Journal of Chromatography, 284 (1984) 125–142
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,313

NUCLEOTIDE COMPOSITION OF CELL EXTRACTS ANALYZED BY FULL-SPECTRUM RECORDING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ERNST FREESE*, ZOFIA OLEMPSKA-BEER and MARK EISENBERG
Laboratory of Molecular Biology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Building 36, Room 3D02, Bethesda, MD 20205 (U.S.A.)
(Received September 20th, 1983)

SUMMARY

A rapid full-spectrum recording spectrophotometer was used to analyze the nucleotide composition of a yeast cell extract by high-performance liquid chromatography. Throughout the run, which lasted 47 min, a UV spectrum with 1-nm resolution was transmitted to a minicomputer every 2.5 sec. The recorded data were later evaluated by several computer programs. One program (HPMAX) plots the position of the absorption maximum for each time point. Another program (HPSPEC) displays three spectra at any chosen time points and enables their normalization (allowing comparison with standards and analysis of the purity of peaks) and the display of the logarithm of the ratio of the values (used to determine the best wavelength for integration of overlapping peaks). A third program (HPEAK) displays the absorption at any desired wavelength against time, allows cursor positioning to mark the position of a slanting baseline as well as the beginning and end of a peak, and the integration of the peak. The components in overlapping peaks can be separately integrated. The method is generally applicable to all compounds that are detectable by spectrophotometric means.

INTRODUCTION

High-performance liquid chromatography (HPLC) has become a major technique for the separation and quantitation of cellular nucleotides. Numerous methods for separation of standard nucleotide mixtures on different types of anion-exchange column[1–7] and by reversed-phase[8–10] or ion-pair reversed-phase HPLC[5,11–14] have been published.

Cell extracts contain many UV-absorbing compounds[3,15–17]. Unknown peaks are frequently identified by comparing their retention times with those of standards and by coelution with added standards. However, if identification is limited to these two methods, some peaks could be easily misidentified and mixed peaks could be regarded as one; therefore, Brown *et al.*[18,19] stressed that these methods should be used only for tentative identification. To identify positively the compounds in com-

plex biological extracts, additional methods should be used, such as chemical or enzymatic modification of the components or spectral analysis of the HPLC peaks.

In this paper we describe how whole UV spectra can be continually accumulated and stored in a computer throughout a long HPLC run and how the data can be evaluated so as to identify peaks, to evaluate the composition of overlapping or coeluting peaks, and to quantitate the extracted compounds at optimal wavelengths. For this purpose, we used the Hewlett-Packard Model 8450 spectrophotometer, which employs a diode array to measure a whole UV–VIS spectrum from 200 to 800 nm in 1 sec. This spectral information can be transmitted to a computer every 2.5 sec (at 9600 baud). Later, the data can be evaluated in several ways described in this paper. Using this approach we have identified and quantitated most of the nucleotide components of yeast cell extracts. Although our studies concentrated on extracts of yeast cells, the same spectral approach is suitable for the HPLC analysis of mixtures of other light-absorbing compounds.

MATERIALS AND METHODS

Chromatographic equipment

The chromatographic and computer arrangement is schematized in Fig. 1. The chromatography equipment consisted of two pumps (Model 100 A), a flow-rate controller (Model 421), and a gradient mixer (Altex Scientific, Berkeley, CA, U.S.A.). The controller contained a pneumatic board enabling the control of pneumatic valves

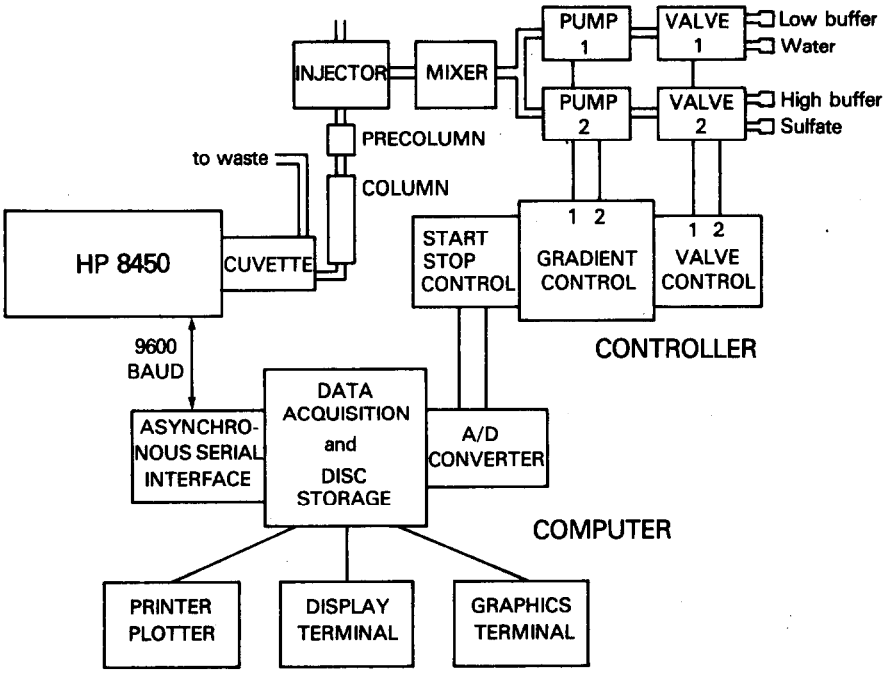

Fig. 1. Diagrammatic sketch of the equipment used for HPLC, absorption measurements and computer storage of the data.

(Model 5302, Rheodyne, Berkeley, CA, U.S.A.) for automatic washing procedures. Samples were injected with a WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.). For comparison, the UV absorption was monitored with two parallel detection systems: (1) the Spectroflow monitor SF770 of the Schoeffel Instrument Division (Kratos, Westwood, NJ, U.S.A.) connected to an SP4100 computing integrator of Spectra-Physics (San Jose, CA, U.S.A.); (2) the 8450 UV–VIS spectrophotometer of Hewlett-Packard (Palo Alto, CA, U.S.A.) was equipped with a flow cell of 10.2-mm path length, 1-mm I.D. and 8-μl volume (Hellma, Forest Hills, NY, U.S.A.) and connected via the serial interface to a PDP 11/10 (16 bit/word) digital computer from Digital Equipment (Maynard, MA, U.S.A.). The computer was equipped with a 64K byte memory, an analog-to-digital (A/D) converter to enable the Altex controller to turn data collection on and off, and a DL11W asynchronous serial interface to control the HP 8450 and to collect its spectral data at 9600 baud.

