A DIRECT SPECTROPHOTOMETRIC ASSAY FOR ARGINASE

by

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Approved by:

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Dr. M. Friedman, Coordinator
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Dear Dr. Friedman,

I submit the accompanying thesis entitled A Direct Spectrophotometric Assay for Arginase as the final requirement of the University Undergraduate Fellows Program.

This report presents the plan of development for a simple and accurate direct spectrophotometric assay for arginase. The report may be helpful to persons studying steady-state kinetics of arginase.

I would like to thank you, and other persons involved with the Undergraduate Fellows Program for giving me an opportunity to take part in such a valuable program.

Sincerely,
Andres L. Buonanno
Andres L. Buonanno

ACKNOWLEDGMENTS

I wish to thank Dr. C.N. Pace for his technical assistance, and thank him for the many hours he spent in helping me understand parts of this project.

ABSTRACT

This report describes the develpment of a direct, spectrophotometric assay for arginase. The assay may be conveniently used at high substrate concentrations (8Km) for assaying crude sources of arginase, and for steady-state kinetic studies of the enzyme. The assay has the advantage over other conventional colorimetric assays, in that it is fast and accurate.

LIST OF ILLUSTRATIONS

Figures

Tables

TABLE OF CONTENTS

A DIRECT SPECTROPHOTOMETRIC ASSAY FOR ARGINASE *

INTRODUCTION

Liver enzymes are of special interest when studying mechanisms and control of enzyme degradation, since 40% of the enzymes in the liver are replaced daily. Arginase, an enzyme found in the liver of ureotelic organisms, proves to be a good model for studying protein turnover since it has been shown that its rate of degradation in the rat liver may be varied considerably depending on the amount of protein in the rat's diet (1). One major problem in working with arginase, is that there is no convenient and accurate assay for the enzyme.

Arginase catalyses the cleavage of arginine to ornithine and urea. Many indirect, colorimetric techniques have been developed for assaying arginases, the most popular of these measures the formation of urea (2). The problem with these assays is that they require a 30 (3) or 60 (4) minute boiling step and that the colored complexes formed are light labile, thus requiring special precautions during the assays. The variation of Michaelis constants reported for bovine liver arginase, ranging from 2.6 (5) to 7.0 mM (6), are possibly a reflection of the assays available. Ward and Srere reported a direct, spectrophotometric assay for arginase based on the difference in absorption between products and reactants at 205.7 nm. The assay is useful for routine assays, but it may not be used for steady-state kinetic studies for arginase since it can only be used at substrate concentrations of approximately 15% Vmax (less than 2mM). This report is an extension of Ward andSrere's method which allows it to be used on cruder sources of arginase, and for studying the steady-state kinetics of arginase.

*This theses follows the format recommended by the Journal of Biological Chemistry.

EXPERIMENTAL TECHNIQUES

Materials

'Grade A' hydrochlorides of L-arginine (Lot# 700326) and L-ornithine (Lot# 601361) were purchased from Calbiochem, and 'Ultra Pure' urea (Lot# W1094) was purchased from Schwarz/Mann. Beef liver arginase was purchased from Boehringer Manheim (Lot# 1276508/1) and Worthington Biochemical Co.(Lot# AR36C869).

All spectral measurements were done with a Cary Model 15 recording spectrophotometer using quartz cuvettes(pathlength 1.0 cm) made by Pyrocell Manufactoring Co.. All pH measurements were taken with a Radiometer Model 26 pH meter.

Methods

Solutions of 0.5mM L-arginine,.2mM L-ornithine, and 50mM urea were set to pH 9.5 and pH 7.5 with 0.1M NaOH. These solutions were scanned between 215 and 204nm at a fixed slitwidth of 0.5mm.

