Biochimica et Biophysica Acta, 327 (1973) 336–344

BBA 67090

AN INTERACTION BETWEEN *S*-ADENOSYL-L-METHIONINE AND PYRIDOXAL 5′-PHOSPHATE, AND ITS EFFECT ON *SACCHAROMYCES CEREVISIAE*

R. W. TREWYN, K. D. NAKAMURA*, M. L. O'CONNOR AND L. W. PARKS
Department of Microbiology, Oregon State University, Corvallis, Oreg. 97331 (U.S.A.)

(Received July 16th, 1973)

SUMMARY

S-Adenosyl-L-methionine (SAM) interacts with pyridoxal 5′-phosphate (PLP) as determined by spectrophotometeric analysis, and this interaction can cause inhibition of PLP-requiring enzymes *in vitro*. SAM inhibition of tyrosine aminotransferase from *Saccharomyces cerevisiae* is of a competitive nature with regard to PLP. The apparent K_M for PLP is $1.2 \cdot 10^{-5}$ M, and the apparent K_I for SAM is approximately $1 \cdot 10^{-3}$ M. Evidence of SAM interacting with PLP *in vivo* was obtained utilizing *S. cerevisiae* grown in excess methionine. *S. cerevisiae* overproduces SAM and exhibits growth inhibition when cultured in a medium containing an excess of the amino acid. An analysis of the cofactor and enzyme levels indicates that SAM inhibition of PLP-dependent enzymatic reactions could cause, or contribute to, the growth inhibition.

INTRODUCTION

The regulation of methionine biosynthesis in yeast has been studied in our laboratory. Studies involving serine transhydroxymethylase resulted in the elucidation of certain regulatory properties of S-adenosylmethionine (SAM)[1]. Additional work on this pyridoxal 5′-phosphate (PLP)-requiring enzyme made it doubtful, however, that all of the inhibitory properties ascribed to SAM could be explained simply by feedback inhibition. Spectrophotometric analysis described in the previous paper[2], indicated that SAM was interacting with the enzyme cofactor PLP, and therefore, the possibility arose that this was contributing to inhibition of serine transhydroxymethylase.

Numerous examples have been reported for the inhibition of PLP-dependent enzymatic reactions by sulfhydryl containing compounds[3–5]. Although it seems un-

Abbreviation: PLP, pyridoxal 5′-phosphate; SAM, *S*-adenosyl-methionine.

* Present address: Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill. 60439, U.S.A.

likely that SAM would form a thiazine ring with PLP, as is the case with homocysteine[6], our spectrophotometric data led us to suspect that SAM was interacting with PLP in a direct way.

An interaction between SAM and PLP *in vivo* could offer an explanation for methionine inhibition of *Saccharomyces cerevisiae*. It is known that the growth rate of *S. cerevisiae* is severely depressed by culturing in high exogenous methionine concentrations[7,8]. The mode of action by which methionine inhibition occurs has not been established, but a related phenomenon observed in yeast, SAM accumulation, may be involved. Culturing *S. cerevisiae* in the presence of excess methionine results in high levels of SAM being produced and stored within the cells[7,8]. It had been thought that a depletion of the ATP pools would accompany the extensive conversion of methionine to SAM, thereby causing the observed inhibition by methionine[9,10]. However, it was recently shown that the intracellular ATP pools were not depleted during culturing in excess methionine[11]. As a result, methionine inhibition in *S. cerevisiae* is still not understood.

The present communication describes an interaction between SAM and PLP, and reports the *in vitro* effects of SAM on the PLP-requiring enzyme tyrosine aminotransferase. In addition, evidence is presented which suggests that SAM may interact with PLP *in vivo* and thereby contribute to methionine inhibition of *S. cerevisiae*.

MATERIALS AND METHODS

Organisms and cultural conditions

S. cerevisiae 5036-D (*ade*$^-$, *his*$^-$, *trp*$^-$, *met*$^-$), a haploid strain from the Oregon State University active culture collection, was used throughout this investigation.

