THE APPLICABILITY OF FORMOL TITRATION TO THE PROBLEM OF END-GROUP DETERMINATIONS IN POLYNUCLEOTIDES. A PRELIMINARY INVESTIGATION

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SUMMARY

The reaction between cytidine-5′-phosphate and formaldehyde has been investigated in order to determine whether or not titration in the presence of added formaldehyde can be used as a means of measuring the length of polynucleotide chains. In 2 *M* formaldehyde the reaction with the nucleotide comes to equilibrium within 40 min at room temperature. The extent to which the pK_a of the cytidylic acid amino group is lowered by increasing formaldehyde concentrations has been measured. In potentiometric titration of cytidine-5′-phosphate in the presence of formaldehyde, good agreement was obtained between the amount of phosphate calculated from the titration curve and that determined by phosphorus analysis. When four molar equivalents of cytidine were also present, however, (a mixture approximately equivalent to a solution of a penta-cytidylic acid), the best estimate of phosphate made from the titration curve exceeded the true value by about 19 %.

INTRODUCTION

A frequently recurring problem in polynucleotide and nucleic acid chemistry is that of determining the number of chain ends in a sample. It is generally accepted that many of the ends will carry singly-esterified phosphoric acid residues, and attempts have been made to estimate the number of such groups either by determining the amount of inorganic phosphate liberated by a purified phosphatase preparation or by analyzing the titration curves of the undegraded polymers. The enzymic approach has the disadvantage that the specificity of the phosphomonoesterase employed may not permit it to remove all of the terminal phosphoric acid residues. At the same time, the titration curves of polynucleotides represent the summation of many different dissociations, and it is necessary to assign dissociation constants to the various groups before an estimate of the number of terminal phosphate residues can be made. It is almost certain that values of the constants for the various types of ionizing group in a polynucleotide differ from those for similar groups in the mononucleotides. In particular, possibilities for hydrogen-bonding and neighboring-group charge effects are likely to raise the pK_a values of the amino groups of the nucleotide residues within a polynucleotide chain to the point where they will be mistaken

for secondary phosphate groups[1]. Interference of this type is to be most expected from the cytidylic (or deoxycytidylic) acid residues, since the pK_a's of the amino groups of these nucleotides are appreciably higher than those of the corresponding groups of adenine or guanine nucleotides. Such interference is likely to be particularly serious in polynucleotides of biological interest, where the ratio of secondary phosphoryl dissociations to interfering amino group dissociations is almost certain to be small.

The classical formol titration of Sørensen has been used in the fields of amino acid and protein study for many years. In this procedure formaldehyde in aqueous solution is used to lower the dissociation constants of the amino groups of α-amino acids (or of protein side-chains) so that they titrate in a pH range where a conventional indicator can be used[2]. It seemed plausible, therefore, that formaldehyde could also be used to lower the pK_a values of the amino groups of nucleotides to the extent necessary to "uncover" the dissociations of the terminal phosphoric acid residues. Nucleosides and nucleotides that have amino groups are known to give a reaction with formaldehyde that can be detected spectrophotometrically[3]. It is thus possible to verify, in any instance, the completion of the reaction between the amino groups of a nucleotide and formaldehyde. The alteration of the titration curve of polyadenylic acid by formaldehyde, ascribed to the lowering of the pK_a values of the amino groups, has been reported[1]. The titration of a nucleic acid preparation in the presence of formaldehyde is also mentioned in the literature[4], but as yet no systematic study of the possible utility of formaldehyde for the determination of end-groups in polynucleotides has been made. The present paper is a report of an investigation of the behavior of the dissociation of the amino group of cytidylic acid in the presence of formaldehyde.

MATERIALS AND METHODS

Cytidine-5'-phosphate was obtained from Pabst Laboratories, Milwaukee, Wisconsin.

Cytidine sulfate monohydrate came from the California Corporation for Biochemical Research, Los Angeles.

Inorganic salts were reagent grade.