The HPLC column contained the strong anion exchanger Partisil 10-SAX (25 cm × 4.6 mm I.D.) and was preceded by a Pellionex SAX guard column (Whatman, Clifton, NJ, U.S.A.).

Reagents, strains and media

Chromatographic eluents were prepared from analytical grade potassium dihydrogen phosphate (Mallinckrodt, St. Louis, MO, U.S.A.) and certified A.C.S. sodium sulfate (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The pH was adjusted with either phosphoric acid or potassium hydroxide. To remove any particulate matter, the solutions were filtered through glass microfiber GF/F filters (Whatman, Clifton, NJ, U.S.A.). Nucleotides and their bases were purchased from Sigma (St. Louis, MO, U.S.A.). The nucleotides were stored at −70°C as 10 m*M* solutions in water and used in appropriate dilutions.

Two homothallic strains of *Saccharomyces cerevisiae* were used; both were constructed by crosses with spores of the homothallic strain Y55[20]. One strain required guanine (strain 30061) and the other required both guanine and uracil (strain 30204) for growth in a synthetic medium. This medium (MNA) contained 200 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma), adjusted to pH 5.5 by potassium hydroxide, 6.7 mg of yeast nitrogen base without amino acids (Difco Labs., Detroit, MI, U.S.A.) per ml, 150 m*M* A.C.S. potassium acetate (Fisher Scientific), adjusted to pH 5.5 with hydrochloric acid, 0.15 m*M* guanine, and for strain 30204 also 1 m*M* uracil.

Preparation of yeast cell extract

A yeast strain was grown overnight at 30°C on a plate containing 1% yeast extract, 2% peptone, 1% potassium acetate, 0.1% glucose, 0.15 m*M* guanine, and for strain 30204 0.4 m*M* uracil. The cells were inoculated at an optical density at 600 nm (OD_{600}) of 0.08 into MNA medium and grown to OD_{600} of 1. Cells of 100 ml culture were rapidly collected on a BA85 membrane filter, pore size 0.45 μm, 100-mm diameter (Schleicher & Schuell, Keene, NH, U.S.A.). The clamp of the filtration unit was left off so that the filter could be rapidly placed upside down onto a Petri plate containing 2 ml of ice-cold 1 *M* formic acid saturated with 1-butanol[21]. It took less than 8 sec to collect the cells and immerse the filter in the formic acid. After 30 min on ice, the cells were removed by centrifugation, the supernatant was freeze-

dried, and the residue was dissolved in 200 μl of water. The small amount of insoluble material was removed by centrifugation and the supernatant was stored at −70°C.

Chromatographic conditions

The chromatographic column was operated at room temperature at a flow-rate of 1 ml/min. The injected sample volume was always 60 μl. Good separation of nucleotides was obtained by initial isocratic elution with 7 m*M* potassium dihydrogen phosphate (pH 4) for 12 min, then a linear gradient in which the first buffer was mixed with a high ionic strength buffer containing 0.5 *M* potassium dihydrogen phosphate and 0.5 *M* sodium sulfate, adjusted to pH 5 by potassium hydroxide. The eluent reached 50% of the high ionic strength buffer after 33 min. The slope of the gradient was then increased so that 100% of the high ionic strength buffer was reached after 43 min and maintained for another 7 min. To quasi-equilibrate the column for the next run, the gradient was reversed so that the initial buffer strength (7 m*M* potassium dihydrogen phosphate) was reached after 5 min; the column was then eluted with the initial buffer for 20 more minutes. The column was regenerated after not more than five chromatograms by passage of 140 ml of 0.5 *M* sodium sulfate, adjusted to pH 2 with sulfuric acid, followed by 45 ml of the high ionic strength buffer. This procedure removed highly ionized, strongly bound impurities. To obtain the same column conditions as after each sample elution, the whole gradient and equilibration program was run once without any sample before the first sample was injected. This regeneration program was run overnight. After *ca.* 30 chromatograms, adsorbed organic impurities were removed by washing the column with HPLC grade methanol (Fisher Scientific).

Collection and evaluation of data using a Hewlett-Packard 8450 spectrophotometer and a PDP 11 computer

The HP 8450 controls were adjusted so that the spectrophotometer collected a spectrum every 2.5 sec and transmitted it via a DL11W asynchronous serial interface at 9600 baud to a PDP 11/10 computer. The HP 8450 needs *ca.* 1 sec to acquire a spectrum. Because it sends 1664 bytes of information for each data point, the data transmission to the computer (at the top rate of 9600 baud) requires another 1.4 sec. Thus, a spectrum could be read and transmitted every 2.4 sec. Taking into account occasional delays, the use of 2.5 sec appeared optimal and has consistently worked. The internal computer of the HP 8450, which had not been designed for HPLC, could convert the measured transmittance data into aborbance = −log (transmittance) when so requested; but it automatically stopped all data acquisition and data transfer if electronic noise caused the measured transmittance to be slightly above the originally established level for a buffer-filled cuvette because such transmittance would have resulted in a negative absorbance value. This problem was avoided by sending transmittance data to the PDP 11.

To handle HPLC data, we have written the computer programs: HPGET, HPDEC, HPMAX, HPEAK, and HPSPEC in PASCAL using the OMSI Pascal-1 compiler of Oregon Software (Portland, OR, U.S.A.) under the RT11 operating system. The programs can be obtained from us without cost by sending an 8-in. single-sided floppy disk to us. It should be easy to modify the peripheral addresses and make other minor changes to accommodate the programs to other computers using standard PASCAL.

The first program, HPGET, enables the user to start data collection automatically at the moment of injection or later (if the beginning of a chromatogram is not of interest). This can be done by programming the 421 Altex controller so that it closes a contact immediately or at a certain time after the start of the run. The contact closure is detected by an A/D converter of the PDP 11/10 which in turn initiates the transmission of spectra from the HP 8450. Because the HP 8450 produces for each wavelength point 4 bytes of data (in a special Hewlett-Packard format), a total of 128 wavelength values can be packed into one 512 byte long record without any data conversion. For the convenience of later calculations, the HPGET program initially asks the user from which lowest wavelength he wants to store data (the HP 8450 enables data collection from 200 to 800 nm with spacing of 1 nm for 200 to 400 nm and 2 nm for 400 to 800 nm). The program then displays on the terminal the highest wavelength to which data will be collected and selects the desired 128 wavelength values from the total of 1664 bytes sent for each time point by the HP 8450 (the first 60 bytes contain information other than wavelength data). Whenever it is not busy receiving data, the computer checks the A/D converter and the computer terminal for a signal indicating the termination of the HPLC run.