Cells were grown in the complete medium of Wickerham[12] *minus* tyrosine and pyridoxine. Methionine concentrations of 0.03 and 5.0 mg/ml were utilized. Inoculations were made from stationary phase starter cultures that had been grown in medium containing 0.03 mg methionine per ml. A 1% inoculum (into 1 l of medium in a 2-l flask) was used in all cases except when lag phase cells were to be harvested, and then a 5% inoculum was used. Cells were grown at 30 °C on a rotary shaker. For the determination of tyrosine aminotransferase levels throughout the culture cycle, cells were grown in a 10-l carboy at 30 °C with vigorous aeration, and samples were removed and harvested periodically.

Preparation of crude cell extracts

Crude cell extracts were prepared in 0.1 M phosphate buffer (pH 7.0) by the method of Thompson and Parks[13]. The 25 000 × *g* supernatant was dialyzed overnight against 2 l of 0.01 M phosphate buffer (pH 7.0) with three changes of buffer. However, when tyrosine aminotransferase was to be assayed, a 4–5 ml portion of the 25 000 × *g* supernatant was removed and stored frozen for at least 48 h before dialysis. Denatured protein was removed by centrifugation at 25 000 × *g* for 20 min. The amount of soluble protein remaining was determined by the method of Lowry *et al.*[14], using bovine serum albumin as the standard.

Enzyme assays

Tyrosine aminotransferase (EC 2.5.1.5) was assayed by the method of Granner

and Tomkins[15] with the following modifications. A 2–3 min preincubation period was utilized, and for the inhibition studies, the SAM was not added to the "zero time" controls until immediately after the 12-min reaction time. For the usual assay procedure, 0.06 ml of the $5 \cdot 10^{-3}$ M PLP solution was utilized instead of 0.01 ml. In all cases, the reaction was started by the addition of 0.05 ml of the diluted crude extract. Spectrophotometric measurements were made with a PMQ II Zeiss spectrophotometer.

Ornithine aminotransferase (EC 2.6.1.13) was assayed by the method of Jenkins and Tsai[16] with only two minor modifications. In the assay mixture, 30 mM phosphate buffer (pH 7.0) was substituted for $Na_4P_2O_7$, and the incubation time was 45 min instead of 20. For ornithine aminotransferase from *S. cerevisiae*, the reaction rate was linear beyond 45 min.

SAM and PLP determinations

Cells were harvested by filtration after which they were extracted with perchloric acid by the method of Schlenk *et al.*[17].

SAM concentrations were determined by the chromatographic procedure of Schlenk *et al.*[17], and PLP concentrations were determined by the method of Chabner and Livingston[18] with minor modifications. Folded filter paper squares saturated with phenethylamine (0.2 ml) were utilized in the well to collect CO_2. After complete evolution of CO_2, the filter paper was placed into 10 ml of Bray's scintillation cocktail[19] and counted in a Packard Tri-Carb liquid scintillation spectrometer. DL-[1-^{14}C] Tyrosine was utilized in place of L-[1-^{14}C] tyrosine, but it was diluted to the same specific activity with respect to L-tyrosine as recommended by Chabner and Livingston[18]. For the determination of PLP, dilutions of the $HClO_4$ extracts were made with 0.1 M sodium acetate buffer (pH 5.5).

Spectrophotometric analysis

Complex formation between PLP and SAM was monitored with a Model 11 Cary recording spectrophotometer. To eliminate any pH change upon addition of SAM, the system was buffered with 0.1 M phosphate buffer (pH 7.0)

Chemicals, enzymes and isotopes

SAM (HSO_4) and *S*-adenosyl-L-homocysteine were obtained from Boehringer Mannheim. PLP, pyridoxal·HCl, pyridoxine·HCl, ATP, α-ketoglutaric acid, D-glucose 6-phosphate, NADP, L-tyrosine, and tyrosine decarboxylase apoenzyme were purchased from Sigma Chemical Co. L-Ornithine·HCl was obtained from Calbiochem, and *o*-aminobenzaldehyde was obtained from K and K Laboratories. DL-[1-^{14}C] Tyrosine was purchased from International Chemical and Nuclear Co.