Formaldehyde was the reagent-grade 36–38 % aqueous solution containing 10–15 % methanol preservative. Prior to use it was passed through a column of Permutit CCG anion-exchange resin (Permutit Company, 330 W. 42nd St., New York, N. Y.), which lowered the formic acid concentration from 0.013 % to a negligibly small value. The resin treatment was found to introduce detectable buffering at about pH 6 in the effluent, probably through the introduction of bicarbonate ion, but the amount so introduced could be kept small by the use of 1 *M* ammonium hydroxide to regenerate the column. In titration experiments correction was made for this contamination by means of a control titration. Formaldehyde solutions treated with the resin in the manner described showed virtually no accumulation of formic acid after eight days storage at room temperature, nor could any accumulation be detected after incubation at pH 8.5 for up to 4 h at 37°. Following removal of the formic acid the molarity of each formaldehyde solution was calculated from its formaldehyde content, as determined by titration in the presence of sodium sulfite[5].

Formaldehyde concentrations in dilute solutions were calculated by assuming in each case that the volumes of concentrated formaldehyde reagent and diluent were additive.

Attainment of equilibrium between cytidine-5'-phosphate and formaldehyde: The spectra of samples of a solution $2.79 \cdot 10^{-3}$ *M* with respect to cytidine-5'-phosphate, 2.4 *M* in formaldehyde, buffered to pH 8.3 with 0.1 *M* phosphate were measured on a Beckman Model DK2 Spectrophotometer after periods of incubation at either room temperature or 37°. Following determination of the spectrum of each sample, its pH was checked on a Beckman Model G pH meter.

Effect of formaldehyde on the dissociation of the amino group of cytidine-5'-phosphate: The pK_a of the cytidylic acid amino group in the presence of formaldehyde (the pG_f) was determined in a manner analogous to that used by LEVY[6] for amino acids, except that reagent-grade aqueous 36–38 % formaldehyde solutions purified in the manner described above were used rather than solutions of the freshly-distilled gas dissolved in water. A series of solutions of cytidine-5'-phosphate and sodium chloride ($4.4 \cdot 10^{-3}$ *M* and 0.035 *M*, respectively) varying in formaldehyde concentration from 0 to 10.42 *M* were prepared by diluting aliquots of a stock solution of cytidine-5'-phosphate at pH 4.45 containing sodium chloride with appropriate volumes of boiled deionized water and 12.72 *M* formaldehyde. Under these conditions, the pH of a solution diluted only with the water was 4.50. The pH of each solution was measured at room temperature on a Beckman Model G pH meter after 3 h incubation at 37° in a tightly closed container. No additional change in the pH of any of the solutions was observed after 20 h further incubation at 37°.

Potentiometric titrations: Titrations of 1.25-ml samples were conducted under a slow stream of nitrogen gas in a closed (but not airtight) vessel equipped with small magnetic stirrer (Tri-R Instruments, 144-13 Jamaica Avenue, Jamaica 35, N. Y.). Inserted in the vessel were the tip of a capillary microburet of the Kirk-Grunbaum type (Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, Calif.) and the glass and calomel electrodes of a Beckman probe assembly. The electrodes were connected to a Beckman Model G pH meter. After standardization of the meter with pH 7.0 buffer, a measured volume (1.00 ml) of test solution (cytidine-5'-phosphate or cytidine-5'-phosphate plus four molar equivalents of neutralized cytidine sulfate, sodium chloride and formaldehyde, pH 8.5) was introduced into the chamber. At the outset of each experiment, before dilution with formaldehyde, the cytidine-5'-phosphate concentration in each case approximated $3 \cdot 10^{-3}$ *M*; that of sodium chloride 0.07 to 0.08 *M*. Prior to its introduction into the chamber each test solution had been made approximately 2 *M* with respect to formaldehyde by the addition of a measured volume of the concentrated reagent, and had undergone a period of incubation of from 40 to 50 min at room temperature (in one case, 37°) to insure establishment of equilibrium between the substrate and formaldehyde. In order to insure immersion of the electrodes to an adequate depth at the outset of the titration, 0.25 ml of formaldehyde diluted with water to the same concentration as that of the test solution was added, plus sufficient carbonate-free standard sodium hydroxide (10–25 μl) to bring the pH of the solution up to 9. The solution was then titrated with standard hydrochloric acid, the pH being recorded after the delivery of each small volume from the microburet. Stirring was momentarily discontinued at those times when the pH was measured, but resumed during and for a short time

after the introduction of the next increment of acid. After the completion of any titration, the standardization of the meter was rechecked with pH 7.0 buffer. Results of the titration were disregarded if the pH of the buffer failed to read 7.00 ± 0.02.