RESULTS

The absorption spectra of arginine, ornithine, and urea at pH 9.5 are shown in Figure 1. The difference absorption coefficients, $\Delta\varepsilon = \varepsilon_{ARG} - \varepsilon_{ORN} - \varepsilon_{UREA}$,are also shown and can be seen to increase sharply below 215nm. Consequently, the absorbance change accompanying the conversion of arginine to ornithine plus urea will be maximized by using the lowest possible wavelength.

In a typical assay we used 2.5ml of L-arginine solution with concentrations ranging from 0.10 to 15.0mM. The solution had been adjusted to a pH of 9.5 with 0.1MNaOH. To initiate the reaction approximately one unit of arginase was added to the substrate. A wavelength was selected so that the absorbance was still in the usable range of the spectrophoto-

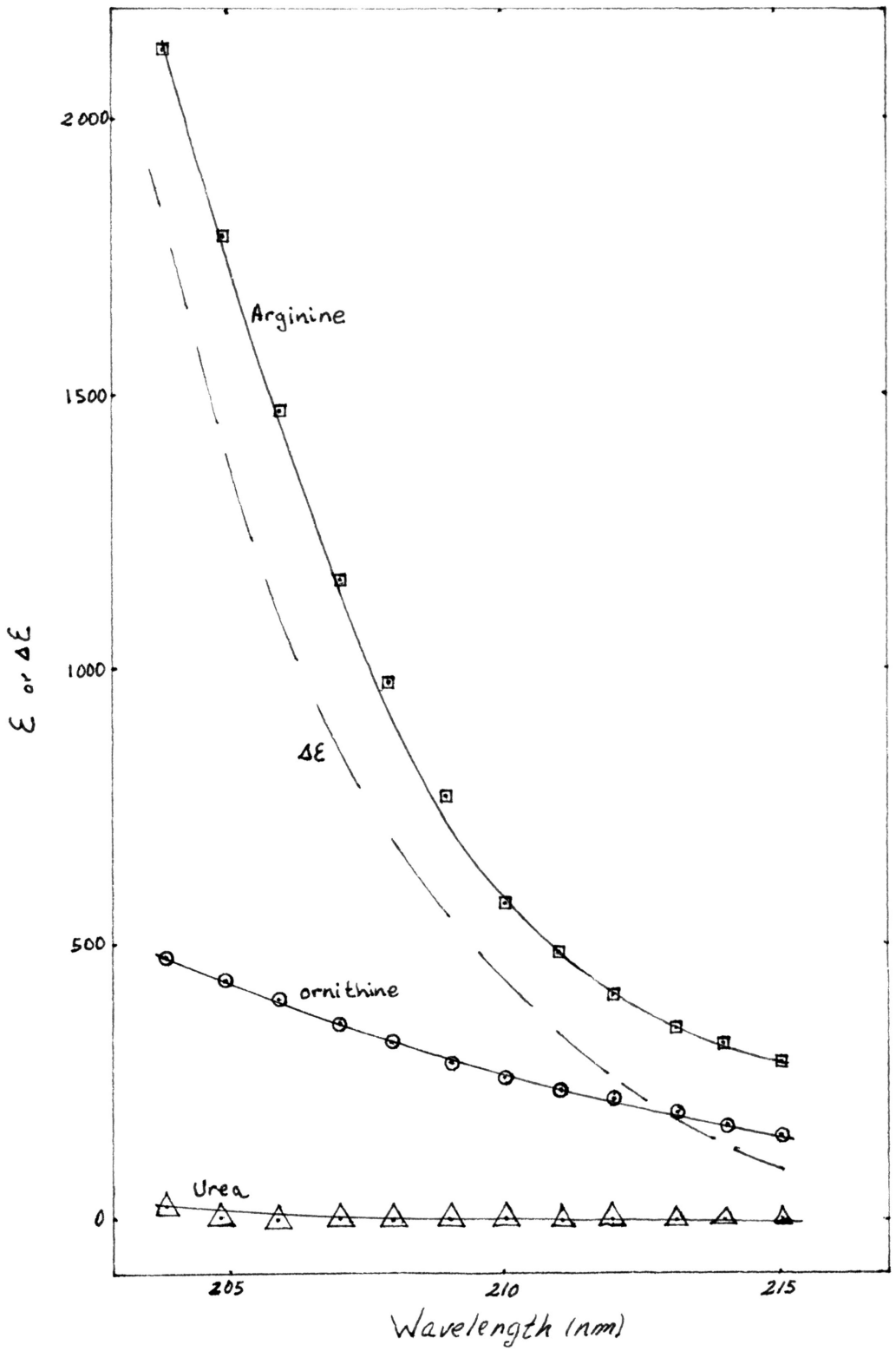

Figure 1. Individual Absorption Spectra at pH 9.5.

TABLE I. Molar Absorption Coefficients for Arginine, Ornithine, and Urea at pH 9.5 and pH 7.5.

Wavelength	Molar Absorption Coefficient[a]			$\Delta\varepsilon$ [b]
	Arg	Orn	Urea	
				pH 9.5
215nm	239.5	139.2	2.6	98
214	277.6	158.0	2.9	117
213	333.7	175.3	3.3	155
212	395.8	192.8	3.8	199
211	478.9	213.1	4.5	261
210	593.2	236.0	5.4	352
209	734.4	260.1	6.7	468
208	914.8	286.4	8.4	620
207	1140.3	314.2	10.6	816
206	1420.8	345.2	13.7	1062
205	1754.0	375.4	17.7	1361
204	2193.4	411.7	23.7	1758
				pH 7.5
215nm	78.52	35.95	1.02	41.55
214	103.17	41.06	1.25	60.87
213	133.54	46.69	1.50	85.35
212	177.34	52.54	1.91	122.89
211	234.75	58.70	2.35	173.70
210	314.48	65.68	3.24	245.56
209	423.07	74.40	4.33	344.34
208	564.46	81.24	5.80	477.42
207	740.81	89.96	7.87	642.98
206	972.49	99.16	10.69	862.64
