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Assay of Thymidine Phosphorylase in Whole Cell Suspensions of Escherichia coli

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Abstract

A simple *in situ* assay for thymidine phosphorylase activity (E.C.2.4.2.4) in intact *E. coli* cells is described which requires only standard spectrophotometric equipment. The assay is based upon a characteristic hyperchromic shift for the product thymine under a change from neutral to alkaline conditions. Conversion reactions can therefore be quantitated in terms of the increased absorption at 290 nm following addition of NaOH.

The enzymic activity may be measured in actively growing or non-growing cells. Maximum rates of activity (μmoles thymine min^{-1}. $A_{600}{}^{-1}$) have been recorded at pH 5.5 and 47°C for non-growing cells and at pH 6.5 and 37°C for actively growing cells. The assay is sufficiently sensitive and convenient to permit the measurement of thymidine conversion in stirred tank or continuous flow reactors without the need for cell disruption.

Introduction

Trends toward the use of immobilized microbial cells as biocatalytic converters have necessitated the development of *in situ* assays of enzymatic activities. In studies of the interconversion of nucleosides and nucleotides by *Escherichia coli* we required a convenient means of monitoring the activity of the enzyme thymidine phosphorylase (EC.2.4.2.4) in intact cells. The enzyme (TPPase), first demonstrated in *E. coli* by Manson and Lampen (1) and purified and characterized by Razzel and Khorana (2) and Razzel and Casshyap (3), catalyses the phosphorolytic catabolism of thymidine (TdR) with the formation of deoxyribose-1-phosphate (dR1P) and thymine (T):

Thymidine + inorganic phosphate ------> Thymine + deoxyribose-1-phosphate. Methods for assaying this reaction have been based upon measurements of dR1P or T by chemical tests, (4, 5) or by incorporation of radioisotopically labelled precursors (6). In either method, purified enzyme or cell free systems were employed (7). For whole cell assays radioisotope exchange has been the method of choice (8). These latter techniques are time consuming and require the chromatographic separation of the nucleoside, TdR, from the pyrimidine base, T, or require materials which are expensive or not readily available.

We have developed an *in situ* assay for TPP activity which requires only standard spectrophotometric equipment. The assay is based on a characteristic hyperchromic shift specific for thymine under a change from neutral to alkaline condition (4). This shift is absent in the case of the nucleoside, TdR. Thus, conversion reactions can be quantitated in terms of the shift in absorption at 290 nm following the addition of NaOH to the reaction mixture. The assay system is sufficiently selective and sensitive to permit the measurement of thymine in the presence of other nucleoside and nucleotide bases and has been used to measure TPP activities of intact cells in stirred tank or continuous-flow reactors.

Materials and Methods

Thymine and thymidine were obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). Yeast extract was obtained from Difco Laboratories (Detroit, Michigan, USA). All other chemicals were from Fisher Scientific Co. Ltd., Canada.

E.coli (strain GK402) was maintained on yeast extract (0.1%) agar slants at 5°C. For experimental purposes the bacteria were grown in 5 ml of 1% yeast extract - 0.1 M potassium phosphate medium (pH 7.0) contained in a 50 ml Erlenmeyer flask on a Model G24 Environmental Incubator Gyrotary Shaker (New Brunswick Scientific Co. Ltd., New Jersey, USA) at 37C. Cell growth was measured optically as absorbance at 600 nm A_{600} in a Gilford Spectrophotometer (Gilford Instruments Laboratory Inc., Oberline, Ohio USA) or harvested for biomass determinations using a Sorvall RC-5B refrigerated centrifuge at 12000 g.

Assay for Thymidine Phosphorylase Activity

Assays were performed on non-growing (stationary phase) cells or on actively growing (logarithmic phase cells).

The cells were harvested by centrifugation and resuspended in 0.1M potassium phosphate buffer pH 7 to provide an appropriate cell density. Thymidine was then added to effect a 1 or 2 mM concentration. At intervals, 0.1 ml samples were removed from the reaction mixture, filtered through a 0.22 μm millipore filter to remove the cells.
The filtrate or supernatant sample was then diluted (1:50 or 1:100) with distilled water and assayed for thymine content.

In assays of TPP activity in actively growing cells, thymidine was added directly to cell suspension growing on yeast extract-potassium phosphate medium. Samples were removed at appropriate intervals and after filtration or centrifugation, diluted 1:50 or 1:100 with distilled water and assayed for thymine. Dilution of the sample was necessary to eliminate background absorption by the medium components and to effect a suitable range for direct measurement.

The absorption of the sample at 290 μm was read at neutral pH and after treatment with concentrated alkali (12 N NaOH). Addition of alkali to the sample caused a characteristic shift in the absorption spectrum (Fig. 1). After appropriate volume corrections for the alkali addition the difference in absorption (A_{290}^{alk} - A_{290}^{neut}) was found to be proportional to thymine concentration (Fig. 2).

For routine purposes TPPase activity was expressed as μmoles thymine produced per min per unit A_{600}. Temperature experiments were performed using a Thermocon Temperature Gradient Incubator (Scientific Industries Inc.). pH experiments were carried out using a 0.1M K_2HPO_4-KH_2PO_4 buffer system.

Results and Discussion

The rate of production of thymine was found to be linear with respect to time (Fig. 3a) until about 40% of the substrate was converted. The amount of thymine produced within a 60 min assay period was directly dependent upon the amount of cell biomass in the reaction mixture (Fig. 3b) confirming the validity of the assay system. A minimum cell density (A_{600}) of 0.15 was usually required for detection of TPP activity and specific enzyme activities (μmoles thymine min^{1} A_{600}^{-1}) were of the order of 4.0 for non-growing cells.

