AN ABSTRACT OF THE THESIS OF

JOHN HUNTINGTON LEETE for the degree DOCTOR OF PHILOSOPHY
(Name) (Degree)

in BIOCHEMISTRY AND BIOPHYSICS presented on July 9, 1974
(Major department) (Date)

Title: THE INFLUENCE OF MONOVALENT CATIONS ON TRYPTOPHANASE AND PYRUVATE KINASE AS STUDIED BY FLUORESCENCE, CIRCULAR DICHROISM, AND KINETIC METHODS

Abstract approved: Redacted for Privacy
Robert R. Becker

Redacted for Privacy
Harold J. Evans

Two spectrally different species of holotryptophanase were obtained from fluorescence and circular dichroism measurements. One is predominant in solutions containing certain enzyme activating monovalent cations while the other predominates in solutions of various inhibiting monovalent cations. Relative magnitudes of the cation sensitive fluorescent intensity maxima or circular dichroism maxima are approximately proportional to the effectiveness of the monovalent cations as activators or inhibitors of tryptophanase activity.

The tryptophanase coenzyme, pyridoxal 5'-phosphate, is required in order to observe any monovalent cation effects on the enzyme's fluorescence or circular dichroism spectra. Fluorometric titrations indicated that the coenzyme binds to apotryptophanase more tightly in the

presence of K^+ or NH_4^+ than in Na^+ solutions. Similarly, low temperature circular dichroism measurements of the holoenzyme indicated that the coenzyme dissociated more readily in Na^+ than in K^+ systems.

Activating and inhibiting monovalent cations had essentially similar effects on the binding of the substrate analog, $1,N^6$-ethenoadenosine diphosphate (εADP), to pyruvate kinase. Likewise, no differential cation affects were observed for the binding of indole to apotryptophanase.

Cation effects on the fluorescence anisotropy of tryptophan residues was found to be negligable for all enzymes tested except pyruvate kinase. Addition of activating as well as inhibiting cations to solutions of pyruvate kinase resulted in nearly identical increases in tryptophan anisotropy. It was demonstrated that the anisotropy increase was not caused by a decrease in energy transfer between tryptophan residues.

Monovalent cation binding site models are discussed. Evidence in support of allosteric models is lacking. The current results as well as recent work reported in the literature support a functional role for the activating monovalent cation at the pyruvate kinase or tryptophanase active site.

The Influence of Monovalent Cations on Tryptophanase and Pyruvate Kinase as Studied by Fluorescence, Circular Dichroism, and Kinetic Methods

by

John Huntington Leete

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed July 9, 1974

Commencement June 1975

APPROVED:

Redacted for Privacy

Professor of Biochemistry

and

Redacted for Privacy

Professor of Botany

jointly in charge
of major

Redacted for Privacy

Chairman of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

Date thesis is presented July 9, 1974

Typed by Darlyne Leete for John Huntington Leete

ACKNOWLEDGEMENT

My thanks are due to my joint major professors, Dr. Robert R. Becker and Dr. Harold J. Evans, for constant encouragement and helpful discussion.

My thanks are also due to Drs. Irvin Isenberg, Kensol Van Holde, and Sonia Anderson for the use of their instruments and for stimulating discussions, and Mr. Robert Howard for practical guidance in the laboratory.

This study was supported by Research Grant AM-08123-11 from the U.S. Public Health Service and Research Grant AT(45-1)2227 from the Atomic Energy Commission.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

THE INFLUENCE OF MONOVALENT CATIONS ON TRYPTOPHANASE AND PYRUVATE KINASE AS STUDIED BY FLUORESCENCE, CIRCULAR DICHROISM, AND KINETIC METHODS

Part A: COENZYME DEPENDENT CATION EFFECTS ON TRYPTOPHANASE

I. SUMMARY

The alkali metal cations and various multiatom monovalent cations were found to have differing effects on the fluorescence spectra, circular dichroism spectra, and enzyme kinetics of holotryptophanase from Escherichia coli. Cation induced differences were not observable for apotryptophanase indicating that the coenzyme, pyridoxal 5'-phosphate, plays an active role in the physical expression of cation effects. Morino and Snell have shown that 0.02 M imidazole-HCl (pH 8.0) solutions of holotryptophanase have an absorbance maximum at about 337 nm in the presence of 0.1 M KCl and a maximum at 420 nm in solutions of 0.01 M NaCl (1). The relative effectiveness of the various alkali metal cations to induce changes in the 340 nm absorbance or 340 nm fluorescence excitation with 385 nm emission of the holoenzyme was reversed with either 420 nm absorbance or 420 nm excitation and 515 nm fluorescence emission. No such reversal of salt effects with change in wavelength was found with several multiatom monovalent cations. Ammonia was unique in its effect upon the fluorescence and circular dichroism maxima for the 340 nm and 420 nm absorbing

species of holotryptophanase. The unique effect of NH_4^+ is consistent with its dual function as an activator in tryptophan degradation (2) and as a substrate in tryptophan synthesis (3).

Evidence for competitive inhibition of K^+ activation of tryptophanase by Na^+ was observed. The effectiveness of several cations to inhibit the K^+ activated enzyme was paralleled by their effectiveness to induce a fluorescence or circular dichroism maxima in the 420 nm absorbing species.

A temperature decrease from 22° to 5° was shown to weaken the binding of the coenzyme to apotryptophanase when in Na^+ solution but was found to have much less effect on binding in K^+ solution. Essentially no monovalent cation effects and only small temperature effects on enzyme helical structure were measured by circular dichroism spectra. A possible binding site for an activating cation which is consistent with the effects observed was suggested between apotryptophanase and coenzyme.

II. INTRODUCTION

Tryptophanase from Escherichia coli B/1t7-A requires both a coenzyme, pyridoxal phosphate (pyridoxal-P)[1], and a monovalent cation, either K^+, Rb^+, or NH_4^+ (2, 4) for activity. Na^+ does not activate holotryptophanase (4). Professor Esmond E. Snell's laboratory has carried out extensive studies on this enzyme from the isolation of the crystalline apoenzyme (4) to partial sequencing of the enzyme (5). Some of these studies showed that absorbance spectra of the holoenzyme were pH dependent and that the low pH (<pH 6.7) spectra obtained in 0.1 M KCl were similar to absorbance spectra obtained at pH 8 in 0.1 M NaCl solutions (1). The spectra of the low pH form and the Na^+ form at pH 8.0 are ascribed to an inactive form of holotryptophanase. Other studies by Snell's group indicated that at 4° in 0.02 M Tris-HCl (pH 8.0) the tetrameric active form of holotryptophanase was stabilized by 0.1 M K^+ but dissociated to inactive dimers in 0.1 M Na^+ (6).