205	1264.3	108.88	14.72	1140.7
204	1615.4	119.40	20.33	1475.7

[a] liter $mole^{-1} cm^{-1}$

[b] $\Delta\varepsilon = \varepsilon_{Arg} - \varepsilon_{Orn} - \varepsilon_{Urea}$

meter once the enzyme was added. The absorbance due to the enzyme solution must be determined independently, since its absorption depends on the source of arginase. For example, from the data shown in Figure 2 the initial velocity at the highest substrate concentration (10.3mM) was determined at 215nm where the absorbance was 2.47, while the initial velocity at the lowest substrate concentration (0.41mM) was determined at 204nm where the absorbance was 0.90. Note that if the 10.3mM solution was run at 204nm, it would have an absorbance of 22.6; for this reason the initial velocity was determined at 215nm.

Calculating Initial Velocities and the Michaelis Constant

Absorbance was recorded for about one minute after the enzyme was added to the substrate. Initial velocities were determined by measuring the absorbance change during the first 30 seconds of the reaction. These rates were converted to initial velocities expressed as substrate concentration per time, by dividing by the $\Delta\varepsilon$ values given in Table 1. Initial velocities were measured to better than ± 4% at the highest substrate concentration, to better than ± 2% at substrate concentrations near Km (2.1mM), and only at the lower substrate concentrations was the uncertainty over ± 5%.

Michaelis constants were obtained from Lineweaver-Burk plots such as that shown in Figure 2. The average Michaelis constant from six independent determinations using two sources of arginase was 2.10 ± 0.16 mM.