TPP activity could also be measured in logarithmic phase cells if appropriate corrections were made for biomass increase (Fig. 4). During the assay period, cell density increased exponentially from $0.15A_{600}$ - 2.0 A_{600} and conversion of the substrate was complete after 240 min. Maximum specific activities of 11.5 µmoles thymine $min^{-1}.A_{600}{}^{-1}$ were recorded for actively growing cells.

The effect of temperature on TPPase activity differed in growing and non-growing cells. (Fig. 5). Maximal rates of TPPase activity were recorded for cells resuspended in buffered substrate at 47°C but decreased at higher temperatures. Actively growing cells were optimally active at 37°C, coincident with their growth maximum, but exhibited activity levels approximately one half of the maximal rates determined with non-growing cells.

The highest activity of the enzyme in non-growing cells (16 µmoles thymine. min^{-1}. $A_{600}{}^{-1}$) was reached when the pH of the reaction mixture was maintained at pH 5.5. A higher pH optimum (pH 6.5) was observed for cells in the active growing state although pH 8 favoured biomass production.

These observations are consistent with data obtained by others using purified enzyme on cell-free systems. Schwartz (7) using <u>E.coli</u> K_{12} indicated a pH optimum of 6.3 for thymidine cleavage while the enzyme rapidly lost activity at 50°C. Similar results were also noted for <u>E.coli</u> K_{12} by Rachmeler <u>et</u> <u>al</u>. (9) suggesting a pH optimum for the enzyme of between 5.9 and 6.1.

Thus we were able to assay spectrophotometrically the thymidine phosphorylase activity in whole cell preparations. In general the activity expressed by actively growing cells was three (3X) times that obtained with stationary phase cells and maximum activity appeared to reside with cultures in the mid exponential phase of growth. Disruption of the cells by sonication or osmotic shock did not increase the measured activity suggesting that the total capability of the cells was being measured by the procedure. The potential of these cells for biocatalytic conversions is currently under investigation.