RESULTS

Spectrophotometric analysis

Spectrophotometric analysis of PLP in the visible range (325–500 nm) was carried out to determine if any alterations could be observed upon addition of SAM. At neutral pH, the aldehyde function of PLP has a characteristic absorption peak at 388 nm (ref. 20). A $5 \cdot 10^{-4}$ M solution of PLP gave the characteristic pattern shown in

Fig. 1 (Curve No. 1). It can be seen in Curve No. 2 ($5 \cdot 10^{-4}$ M PLP + $9 \cdot 10^{-4}$ M SAM) and Curve No. 3 ($5 \cdot 10^{-4}$ M PLP + $5 \cdot 10^{-3}$ M SAM) that the PLP peak was depressed and red shifted by increased concentrations of SAM. The absorption spectrum obtained with the higher SAM concentration (Curve No. 3) exhibits one peak near 410 nm and another peak near 335 nm. A similar alteration was not observed when S-adenosylhomocysteine was substituted for SAM, or when the SAM was converted to 5'-methylthioadenosine by boiling[21]. In addition, pyridoxine exhibited no reaction with SAM.

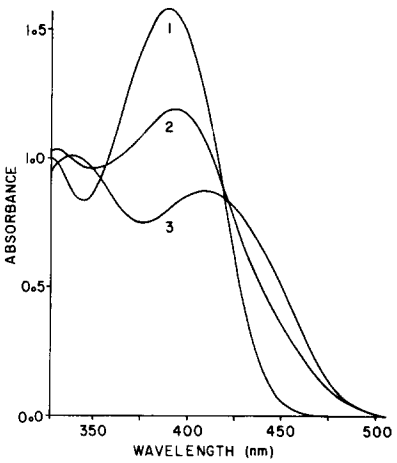

Fig. 1. Absorption spectrum of $5 \cdot 10^{-4}$ M PLP containing: (1) no SAM, (2) $9 \cdot 10^{-4}$ M SAM and (3) $5 \cdot 10^{-3}$ M SAM.

The time course of complex formation is shown in Fig. 2. The reaction between SAM and PLP, as measured by the change in absorbance at 388 nm, was complete within 30 s when incubated at 37 °C. Neither methionine nor adenosine (at concentrations equivalent to those used for SAM) had any effect on the PLP 388 nm peak, even when incubated with PLP at 37 °C for up to 1 h.

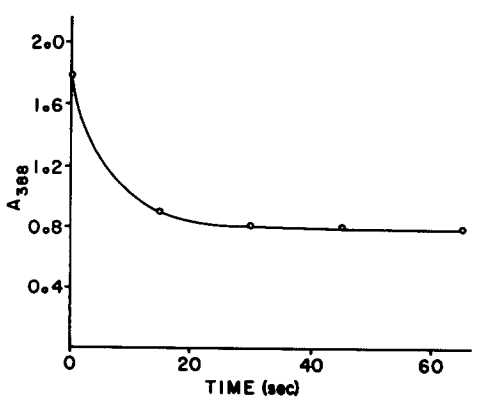

Fig. 2. Time course of complex formation between SAM and PLP. The change in absorbance at 388 nm was monitored when SAM was added to PLP. Incubation was at 37 °C, and the concentrations of SAM and PLP were $5 \cdot 10^{-3}$ M and $5 \cdot 10^{-4}$ M, respectively.

Inhibition of tyrosine aminotransferase in vitro

The interaction of SAM and PLP suggested the possibility of the sulfonium compound acting as a generalized inhibitor of PLP-dependent enzymatic reactions. To pursue this possibility further, tyrosine aminotransferase, a PLP-requiring enzyme unrelated to SAM biosynthesis, was assayed in the presence and absence of the prospective inhibitor. When low levels of PLP were used in the reaction mixtures along with $2 \cdot 10^{-3}$ M SAM, inhibition of up to 70% was obtained.

Enzyme kinetics were investigated to verify the mode of action by which SAM inhibits tyrosine aminotransferase. Using the method of Lineweaver and Burk[22], an apparent K_m for PLP of $1.2 \cdot 10^{-5}$ M was established. As can be seen in Fig. 3, with SAM concentrations between $5 \cdot 10^{-4}$ and $2 \cdot 10^{-3}$ M, inhibition of a competitive nature was obtained. Establishment of a K_I was accomplished with a Dixon plot[23], and a K_I for SAM of approx. $1 \cdot 10^{-3}$ M was apparent.