The current studies were designed to reveal more information about the location of monovalent cation binding to the holoenzyme and about how the activating cation functions in the degradation of tryptophan. The comparative effects of monovalent cations on the binding of pyridoxal-P to apoenzyme and on tryptophan residue orientation

[1]Pyridoxal 5'-phosphate is abbreviated pyridoxal-P.

were investigated using the method of fluorescence anisotropy (see "Part B"). Effects of various monovalent cations on tryptophanase fluorescence spectra, circular dichroism spectra, and kinetics of tryptophan degradation are reported in "Part A". Experiments using multiatom univalent cations to estimate the number and orientation of binding site ligands (7) were complicated by the tendency of pyridoxal-P to react with some of the nitrogen containing cations (8).

III. EXPERIMENTAL PROCEDURE

Materials

Fluorimetric grade imidazole, pyridoxal 5'-phosphate, bovine serum albumin, and lactic dehydrogenase were purchased from Sigma Chemical Company. The monohydrochloride salts of methylamine, dimethylamine, guanidine, hydrazine, hydroxylamine, formamidine, and the sulfate of hydroxyguanidine were a gift from Professor George Eisenman. Other chemicals were of reagent grade.

Disc Gel Electrophoresis

The procedure and equipment used were previously described by Howard and Becker (9) and are essentially the same as those of Davis (10).

Preparation of Tryptophanase

Slant cultures of E. coli B/1t7-A were a gift from Professor Esmond E. Snell. Tryptophanase was prepared on a large scale as previously described by Watanabe and Snell (3) with the following modifications: (a) Wet cells, 208 grams obtained from 103 liters of medium, were washed in 400 ml of 0.1 M potassium phosphate, 2 mM EDTA, 0.2 mM dithiothreitol buffer (pH 7.0) and frozen in plastic bags until needed for preparation. (b) The active precipitate from the second ammonium sulfate fractionation was

dissolved in 0.06 M potassium phosphate, 1 mM EDTA, 0.1 mM dithiothreitol, 0.04 mM pyridoxal-P and passed through a 2x80 cm Sephadex G-200 column (4) to remove several faint bands noted by acrylamide gel electrophoresis. Tryptophanase was applied to a 1.5x5 cm DEAE-cellulose column equilibrated with the same buffer (2). Apotryptophanase was crystallized by the published method (3) to a final specific activity of 28 to 35 μmoles pyruvate formed per mg of enzyme per min at 37°. The purified enzyme was essentially homogeneous as judged by acrylamide gel electrophoresis (Figure 1) and the amino acid analysis was consistent with previously reported analyses (Table I).

Tryptophanase Assay

Enzyme activity was measured (in μmoles product formed per mg of protein per min) either by determining pyruvate production from L-tryptophan with DPNH and lactate dehydrogenase (13, 14) or by measuring indole production using ρ-dimethylamino-benzaldehyde in Newton and Snell's version of the Ehrlich test (15). Pyruvate assays were routinely carried out in 1.5 ml cuvettes in a Cary 11 recording spectrophotometer with 0-0.1 slide wire and a 37° water jacketed cuvette compartment. The following modifications to the lactic dehydrogenase coupled assay gave higher apparent specific activities than reported by Watanabe and Snell (3): (a) Tryptophanase was incubated in

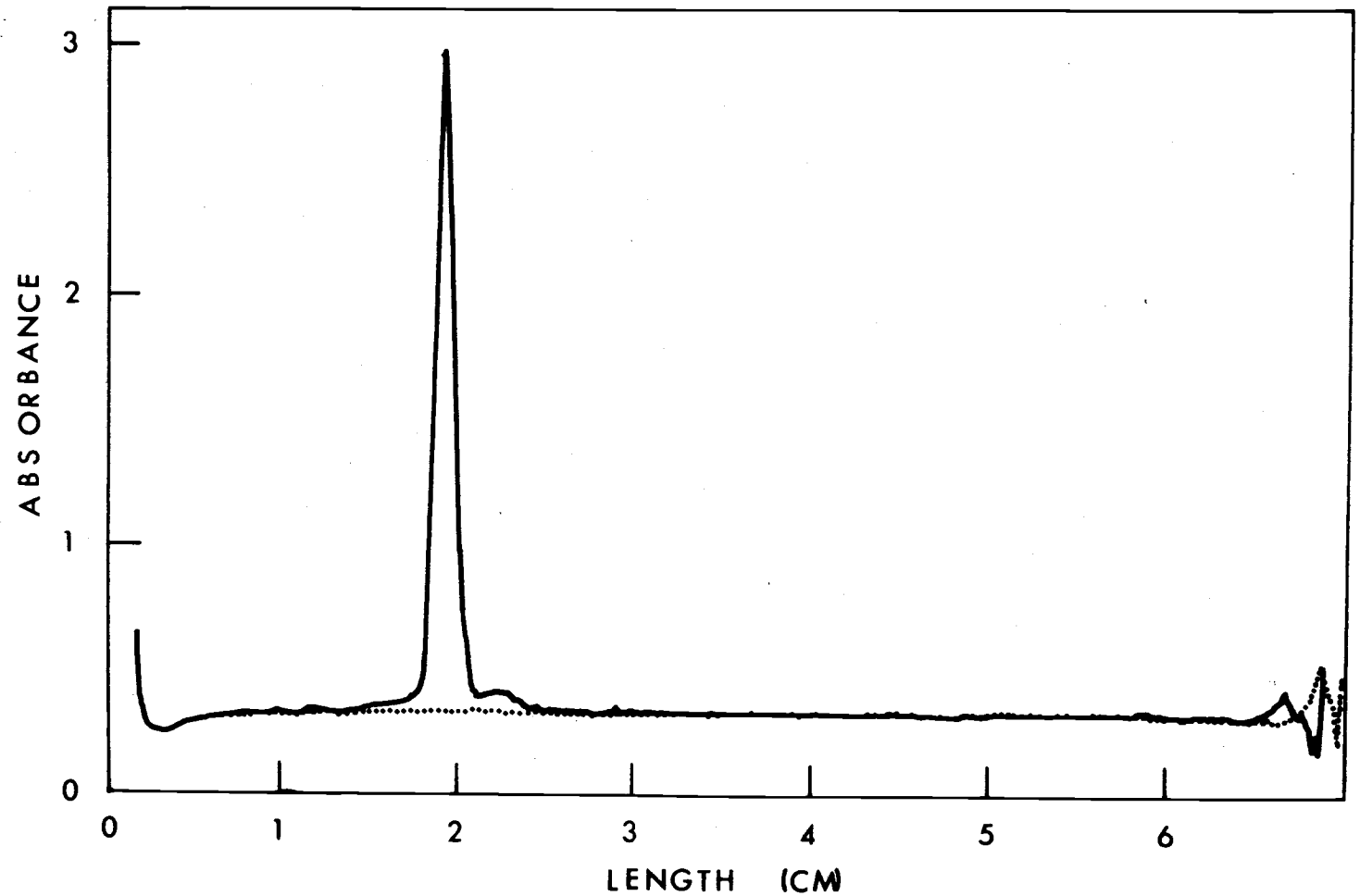


Figure 1. Absorbance scan of an Amido schwarz stained polyacrylamide gel of apotryptophanase. Sample contained 22 µg protein in 40% sucrose, (———); sucrose blank, (·····).