References

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4. Hotchkiss, R.D. (1948). J. Biol. Chem. 175; 315-332.
5. Burton, K (1956). Biochem. J. 62; 315-323.
6. Munch-Peterson, A. and Mygind, B. (1976) J. Cell Physiol., 89; 551-560.
7. Schwartz, M. (1971). Eur. J. Biochem. 21: 191-198.
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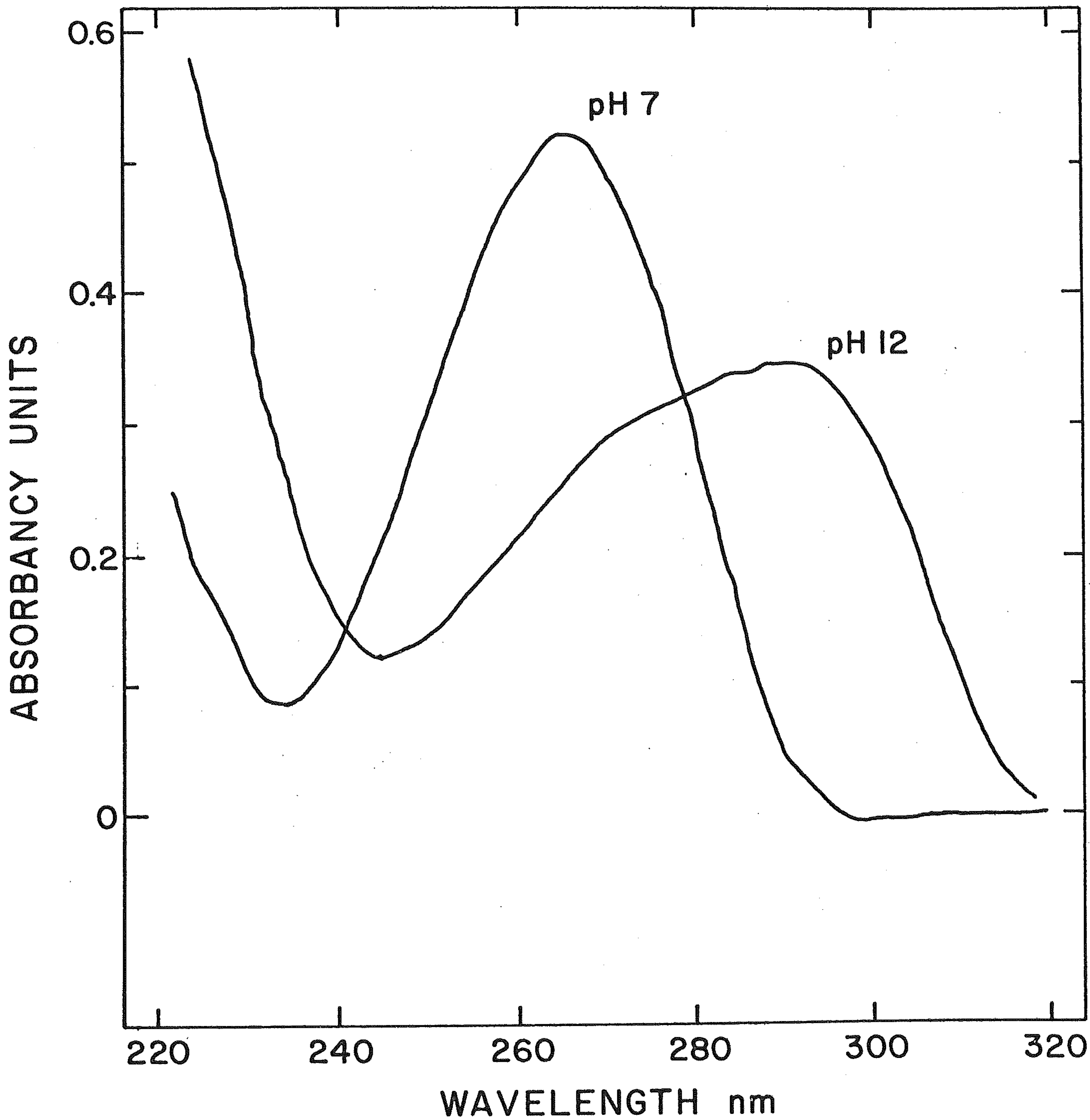
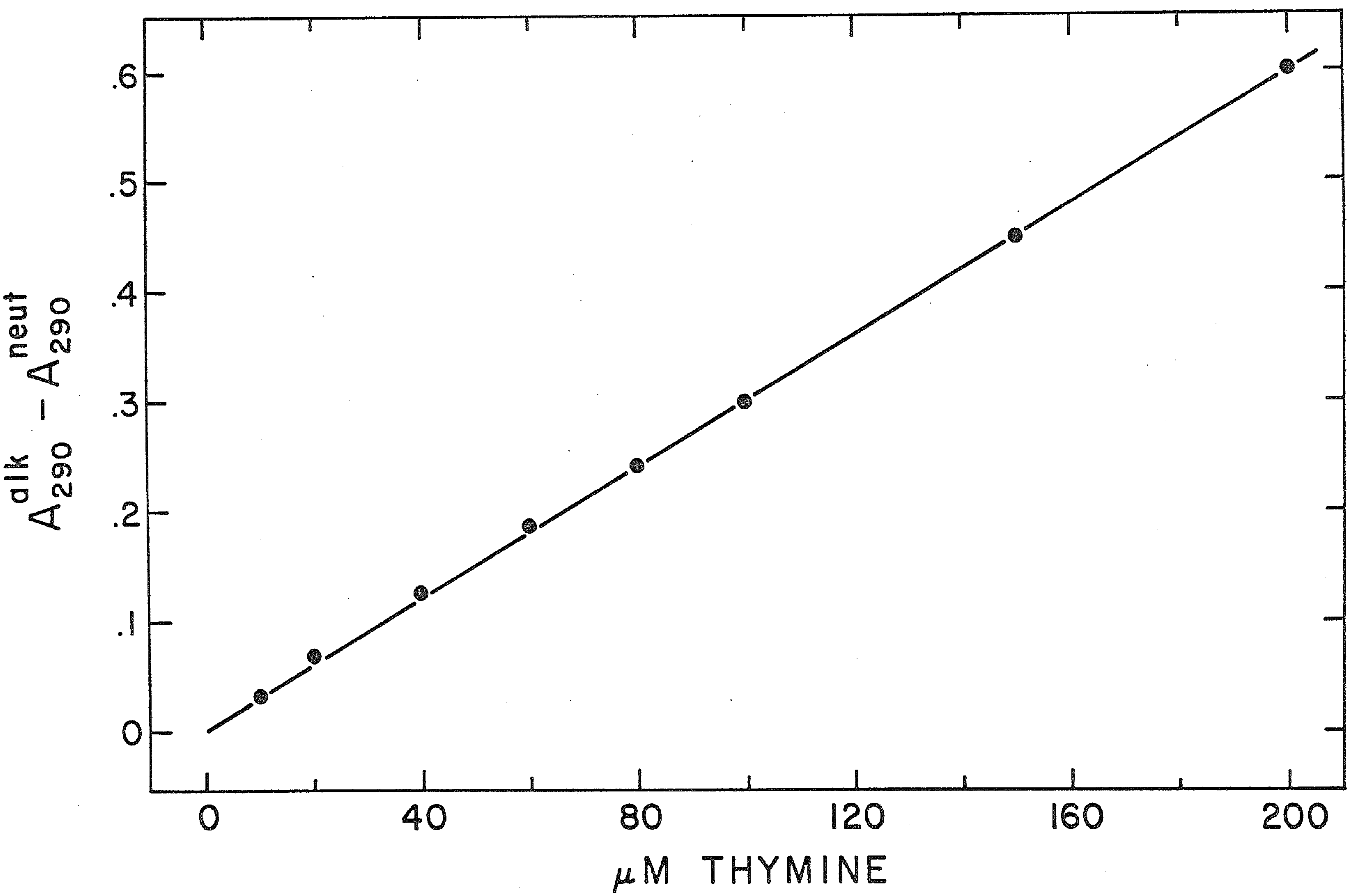
Acknowledgments