Comparison of Results Between Colorimetric and Direct, Spectrophotometric Assays

The arginase reaction was followed using Archibalds' colorimetric urea assay (4) and our direct, spectrophotometric assay in order to compare results obtained by both methods. The results, presented in Figure 3, show the close

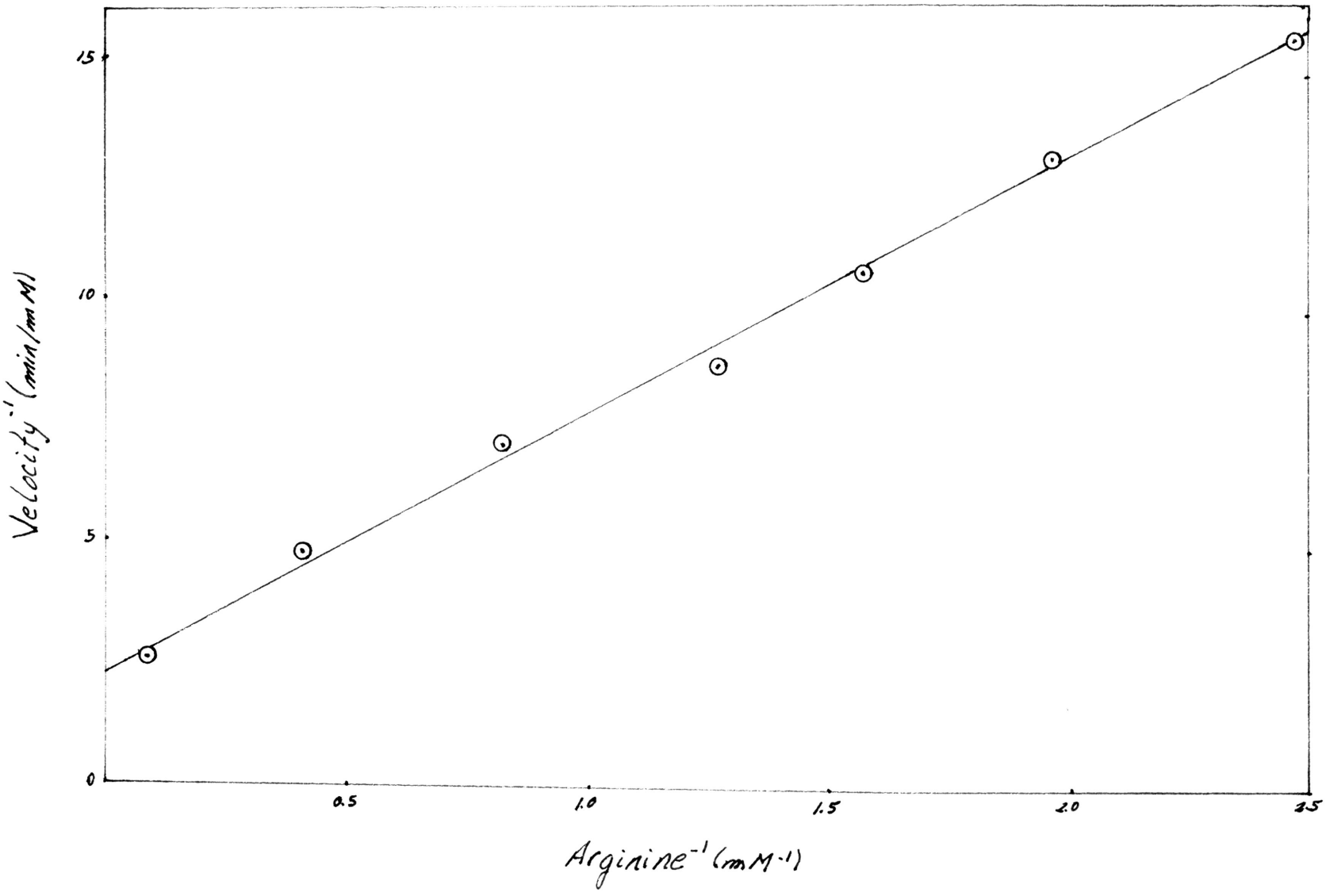

Figure 2. Lineweaver-Burk Plot for Arginase at pH 9.5.