In each experiment a blank, consisting of a solution of sodium chloride and formaldehyde alone carried through an identical incubation procedure was titrated, and in most cases a control titration was performed on a solution consisting of the nucleotide or nucleotide–nucleoside mixture, salt, a volume of water equal to the volume of formaldehyde solution added to the experimental solution, plus whatever volume of standard sodium hydroxide that had been found necessary to bring the initial pH of the corresponding experimental solution to 9.

The amount of cytidine-5′-phosphate in the solution titrated was calculated on the basis of the concentration of total organic phosphorus in the test solution prior to its dilution with formaldehyde, assuming the volumes to have been additive on dilution. Total organic phosphorus was determined after hydrolysis to inorganic phosphate with perchloric acid according to the procedure of ALLEN[7], followed by a phosphomolybdate determination of inorganic phosphate using ascorbic acid as the reducing agent[8,9].

Cytidine concentrations were calculated from the absorbance at 280 mμ of a sample diluted with pH 2 buffer, assuming an ε_{280} of $13.0 \cdot 10^3$ cm/M (ref. 10).

RESULTS AND DISCUSSION

In Table I are recorded the results of the experiment on the time required for the attainment of equilibrium between cytidine-5′-phosphate and formaldehyde. From the table it is evident that the reaction has reached equilibrium within 40 min at room temperature at pH 8.3. The errors of the experiment are such that it cannot be said with certainty that equilibrium at room temperature is reached within 10 min. It seems probable that the reaction between formaldehyde and cytidine-5′-phosphate is significantly slower than that between formaldehyde and the amino acids, which is reported[11] to reach equilibrium in periods of the order of 30 sec to 2 min. In the titration experiments subsequently carried out the nucleotide derivatives were incubated with formaldehyde for at least 40 min under conditions of temperature and pH which had been demonstrated to convert no significant amount of formaldehyde to formic acid.

TABLE I

ABSORBANCE RATIOS FOR CYTIDINE-5′-PHOSPHATE AFTER REACTION WITH FORMALDEHYDE AT pH 8.3

Formaldehyde concentration	*Time (min)*	*Incubation temperature*	A_{230}/A_{275}	A_{250}/A_{280}
0	—	—	0.905	0.847
0	—	—	0.906	0.860
2.4	10	25°	0.707	0.825
2.4	40	25°	0.688	0.797
2.4	30	37°	0.686	0.803
2.4	90	37°	0.686	0.803
2.4	150	37°	0.689	0.802
2.4	270	37°	0.689	0.802

The results of the experiment on the effect of formaldehyde concentration on the pK_a of the cytidylic acid amino group are plotted in Fig. 1. These results are of some theoretical interest in addition to their practical significance with regard to the

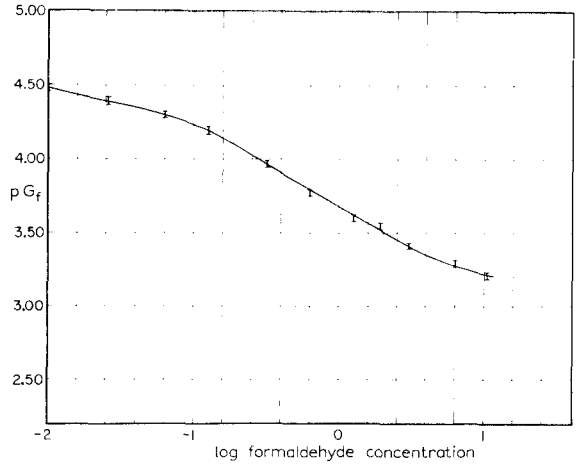

Fig. 1. Effect of formaldehyde on the dissociation of the amino group of cytidine-5′-phosphate. The abscissa represents the logarithm (to the base 10) of the formaldehyde concentration; the ordinate the pK_a (pG_f) of the amino group of cytidine-5′-phosphate at the corresponding formaldehyde concentration. Cytidine-5′-phosphate concentration, $4.4 \cdot 10^{-3}$ M; sodium chloride, 0.035 M.