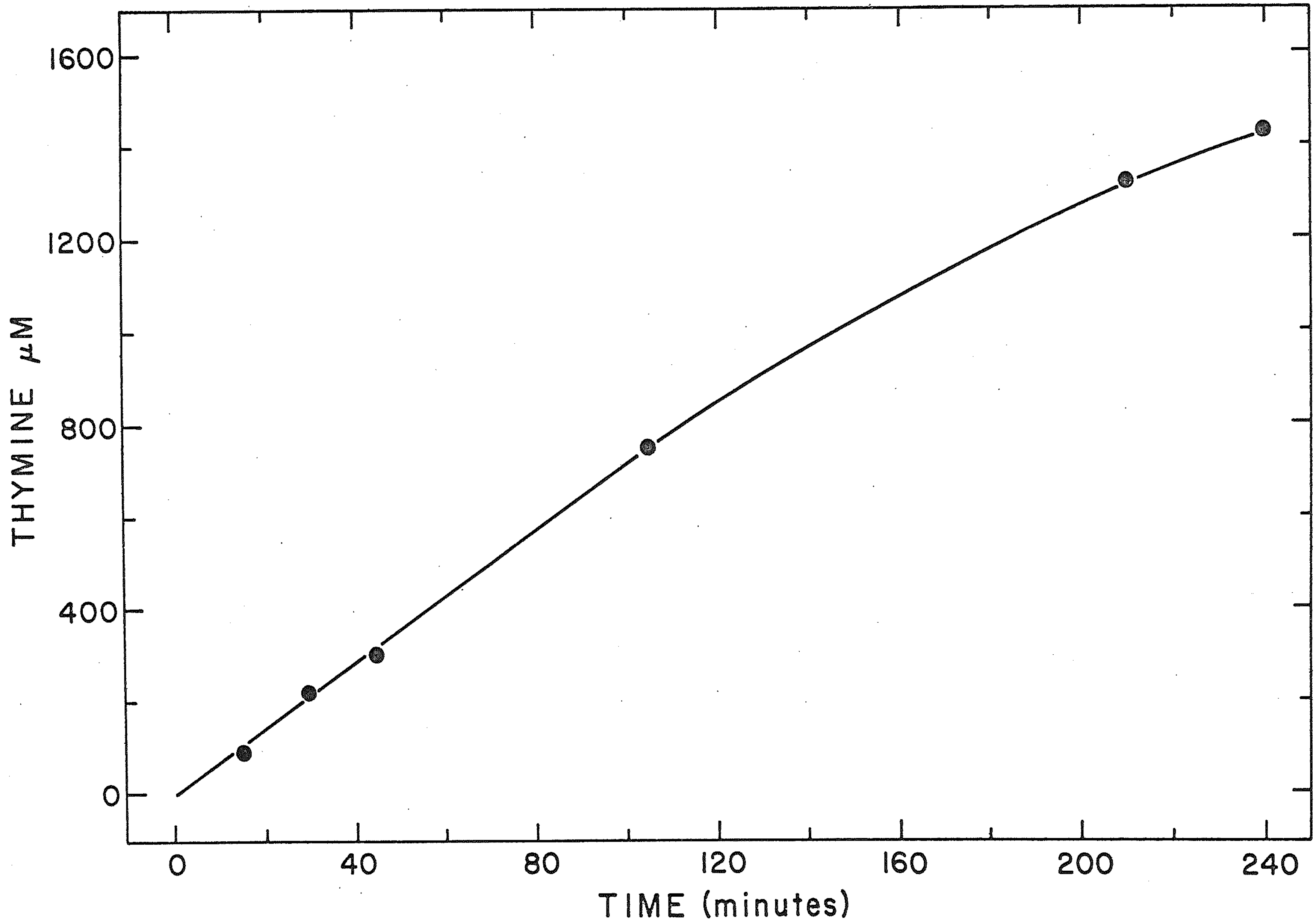
We gratefully acknowledge the capable technical assistance of Ms. C. Knapp and Mrs. D. Bergerman.

