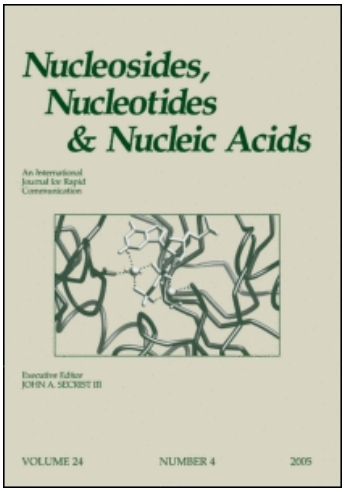

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Quantitation of Cytidine 3',5'-Cyclic Monophosphate in Aqueous Solution by UV Absorption Spectrophotometry Independent of Direct Determination of Solution pH

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QUANTITATION OF CYTIDINE 3',5'-CYCLIC MONOPHOSPHATE IN AQUEOUS SOLUTION BY UV ABSORPTION SPECTROPHOTOMETRY INDEPENDENT OF DIRECT DETERMINATION OF SOLUTION pH.

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ABSTRACT. uv absorbance spectrophotometry is the routine method of determining nucleotide concentrations in solution. To obviate the need for determining solution pH a method is described whereby cyclic CMP concentration in aqueous solution is calculated from absorbances at four wavelengths: the rationale is of general applicability to nucleosides and nucleotides.

Cyclic nucleotides perform a vital function in metabolic regulation, their role in signal transduction across cell membranes leading to their description as intracellular secondary messengers, and to the use of the enzymes which metabolize cyclic nucleotides as pharmacological targets. Adenosine 3',5'-cyclic monophosphate (cyclic AMP) mediates the action of a wide range of extracellular signals in the form of mammalian hormones and neurotransmitters[1], and guanosine 3',5'-cyclic monophosphate (cyclic GMP) is involved in the transduction of the visual signal in the mammalian eye[2] and in the regulation of the water/electrolyte balance in the bloodstream[3] and the intestine[4]. While the natural occurrence and functions of these two cyclic nucleotides are well established, more recently cytidine 3',5'-cyclic

monophosphate (cyclic CMP), uridine 3',5'-cyclic monophosphate (cyclic UMP), inosine 3',5'-cyclic monophosphate (cyclic IMP) and thymidine 3',5'-cyclic monophosphate (cyclic dTMP) have been shown to be endogenous components of both mammalian[5,6] and plant tissues[7], with strong evidence supporting the concept that cyclic CMP at least fulfills a similar regulatory function[8,9,10] to those of cyclic AMP and cyclic GMP. Thus these latterly-demonstrated cyclic nucleotides and the enzymes regulating their levels are of much current research interest as potential pharmacological targets with, for example, a pro-drug being converted into the anti-AIDS agent cidofovir by cyclic CMP phosphodiesterase in the liver[11].

The cellular concentrations of these compounds are in the pmol/g tissue range and below[12,13]; thus in making up standards for immunoassays and as part of enzyme assay incubations it is necessary to prepare small volumes of solutions in which the analyte concentration is determined spectrophotometrically, since the quantities involved are too small to be determined accurately by weight. Cyclic nucleotides have the same characteristic UV absorbance spectra as their constituent bases, and the molar absorbance coefficient varies with pH, thus normally the compound is made up in solution in a buffer of known pH. However, in estimating the concentration of microlitre quantities of cyclic nucleotide solutions it is often not practicable to measure the pH of the solution or to adjust it to a pH for which molar absorbance is known. For example it is necessary to have, as homogeneous solutions, nanogram quantities of cyclic nucleotides available as standards for quantitation of cyclic nucleotide-related enzymes by mass spectrometry[14,15], and microgram quantities of

derivatized cyclic nucleotides for radioiodination for use in radioimmunoassay[13]. Here we describe the development of a procedure for estimating a cyclic nucleotide concentration in the microgram range without prior determination of its pH: a computation was carried out which enables the pH of the solution to be determined from the UV absorbance spectrum of the cyclic nucleotide solution, and the concentration can then be determined simply by application of the Beer-Lambert realationship.