TABLE I. AMINO ACID COMPOSITION OF TRYPTOPHANASE FROM E. COLI

Amino acid	Integral value[a]	Integral value from literature (11)	Integral value from literature (12)
Lysine	31	29	34
Histidine	8	8	10
Arginine	24	24	28
Tryptophan	b	2	2
Aspartic acid	44	40	40
Threonine	28	27	27
Serine	20	19	21
Glutamic acid	59	56	56
Proline	22	19	20
Glycine	44	37	37
Alanine	47	46	45
Half-cystine	b	6	6
Valine	30	31	30
Methionine	15	16	16
Isoleucine	26	28	28
Leucine	39	37	37
Tyrosine	24	24	24
Phenylalanine	23	25	24

[a]Residues are based on a molecular weight of 55,000 with aspartic as key amino acid. Values are the amount of amino acid present in 48-hour acid hydrolysates.

[b]Not determined.

a 0.5 mM pyridoxal-P solution at 37° for a minimum of one hour before being added to the reaction mixture and (b) toluene was layered over the surface of the cuvette reaction mixture as in the Ehrlich test (15). Studies ("Part B" of this paper) indicate an apparent dissociation constant of 3.8 μM for pyridoxal-P to tryptophanase. Thus, 1.0 nM pyridoxal-P binding sites require at least 380 μM pyridoxal-P to achieve a 0.99 fraction of sites saturated. Earlier assays report 0.01 mM to 0.04 mM pyridoxal-P in assay solutions (13, 15) indicating that their assay conditions were probably not optimal. The surface layer of toluene seemed to eliminate much of the drift observed at 340 nm absorbance after mixing enzyme and solution. Also, inhibition by indole may be retarded. Protein was determined either by the method of Lowry (16) for the crude tryptophanase preparations and the holoenzyme, or from the absorbance at 277 nm using an extinction coefficient of 0.795 liter g^{-1} cm^{-1} (6) for apotryptophanase.

Effect of Cations on Tryptophanase Activity

The DPNH and lactate dehydrogenase coupled assay for pyruvate was revised for measuring the activation or inhibition of tryptophanase activity by monovalent cations. Reactions were run in 1.5 ml cuvettes held at 23±.5° by circulating water from a constant temperature bath. Lactic dehydrogenase and tryptophanase were dialyzed in 1 ml

volumes against 3 volumes of 500 ml 0.2 mM dithiothreitol, 0.15 M imidazole-HCl (pH 7.8). Other components of the reaction mixture were used without purification. The 1.1 ml reaction mixtures contained various amounts of chloride salts, 1 mM reduced glutathione, 0.05 mM pyridoxal-P, 18 µg bovine serum albumin, 0.1 mM DPNH, excess lactate dehydrogenase (3 to 4 µg, 2 to 2.7 international units at 37°), tryptophanase (0.7 to 1.0 µg, 0.02 to 0.03 international units at 37°), and 0.15 M imidazole-HCl buffer (pH 7.8 at 24°). Reaction mixtures were incubated one hour at 37° followed by 2 hours at 23±.5° before layering the solutions with 0.3 ml toluene and initiating the reactions with the addition of 50 µl of 49 mM L-tryptophan solution. The L-tryptophan solution was prepared in 0.15 M imidazole-HCl buffer (pH 7.8) and contained 13 µM DPNH and about 5 µg/ml lactic dehydrogenase to remove traces of pyruvate. Initial velocities were determined for the first one min after addition of L-tryptophan. A measurable decrease in rate was observable after an absorbance decrease of about 0.025 units (formation of about 4 µM pyruvate) due to indole inhibition. The tryptophanase concentration was chosen to keep the rate within the limits of 0.018 to 0.006 absorbance units per min for all the inhibition experiments.

Measurement of Circular Dichroism

Circular dichroism spectra were measured with a Durrum-Jasco Model J-10 CD-SP recorder. A 2 ml volume cell with a 0.1 mm optical path was used for measuring at wavelengths below 250 nm and a water jacketed 0.5 ml cell with a 1.0 cm optical path was used above 250 nm. A water jacketed cell holder was used with the 0.1 mm cell. Temperature was maintained within a half degree of 37°, 22°, or 5° with a Lauda K-2/R constant-temperature circulator. The cell compartment was flushed with nitrogen while measuring spectra to prevent condensation at 5° and to lessen oxygen absorbance below 210 nm. Circular dichroism units are reported in liters per cm per mole-of-pyridoxal-P where $\Delta\varepsilon = \varepsilon(\text{left}) - \varepsilon(\text{right})$ for spectra measurements above 250 nm. Below 250 nm, circular dichroism results were expressed as the mean residue ellipticity, $[\theta]$, in units of deg cm^2 $dmole^{-1}$. The mean residue weight was taken as 115.

Concentrations of apotryptophanase and pyridoxal-P were determined by absorbance prior to forming the holoenzyme. Pyridoxal-P was determined from absorbance measurements at 388 nm using an extinction coefficient of 6.60×10^3 liter cm^{-1} $mole^{-1}$ in 0.1 M NaOH (8) which was found to correspond to an extinction coefficient of 5.63×10^3 liter cm^{-1} $mole^{-1}$ in 0.02 M imidazole-HCl (pH 8 at 22°)

buffer. Samples for circular dichroism measurements were prepared by adding 0.2 ml of 0.1 M chloride salt solution to 1.8 ml of holoenzyme solution. The 2.0 ml mixture contained 9.1 μM apotryptophanase (tetramer molecular weight 220,000) (6), 36 μM pyridoxal-P (1 mole per 55,000 g of protein), 0.1 M chloride salt, 0.2 mM dithiothreitol, and 0.02 M imidazole-HCl (pH 8.0 at 22°). Circular dichroism measurements were made at three temperatures on samples incubated in the following sequence for the following times: 30 min at 37° and then one hour at 22° followed by a circular dichroism scan, overnight at 5° followed by a circular dichroism scan, and one hour at 37° followed by a circular dichroism scan.