Figures

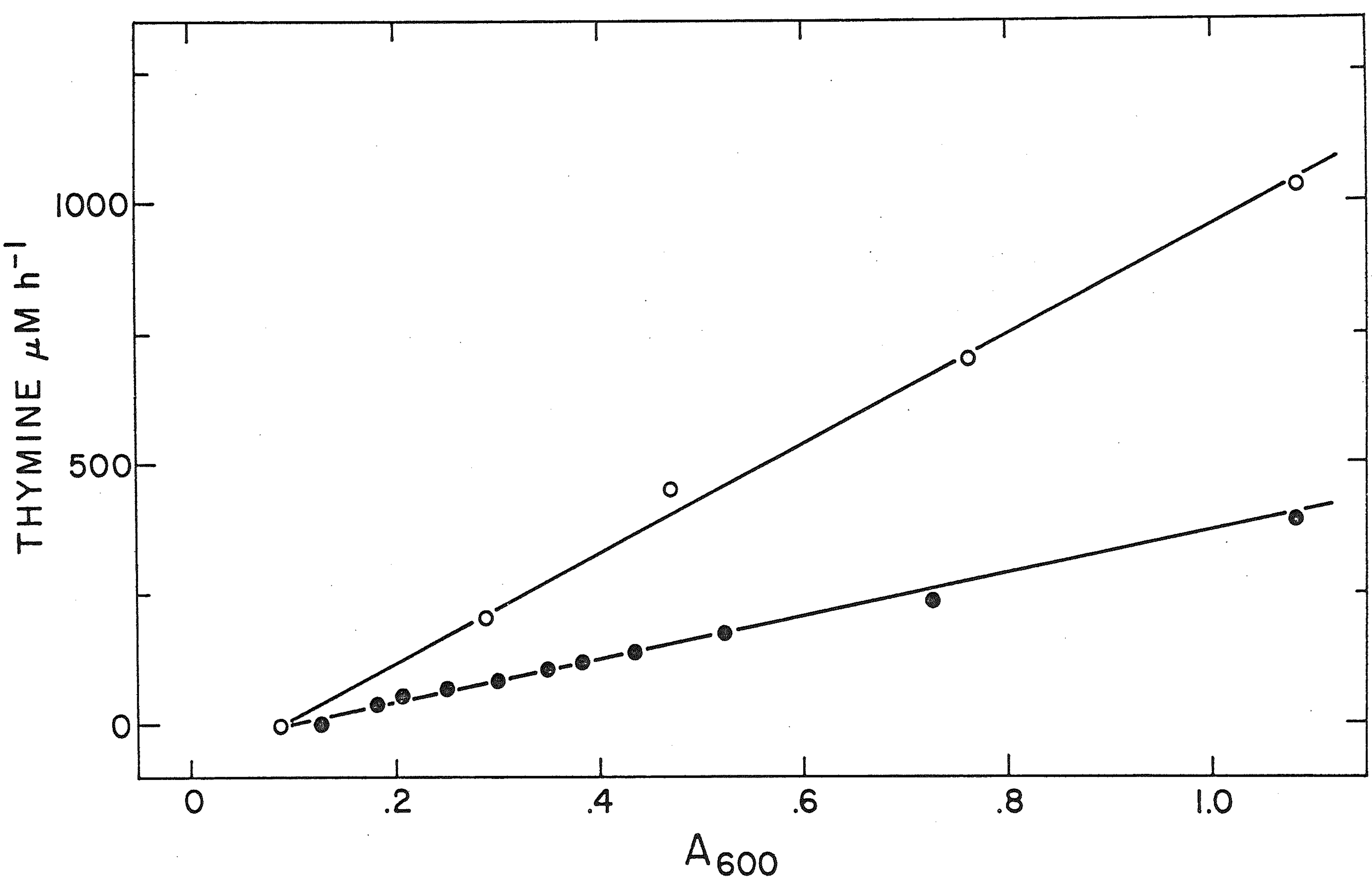

0.6
0.4
0.2
0
ABSORBANCY UNITS
pH 7
pH 12
220
240
260
280
300
320
WAVELENGTH nm

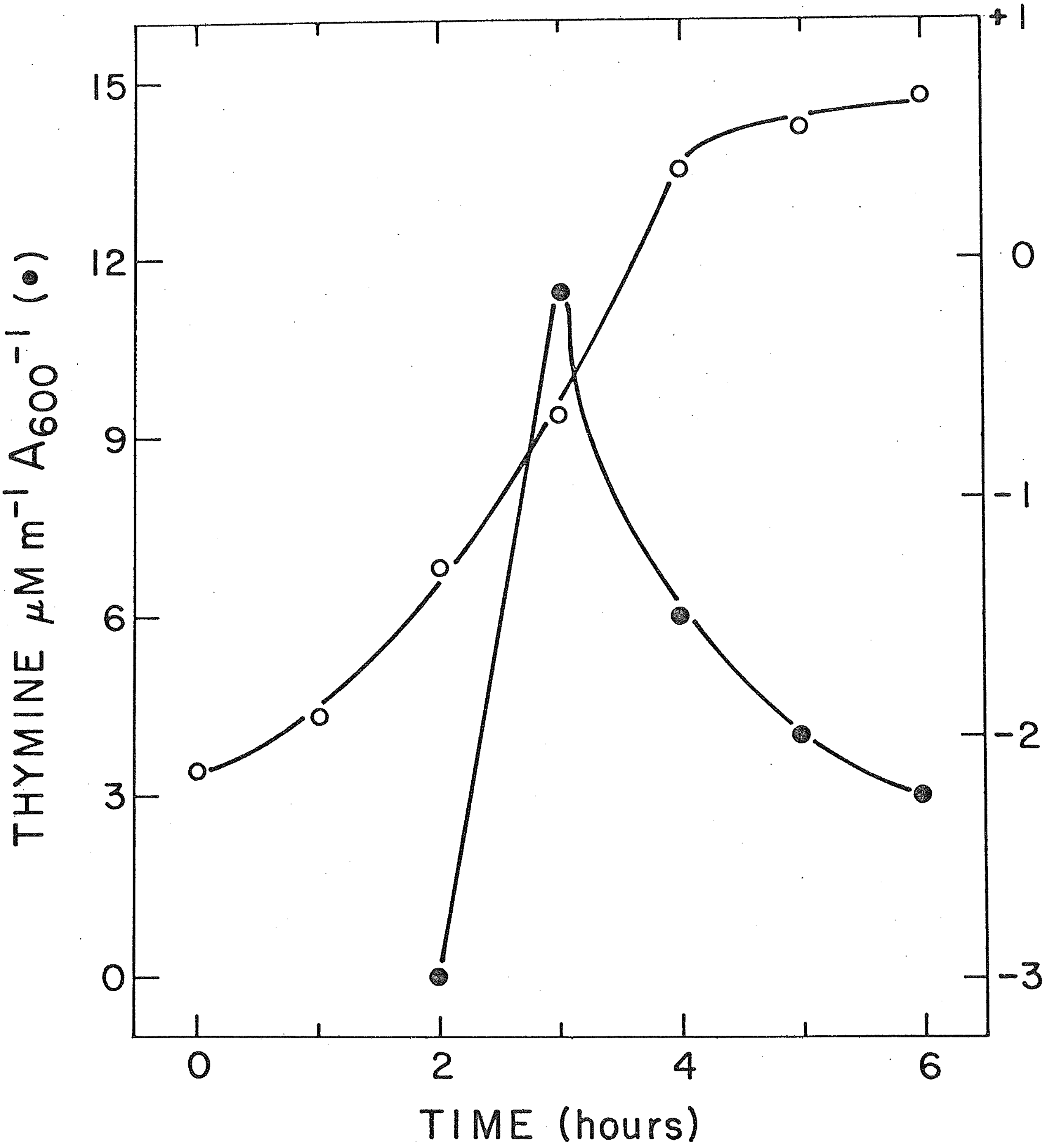


.6
.5
.4
.3
.2
.1
0
$A_{290}^{alk} - A_{290}^{neut}$
0
40
80
120
160
200
μM THYMINE