The basic rationale used was to produce a series of spectra at different pH values and transpose the data into software files readily amenable to mathematical manipulation. The latter were then processed to select reference wavelengths which can be computed into a function which varies with pH in a mathematically defined manner. Standard solutions of cyclic CMP were prepared at concentrations of 30-60 ug/mL in distilled water and the pH adjusted with either HCl or NH_4OH to give a range of pH 2-11. Each standard solution was scanned by a Hewlett-Packard diode array spectrophotometer model HP8452A between 200 and 300nm at 2nm intervals and the data stored as ASCII files. Representative spectra are depicted in FIG. 1 in which the variation in the UV absorbance spectrum with pH is clearly apparent. Also apparent from these spectra is that there is no clear isobestic point, which could theoretically be used as an alternative single reference wavelength with a molar absorbance coefficient insensitive to pH change. The closest that is observed to an isobestic point is 265nm, calculated as the mean of the points of intersection, but these points of intersection range from 263.8 to 266.1nm.

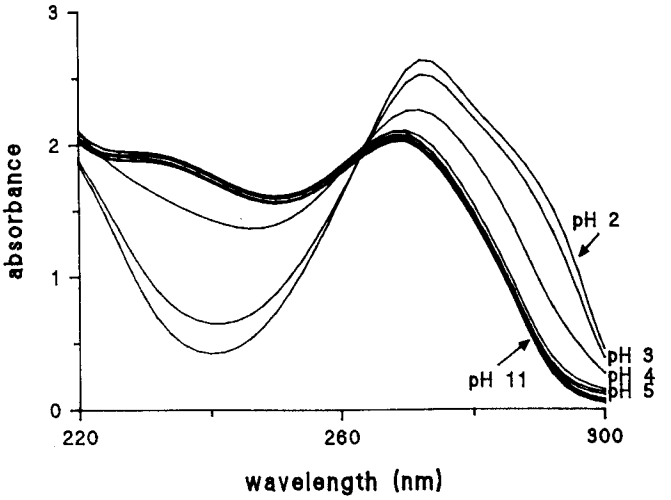

FIG. 1. UV absorbance spectra obtained from 4mL cyclic CMP solutions in 1 cm path length quartz cell at pH 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11.

The spectra were transposed via x,y ASCII files into Fig. P graphics and data analysis software (Oxford University Press Electronic Publishing, Oxford, U.K.) and the spreadsheet used to generate plots of the ratio of absorbance at each wavelength to the absorbance at each other wavelength, at each pH. Representative plots are depicted in FIG. 2 at pH 2,5,8 and 11. At pH 2 the maximum and minimum for each curve were at 272 and 240nm respectively, at pH 5, 270 and 248nm, at pH 8, 269 and 249 nm, and at pH 11, 268 and 251nm respectively. These maxima and minima at the extremes of the practicable pH range identify the reference wavelengths which show the clearest variation with pH and are used to calculate solution pH indirectly.

A variety of functions incorporating these reference wavelengths were examined and the best correlation with pH was observed with $(A_{240}/A_{250}) + (A_{268}/A_{272})$ as shown