The HPDEC program decodes the data from the Hewlett-Packard format into the Digital Equipment format of real numbers, calculates the absorbance = $-\log$ (transmittance), and converts that number into an integer = round (1000*absorbance). Because this integer requires only one word of memory, the output of this program uses only 256 bytes for each time point —saving file space.

The HPMAX program calculates for each time point of an HPLC run the maximal absorption value of the selected wavelength range (which in our case was always from 200 to 328 nm). A certain trade-off was necessary between the rejection of noise and the detection of small peaks. The program searches, from the lowest to the highest wavelength, for points whose absorbance values increase, thereby identifying the beginning of a peak. When it has encountered a beginning peak, the search continues until it finds four points whose values decrease, *i.e.*, the end of the peak. The routine then backtracks until it again finds four decreasing values, *i.e.*, the beginning of the peak. The wavelength of the maximum absorption is taken as the midpoint of the highest values on the upslope and the downslope. The program continues this process to the highest wavelength and could in this way detect up to three peaks. The results are plotted using absorbance as one dimension and time as the other (Fig. 2). For each time point for which a peak is detected above the noise level an "*" is printed at the appropriate wavelength. Thus, the user can see the changes in maximal absorption values during an HPLC run, verify the location of known compounds in the chromatogram, and obtain the UV absorption maxima for an unknown compound.

The HPEAK program displays the absorption data *versus* time of the HPLC run at a selected wavelength on a cathode ray tube with 512 × 512 pixel resolution (using the MLSI-512 Video Graphics Controller, Matrox Intl., Mooers, NY, U.S.A.). The display can advance and backspace in time, and its horizontal and vertical dimensions can be expanded and compressed. At any stage of these manipulations the displayed image can also be transferred to a file for subsequent plotting. Examples of such plots are shown in Figs. 3 and 4 (see Results). A cursor can be used to set the beginning and end point of the baseline on the curve displaying the absorption

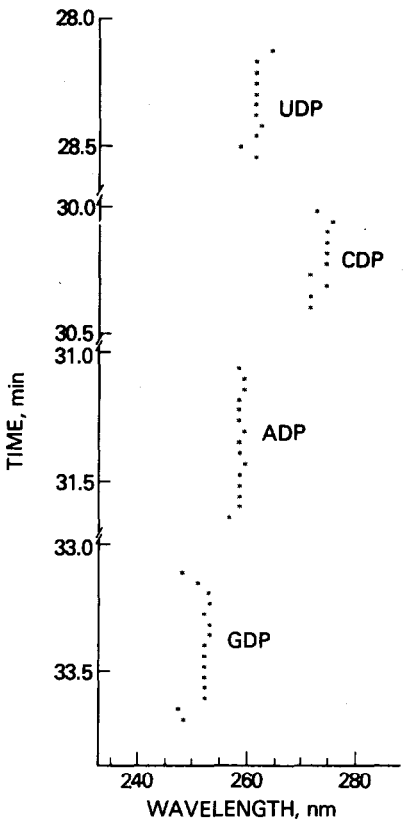

Fig. 2. Absorption maxima of standard nucleoside diphosphates. The plot was obtained with HPMAX program. It represents the fragment (28–33.7 min of elution) of a chromatogram shown on Fig. 3.

against time (this allows integration above the baseline shifts) and mark on the display curve the beginning and end of the particular peak to be integrated. The integral of the peak above the baseline is then calculated by the sums of trapezoids from time point to time point, the value is displayed on both the video terminal and on the graphics screen. Although this integration required somewhat more time than the automatic integration, *e.g.*, by the Spectra-Physics unit, we found it much more accurate.

The HPSPEC program can be used to display spectra at different time points, to check the purity of individual peaks, and to determine the optimal wavelength for integration. The program allows simultaneous display of three spectra, corresponding to three selected time points (Fig. 5). Two spectra are displayed at two vertically displaced positions on the left side of the screen and one, of reduced width, on the right side. The spectra can be entered and rotated sequentially (lower left, upper left, right). The two spectra on the left can be manipulated in various ways: their horizontal or vertical scale can be expanded and contracted (if the horizontal expansion uses the whole width of the screen, the third spectrum disappears), their vertical position can be adjusted, they can be exchanged or normalized (so that the peak maxima have the same value and are localized near the top of the display), they can

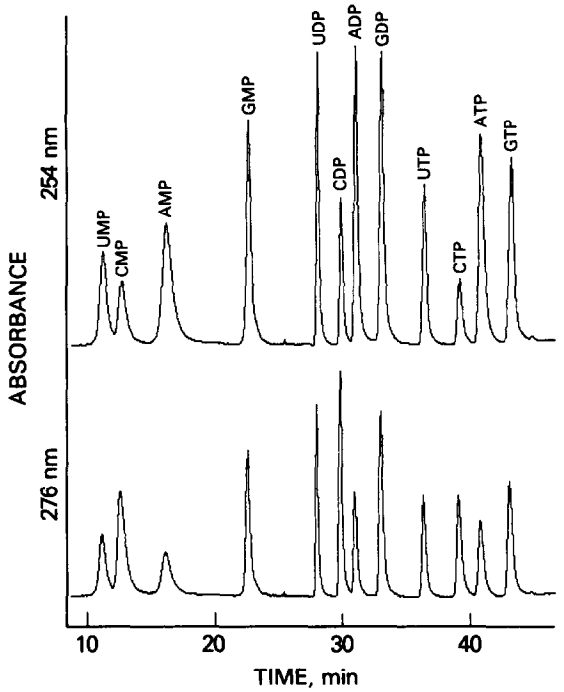

Fig. 3. Separation of a standard mixture of nucleotides at 254 and 276 nm.

be joined at any wavelength to compare how similar they are, and the logarithm of the ratio of the absorbance values (at each wavelength) can be displayed. At any stage of these manipulations the display can also be transferred to a file for subsequent plotting. To identify an unknown compound, one can display a spectrum at a selected time point of a peak, *e.g.*, at a maximum, print it out, and compare it with spectra of known compounds. To determine whether a peak is pure, one can select two time points, *e.g.*, one on the upslope and the other on the downslope of the peak and display the two spectra. If the spectra coincide, after being normalized or joined at one point, the peak represents a pure compound. Occasionally, two peaks overlap. If the spectra of the two corresponding compounds differ, one can maximize the contribution of one compound to the integral of the peak. For this purpose, one can display the log of the ratio of absorbance at each wavelength value of the two spectra. The minima and maxima of this ratio mark the two wavelength values at which the contribution of one compound to the integral is maximized. If the contribution of the minor compound to the integral is still too high, one can use a more refined method of quantitating both components, which is described in Results.