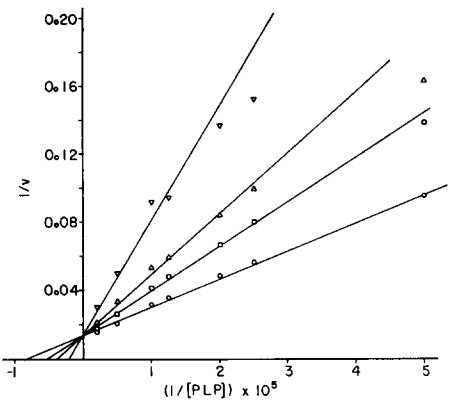

Fig. 3. Lineweaver–Burk plots of tyrosine aminotransferase at different SAM concentrations. ○, no SAM; □, $5 \cdot 10^{-4}$ M SAM; △, $1 \cdot 10^{-3}$ M SAM; ▽, $2 \cdot 10^{-3}$ M SAM. The results from the experiment presented here are representative of numerous enzyme assays.

Enzyme levels in cells grown in low and high methionine concentrations

Tyrosine aminotransferase. The levels of tyrosine aminotransferase were measured throughout the growth cycle for cells cultured in low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations. The results are presented in Fig. 4. As can be seen, tyrosine aminotransferase attained much higher levels in the slower growing (methionine inhibited) culture.

Ornithine aminotransferase. The levels of ornithine aminotransferase in both late exponential and early stationary phase cells are presented in Table I. The pattern indicated for ornithine aminotransferase suggests that it behaved much like tyrosine aminotransferase.

PLP and SAM levels in vivo

The PLP and SAM levels in *S. cerevisiae* were also determined at different periods during the growth cycle for cells grown in the two methionine concentrations. The results are presented in Table II. The levels observed during exponential growth appear to be relatively independent of the time of harvest, *i.e.* both the PLP

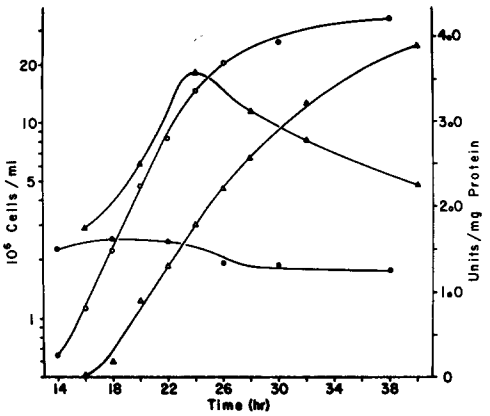

Fig. 4. Tyrosine aminotransferase activity in cells grown in medium containing low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations. ○, (cells/ml) × 10^6 in low methionine; △, (cells/ml) × 10^6 in high methionine; ●, tyrosine aminotransferase activity in cells grown in low methionine; ▲, tyrosine aminotransferase activity in cells grown in high methionine. Tyrosine aminotransferase activity is expressed in I.E. units, *i.e.* μmoles/min/mg protein.

TABLE I

ORNITHINE AMINOTRANSFERASE LEVELS IN CELLS GROWN IN MEDIUM CONTAINING LOW AND HIGH METHIONINE CONCENTRATIONS

Exponential represents late exponential phase and stationary represents early stationary phase. Approximately two doublings of a culture separate the two times. Enzyme activity is expressed in International Units, *i.e.* μmoles/min/mg protein.

Growth phase at time of harvest	*Methionine concr. in growth medium:*	*0.03 mg/ml (control) Enzyme activity*	*5.0 mg/ml Enzyme activity*	*% of control*
Exponential		$4.63 \cdot 10^{-4}$	$9.54 \cdot 10^{-4}$	206
Stationary		$4.61 \cdot 10^{-4}$	$6.19 \cdot 10^{-4}$	134

and SAM levels appear to be at relatively steady-state concentrations throughout exponential phase. Elevated levels of both compounds were observed in cells grown in high methionine concentrations, and the higher steady-state levels were apparently attained quite early as indicated by the lag phase values. It can also be seen in

TABLE II

PLP AND SAM LEVELS IN CELLS GROWN IN MEDIUM CONTAINING LOW AND HIGH METHIONINE CONCENTRATIONS

Methionine in growth medium (mg/ml)	*Growth phase at time of harvest*	*Cellular content (nmoles/g wet wt)*	
		PLP	*SAM*
0.03	Lag	13.3	1300
	Exponential	6.5	1100
	Stationary	25.0	1600
5.0	Lag	17.7	2100
	Exponential	18.9	1900
	Stationary	24.0	8600

Table II that the characteristic accumulation of SAM did not occur until stationary phase.

