-water, a considerable amount of purines, pyrimidines and nucleosides is extracted. The possibility that these substances originated from the pool of nucleotides does not seem very likely. However, there are several other possibilities. The relatively large amounts of bases and nucleosides could be present as such in the cells, but were for some reason, not extractable with perchloric acid.

Another possibility is this: The process of heating liberates the above mentioned substances, not from the nucleotide pool, but from other parts of the cell. In this connection it is interesting that incubation of *E. coli* ribosomes at 37° followed by ethanol-water at room temperature results in the liberation of bases and nucleosides.[13]

The extraction with perchloric acid results in the presence of greater quantities of AMP, GMP, CMP, and UMP (Table 1) than with ethanol-water. Because of the acid lability of the nucleoside polyphosphates, it seems a fair assumption that the greater amount of the nucleoside monophosphates is due to some breakdown of these substances. Thus, from the point of view of getting a more correct quantitative picture of the nucleotide pool, extraction with ethanol-water is probably preferable.

The amount of CMP found is small compared to the amount of the other nucleoside monophosphates. This is in agreement with the work of O'Donnell *et al.*[2] on *E. coli.*

dAMP and dCMP are found in both extracts, a finding which is in contrast to the work of O'Donell *et al.* who did not detect any deoxyribotides in *E. coli.*

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