Integration using the Spectra-Physics 4100 computing integrators

The damped voltage produced by the Spectroflow monitor SF770 of Schoeffel Instruments (full scale sensitivity of 0.01–2.0 absorbance units) was entered into the SP4100 computing integrator. Automatic integration parameters, which are the defaults for peak detection and integration, were unsatisfactory for handling our chro-

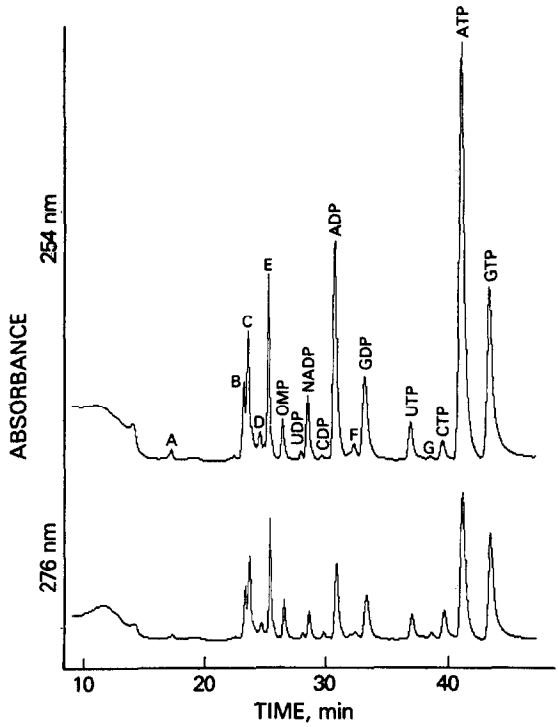

Fig. 4. Separation of nucleotides of a yeast extract at 254 and 276 nm. Strain 30204 was grown in MNA medium supplemented with guanine and uracil.

matograms. Therefore, we reduced the number of peak detectors to four by introducing the time function H31, while leaving the PW (peak width) parameter at its default level 6. The PT (peak threshold) value was set at 350, which was higher than the level obtained by automatic PT evaluation, in order to filter out small peaks. We also inhibited integration for the first 21 min of elution thus allowing the instrument

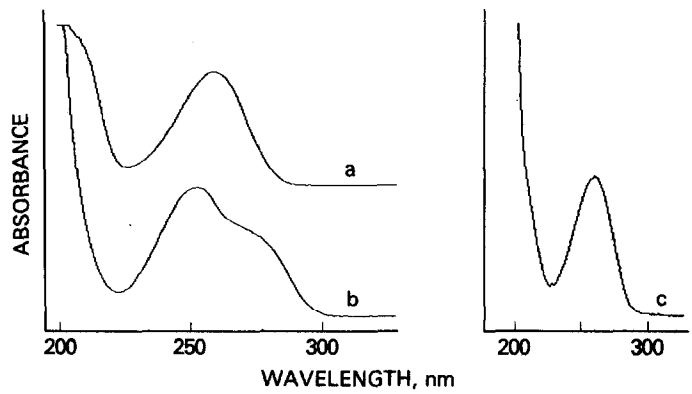

Fig. 5. Spectra of standard ATP (a), GTP (b), and UTP (c) obtained with the HPSEC program at time points corresponding to peak maxima.

to set a baseline shortly before elution of the peaks of interest. The time function "TP1" was introduced to disable the recognition of tailing peaks.

Quantitation of peaks

Peak areas of individual compounds were determined at the wavelength of (or near) maximal UV absorption using the HPEAK program. For example, the peaks of adenine, guanine, and uracil nucleotides were integrated at 254 nm and those of cytosine nucleotides at 276 nm. For peaks overlapping with others, the optimal wavelengths giving the least interference of one peak by the other were used. The concentrations of known compounds in the yeast cell extract were determined from calibration plots of standard compounds: the peak areas obtained by the HPEAK program for different amounts of a standard were plotted against the amount. The best straight line through the points was determined by a linear regression analysis. When the Spectroflow monitor was used, the absorption was recorded at 254 nm and the peaks were integrated by the SP4100 computing integrator.

RESULTS

We first show how nucleotides and other compounds were separated by the column used here and how they could be tentatively identified by retention time, coelution, and absorption maxima. The remainder of the Results is subdivided into two parts: (1) The properties of isolated peaks are examined; this includes verification that the peak is homogeneous, identification of the compound producing the peak, and integration of the peak. (2) The properties of overlapping peaks are characterized; this includes identification of components, determination of the best wavelength for integration, and calculation of the amounts of each compound in two overlapping peaks.

Separation of nucleotides

Fig. 3 shows the separation of twelve standard nucleotides using the column and solvent program described in the Materials and methods section. The retention times of these compounds as well as those of IMP, NADP, and orotidine-5'-phosphate (OMP) are given in Table I. The computer generated drawing shows the stepwise changes in the absorption which result from the fact that the Hewlett-Packard 8450 unit can transmit one spectrum every 2.5 sec. Although these curves are not as smooth as those obtained by direct recording of the absorbance (*e.g.*, in the Spectra-Physics 4100 unit), they contain enough steps that the peaks can be reliably integrated. However, a much faster separation of compounds into narrower peaks would not permit integration with the HP 8450 unit. The sensitivity of this unit was also lower than that of more conventional spectrophotometers used for HPLC, but we did not find this to be a significant problem because it is difficult anyway to accurately integrate tiny peaks that overlap large peaks.

In contrast to the fully separated peaks of the mixture of standard nucleotides, the peak pattern of a yeast cell extract was complex and showed numerous overlapping peaks (Fig. 4). By use of the appropriate buffer gradient, it might be possible further to separate peaks of particular interest. For example, if one wanted to separate the mononucleotides from other components, one would have to shift the onset

TABLE I

RETENTION TIMES OF STANDARD NUCLEOTIDES AND OF NUCLEOTIDES IN YEAST EXTRACTS

The standard mixture contained 5 n*M* of each nucleotide in 60 μl of sample injected. The yeast extract was prepared as described in the Materials and methods section from cells of strain 30204 grown to OD_{600} = 1.5 in MNA medium supplemented with guanine and uracil.