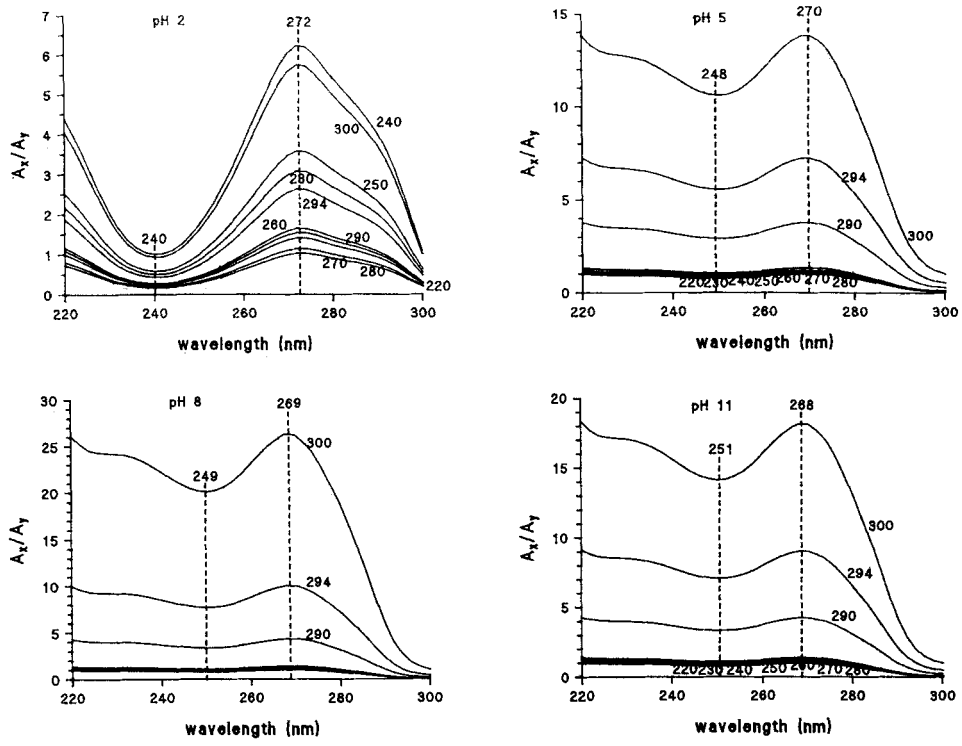

FIG. 2. Spreadsheet-generated plots of ratios of absorbance at each wavelength to the absorbance at each other wavelength, at pH 2, 5, 8 and 11. Dotted lines indicate maxima and minima for each plot.

in FIG. 3. To calculate pH this can be used as a standard curve or alternatively the polynomial expression, obtained by a curve-fitting of the data in FIG. 3,

$y = 1.68014 - 6.1028*10^{-1}x + 3.82003*10^{-1}x^2 - 7.55116*10^{-2}x^3 + 6.30579*10^{-3}x^4 - 1.9165*10^{-4}x^5$, where x is pH and y is $(A_{240}/A_{250}) + (A_{268}/A_{272})$, can be employed in a computation.

Determination of the pH of a cyclic CMP solution as above allows selection of the molar absorbance coefficient appropriate for this pH, required for application of

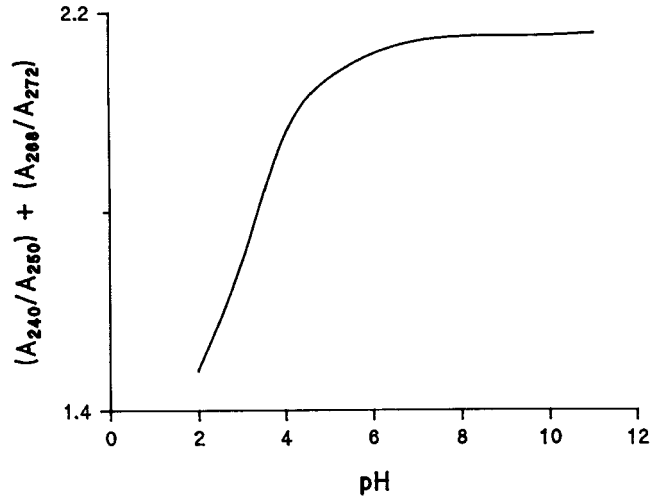

FIG. 3. Plot of reference wavelengths function against pH.
The ratio of absorbances at 240 and 250nm plus that at 268 and 272nm is plotted against pH.

the Beer-Lambert equation. The established molar absorbance coefficient for cCMP is at 272nm, which is the maxima at pH 2[12]. As the coefficient varies with pH, a plot of molar absorbance against pH was constructed by plotting ($A_{272,pHx}/A_{272,pH7}$) * molar absorbance at pH 7, against pH, as shown in FIG. 4. Again this figure can be used as a standard curve or a curve fit employed to generate the polynomial expression below which can be incorporated into an automated computation:

$A = 1.575563*10^4 - 2.11267*10^3x + 2.231049*10^2x^2 - 7.844x^3$

where A = molar absorbance and x = pH.