question of the extent to which the pK_a is lowered. According to the theoretical treatment of the reaction between formaldehyde and amino acids made by LEVY[6], those amino acids which are primary amines (alanine, valine, etc.) at high formaldehyde concentrations obey the equation:

$$pK_a - pG_f = \log L_2 + 2 \log [HCHO]$$

where pK_a has its usual significance, being the negative logarithm (to the base 10) of the dissociation constant of the charged amino group in aqueous solution, while pG_f represents the analogous quantity in a solution containing formaldehyde, [HCHO] represents the molarity of formaldehyde in solution, and L_2 is a constant. Thus a plot of pG_f *versus* the logarithm of the formaldehyde concentration in the case of most of the simple amino acids approaches a straight line with slope of —2 at high formaldehyde concentrations. In the case of the amino acids that are secondary amines (such as proline or sarcosine) however, the theoretical equation obeyed at high formaldehyde concentrations is:

$$pK_a - pG_f = \log L_1 + \log [HCHO]$$

L_1 being a constant. The curve given by plotting pG_f against log [HCHO] at high formaldehyde concentrations approaches a straight line with slope —1.

Fig. 1, the curve given by the nucleotide, appears at formaldehyde concentrations greater than about 0.1 M, closely to approximate a straight line with slope of —0.6. Since there is a suggestion of decrease in the slope at the higher of the formaldehyde concentrations investigated, it is possible that buffering impurities present in the formaldehyde may have served to reduce the limiting slope of the curve somewhat.

It seems unlikely that much of the flattening of the curve is due to buffering by the primary phosphoryl dissociation of the nucleotide, which has a pK_a value of[12] approximately 0.8, except possibly at the very highest of the formaldehyde concentrations employed. The curve seems to indicate that cytidine-5'-phosphate combines with one rather than with two moles of formaldehyde, *i.e.*, it behaves more as though it had a secondary, rather than a primary amino group.

The extent of the depression of the pK_a of the cytidylic acid amino group produced at workable concentrations of formaldehyde was much less than has been observed with the amino acids. Nevertheless it was felt that the shift observed at high formaldehyde concentrations was sufficient to permit the determination of phosphate end-groups by formol titration, at least in some cases.

The titration experiments were performed on solutions which approximated the concentration of monoesterified phosphate that would be present in a decanucleotide at a concentration of 1 mg phosphorus/ml. Titration studies with cytidine-5'-phosphate alone would then be equivalent to titration of a decanucleotide with but a single cytidylic acid residue; when four moles of cytidine/mole of cytidylic acid were also present the solution approximated a decanucleotide containing five cytidylic acid residues per molecule. (This latter solution, of course, also approximates a pentacytidylic acid at half as high a concentration.)

The result of the titration of cytidine-5'-phosphate alone in 2 *M* formaldehyde, plotted as pH *vs* milliequivalents of acid consumed from pH 8.5 to pH 2.25 is given in Fig. 2. (The titration curves of all solutions containing formaldehyde coincide within experimental error above pH 8.5.) In addition to the experimental curve (curve 1), the curve given by the corresponding control solution (substrate but no formaldehyde, curve 2) and those given by the two blank solutions (one with formaldehyde, curve 3, and the other without, curve 4) are shown. Upon comparing the titration curves of the nucleotide with and without formaldehyde (curves 1 and 2) it is obvious that the presence of formaldehyde has effected a detectable separation of the amino and secondary phosphoryl dissociations.

In Fig. 3 are shown the results of the titration of cytidine-5'-phosphate in 2 *M* formaldehyde corrected for the acid consumed in titrating the blank alone (curve 1), and the corresponding curve given when cytidine-5'-phosphate plus four molar equivalents of cytidine are titrated under similar conditions (curve 2). In both experiments the milliequivalents of acid consumed in titrating the substrates from pH 8.5 to 5.0 and from pH 8.5 to 4.5 can be read directly from the curves, and are recorded in Table II. Also recorded in Table II are the milliequivalents of monoesterified phosphate calculated to have been present from the phosphorus analysis of the solutions.

The agreement between the milliequivalents of acid consumed in the titration and the milliequivalents of secondary phosphate present is close in the case where the mononucleotide alone is the substance titrated. The inflection point of the curve appears to be located at pH 4.5 or slightly below, and the milliequivalents of acid consumed in titrating to this pH exceeds the milliequivalents of secondary phosphate calculated to have been present from the phosphorus analysis by only about 5 % (close to the limit of accuracy of the method of phosphorus analysis employed). The pH at which the milliequivalents of acid consumed is stoichiometrically equivalent to the secondary phosphate present lies slightly below pH 5.0.