	*Standard**	*Extract**	*Extract***
AMP	16.33	–	–
ADP	31.25	31.29	32.17
ATP	41.08	40.50	42.50
GMP	22.83	–	–
GDP	33.33	32.87	34.58
GTP	43.08	43.21	44.75
CMP	12.83	–	–
CDP	30.08	30.25	31.08
CTP	39.42	38.92	41.00
UMP	11.33	–	–
UDP	28.25	28.37	29.33
UTP	36.67	36.17	38.33
IMP	18.03	–	–
OMP	27.50	26.96	27.83
NADP	29.68**	29.33	29.92

* Chromatographed on column No. 1.
** Chromatographed on column No. 2.

of the gradient to a later time and decrease the rate at which the gradient increases. However, not only would the chromatographic run take more time but the peaks of the more highly phosphorylated compounds would also spread much wider, making it more difficult to integrate small, late-eluting peaks accurately if they did not increase much above background. This is particularly pronounced for the late-eluting, highly phosphorylated nucleotides such as ppGpp and pppGpp, which were observed in bacterial extracts[22] but not in our yeast extracts. Since for our purposes the concentrations of nucleoside di- and triphosphates were particularly important, we decided to condense the initial part of the HPLC run and not to evaluate the early peaks including the mononucleotides.

Identification of major extract components

The retention times of nucleoside di- and triphosphates, measured in two cell extracts, agreed well with the retention times of standard nucleotides run separately (Table I). Even when another column (of the same type and from the same company) was used, the differences in retention times were only minor. The retention times of other compounds, such as NADP, were obtained by adding the compound to the mixture of standards. A small peak, A (see Fig. 4), eluting at 18.7 min was obtained even when an empty BA85 membrane filter was extracted; thus it represents an impurity in that filter. Peaks B, C, D and E were not well separated but that was of no concern for our purpose. Peaks B and C eluted at about the same time as UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, and CDP-glucose when added to the extract. When examined with the HPSPEC program, peaks B and C produced

spectra whose shapes and maxima were characteristic for UDP. Peak D coeluted with XMP. It could be positively identified as XMP when the guanine requiring strain (30061; deficient in GMP synthetase) was starved for guanine; the cells then accumulated large amounts of XMP, the biochemical precursor of GMP (chromatogram not shown). Peak E coeluted with GDP-glucose and GDP-mannose. From peak area ratios at different wavelengths (see later) peak E was found to be not homogeneous. Spectra displayed for different time points along the peak confirmed the presence of a GDP-sugar as one of the peak components. The other component, eluting immediately after the GDP-sugar, had an absorption maximum below 200 mm. Presumably, the GDP-sugar is GDP-mannose which is a precursor in the cell wall synthesis of yeast[23]. Peak E was followed by a peak for OMP. The first eluted nucleoside diphosphate was UDP. It was followed by NADP. The compound of the remaining peaks were identified as nucleoside di- and triphosphates (see later). The compounds producing two small peaks, F and G, remain unknown. They did not coelute with any of the tested compounds, which included, in addition to already mentioned FAD, Ap_4A [p^1p^4-di-(adenosine 5') tetraphosphate], ITP, IDP, and deoxy-nucleoside triphosphates. The chromatograms of the standard nucleotide mixture and the extract were also screened with the HPMAX program to identify (by an "*") the wavelengths of maximum UV absorption for each time point. This is demonstrated for portions of the chromatogram of the standard nucleotide mixture in Fig. 2. It is apparent, *e.g.*, that the compound with maximal absorption at 276 nm must be CDP and cannot be one of the other nucleoside di- or triphosphates. The difference in the absorption of the cytosine peaks is also apparent when one compares the elution profiles at 254 and 276 nm (Fig. 3). In the chromatogram of the cell extract, the peaks which appeared at the same time as and coeluted with known standards all produced absorption maxima at the expected location. The HPMAX plot also suggested that the UV-absorbing compounds that eluted early (within the first 20 min) were mainly adenine or its derivatives.

Integration of isolated peaks

To examine our method of peak integration, we determined the peak areas of the nucleoside di- and triphosphates under different conditions. We found that peak areas depended less on the column condition (age and buffer equilibration) and were more reproducible than peak heights. Linear regression analysis of area *vs.* amount data showed correlation coefficients of 0.996, 0.997, 0.996, and 0.995 for ATP, CTP, GTP, and UTP, respectively. For nucleoside diphosphates, the correlation coefficients were within the range 0.995–1.00. The detector response for ATP was no longer linear if more than 60 nmoles of a nucleotide were injected. To avoid working in a non-linear range we maintained the ATP concentration below that limit (usually at about 40 nmole per injection) by diluting the extract if necessary. (One could rapidly determine the amount of ATP by a phosphorescence assay using luciferase.) Because the concentration of all other nucleotides was always lower than that of ATP, all peaks were in the linear range of detector response.

Determination of the purity of isolated peaks by comparing spectra

Using the HPSPEC program, the spectra of three time points of an HPLC run could be displayed simultaneously and those of two time points could be used for

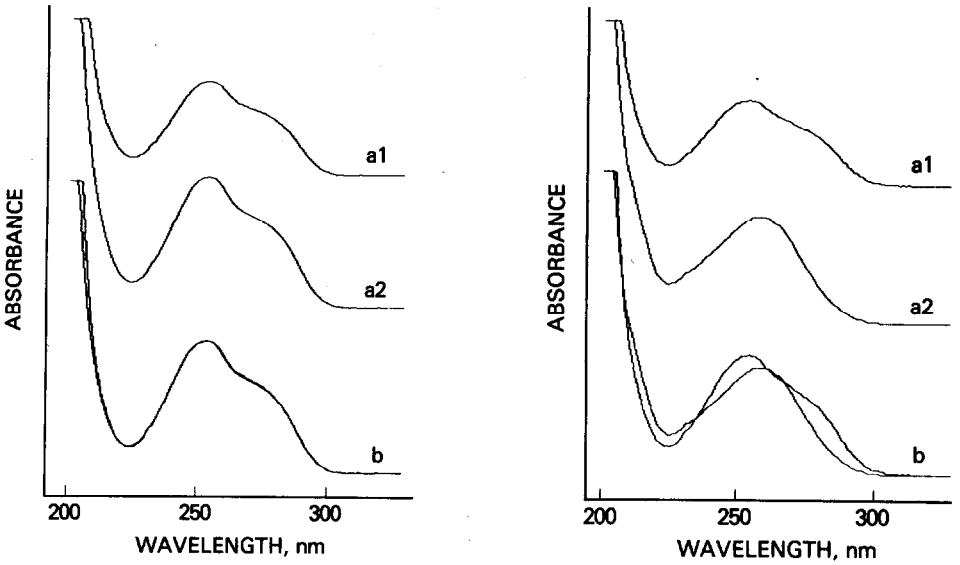

Fig. 6. Spectra of the GTP peak in a yeast extract. Strain 30061 was grown in MNA medium supplemented with guanine. (a1) and (a2) spectra correspond to the two time points, at *ca.* 1/2 maximal absorption on the upslope and the downslope of the peak respectively; (b) represents both spectra joined at their absorption maximum (254 nm). Because the joined spectra coincide, the GTP peak is pure.