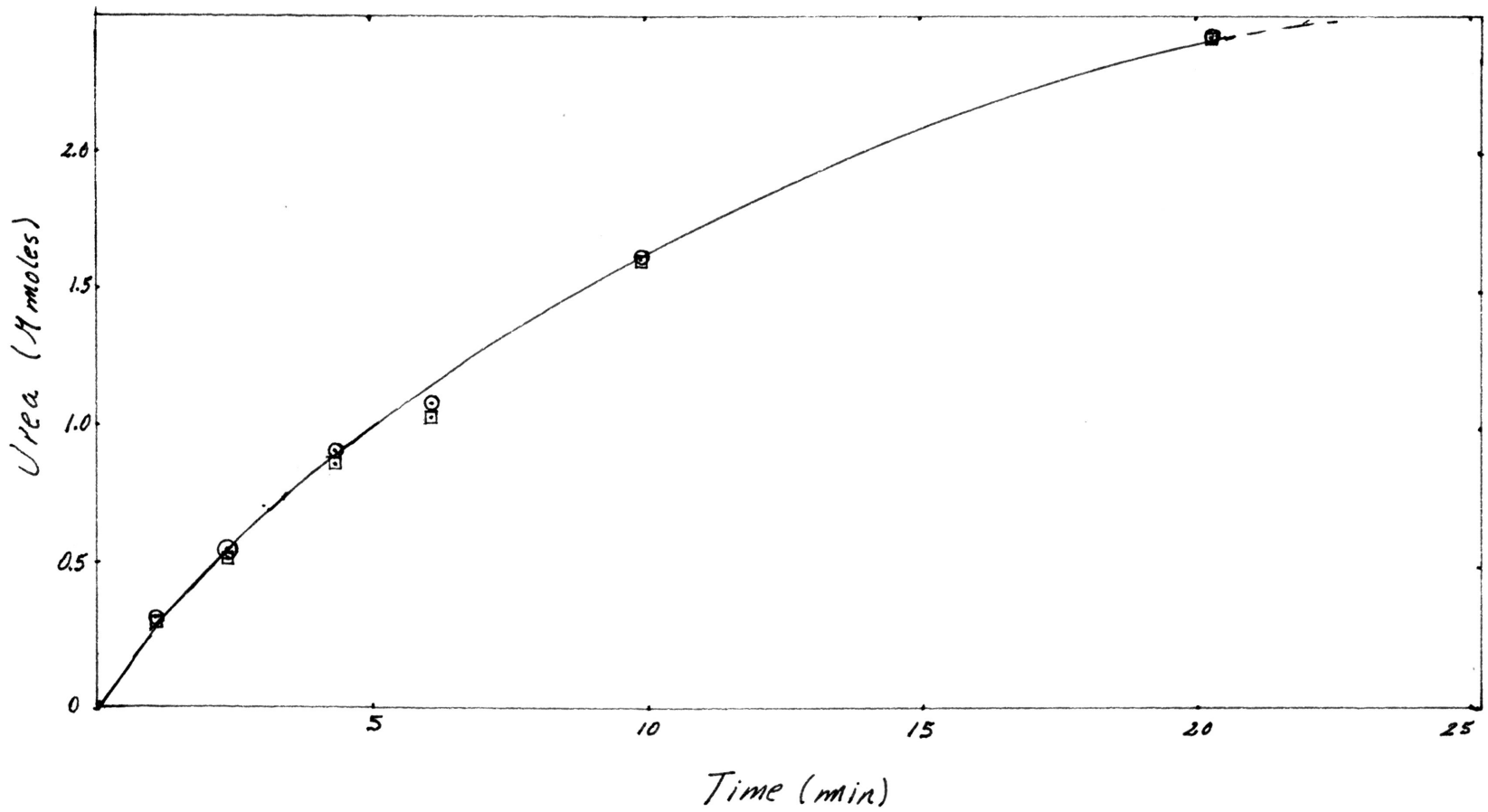

Figure 5. Comparison of Colorimetric and Direct Assay of Arginase at pH 9.5.

agreement between the two assays. The direct assay was done at 211nm with an arginine concentration of 0.4mM, but at intermediate substrate concentrations several different wavelengths can be used with equivalent results.