The PLP and SAM values obtained for lag and stationary phase cells were dependent on the exact time of harvest during the particular phase. For this reason, stationary phase cells were harvested late in stationary, and the values in the table represent the average of numerous determinations. However, the lag phase values represent specific experiments when the cells were harvested 3 h after inoculation. This time represents the approximate midpoint in lag phase with the conditions utilized (a 5% inoculum yielding an initial concentration of approx. $2 \cdot 10^6$ cells/ml).

DISCUSSION

The data presented establishes SAM as a potential inhibitor of PLP-dependent enzymatic reactions. The exact nature of the complex formed between SAM and PLP is not known, but the involvement of certain functional groups has been established. The fact that the interaction altered the PLP 388-nm peak and that pyridoxine was unreactive with SAM indicates that the aldehyde group on PLP is involved. The red shift of the 388-nm peak when SAM was added is characteristic of the formation of a Schiff base[6], so this would tend to implicate the amino group on the methionine moiety of SAM as being the group that interacts with the aldehyde on PLP. However, the fact that methionine did not cause a red shift of the PLP peak suggests that more than simple nonenzymatic Schiff base formation is involved. The fact that the depression of the 388-nm peak and the subsequent increase in the 330-nm range observed when SAM was added is characteristic of the formation of a ring compound[6] adds further evidence for the complexity of the interaction. This complexity was partially resolved by the failure of adenosine, 5'-methylthioadenosine, and *S*-adenosylhomocysteine to elicit a spectral alteration of the PLP peak, thereby implicating the sulfonium function of SAM. It seems likely that the sulfonium function may interact with PLP at some site other than the aldehyde group, and in doing so, it allows or accelerates the formation of the Schiff base.

Additional experimentation suggested that the binding strength of SAM to PLP may be relatively weak. The formation of the highly stable, covalently linked thiazine and thiozolidine (involving 1-amino-2-thio compounds) complexes requires many minutes when incubated at 37 °C (refs. 3 and 4), while the reaction between SAM and PLP was complete in less than 30 s. In addition, numerous chromatographic techniques utilized to isolate the complex have proven unsuccessful. All indications at this time are that the reaction may involve strictly an equilibrium process making isolation of the complex exceedingly difficult.

The competitive inhibition of tyrosine aminotransferase with regard to PLP is as expected for an SAM–PLP complex, and the fact that this enzyme which is unrelated to SAM biosynthesis is inhibited *in vitro*, establishes that SAM may act as a generalized inhibitor of PLP-dependent enzymatic reactions *in vivo*. Such inhibition has been reported for other PLP-binding compounds. Tyrosine aminotransferase has been studied quite extensively in mammalian systems, and along with numerous other PLP-dependent enzymes, has been shown to be inhibited by a wide variety of compounds which interact with PLP[3,4,24,25]. Most of the work has involved *in vitro* studies, but *in vivo* evidence of inhibition has also been obtained[3,4].

An important question that arises is whether or not SAM inhibition of PLP-dependent enzymatic reactions occurs *in vivo*, and if this could be the cause of methionine inhibition observed in yeast. A recent report mentioned that pyridoxine starvation of pyridoxine-deficient mutants of *E. coli* results in a substantial increase in the formation of PLP-dependent apoenzymes[26]. The data obtained in this study for the *in vivo* levels of tyrosine aminotransferase and ornithine aminotransferase are indicative of similar results being obtained for *S. cerevisiae* cultured in excess menthionine. The analogy between the two systems is recognizable quite readily if, in fact, the inhibition of PLP-dependent enzymatic reactions by SAM is occurring in the methionine inhibited yeast, *i.e.* SAM binding to PLP would be imposing a state of PLP starvation on the cells.