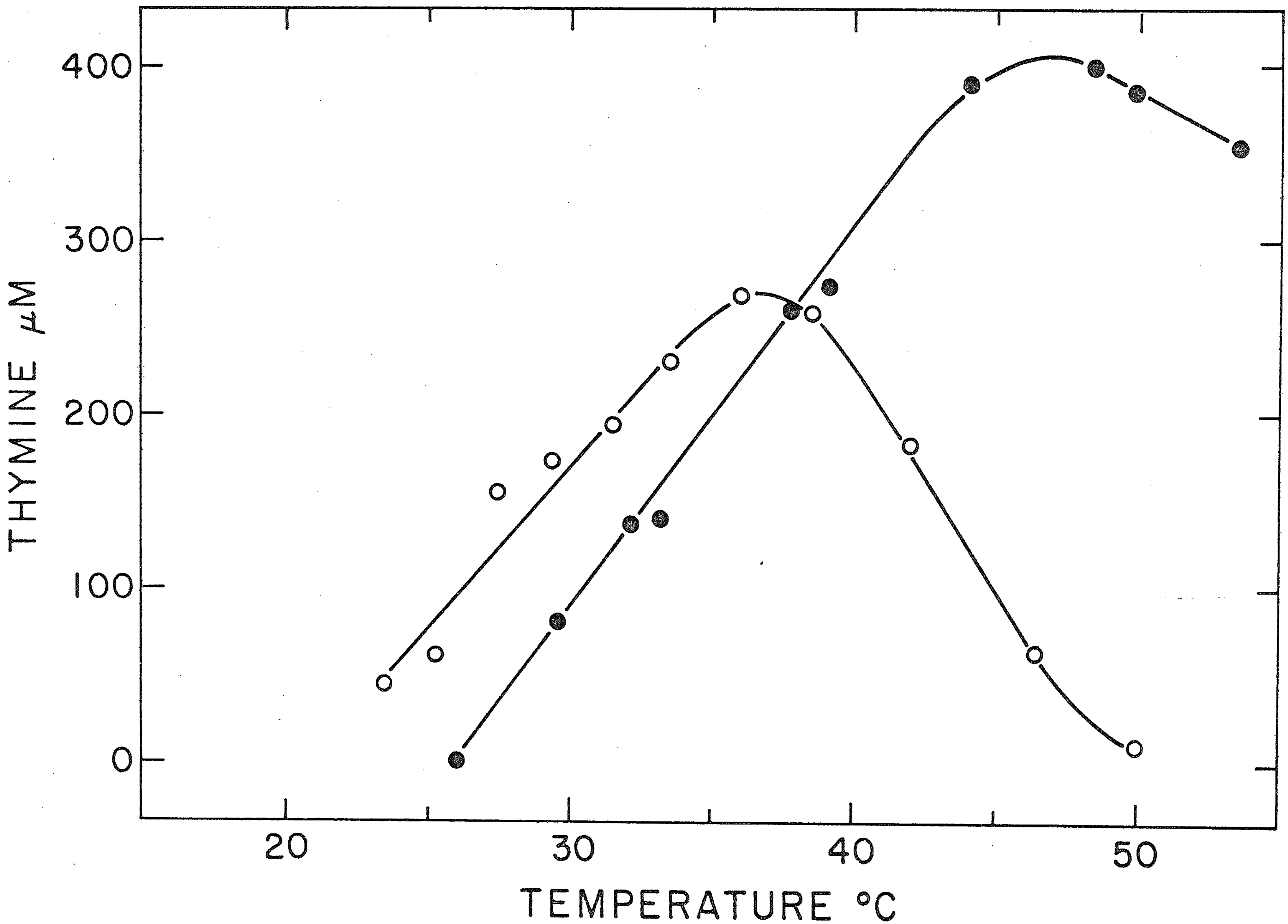


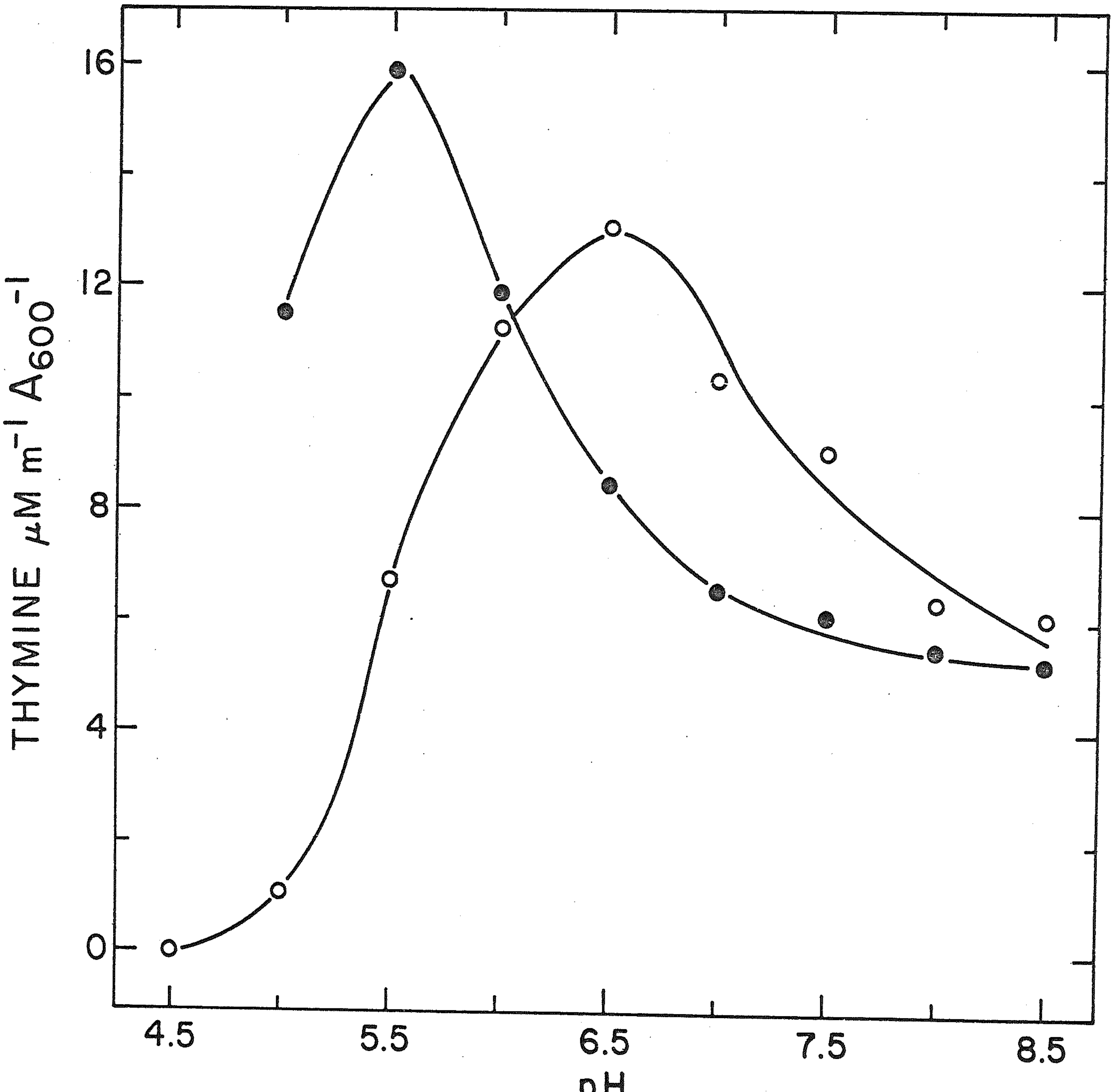
1600
1200
800
400
0
THYMINE µM
0
40
80
120
160
200
240
TIME (minutes)