DISCUSSION

In determining the Michaelis constant and the maximal velocity for an enzyme catalyzed reaction, Clealand (8) recommends the initial velocities to be measured over a substrate range from 0.2Km to 5Km. The value of $\Delta\varepsilon$ at 215nm is large enough to follow the kinetics of arginase reaction and at this wavelength substrate concentrations of about 8Km can be used. At 205.7nm, the wavelength recommended by Ward and Srere (7), the highest substrate concentration which can be used is approximately equal to Km. It is clear from Figure 1 that we could have gotten larger values of $\Delta\varepsilon$ by extending the studies to lower wavelengths. This was not done, however, since substrate concentrations as low as 0.05Km can be run at 204nm. Thus, steady-state kinetic studies of arginase can be conveniently carried out using the data in Table1 and the approach described in this paper.

A major disadvantage of the assay described here is that it is pH dependent; the absorption spectra of both arginine and ornithine vary with pH. To apply this assay at different pH's requires redetermining the absorption spectra at the pH of interest. Fortunately, hepatic arginases have a wide range of pH optima, occuring between pH 9.3 and 10.5 (9).

Borate seemed like an ideal buffer since it has good buffering capacity at pH 9.5, and unlike most buffers, it absorbs very little at the wavelength range being used. But borate can not be used in the assay, since it is a potent inhibitor of arginase (10). Although, the pH of the mixture decreases as the reaction proceeds, the decreased occuring during the time required to measure initial velocities is

not significant.

In recent studies we have tried to apply our assay to study the role of manganese in the activation of arginase. There were difficulties in applying the assay at pH 9.5, in that at high pH's manganese oxides are formed. The brown, manganese oxide precipitate absorbs light, thus it interferes with the spectrophotometric assay.

Values for the difference molar absorption coefficients were determined at pH 7.5, since manganese oxides are not formed at this pH (refer to Table 1). The disadvantage in working at a pH of 7.5 is that the activity of arginase is at best about 40% of that at a pH of 9.5 (11). This, plus the fact that the difference absorption coefficients are lower leads to a less sensitive assay for arginase at pH 7.5. Thus far we have not obtained useful data concerning the role of manganese in the activation of arginase.

SUMMARY

The direct, spectrophotometric assay reported here is of general applicability, and is faster and more accurate than the conventional colorimetric procedures generally used for assaying arginase.

REFERENCES

1. Schimke, R.T. (1964). J. Biol. Chem. 239, 3808-3817.

2. Archibald, R.M. (1945). J. Biol. Chem. 157, 507-517.

3. Schmke, R.T. (1970). Methods Enzymol., XVIIA, 313-317.

4. Archibald, R.M. (1945). J. Biol. Chem. 157, 507-517.

5. Roholt, O.A.,Jr., and Greenberg, D.M. (1956). Arch. Biochem. Biophys., 62, 454-470.

6. Buch, S.J., and Killip, J.D. (1960). Biochem. J., 77, p7.

7. Ward, R.L., and Srere, P.A. (1967). Anal. Biochem., 18,102-106.

8. Cleland, W.W. (1967). Adv. Enzymol. 29, 1-33.

9. Hirsch-Kolb, H., Heine, J.P., Kolb, H.J. and Greenberg, D.M. (1970). Comp. Biochem. Physiol., 37, 345-359.

10. Kaysen, G.A., and Strecker, H.J. (1973). Biochem. J., 133, 779-788.

11. Mohamed, M.S., and Greenberg, D.M. (1945). Arch. Biochem., 8, 349.