Fluorescence and Absorbance Spectra

Fluorescence emission spectra were measured on a Perkin-Elmer Model MPF-2A Spectrophotometer equipped with an Hitachi Model QPD 33 Recorder. The spectra were corrected for the wavelength dependence of photomultiplier response and grating transmission with reference spectra of quinine in 0.1 N H_2SO_4. Sample solutions were prepared as for the circular dichroism experiment and measurements were made at 22°. Absorbance spectra were measured using a Cary 11 recording spectrophotometer which was equipped with a thermostated, water jacketed cuvette compartment.

IV. RESULTS

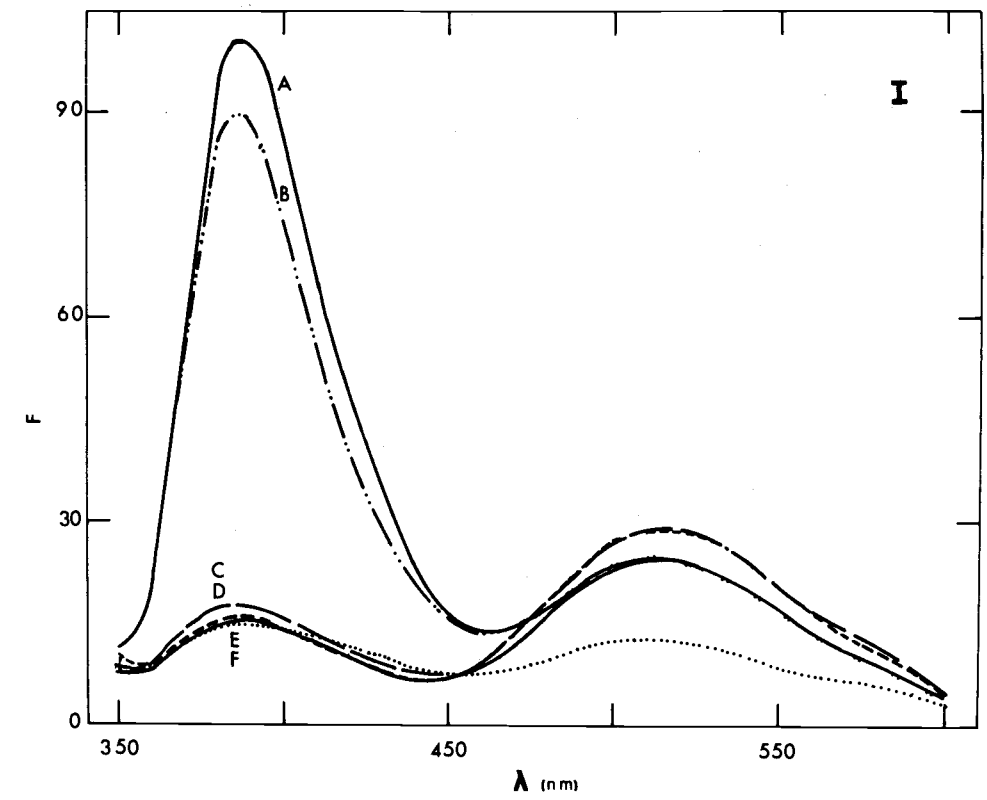
Fluorescence Spectra

Fluorescence emission spectra of holotryptophanase with excitation at 340 nm had emission maxima at 385 nm and at 515 nm (Figure 2). Only the 515 nm maxima was observed with excitation at 420 nm (Figure 3). The order of effectiveness for inducing an emission maximum with 340 nm excitation and 385 nm emission for several of the monovalent cation salts was $K^+ > Rb^+ > C(NH_2)_3^+ > Cs^+ > Li^+$. This order was reversed with 420 nm excitation and 515 nm emission giving $Li^+ > Cs^+ > C(NH_2)_3^+ > Rb^+ > K^+$ (Table II). Holoenzyme solutions containing the polyatomic cations, $C(NH_2)_3^+$ and $(CH_3)_2NH_2^+$, gave emission spectra similar to the spectra of the Na^+ containing solution with either 340 nm or 420 nm excitation. Ammonium stood out from the other monovalent cations in that its 385 nm emission maximum was stronger than all but K^+ or Rb^+ and its 515 nm emission maximum was the largest of all the monovalent cations tested. The emission spectra measured after the addition of $CH_3NH_3^+$ or $NH_2NH_3^+$ to holotryptophanase were of low intensity with weak or no measurable maxima.

Circular Dichroism Spectra

The circular dichroism curves for holotryptophanase in either 0.1 M KCl or 0.1 M NaCl solutions were nearly

Figure 2. The comparative effects of monovalent chloride salts on the fluorescence emission spectra of holotryptophanase. The excitation wavelength of light was 340 nm. Each 2.0 ml sample contained 4.0 mg enzyme, 0.1 M perturbing cation, 0.2 mM dithiothreitol, and 0.02 M imidazole-HCl (pH 8.0) at 22°. Fluorescence units are relative to the maximum peak height. Figure (2-I): (A), K^+; (B), Rb^+; (C), Cs^+; (D), Li^+; (E), Na^+; and (F), without salt addition. Figure (2-II): (A), NH_4^+; (B), $C(NH_2)_3^+$; (C), $(CH_3)_2NH_2^+$; (E), $NH_2NH_3^+$; (F), $CH_3NH_3^+$; and (G), coenzyme in buffer.


I
A
B
C
D
E
F
90
60
30
0
F
350
450
550
λ (nm)