Thus, in order to calculate an unknown cyclic CMP concentration, the UV absorbance spectrum is obtained and the absorbance at 240, 250, 268 and 272 nm

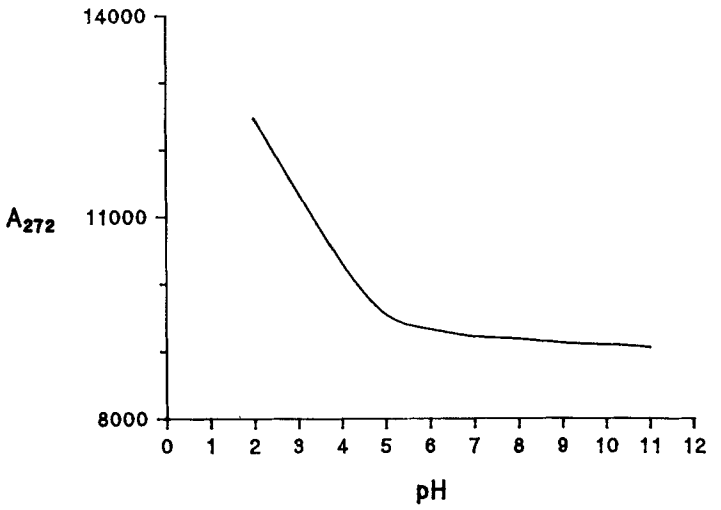

FIG. 4. Plot of absorbance at 272nm against pH for a molar solution of cCMP. The molar absorbance is calculated by multiplyng the molar absorbance coefficient at pH 7 by the ratio of the absorbance at 272nm at the specified pH to the absorbance at 272nm at pH 7. The plot is thus equivalent to a plot of molar absorbance coefficient against pH.

abstracted from it. Input of these data into a simple macro, BASIC programme or a similar computation enables determination of the pH of the solution which is then used to calculate the appropriate A value at 272 nm, based upon A = 9300 at pH 7.0[12] and thence the cyclic CMP concentration calculated.

To validate the above protocol a series of aqueous solutions of cyclic CMP of different concentration and pH were prepared by accurate weighing and serial dilution, then blind estimations of them carried out by (a) sole use of the absorbance at 265 nm, which is the closest one observes to a pH-independent isobestic point (FIG. 1), (b) the cyclic CMP radioimmunoassay[13], and (c) the above spectrophotometric

TABLE 1. Determination of cyclic CMP by alternative procedures.

	Method of estimation		
Quantity	(a)	(b)	(c)
1.2 ug	0.7 +/- 1.8 ug	1.1 +/- 0.1 ug	1.3 +/- 0.1 ug
3.8 ug	5.9 +/- 3.7 ug	3.9 +/- 0.36 ug	3.7 +/- 0.35 ug
5.7 ug	6.3 +/- 2.1 ug	5.6 +/- 0.39 ug	5.7 +/- 0.41 ug
8.9 ug	9.4 +/- 2.0 ug	8.7 +/- 0.35 ug	9.1 +/- 0.45 ug
10.4 ug	9.9 +/- 2.4 ug	10.2 +/- 0.43 ug	10.2 +/- 0.41 ug
12.9 ug	12.6 +/- 3.2 ug	12.6 +/- 0.48 ug	13.1 +/- 0.44 ug

Quantity of cyclic CMP obtained after serial dilution of weighed sample. Estimations carried out by (a) spectrophotometry using 265 nm as pH-independent isobestic point, (b) radioimmunoassay, and (c) spectrophotometry using the procedure described above. Data are expressed as the means of six replicates and the standard error of the mean.

protocol. As can be seen from TABLE 1, with six replicates, the use of 265 nm as an isobestic point gives inaccurate data of lower precision than the radioimmunoassay and the protocol described above. The data obtained with the latter two procedures are very similar in quality, and show good agreement with concentrations calculated from the weighed samples. These data, and the rapidity with which the uv spectrum can be obtained relative to the time and expense of an immunoassay, together with the fact that the sample in the radioimmunoassay is consumed whereas that determined by spectrophotometry is not, indicate the value of the procedure developed.

The procedure described above provides us with a rapid means of determining cyclic CMP concentrations in small aliquots by UV absorbance spectrophotometry without the need to separately determine the pH of the analyte solution. With the use of large numbers of solutions of different concentration, usually containing submicrogram quantities of solid and thus not being amenable to determination by weighing, the procedure was developed primarily for use with analyses involving radioimmunoassay[12], chemical derivatization and mass spectrometric analyses relating to cyclic CMP and other cyclic nucleotides. However the problems of pH effects upon absorbance coefficients and the availability of only restricted quantities of analyte are not parochial to studies of cyclic nucleotides, and the approach used here will also be of value in similar analyses of nucleotides, nucleosides and heterocyclic bases.

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