Journal of Chromatography, 316 (1984) 43–52
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 496

ENZYMATIC ASSAY PROCEDURES THAT EMPLOY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: COMPETITION BETWEEN PHOSPHORIBOSYLTRANSFERASES FOR A COMMON SUBSTRATE

DONALD L. SLOAN*, LINDA Z. ALI, DIOGENESE AYBAR-BATISTA, CONG YAN and SUSAN L. HESS

Department of Chemistry and Program in Biochemistry, City College of the City University of New York, New York, NY 10031 (U.S.A.)

SUMMARY

A survey of the phosphoribosyltransferase (PRTase) activities in yeast has been accomplished using reversed-phase high-performance liquid chromatographic assay procedures. The following bases were observed to be utilized during phosphoribosyl pyrophosphate (PRibPP)-dependent nucleotide syntheses: adenine, xanthine, hypoxanthine, guanine, uracil, orotate, nicotinamide, nicotinate and quinolinate. Gradient elution procedures have also been perfected that allow the separation of the two following sets of PRTase assay components: (1) adenosine monophosphate, nicotinate mononucleotide, orotate, adenosine triphosphate, nicotinate, adenosine diphosphate, inosine monophosphate and hypoxanthine, and (2) nicotinate mononucleotide, nicotinamide mononucleotide, adenosine triphosphate, nicotinate, adenosine diphosphate and nicotinamide. Separation 1 has been employed to examine the PRibPP allocation among the hypoxanthine PRTase, orotate PRTase and nicotinate PRTase catalyzed reactions, whereas separation 2 has been employed to define the role that ATP plays in the nicotinamide PRTase-catalyzed reaction along with the allocation of nicotinamide between the reactions catalyzed by nicotinamide PRTase and nicotinamide deamidase.

INTRODUCTION

High-performance liquid chromatographic (HPLC) assay procedures provide the required sensitivity for detailed kinetic analyses of many enzyme-catalyzed reactions[1]. We have designed several assay procedures recently[2–5] that have been employed to characterize the kinetic mechanism of hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) and nicotinate phosphoribosyltransferase (N_aPRTase) purified from yeast. In addition, we have isolated yeast orotate phosphoribosyltransferase (OPRTase) and characterized the kinetic mechanism of this enzymatic reaction[6,7]. These three enzymes are part of a class of ten enzymes[8] that catalyze the transfer of the phosphoribosyl portion of phosphoribosyl α-1-pyrophosphate (PRibPP) to the appropriate nitrogenous base (Fig. 1).

0021-9673/84/$03.00

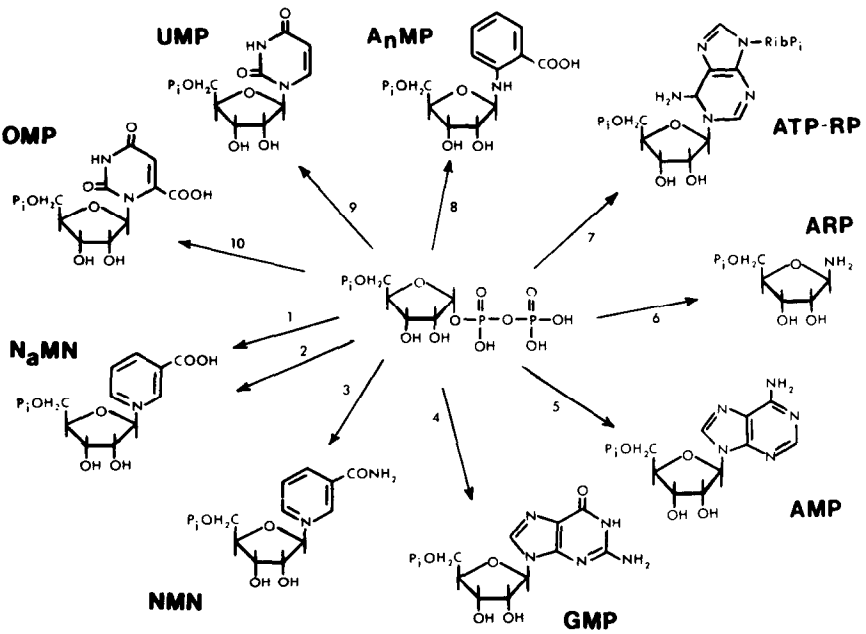

Fig. 1. Phosphoribosyltransferase (PRTase)-catalyzed reactions and the metabolic fate of phosphoribosyl α-1-pyrophosphate. The following enzymes are represented: (1) nicotinate PRTase, (2) quinolinate PRTase, (3) nicotinamide PRTase, (4) hypoxanthine-guanine PRTase, (5) adenine PRTase, (6) glutamine-dependent aminotransferase, (7) ATP-PRTase, (8) anthranilate PRTase, (9) uracil PRTase and (10) orotate PRTase.