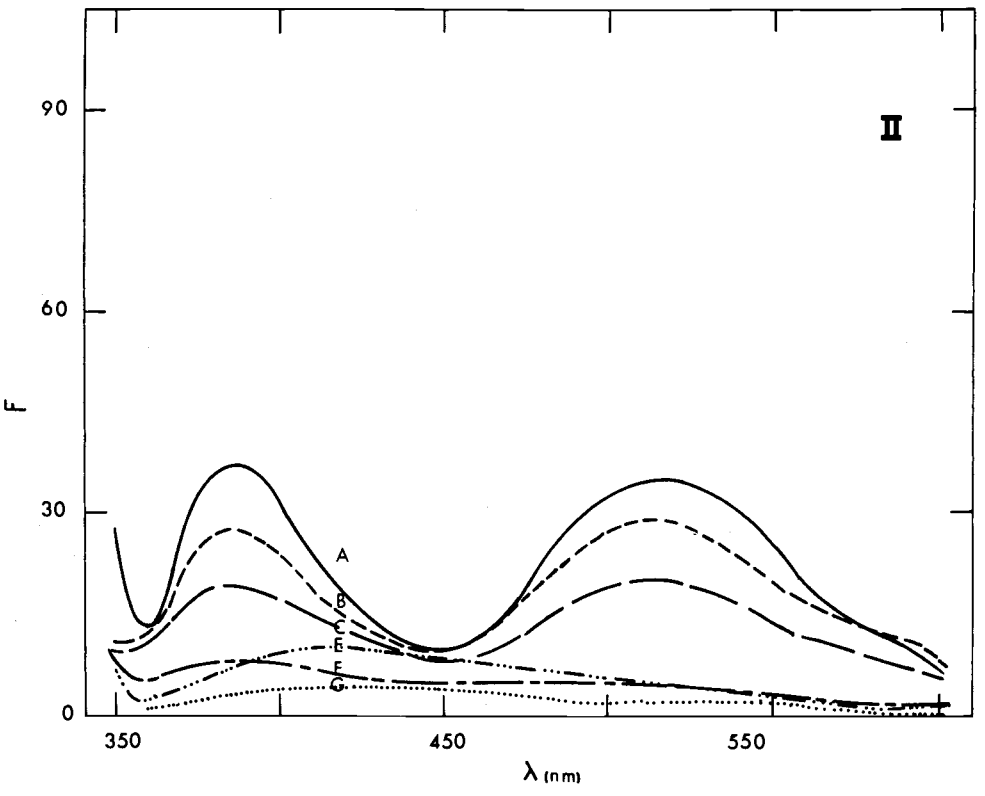
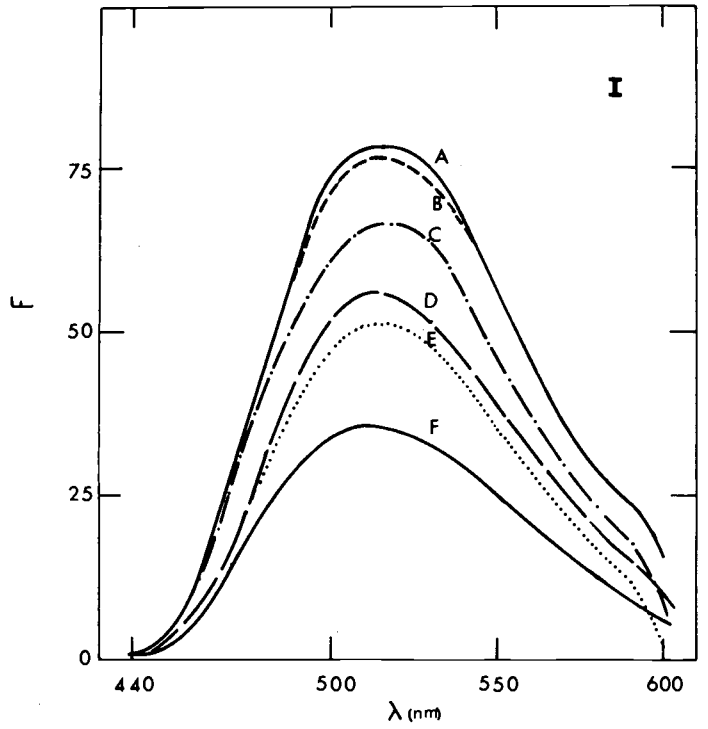

II
A
B
C
E
F
G
90
60
30
0
F
350
450
550
λ (nm)

Figure 3. Monovalent chloride salt effects on the fluorescence emission spectra of holotryptophanase when excited at 420 nm. Samples were as for Figure 2. Figure (3-I): (A), Li^+; (B), Cs^+; (C), Na^+; (D), Rb^+; (E), K^+; and (F), without salt addition. Figure (3-II): (A), NH_4^+; (B), $C(NH_2)_3^+$; (C), $(CH_3)_2NH_2^+$; (D), $CH_3NH_3^+$; (E), coenzyme in buffer; and (F), $NH_2NH_3^+$.


I
A
B
C
D
E
F
75
50
25
0
F
440
500
550
600
λ (nm)

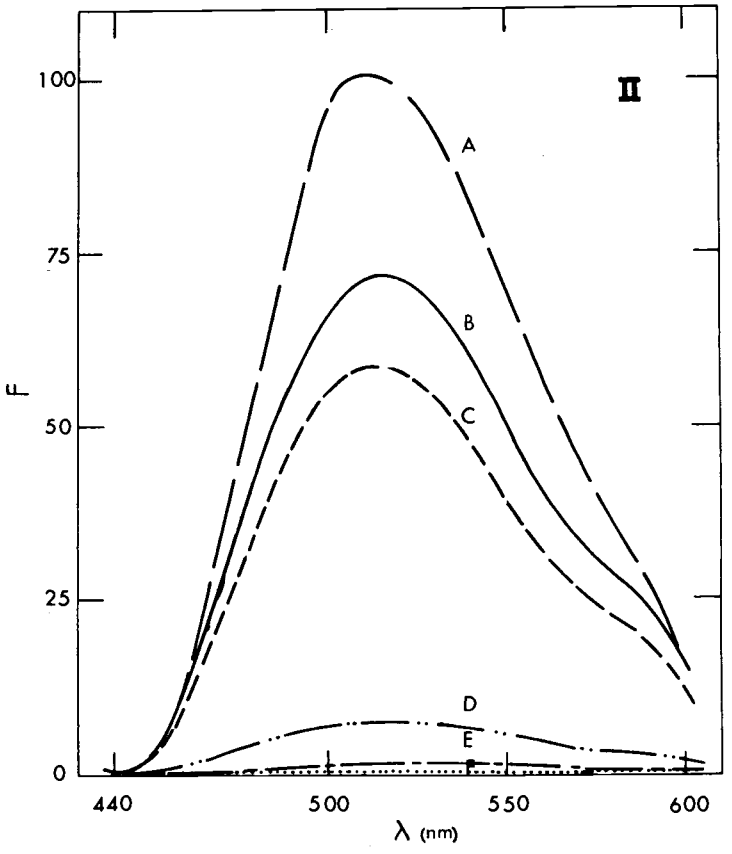

II
A
B
C
D
E
100
75
50
25
0
F
440
500
550
600
λ (nm)

TABLE II. COMPARISON OF 0.1 M CHLORIDE SALT EFFECTS ON HOLOTRYPTOPHANASE

Molar circular dichroism values, $\Delta\varepsilon$, were measured at 340 nm and 420 nm. Relative fluorescence intensity, F, was measured either with 340 nm excitation and 385 nm emission or with 420 nm excitation and 515 nm emission (data are from Figures 2, 3, 5, 6, and 7-II). Note that data is arranged in descending orders of magnitude and that arrows and boxes illustrate the changes in relative fluorescence intensity and circular dichroism for the various cation solutions with 340 nm or 420 nm wavelength light (see text for discussion).