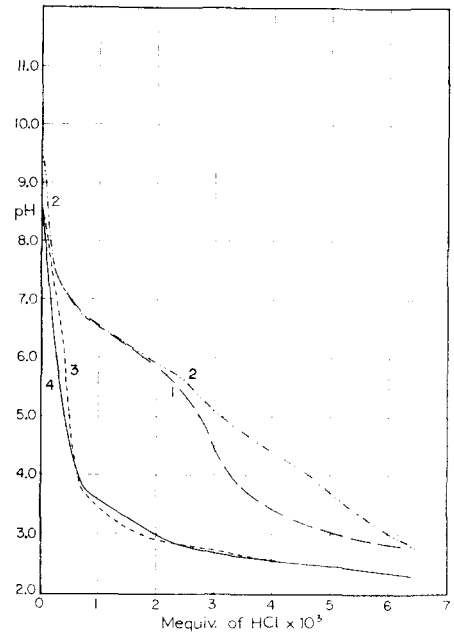

Fig. 2. Titration of cytidine-5′-phosphate in the presence and absence of 2 M formaldehyde. Curve 1: $1.84 \cdot 10^{-3}$ M cytidine-5′-phosphate, 0.043 M in NaCl, 2.30 M in HCHO, pH 8.5. Curve 2: $1.84 \cdot 10^{-3}$ M cytidine-5′-phosphate, 0.043 M in NaCl, no formaldehyde present, alkali equivalent to that necessary to bring the pH of the formaldehyde-containing solution to pH 8.5. Curve 3: (blank for Curve 1): 2.30 M formaldehyde, 0.043 M in NaCl, pH 8.5. Curve 4: (blank for Curve 2): 0.05 M NaCl, same initial pH as curve 2.

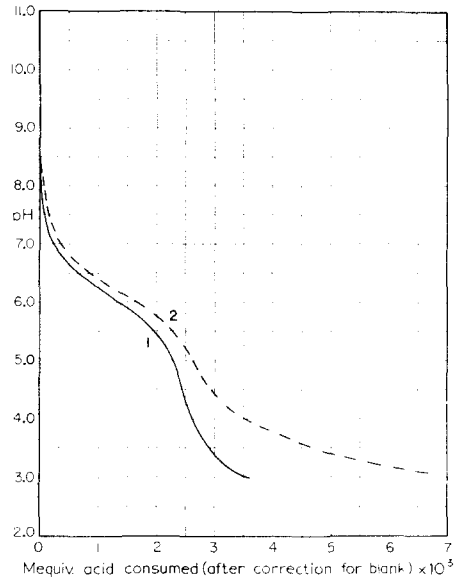

Fig. 3. Titrations conducted in 2 M formaldehyde. Curve 1: $1.84 \cdot 10^{-3}$ M cytidine-5′-phosphate, 0.043 M in NaCl, 2.30 M in HCHO. Curve 2: $1.91 \cdot 10^{-3}$ M cytidine-5′-phosphate, $7.83 \cdot 10^{-3}$ M cytidine 0.044 M NaCl, 2.26 M HCHO.

TABLE II

DETERMINATION OF NUCLEOSIDE PHOSPHATE BY TITRATION IN 2.3 M FORMALDEHYDE

Substances titrated	*Mequiv. nucleoside phosphate titrated* 10^3*	*Titration to pH*	*Mequiv. acid consumed 10^3*	*Per cent difference*
Cytidine-5′-phosphate	2.31	5.0	2.27	−1.73
		4.5	2.42	5.19
Cytidine-5′-phosphate plus cytidine	2.21	5.0	2.62	18.6
		4.5	2.95	33.5

* Calculated from phosphorus analysis.

When four molar equivalents of cytidine are present in addition to the cytidylic acid, however, the disagreement between the milliequivalents of acid consumed in the titration and the value calculated from the phosphorus analysis is substantial. The inflection point appears to be located between pH 4.5 and 5.0, yet the milliequivalents of acid consumed in titrating to pH 5.0 exceeds the milliequivalents of nucleotide by nearly 19 %.