THYMINE μM h⁻¹
1000
500
0
0
.2
.4
.6
.8
1.0
A600



THYMINE μM m⁻¹ A600⁻¹ (●)
BIOMASS ln (g/l) (○)
TIME (hours)
15
12
9
6
3
0
+1
0
-1
-2
-3
0
2
4
6


THYMINE μM
400
300
200
100
0
20
30
40
50
TEMPERATURE °C



THYMINE μM m⁻¹ A600⁻¹
16
12
8
4
0
4.5
5.5
6.5
7.5
8.5
pH

ASSAY OF THYMIDINE PHOSPHORYLASE IN WHOLE CELL SUSPENSIONS OF ESCHERICHIA COLI

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KEYWORDS

Thymidine phosphorylase assay

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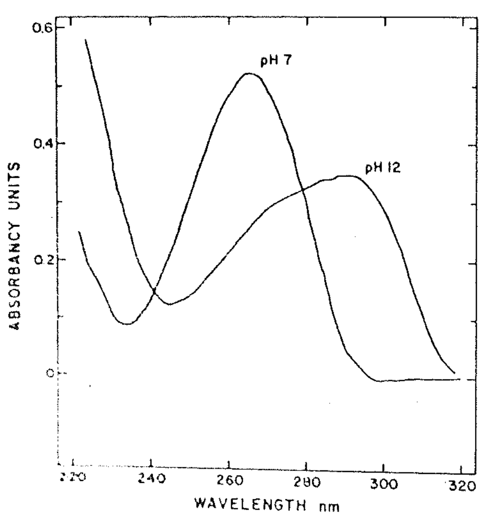

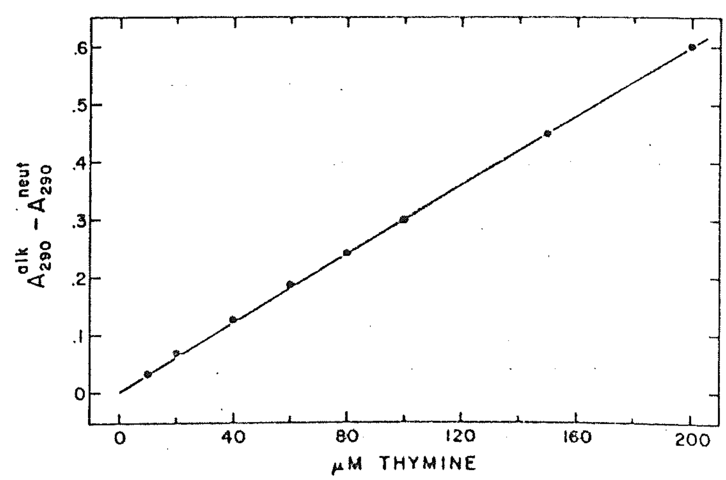

Figure 1. Characteristic hyperchromic shift of thymine from neutral to alkaline conditions.
Figure 2. The relationship between absorption (A_{290}^{alk} - A_{290} ^{neut}) and thymine concentration.