M^+	$\Delta\varepsilon$(340)	F(340)
K^+	4.4	100
Rb^+	3.5	86.9
NH_4^+	2.7	36.6
$C(NH_2)_3^+$	1.8	27.0
Cs^+	1.5	17.7
Li^+	1.3	15.6
Na^+	1.2	14.9
$(CH_3)_2NH_2^+$	1.0	(18.7)
No salt	0.5	14.4
$CH_3NH_3^+$	0.2	8.0
$NH_2NH_3^+$	-0.3	7.3

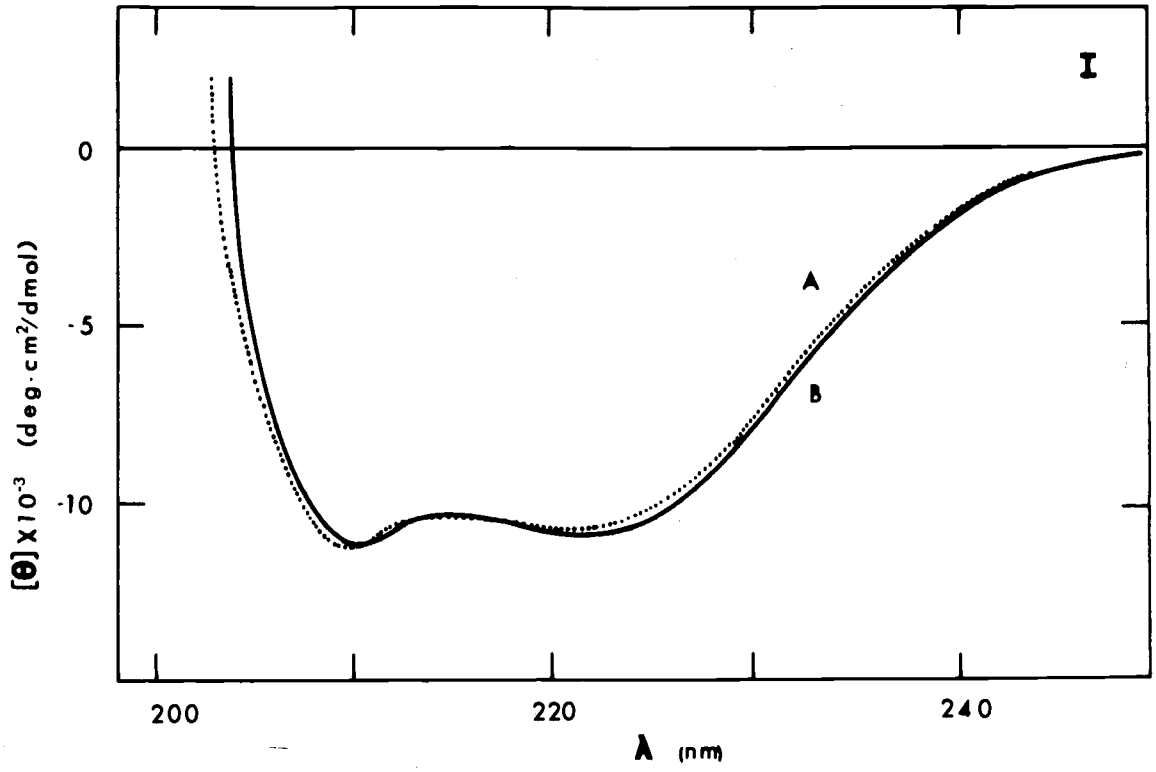
M^+	$\Delta\varepsilon$(420)	F(420)
NH_4^+	9.0	100
Li^+	6.5	77.9
Cs^+	5.5	77.0
$C(NH_2)_3^+$	5.4	70.9
Na^+	4.7	65.8
Rb^+	4.3	55.8
K^+	4.2	55.1
$(CH_3)_2NH_2^+$	3.6	(58.6)
No salt	2.3	35.3
$CH_3NH_3^+$	0.5	7.5
$NH_2NH_3^+$	-0.5	0.6

identical in the 200 nm to 250 nm range when measured at either 22° or 5° (Figure 4). Spectra for both salt solutions had slightly lower ellipticities at 22° in the 208 nm to 210 nm range than spectra measured at 5°. Using the approximation of Greenfield and Fasman (17), this change in ellipticity corresponds to a change in holotryptophanase α-helix content from about 20% at 22° to 17% α-helix at 5°.

Monovalent cation effects on circular dichroism spectra of holotryptophanase are significant in the 250 nm to 500 nm range. These changes in circular dichroism are largest at about 340 nm and 420 nm (Figures 5, 6). Similar to the fluorescence measurements, the order of effectiveness for some of the cations in inducing a circular dichroism maxima at 340 nm was shown to be $K^+ > Rb^+ > C(NH_2)_3^+ > Cs^+ > Li^+$, but was reversed at 420 nm absorbance. NH_4^+ and Na^+ induce larger circular dichroism maxima than K^+ or Rb^+ at 420 nm but not at 340 nm. Holotryptophanase solutions without added salt or with added $(CH_3)_2NH_2^+$, $CH_3NH_3^+$, or $NH_2NH_3^+$ do not show a reversal of 340 nm and 420 nm circular dichroism values with each other or any of the other monovalent cations tested (Table II). Other salt dependent circular dichroism maxima appeared at about 260 nm and 300 nm and a minima at 280 nm (Figures 5, 6).

The circular dichroism curve from 250 nm to 390 nm of K^+ and holotryptophanase shows very little change upon cooling from 22° to 5°. Upon cooling from 22° to 5°, the

Figure 4. The effect of either NaCl or KCl on the 200 nm to 250 nm circular dichroism spectra of holotryptophanase at 22° (Figure 4-I) or 5° (Figure 4-II). Each sample contained 2 mg/ml enzyme in (A) 0.1 M NaCl or (B) 0.1 M KCl, 0.2 mM dithiothreitol, and 0.02 M imidazole-HCl (pH 8.0) (see "Experimental Procedures" for methods and incubation times).