In this paper, we report the results of a survey of various phosphoribosyltransferase activities in yeast that were detected with HPLC. In addition we have defined two competitive assay procedures. One method defines the competition among the OPRTase, N_aPRTase and HGPRTase catalyzed reactions for a limited concentration of PRibPP whereas a second method characterizes an allocation of nicotinamide between the reactions catalyzed by nicotinamide phosphoribosyltransferase (N_mPRTase) and nicotinamide deamidase. We believe that these latest procedures exemplify a major use of HPLC, namely, to monitor several related or coupled enzymatic reactions simultaneously.

MATERIALS AND METHODS

Materials

Bakers' yeast (Budweiser brand) was obtained from Valente Yeast Inc. (Flushing, NY, U.S.A.). PRibPP (sodium salt), hypoxanthine, inosine monophosphate (IMP), orotidine monophosphate (OMP), orotic acid, uracil, uridine monophosphate (UMP), nicotinamide, quinolinic acid, nicotinamide mononucleotide (NMN or N_mMN), adenosine triphosphate (ATP, disodium salt), nicotinate (free acid), nicotinate mononucleotide (NaMN), adenosine diphosphate (ADP, sodium salt) and triethanolamine were supplied by Sigma. All other chemicals were analytical grade.

Enzyme purification

HGPRTase, OPRTase and N_aPRTase were purified from bakers' yeast to apparent electrophoretic homogeneity through the use of published procedure[2,5,6]. N_mPRTase was purified partially from a yeast extract using $(NH_4)_2SO_4$ fractionation, whereas other phosphoribosyltransferases were examined by making use of this extract.

High-performance liquid chromatography

A Waters HPLC instrument equipped with a Model 6000A and a M-45 solvent delivery system, Model 660 solvent programmer, Model U6K sample injector, Model 440 absorbance detector, and a Houston Omniscribe chart recorder was used in the assay procedure. A single 30 cm × 3.9 mm Waters μBondapak C_{18} column (equilibrated with 15 m*M* $NH_4H_2PO_4$, pH 6.0) was placed on-line with the solvent delivery system at a flow-rate of 1.2 ml/min. Two gradient systems were used for the studies presented in this paper:

(1) A 20-min linear gradient ranging from 0% to 100% 25 m*M* $NH_4H_2PO_4$, pH 6.0, was used to separate the bases and nucleotides.

(2) The μBondapak C_{18} column was first equilibrated with 25 m*M* $NH_4H_2PO_4$ buffer, pH 6.0. Thereafter a linear gradient (from 0% to 100%) of a methanol–water (10:90) mixture was employed to separate the bases and nucleotides within a 20-min period. Samples (10 μl) from solutions containing the three enzymes and substrates were injected using a Hamilton 801 microliter syringe. Nucleotides and bases in the eluent were detected at 254 nm with a 0.1 absorbance setting. All of the solvents used in the chromatographic procedures were eluted by vacuum filtration through a 0.45-μm HA Millipore filter.

Enzymatic assay procedures

Measurements of the initial velocities of the HGPRTase, OPRTase and N_aPRTase catalyzed reactions were accomplished using modifications by Hanna and Sloan[4], and by Ali and Sloan[2] of the method described by Flaks[9]. The complete assay mixture consisted of 0.1 ml hypoxanthine (100 μ*M*), 0.1 ml orotate (100 μ*M*), 0.1 ml ATP (100 μ*M*), 0.1 ml nicotinate (100 μ*M*), 0.2 ml PRibPP (100 μ*M*, 40 μ*M*, or 20 μ*M*), 0.1 ml of 10 m*M* $MgCl_2$ and 0.5 ml of 20 m*M* triethanolamine buffer (pH 8.0) in a final volume of 1.2 ml. The mixture was placed in a 38°C water bath and the reaction was initiated by the addition of approximtely 0.01 μg HGPRTase, 0.3 μg OPRTase and 0.7 μg N_aPRTase. Aliquots of this solution were removed at appropriate time intervals and the reaction occurring in each of these aliquots was terminated by heating in a boiling water bath for 2 min. The samples were then clarified first by centrifugation and then by filtration through a 0.45-μm HA Millipore filter prior to HPLC injection. The HPLC elution profiles were employed to determine the time course of the reaction as described previously[2,4]. Variations on this basic assay mixture were also employed in which appropriate bases were either excluded or included. These changes are described in the figure legends.