In the aforementioned experiment on the effect of formaldehyde on the pK_a of the amino group of cytidine-5'-phosphate it had been found that the pK_a value was lowered to 3.46 or below at the formaldehyde concentration employed in the titration experiment (2.27 *M*). It can be calculated from the HENDERSON-HASSELBACH equation that the milliequivalents of acid consumed in titrating to pH 5.0 a solution of the salt of an acid with pK_a 3.50 (at the same concentration as that of the amino groups in the quoted titration experiment) are $3.47 \cdot 10^{-4}$. This amount represents a considerable fraction (nearly 16 %) of the total milliequivalents of nucleoside phosphate present ($2.21 \cdot 10^{-3}$), but should represent an upper limit of error from this source, since the true pK of the amino group is believed to have been below 3.50. The actual error observed is, of course, of this order of magnitude, but is significantly higher than this calculated upper limit.

It will be noted that the milliequivalents of acid consumed in titrating the nucleotide–nucleoside mixture (curve 2, Fig. 3) from pH 8.5 to 7.0 exceeds the milliequivalents consumed in titrating the nucleotide alone (curve 1) to the same pH by about $0.09 \cdot 10^{-4}$. This difference is probably close to within the margin of experimental error, but if any portion of it is due to contamination of the mixture solution with a buffer (such as bicarbonate), it would account in part for the discrepancy.

Another possible explanation for the magnitude of the observed discrepancy is that the pK_a of the amino group of the free nucleoside, cytidine, may not be lowered to the same extent at a given formaldehyde concentration as that of the amino group of cytidine-5'-phosphate, despite the fact that the pK_a of the amino group of the nucleoside in aqueous solution is lower than that of the nucleotide[10,13] (4.22 *vs* 4.5). Whether or not such is the case could be determined by a direct experiment with cytidine and formaldehyde, but the question is irrevelant to the problem of polynucleotide end-group determination, since cytidine itself is not a component of polynucleotides.

The results obtained when cytidine-5'-phosphate-cytidine mixtures were titrated in formaldehyde make it appear doubtful that the titration method with formaldehyde is applicable to many polynucleotides, since most large polynucleotides of natural origin would contain several times as many amino groups as terminal phosphate groups. However, the possibility remains that the behavior of the amino group of the free nucleoside toward formaldehyde is sufficiently different from that of a cytidylic acid residue in a polynucleotide chain for such a method to be useful to a limited extent. Interference due to the dissociation of amino groups should be further reducible by the use of formaldehyde concentrations higher than were used in the titration experiments reported here, although practical considerations place limits on the formaldehyde concentrations that can be used.

An ideal test substrate for the method would be an oligonucleotide of, say, ten residues in length, five of which were cytidylic (or deoxycytidylic) acid residues. It is probable that synthetic methods for such substances will be developed in the relatively near future[14].

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ÉTUDES CINÉTIQUES SUR LA GALACTOSIDE-PERMÉASE D'*ESCHERICHIA COLI*

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SUMMARY

Kinetic studies on the galactoside-permease in E. coli

Galactoside-permease positive *E. coli* accumulates radioactive thiogalactosides in the intracellular space until a steady state is reached. Measurement of the steady state concentration level, of the affinity, and of the rate of turnover in the steady state makes possible the calculation of the activity of the mechanism of uptake and of the rate constant of passive exit. The entry mechanism is catalytic; it is coupled with metabolic energy donors. The energy consumption corresponds to the hydrolysis of one mole of ATP per mole of TMG flowing inward. Passive exit is not by free diffusion, but can be understood as a carrier diffusion.

A model is proposed which is compatible with the observed behaviour of the entry and exit parameters under various conditions.

INTRODUCTION

Un nombre sans cesse croissant de phénomènes de perméation sélective, de transport actif à travers des membranes biologiques pluricellulaires, de concentration ou

Abréviations utilisées: TMG, méthyl-thio-β-D-galactoside; TDG, galactosyl-thio-β-D-galactoside; TPG, phényl-thio-β-D-galactoside; DNP, 2,4-dinitrophénol; *p*CMB, *p*-chloro-mercuribenzoate; ATP, acide adénosine triphosphorique.