I
0
-5
-10
[θ] X10⁻³ (deg·cm²/dmol)
A
B
200
220
240
λ (nm)

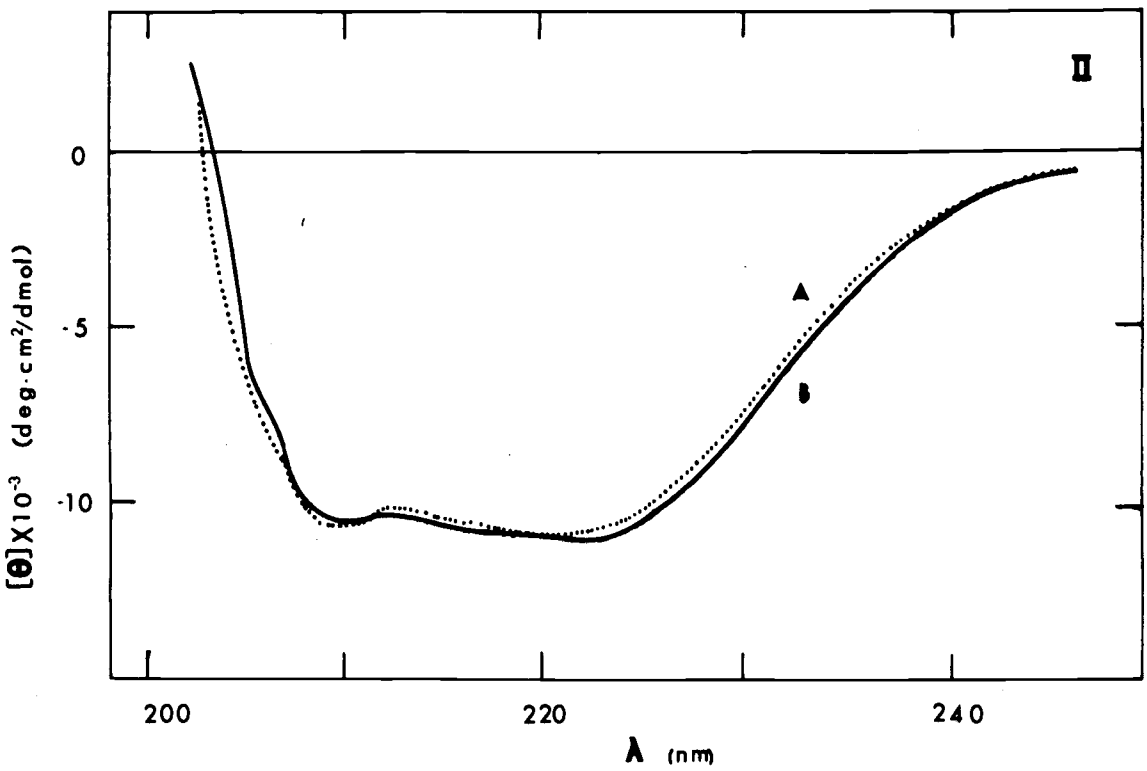

II
0
-5
-10
[θ] X10⁻³ (deg·cm²/dmol)
A
B
200
220
240
λ (nm)

Figure 5. The effects of alkali metal cations on the circular dichroism spectra of holotryptophanase at 22°. See Figure (7-II) for the K^+ and Na^+ 22° spectra. Samples contained 0.1 M concentrations of the following chloride salts: (A), Li^+; (B), Cs^+; (C), Rb^+; (D), without salt addition. Other conditions were as for Figure 4.