The higher level of perchloric acid extractable PLP in exponentially growing, methionine inhibited cells further supports the theorized mode of inhibition. If the additional SAM in these cells binds a portion of the PLP that normally would be precipitated with the protein (PLP tightly bound to protein typically remains bound when the protein is denatured[27,28]), the measurable PLP level could be higher. The additional SAM would not have to remove PLP which already was bound tightly to enzymes to get the observed increase in extractable PLP, but only compete effectively with the newly synthesized apoenzymes for a portion of the available PLP. In these terms, the additional 800 nmoles of SAM per g of cells must compete effectively for only 12 nmoles of PLP that normally would be removed during extraction to get the observed 3-fold increase in PLP. This represents almost 70 times more SAM than PLP, and considering the spectrophotometric results (Fig. 1) and rate of complex formation (Fig. 2), such a possibility is certainly feasible. The increase in the measurable level of PLP is not inconsistent with a state of PLP starvation being imposed on the cells, since the level of PLP available for enzymatic reactions would be decreased.

The increased levels of PLP and PLP-requiring enzymes *in vivo* suggest that SAM inhibition of PLP-dependent enzymatic reactions could play a role in methionine inhibition of yeast. Other, as yet unproposed, explanations can not be ruled out, but the fact that other PLP-binding compounds have been reported to have detrimental effects *in vivo*[3,4] adds credence to the proposed mode of inhibition.

ACKNOWLEDGMENTS

This research was supported by a U. S. Public Health Service grant (AM 11927-03) and a NDEA Title IV Fellowship award (R.W.T.).

REFERENCES

1 Botsford, J. L. and Parks, L. W. (1969) *J. Bacteriol.* 97, 1176–1183
2 Nakamura, K. D., Trewyn, R. W. and Parks, L. W. (1973) *Biochim. Biophys. Acta* 327, 328–335
3 Du Vigneaud, V., Kuchinskas, E. J. and Horvath, A. (1957) *Arch. Biochem. Biophys.* 69, 130–137
4 Pestaña, A., Sandoval, I. V. and Sols, A. (1971) *Arch. Biochem. Biophys.* 146, 373–379
5 Schirch, L. and Mason, M. (1962) *J. Biol. Chem.* 237, 2578–2581
6 Matsuo, Y. (1957) *J. Am. Chem. Soc.* 79, 2011–2015
7 Schlenk, F. and DePalma, R. E. (1957) *J. Biol. Chem.* 229, 1037–1050
8 Schlenk, F. and DePalma, R. E. (1957) *J. Biol. Chem.* 229, 1051–1057
9 Killick, K. A. (1971) *J. Bacteriol.* 106, 931–937

10 Yall, I., Norrell, S. and Knudsen, R. C. (1967) *J. Bacteriol.* 93, 1551–1558
11 Bailey, R. B. and Parks, L. W. (1972) *J. Bacteriol.* 111, 542–546
12 Wickerham, L. J. (1946) *J. Bacteriol.* 52, 293–301
13 Thompson, E. D. and Parks, L. W. (1972) *Biochim. Biophys. Acta* 260, 601–607
14 Lowry, O. H., Rosebrough, N. J., Farr, A. J. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
15 Granner, D. K. and Tomkins, G. M. (1970) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 17, pp. 633–637, Academic Press, New York
16 Jenkins, W. T. and Tsai, H. (1970) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 17, pp. 281–285, Academic Press, New York
17 Schlenk, F., Zydek, C. R., Ehninger, D. J. and Dainko, J. L. (1965) *Enzymologia Acta Biocatalytica* 29, 283–298
18 Chabner, B. and Livingston, D. (1970) *Anal. Biochem.* 34, 413–423
19 Bray, G. (1960) *Anal. Biochem.* 1, 279–285.
20 Peterson, E. A. and Sober, H. A. (1954) *J. Am. Chem. Soc.* 76, 169–175.
21 Schlenk, F. and DePalma, R. E. (1955) *Arch. Biochem. Biophys.* 57, 266–269.
22 Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658–666.
23 Dixon, M. (1953) *Biochem. J.* 55, 170–172.
24 Black, I. B. and Axelrod, J. (1969) *J. Biol. Chem.* 244, 6124–6129
25 Braunstein, A. E. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H. and Myrback, K., eds), Vol. 2, pp. 113–184, Academic Press, New York.
26 Wasmuth, J., Umbarger, H. E., and Dempsey, W. B. (1973) *Biochem. Biophys. Res. Commun.* 51, 158–164.
27 Storvick, C. A., Benson, E. M., Edwards, M. A., and Woodring, M. J. (1964) *Methods Biochem. Anal.* 12, 183–276.
28 Storvick, C. A. and Peters, J. M. (1964) *Vitam. Horm.* 22, 833–854.