RESULTS AND DISCUSSION

Survey of phosphoribosyltransferase activities in yeast

The use of reversed-phase HPLC and isocratic elutions with 10% methanol, allowed us to monitor most of the ten known phosphoribosyltransferases in protein extracts from yeast. Both PRibPP-dependent orotate and uracil utilizations were observed although OMP synthesis predominated under these conditions and the ability of this extract to synthesize UMP directly from uracil was lost over time. The UPRTase activity in yeast and its instability have been observed previously[10].

As shown in Fig. 2, N_aPRTase as well as N_mPRTase and QPRTase activities were all observed. These three enzymes were monitored by including ATP in each of the assay solutions (Fig. 2). Interestingly, very little QPRTase activity was observed under these conditions, whereas considerable concentrations of NMN and NaMN were synthesized. It was during this survey of the pyridine nucleotide productions, that we detected a PRibPP-independent nicotinamide-to-nicotinate transition (Fig. 2) and we have examined this reaction in greater detail (see below). As expected, myokinase activity was also observed in these crude extracts.

The PRibPP-dependent utilizations of several purine bases were characterized. Whereas hypoxanthine, guanine and xanthine are presumed to be substrates for HGPRTase[11], AMP production has been observed to occur through the use of a separate enzyme (APRTase) in yeast[12]. In our survey, the PRibPP-dependent syntheses of IMP, GMP, XMP and AMP were all observed, although relatively small concentrations of XMP and AMP were produced under the conditions of the assay.

Thus we have detected three pyridine-, four purine- and two pyrimidine-nucleotide synthetic reactions in yeast and we are currently isolating several of these newly detected enzymes.

Competititon between nicotinamide phosphoribosyltransferase and nicotinamide deamidase

The protein extract that was observed to contain the three pyridine phosphoribosyltransferases was fractionated through the use of $(NH_4)_2SO_4$ salting-out procedures. A redissolved 40–60% fraction was observed to contain both N_mPRTase and N_aPRTase activities but not to contain any detectable QPRTase. To our knowledge, this marks the first time that N_mPRTase has been detected in yeast. Moreover, we have observed that this enzyme is essentially independent of added ATP for activity (Fig. 3), in contrast to the yeast N_aPRTase-catalyzed reaction during which ATP is substrate[13] and in contrast with the N_mPRTase from mammalian sources which require ATP as an allosteric effector[14]. We observed also that nicotinamide is converted into nicotinate in the presence or absence of PRibPP and that a relatively small portion of nicotinamide reacts to form NMN under these conditions (Fig. 3). The HPLC assay procedure and separation of reactants that were achieved with a methanol–phosphate gradient elution, are illustrated in Fig. 4. These experiments thus provide a means by which the metabolic fate of pyridine bases may be characterized. Kinetic analyses over a range of base and nucleotide concentrations are underway currently.