Fig. 7. Spectra of the GDP peak in a yeast extract. Strain 30061 was grown in MNA medium supplemented with guanine. (a1) and (a2) represent spectra for time points on the upslope and the downslope of the peak respectively. When joined at 254 nm (b), the spectra do not coincide indicating that the peak contains more than one compound.

detailed comparisons. By comparing the spectra on the upslope and the downslope of a peak, the purity of the peak could be determined. This was achieved either by the display of the two normalized spectra or by multiplying the values of one spectrum with a number causing the two spectra to coincide at one particular wavelength (selected by the cursor location). For example, Fig. 6 shows spectra for the GTP peak of a yeast extract. The upper spectrum (a1) represents a time point on the left of the peak maximum, at which the absorption is *ca.* 1/3 of the maximum value and the next lower spectrum (a2) represents the corresponding time point on the right. The lower part of Fig. 6 (b) shows the same spectra but their absorption was made identical at 254 nm. It is apparent that the values coincided also at all other wavelengths, which indicates that the peak was pure (produced by a single compound). The agreement of this spectrum with that of GTP (eluted as a standard or mixed with the extract) showed that the peak represented pure GTP.

A different result was obtained for the GDP peak of the same chromatogram. Although this peak also appeared symmetric when recorded at 254 nm, comparison (Fig. 7) of the spectra on the left and the right of the peak maximum clearly showed that the peak was produced by overlapping elution of two different compounds, one being GDP and the other one unknown. Integration of this peak obviously would not measure the amount of either GDP or the unknown. But we will show below how this overlap can be resolved.

Determination of the purity of isolated peaks by comparing the ratio of peak areas at different wavelengths with the ratio of a standard

If a peak is pure, its area determined at one wavelength should have a constant proportion to the area determined at another wavelength. Furthermore, if the compound producing the peak has been identified, the ratio of these two areas should be the same as the ratio obtained for the corresponding standard compound. This concept enables another verification of the purity of a peak. A problem could arise only if very low wavelengths are used for area determination and if the buffers used to run the sample and the standard can absorb UV at this wavelength and differ slightly in this respect because one buffer contains more of an absorbing contaminant than the other.

To make this comparison, we have determined three such ratios for standard nucleoside di- and triphosphates and OMP (Table II). The table also shows the corresponding area ratios for tentatively identified nucleotide peaks of a chromatogram of yeast extract. It can be seen that the area ratios for most nucleotides agreed well with those of the standards. The exception was GDP. All three area ratios of GDP differed from those of the standard, which suggests the contribution of a second compound to the peak. The 254/240 area ratios for GTP and UTP were lower than those of the standards, which indicates that the extract contained small amounts of other compounds which absorbed at 240 nm (but not at 254 nm or above) and eluted at almost the same time as these two peaks; however, these compounds did not prevent integration of the GTP and UTP peaks as long as that was done at a high enough wavelength, *e.g.*, 254 nm. [The absorption difference at 240 nm does not seem to result from a contamination of one of the buffers, for in that case all 254/240 area ratios (or at least those for all early- or all late-eluting peaks) should have been low.]

A more cumbersome method would be to calculate the peak areas at many

TABLE II

PEAK AREA RATIOS OF STANDARD NUCLEOTIDES AND NUCLEOTIDES IN A YEAST CELL EXTRACT

Strain 30204 was grown to OD_{600} = 1.5 in MNA medium supplemented with guanine and uracil and the cells were extracted as described in Materials and methods.

	*Standard**			*Extract***		
	$\frac{254}{240}$	$\frac{254}{260}$	$\frac{254}{276}$	$\frac{254}{240}$	$\frac{254}{260}$	$\frac{254}{276}$
ADP	2.22 ± 0.06	0.91 ± 0.00	2.84 ± 0.04	2.26	0.94	2.85
ATP	2.35 ± 0.07	0.91 ± 0.02	2.97 ± 0.09	2.30	0.93	2.84
GDP	1.32 ± 0.13	1.21 ± 0.06	1.58 ± 0.02	1.42	1.01	1.99
GTP	1.49 ± 0.02	1.18 ± 0.03	1.62 ± 0.02	1.25	1.14	1.68
CDP	0.99 ± 0.03	0.82 ± 0.01	0.63 ± 0.01	1.00	0.82	0.65
CTP	1.01 ± 0.01	0.85 ± 0.01	0.62 ± 0.03	0.98	0.80	0.60
UDP	2.29 ± 0.02	0.88 ± 0.00	1.55 ± 0.01	2.33	0.88	1.50
UTP	2.36 ± 0.06	0.89 ± 0.02	1.55 ± 0.01	2.24	0.88	1.50
OMP	2.10	0.84	1.00	2.02	0.84	1.09

* Mean of three determinations ± S.D., except OMP (one determination).

** Separation of nucleotides is shown on Fig. 4.

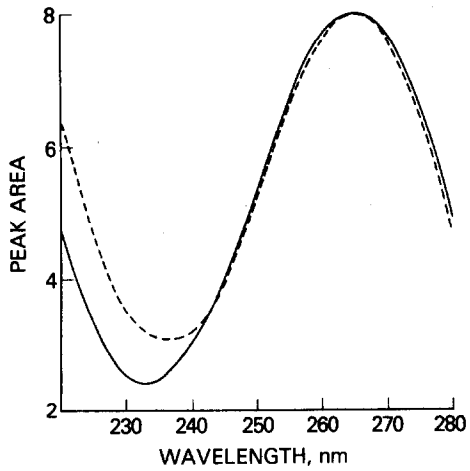

Fig. 8. Peak area plotted against wavelength for standard OMP (solid line) and the OMP peak of a yeast extract. Strain 30204 was grown in MNA medium supplemented with guanine and uracil (broken line). The chromatogram of the extract is shown in Fig. 4.