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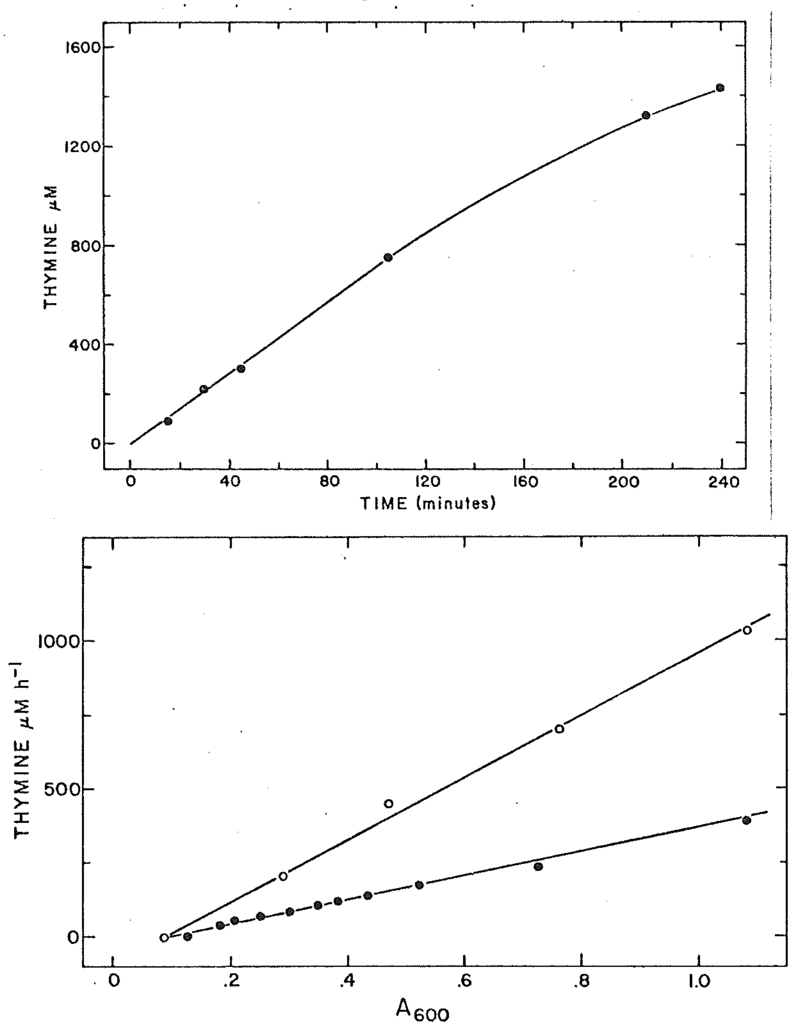
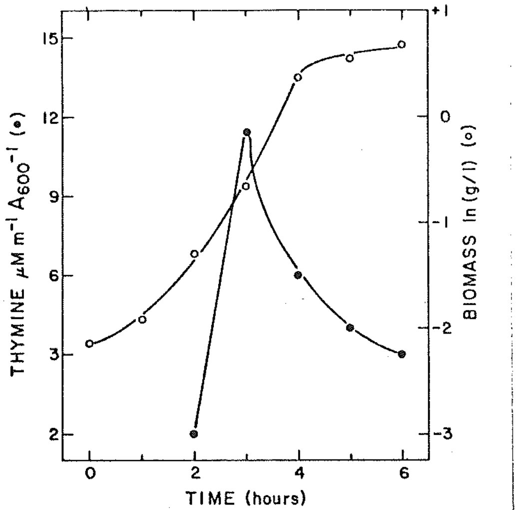
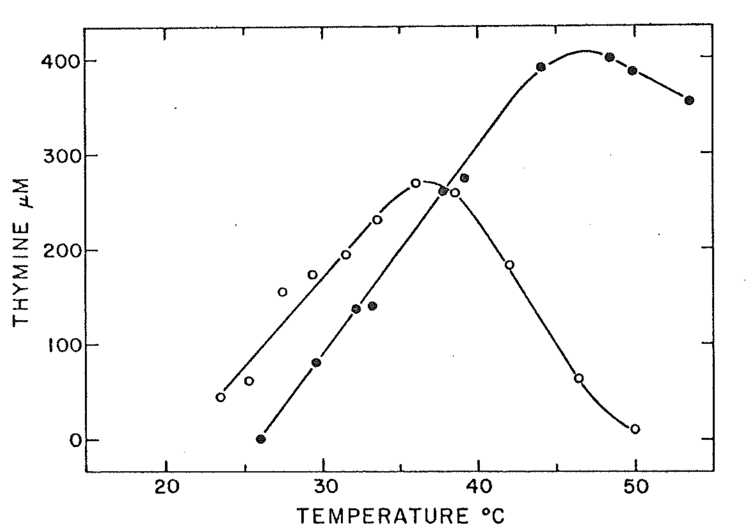


Figure 3. a) The rate of thymine production by non-growing cells (A_{600}=1.76)
b) The effect of biomass on thymidine phosphorylase activity in growing (o) and non-growing cells (●).





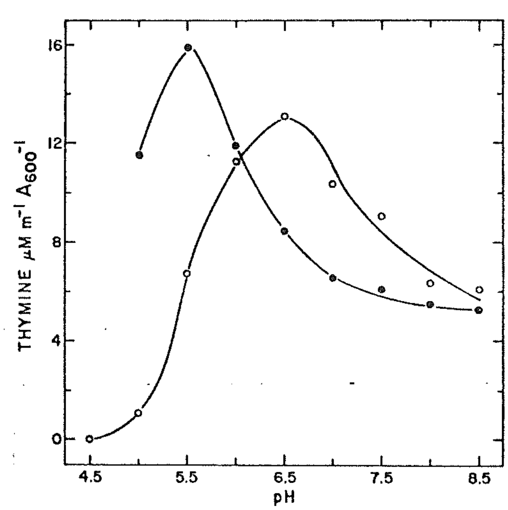


Figure 4. Specific activity of thymidine phosphorylase, in an actively growing culture of *E. coli*.

Figure 5. Effect of temperature on thymidine phosphorylase activity with growing (o) and non-growing (●) cells.

Figure 6. Effect of pH on thymidine phosphorylase activity with growing (o) and non-growing (●) cells.

REFERENCES

1. Manson, L.A. and Lampen J.O. (1951). J. Biol. Chem. 193; 539-547.
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