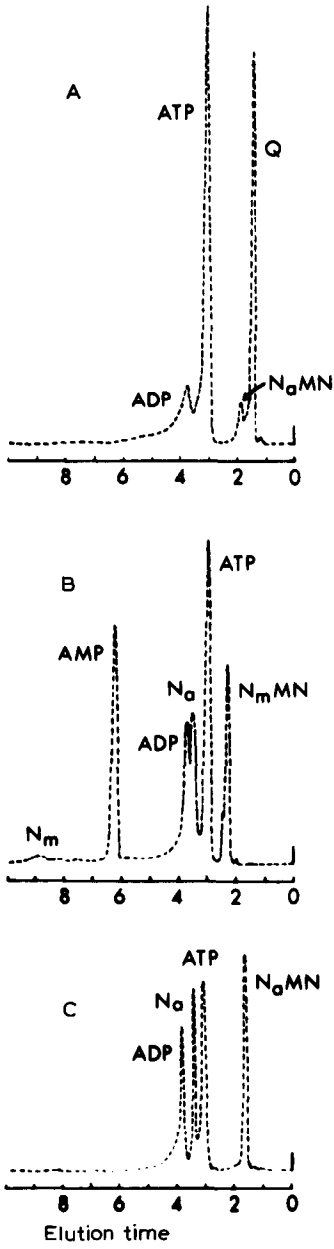

Fig. 2. HPLC assay procedures for the pyridine phosphoribosyltransferases (PRTase). The concentrations of the assay components in the incubation mixture were: 0.05 *M* Tris–phosphate buffer (pH 8), 1 m*M* $MgCl_2$, 1 m*M* ATP (when appropriate), 1 m*M* PRibPP, 1 m*M* nicotinamide or nicotinate or quinolinate and 100 μl of the protein extract. The final solution volume was 0.5 ml. (A) HPLC elution profile after incubation of the extract with quinolinate, (B) this profile after incubation with nicotinamide, (C) this profile after incubation with nicotinate. Elution conditions are as described in the Materials and Methods section. Elution times are in min.

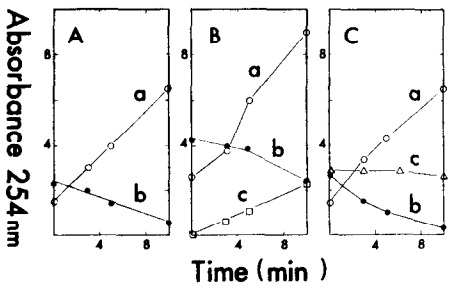

Fig. 3. Rate of appearances of nicotinate (A) and NMN (C) and rate of dissappearance of nicotinamide (B). The assay conditions and incubation mixture were as described in Fig. 2 except that: (A) PRibPP and ATP were not present, (B) 1 m*M* PRibPP was present but ATP was not present and (C) 1 m*M* ATP was present but PRibPP was not present.

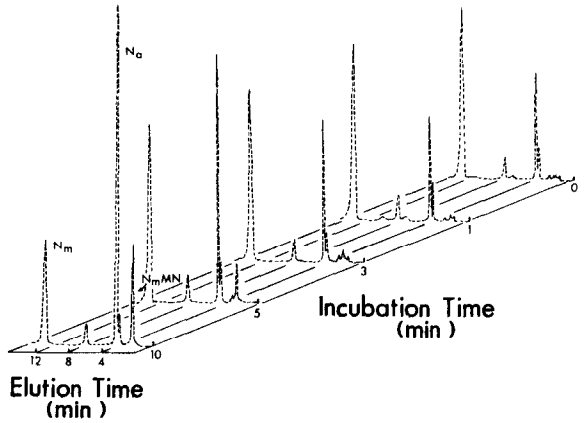

Fig. 4. Elution profiles of the incubation mixture defined in Fig. 2, which included nicotinamide but not ATP, over a 10-min time period. Elution conditions with a methanol–phosphate gradient are as described in the Materials and Methods section.

Competition for PRibPP by more than one phosphoribosyltransferase

The HGPRTase-catalyzed reaction has been characterized as being reversible but favoring phosphoribosyltransfer[15]. In contrast, the OPRTase-catalyzed reaction has been characterized as reversible but favoring PRibPP formation[6], and the N_aPRTase-catalyzed reaction is irreversible, primarily because of its concomitant ATPase activity[5]. In addition these three enzymes have very different specific activities (1300, 80 and 4.4 units/mg for HGPRTase, OPRTase and N_aPRTase respectively). In view of these dissimilarities, we have asked how PRibPP might be allocated among these three enzymatic reactions. Both the initial velocity and time-dependence measurements were to be accomplished.

A competition between HGPRTase and OPRTase was examined first. Listed in Table I are the initial velocities of these two enzyme-catalyzed reactions, where the enzymes appear in the incubation solutions together and separately. The OPRTase concentrations were chosen to be relatively high in order to compensate for the high specific activity of HGPRTase. As shown in Table I, at the lower PRibPP concen-

TABLE I

EFFECT OF THE PRESENCE OF HGPRTase AND OPRTase ASSAY COMPONENTS ON OPRTase AND HGPRTase ACTIVITIES, RESPECTIVELY

Experiment	*PRiPP concentration (μM)*					
	100	*40*	*20*	*100*	*40*	*20*
	OPRTase initial velocity (μmoles OMP/min)			*HGPRTase initial velocity (μmoles IMP/min)*		
pH 8 + OPRTase – HGPRTase 100 μM orotate	23	17	12			
+ OPRTase + HGPRTase 100 μM orotate	24	14	7			
– OPRTase + HGPRTase 100 μM hypoxanthine				36	29	19
+ OPRTase + HGPRTase 100 μM hypoxanthine				30	–	15
+ OPRTase + HGPRTase 100 μM hypoxanthine	24	8	3	29	20	15
pH 6 + OPRTase + HGPRTase 100 μM orotate 100 μM hypoxanthine	13	6	1	28	22	11

tration the presence of OPRTase alone has no effect on the HGPRTase-catalyzed synthesis of IMP, whereas the presence of HGPRTase alone inhibits the initial rate of OMP synthesis. Moreover, in the presence of the complete HGPRTase assay mixture, OMP synthesis is significantly inhibited whereas IMP synthesis is only slightly affected by the presence of all of the OPRTase assay components.