wavelengths and plot these areas *versus* the wavelength. If the compound is pure, the corresponding plot for the standard compound should (after normalization) give the same curve. As an example, the identification of the OMP peak (of Fig. 4) is shown in Fig. 8. The solid curve represents standard OMP, whereas the broken curve represents a compound in the extract which coeluted with OMP. The two curves were normalized at the wavelength of their maximal absorption; this wavelength corresponded to the absorption maximum (265 nm) for standard OMP and had the same value for the unknown compound. If the unknown were the same compound as the

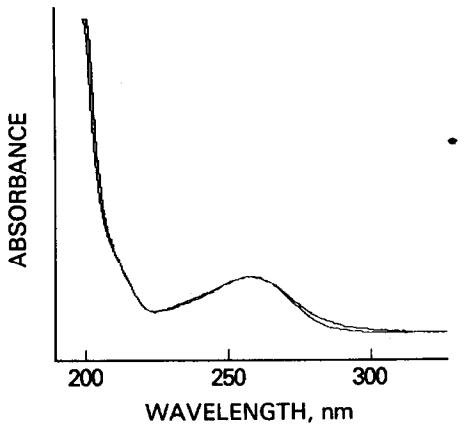

Fig. 9. Joined spectra for the two time points on the upslope and the downslope of a peak representing a compound coeluting with GDP in the extract of strain 30061. The strain was initially grown in MNA medium with guanine, then transferred to the same medium without guanine. Cells were collected and extracted shortly after the growth rate declined due to guanine starvation. The coinciding spectra indicate that the compound is pure. Apparently this was the compound which coeluted with GDP when the strain was grown with guanine (see Fig. 7).

known one, both curves should coincide. This was true except for the wavelength range below 240 nm in which the absorbance of standard OMP was lower than that of the unknown. We concluded that the contamination of the OMP peak would be negligible if one would integrate the peak at 265 nm, the absorption maximum of OMP.

Identification of components in overlapping peaks

If two overlapping peaks show a dip in absorption between them, one can assume that the time points on the far left and the far right of the double peak each represent only one compound whose spectrum can be compared with that of a standard. However, if the overlap is more complete, the spectra of the most distant time points at which they are still observable may still not represent pure compounds. In that case one could use a different solvent program to separate the compounds better, but another approach is to find conditions under which one of the two compounds is no longer present in the extract. This can be achieved by chemical or enzymatic treatment of the extract if one of the compounds is known and can be easily modified. We have, instead, used a biological technique to characterize the compound coeluting with GDP by growing a guanine requiring yeast mutant (30061) in synthetic medium plus 150 μM guanine to $OD_{600} = 1$, transferring the cells to the synthetic medium without guanine and extracting them after the culture had ceased to grow because it had used up the residual guanine. Fig. 9 shows, for the peak which coeluted with GDP, the normalized spectra of a time point on the left and the right of the peak maximum (at about one half maximal absorption). It is apparent from the agreement of the two spectra that this peak was homogeneous, and comparison with the GDP standard showed that the spectrum of this compound was quite different from that of GDP. Although we still do not know which compound produced the peak, the knowledge of its spectrum and of that of GDP made it possible to determine that the outer edges of the mixed peak (Fig. 7), produced by the original yeast extract, each represented essentially only one of the two materials.

Integration of overlapping peaks at optimal wavelengths

To quantitate the amount of each compound in a mixed peak, ideally one should integrate at the two wavelengths at which the contribution of one compound to the integral is maximized (*i.e.*, that of the other compound is minimized). Unless one compound absorbs maximally at a very different wavelength than the other compound, these optimal wavelengths are not identical with the absorption maxima of the two compounds but rather are the wavelengths at which the ratio of the absorption of the two compounds is maximal and minimal. These values can be determined by using the log ratio routine of the HPSPEC program. For the GDP peak, the optimal wavelengths were determined to be (1) 246 nm for GDP and (2) 262 nm for the unknown compound. These wavelengths were close to but not identical with the wavelengths of the absorption maxima of the compounds, which were 254 for GDP and 258 nm for the other compound.

We now describe, for the example of the GDP peak, how the absorption areas of the two compounds, which overlap to form the mixed peak, can be determined. This isolation is possible if the spectrum of each compound can be determined and if the two spectra do not coincide. Integration of the mixed peak produced area

values of $A_1 = 37.0$ at the optimal wavelength for GDP integration (246 nm) and $A_2 = 41.1$ at the optimal wavelength for the unknown compound (262 nm). These two areas can be written as

$$A_1 = a_1 + b_1 \quad \text{and} \quad A_2 = a_2 + b_2$$

where a_n is the contribution of GDP to the integral at wavelength n, and b_n is the contribution of the unknown compound. One can determine all four a_n and b_n values if one can obtain the dimensionless ratios

$$\alpha = a_2/a_1 \quad \text{and} \quad \beta = b_2/b_1$$

For example, in the case of the GDP peak, one can run a chromatogram of standard GDP, which gives the value for α; we found this value to be $\alpha = 37.7/44.8 = 0.84$.

To obtain β, we chromatographed the extract of the *gua* mutant grown in the absence of Gua, in which case the peak represented the unknown material; we obtained the value $\beta = 36.5/28.8 = 1.27$. (Note that α and β do not depend on the amount of the injected compound as long as that is within the linear response range.) Using $a_2 = \alpha a_1$ and $b_2 = \beta b_1$, one obtains:

$$a_1 = \frac{A_2 - \beta A_1}{\alpha - \beta} = 13.8 \quad \text{and} \quad b_1 = \frac{A_2 - \alpha A_1}{\beta - \alpha} = 23.3$$

The corresponding values for a_2 and b_2 can be similarly calculated. As a_1 was 37.3% of A_1, GDP represented 37.3% of the mixed peak. Because 1 nmole of standard GDP produced an absorbance area of 8.96 at the optimal wavelength (246 nm) for GDP integration in the mixed peak, the above value of a_1 corresponds to 1.54 nmole of GDP.

DISCUSSION

The first separation of 21 nucleotides on a microparticle, chemically bonded, strong anion exchanger was reported by Hartwick and Brown[1]. The original separation time of 90 min was later reduced to 50 min[4] by altering the buffer composition and increasing the flow-rate. In our laboratory, this method was modified by replacing potassium chloride with potassium sulfate in the high ionic strength phosphate buffer[24]. This method had the advantage that the always ionized doubly negative sulfate ions force highly phosphorylated nucleotides, especially GTP, ppGpp, and pppGpp, out of the column earlier, yielding much narrower and more accurately integrable peaks than those obtained with phosphate and chloride ions alone. Because phosphate and sulfate are available in highly purified form, baseline drift is also avoided. These ions are also less harmful to the high quality HPLC pumps than are halide ions. An abbreviated version of this method, using a steeper gradient and disregarding the separation of early peaks, was published by Ochi *et al.*[22] and was used in slightly altered form for the present work. The described gradient program allowed excellent separation of standard nucleotides in 47 min.