The time-dependent effects of one PRTase assay solution on another is illustrated in Fig. 5A and B. As expected, the major effect of the OPRTase assay solution is to slow the rate of IMP synthesis after a 10-min incubation. However, the effect of HGPRTase assay components is to reverse the synthesis of OMP until a new reduced equilibrium concentration is reached.

The competition between OPRTase and HGPRTase was repeated in the presence of 1/2 of the OPRTase and 1/5 of the HGPRTase concentrations utilized previously (Table II, Fig. 5A and B). As shown by the initial velocity values listed in Table II, the effect of the addition of nicotinate is to inhibit OMP synthesis both in the presence and absence of ATP. In contrast, the effect of ATP is to activate IMP synthesis. Finally, the effect of the addition of the complete N_aPRTase assay solution has no effect on the initial velocity of IMP synthesis but OMP synthesis is apparently

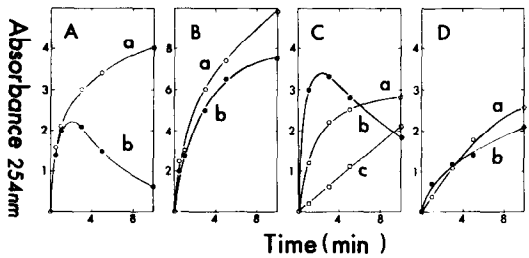

Fig. 5. Rate of appearance of the nucleotides AMP, IMP and NaMN from incubation mixtures defined in the Materials and Methods section and in Experiments 4 and 5 of Table II. (A) Rate of OMP synthesis from an incubation of OPRTase with its substrates in the absence of the complete HGPRTase assay mixture (a) and in the presence of this mixture (b). (B) Rate of IMP synthesis from an incubation of HGPRTase with its substrates in the absence of the complete OPRTase assay mixture (a) and in the presence of this mixture (b). (C) Rate of OMP synthesis from an incubation of OPRTase with its substrates in the absence of any other enzyme (a) and in the presence of both the N_aPRTase and HGPRTase assay mixtures (b). Also shown is the rate of NaMN synthesis (c) under these conditions. (D) Same as (C) except that the HGPRTase-catalyzed rate of IMP formation is illustrated.

TABLE II

EFFECTS OF THE PRESENCE OF N_aPRTase ASSAY COMPONENTS ON OPRTase AND HGPRTase ACTIVITIES

The PRibPP concentration in all experiments is 100 μM.

	Experiment	*Initial velocities*		
		OPRTase (μmoles OMP/min)	*HGPRTase (μmoles IMP/min)*	*N_aPRTase (μmoles NaMN/min)*
(1)	+ OPRTase + HGPRTase 100 μM orotate	11.6	4.3	–
(2)	+ OPRTase + HGPRTase 100 μM orotate 100 μM hypoxanthine 100 μM nicotinate	8.5	4.0	–
(3)	+ OPRTase + HGPRTase 100 μM orotate 100 μM hypoxanthine 100 μM ATP	11.0	8.0	–
(4)	+ OPRTase + HGPRTase 100 μM orotate 100 μM hypoxanthine 100 μM nicotinate 100 μM ATP	8.5	7.5	–
(5)	+ OPRTase + HGPRTase + N_aPRTase 100 μM orotate 100 μM hypoxanthine 100 μM nicotinate 100 μM ATP	27.5	7.5	2.8

activated. This activation is in fact due to an OPRTase contaminant in the N_aPRTase preparation. These two enzymes copurify until the last steps in the N_aPRTase isolation.

The time-dependent effects of the three enzyme assay solutions on one another is illustrated in Fig. 5C and D. The relatively slow synthesis of NaMN (and ADP) is linear and unaffected by the addition of the assay components of the other two enzymes. The effects of the OPRTase and N_aPRTase assay components on IMP synthesis is slight over a 10-min period (Fig. 5D). However, the presence of the N_aPRTase assay solution eventually reverses IMP synthesis toward a new equilibrium concentration. This effect is shown in Fig. 6 where the HPLC assay procedure and the component separation are also illustrated. Moreover, the presence of the N_aPRTase and HGPRTase assay solutions on OMP synthesis is to reverse this synthesis completely. As shown in Figs. 5C and 6, all of the orotate that was added initially is present again after 30 min. Thus OPRTase, which catalyzes the fastest rate of nucleotide synthesis initially, ultimately loses the competition for PRibPP to the irreversible N_aPRTase-catalyzed reaction. Presumably, IMP synthesis would be reversed as well over a longer period of time under these assay conditions. More detailed studies of these competitions have been initiated and kinetic equations which will define these effects quantitatively are being derived.

In conclusion, we have presented assay procedures which characterize the allocation of phosphoribosyltransferase substrates between more than one enzymatic reaction. The HPLC procedure may yet provide more detailed separations of the phosphoribosyltransferase substrates and products and a separation by HPLC of all of the reactants shown in Fig. 1 and would allow a characterization of the PRibPP allocations among all of the phosphoribosyltransferase-catalyzed reactions.

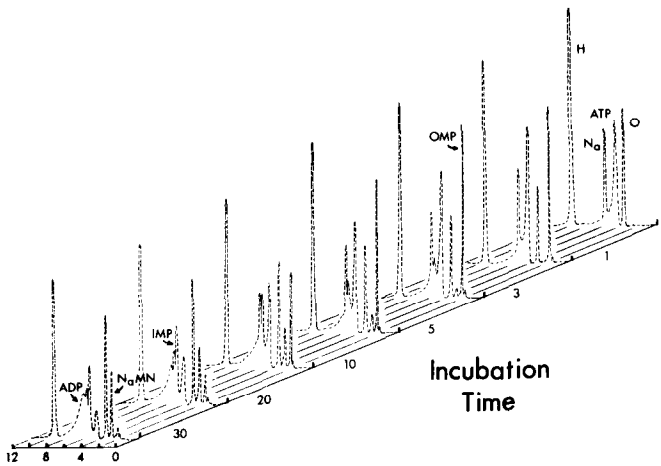

Fig. 6. Elution profiles of the incubation mixture defined in Fig. 5C which illustrates the simultaneous detection of the OPRTase, HGPRTase, N_aPRTase catalyzed reactions over a 30-min time period. Elution conditions with a phosphate gradient are as described in the Materials and Methods section. Elution and incubation times are in min.

ACKNOWLEDGEMENTS

This research was supported by NIH grant No. AM-20183 and by a grant from the City University of New York Research Foundation. We wish to acknowledge the contributions of Kareen Joseph and Sung-Hyun Chung who helped analyze the XPRTase and UPRTase activities.

REFERENCES

1 D. L. Sloan, *Advan. Chromatogr.*, 23 (1984) 97.
2 L. Z. Ali and D. L. Sloan, *J. Biol. Chem.*, 257 (1982) 1149.
3 L. Z. Ali and D. L. Sloan, *Biochemistry*, 22 (1983) 3419.
4 L. S. Hanna and D. L. Sloan, *Anal. Biochem.*, 103 (1980) 230.
5 L. S. Hanna, S. L. Hess and D. L. Sloan, *J. Biol. Chem.*, 258 (1983) 9745.
6 J. Victor, L. Greenberg and D. L. Sloan, *J. Biol. Chem.*, 254 (1979) 2647.
7 J. Victor, A. Leo-Mensah and D. L. Sloan, *Biochemistry*, 18 (1979) 3597.
8 W. D. L. Musick, *CRC Crit. Rev. Biochem.*, 11 (1981) 1.
9 J. B. Flaks, *Methods Enzymol.*, 6 (1963) 144.
10 P. Natalini, S. Ruggieri, I. Santarelli, A. Vita and G. Magni, *J. Biol. Chem.*, 254 (1979) 2558.
11 L. Z. Ali and D. L. Sloan, unpublished results.
12 M. Nagy and A. M. Ribet, *Eur. J. Biochem.*, 77 (1977) 77.
13 A. Kosaka, H. O. Spivey and R. I. Gholson, *Arch. Biochem. Biophys.*, 179 (1977) 334; and references therein.
14 M. C. Powanda, O. Muniz and L. S. Dietrich, *Biochemistry*, 8 (1969) 1869.
15 A. Kornberg, I. Lieberman and E. S. Simms, *J. Biol. Chem.*, 215 (1955) 417.