Although a cell extract contains many UV-absorbing components, some of

which are difficult to separate, the nucleoside di- and triphosphates were separated in the same short time. To identify the compounds in such a complex mixture, measurements of retention times and co-chromatography with reference compounds have to be combined with other identification methods. One can use the shift of a peak resulting from enzymatic or chemical treatment, or the detailed analysis of a collected fraction. One can also compare the spectrum, or at least the absorbance ratio at different wavelengths, of an unknown compound with that of a standard. The spectrum can be obtained by stop-flow techniques[25]; but to collect the spectral information for numerous retention times, the chromatogram has to be repeated several times. Another method is to compare the ratio of peak areas[26] or peak heights[27] at different wavelengths by recording at two wavelengths simultaneously[28]. The value of this method depends on the choice of the two wavelengths, which is usually optimal only for one compound. To avoid these limitations, one can use the full spectral information made available by the 8540 (or the newer 8450A) spectrophotometer of Hewlett-Packard. This spectrophotometer can read the entire spectrum and transmit it to a computer fast enough that a complete file of the three-dimensional chromatographic information (absorption *versus* time and wavelength) is obtained. This information could be plotted in a quasi three-dimensional perspective by shifting the baseline of each spectrum for successive times by a constant along a 45° line. While this produces a pretty picture, the plot cannot be quantitatively evaluated. We found it more useful to use several computer routines that display and plot various types of information in two-dimensional form. In this way, the results of a single HPLC run can be used for numerous types of evaluation. Absorption maxima can be plotted *versus* wavelength and time, enabling tentative identification of peaks. Peaks can be integrated at any wavelength; this also provides data for determining the ratios of peak areas or plots of peak area *vs.* wavelength for comparison with a standard and the evaluation of the purity of a peak. We found our integration method more reliable (due to the possibility of manual baseline setting) when compared with automatic integration, especially when small peaks alternated with large peaks. Spectra for different time points along a chromatogram can be displayed and compared and the display can be plotted. Because nucleotides with different bases have different absorption spectra, the compounds responsible for the major peaks can be easily identified by comparison with reference compounds. By superimposing spectra corresponding to different time points along the same peak, one can verify the purity of a peak and either select optimal wavelengths for the integration of peak components so that the overlapping of peak areas is minimized or calculate the composition of a peak if the spectra of coeluting compounds are known and different. These data support other identification methods. However, if a peak is composed of two or more compounds producing identical or very similar spectra (*e.g.*, coeluting UDP-sugars), additional chemical or enzymatic identification methods are necessary.

Although our results were obtained with UV-absorbing components of yeast cell extracts, the method should have general significance to any chromatographic procedure using the spectral properties of compounds. As one could use the same computer programs also for the much faster chromatograms obtained on reversed-phase columns, we hope that some spectrophotometer will soon be available which allows faster recording and transmission of spectra for a more extended time period than the present Hewlett-Packard units.

REFERENCES

1 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
2 J. X. Khym, *Clin. Chem.*, 21 (1975) 1245.
3 A. Floridi, C. A. Palmerini and C. Fini, *J. Chromatogr.*, 138 (1977) 203.
4 M. McKeag and P. R. Brown, *J. Chromatogr.*, 152 (1978) 253.
5 S. M. Payne and B. N. Ames, *Anal. Biochem.*, 123 (1982) 151.
6 E. Nissinen, *Anal. Biochem.*, 106 (1980) 497.
7 T. L. Riss, N. L. Zorich, M. D. Williams and A. Richardson, *J. Liquid Chromatogr.*, 3 (1980) 133.
8 M. W. Taylor, H. V. Hershey, R. A. Levine, K. Coy and S. Olivelle, *J. Chromatogr.*, 219 (1981) 133.
9 H. Martinez-Valdez, R. M. Kothari, H. V. Hershey and M. W. Taylor, *J. Chromatogr.*, 247 (1982) 307.
10 G. H. R. Rao, J. D. Peller, K. L. Richards, J. McCullough and J. C. White, *J. Chromatogr.*, 229 (1982) 205.
11 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
12 T. F. Walseth, G. Graff, M. C. Moos, Jr. and N. D. Goldberg, *Anal. Biochem.*, 107 (1980) 240.
13 J. H. Knox and J. Jurand, *J. Chromatogr.*, 203 (1981) 85.
14 J. H. Knox and J. Jurand, *J. Chromatogr.*, 218 (1981) 341.
15 J. X. Khym, J. W. Bynum and E. Volkin, *Anal. Biochem.*, 77 (1977) 446.
16 J. X. Khym, M. H. Jones, W. H. Lee, J. D. Regan and E. Volkin, *J. Biol. Chem.*, 253 (1978) 8741.
17 C. D. Lothrop, Jr. and M. Uziel, *Clin. Chem.*, 26 (1980) 1430.
18 P. R. Brown, *High Pressure Liquid Chromatography, Biochemical and Biomedical Applications*, Academic Press, New York, 1973.
19 P. R. Brown, A. M. Krstulovic and R. A. Hartwick, *Advan. Chromatogr.*, 18 (1980) 101.
20 E. B. Freese, Z. Olempska-Beer, A. Hartig and E. Freese, *Develop. Biol.*, in press.
21 Z. Olempska-Beer and E. B. Freese, in preparation.
22 K. Ochi, J. C. Kandala and E. Freese, *J. Biol. Chem.*, 256 (1981) 6866.
23 E. Cabib and R. Roberts, *Ann. Rev. Biochem.*, 51 (1982) 763.
24 E. Freese, unpublished results.
25 A. M. Krstulovic, R. A. Hartwick, P. R. Brown and K. Lohse, *J. Chromatogr.*, 158 (1978) 365.
26 E. Jugengling and H. Kammermeir, *Anal. Biochem.*, 102 (1980) 358.
27 R. A. Hartwick, A. M. Krstulovic and P. R. Brown, *J. Chromatogr.*, 186 (1979) 659.
28 R. A. Hartwick, S. P. Assenza and P. R. Brown, *J. Chromatogr.*, 186 (1979) 647.