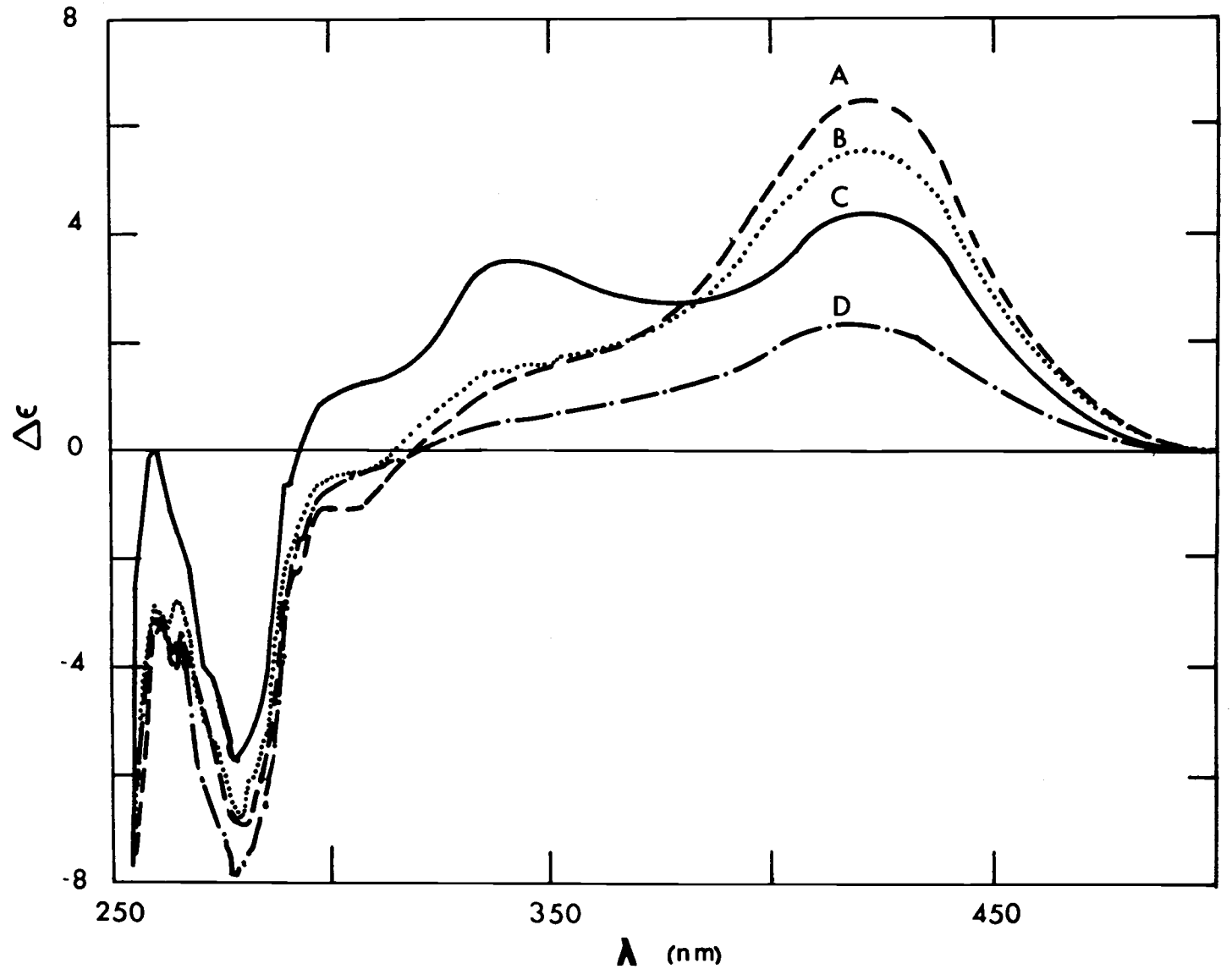
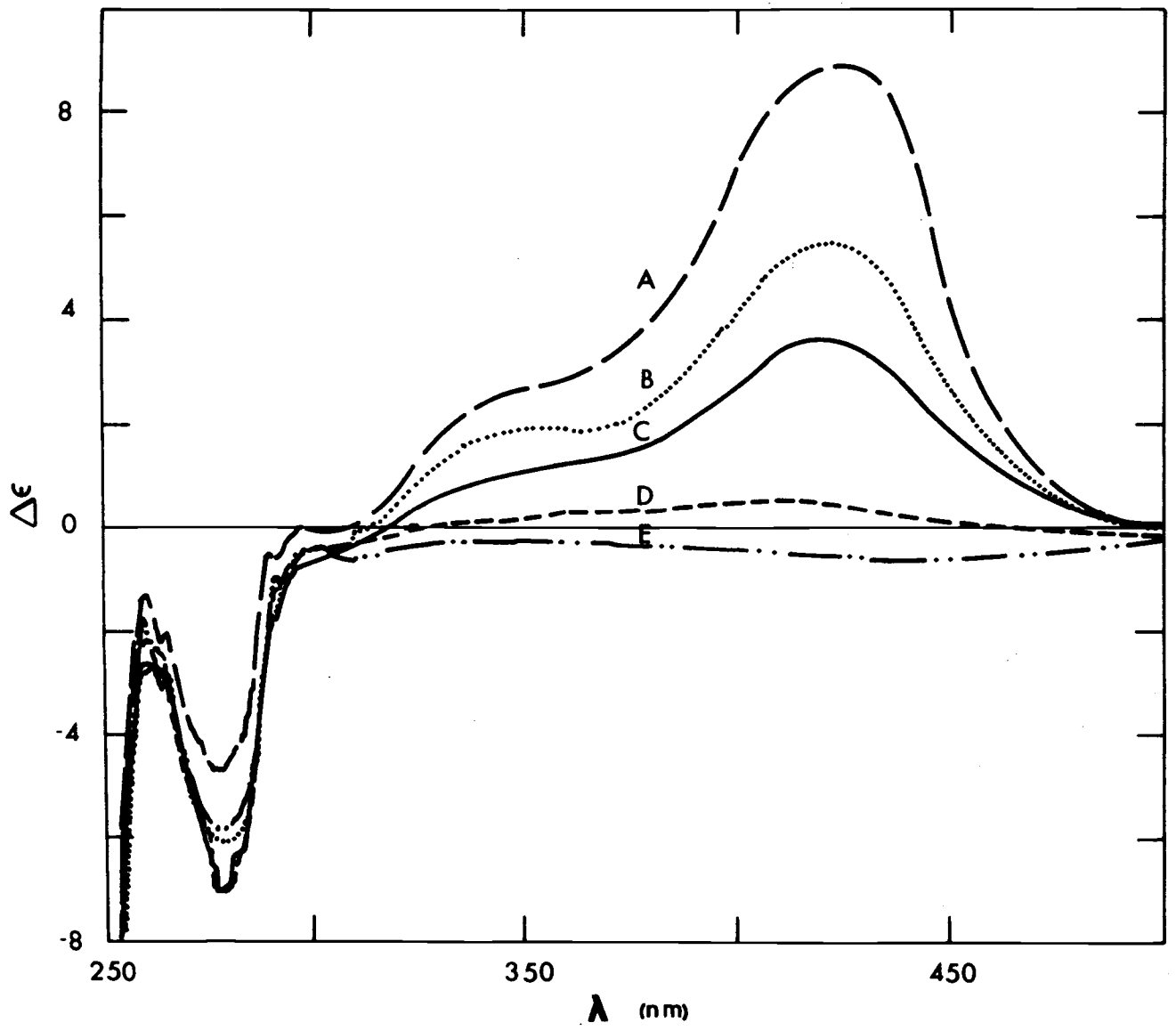

8
4
0
-4
-8
Δε
250
350
450
λ (nm)
A
B
C
D

Figure 6. The circular dichroism spectra of holotryptophanase solutions containing various polyatomic monovalent cations. Salt concentrations were 0.1 M in the following: (A), NH_4^+; (B), $C(NH_2)_3^+$; (C), $(CH_3)_2NH_2^+$; (D), $CH_3NH_3^+$; and (E), $NH_2NH_3^+$. For further details, see legend of Figure 4.

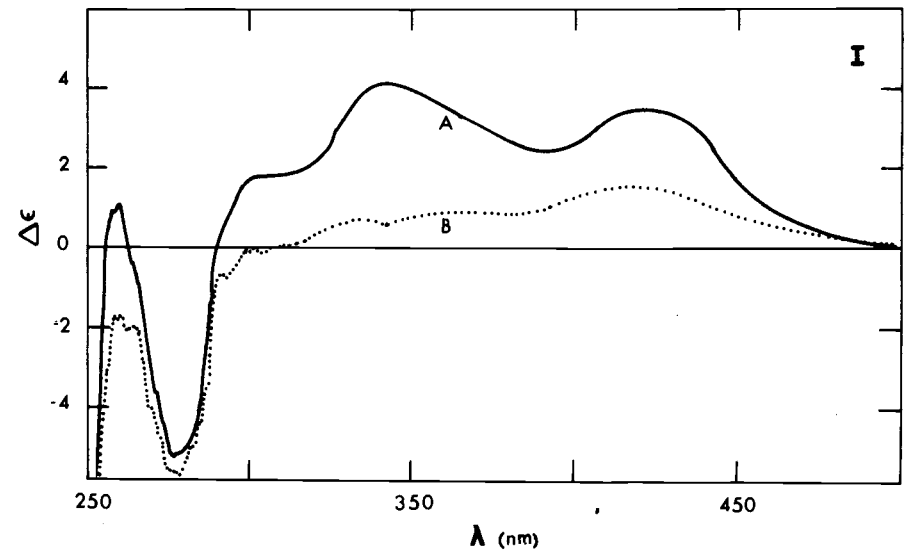

8
4
0
-4
-8
Δε
A
B
C
D
E
250
350
450
λ (nm)

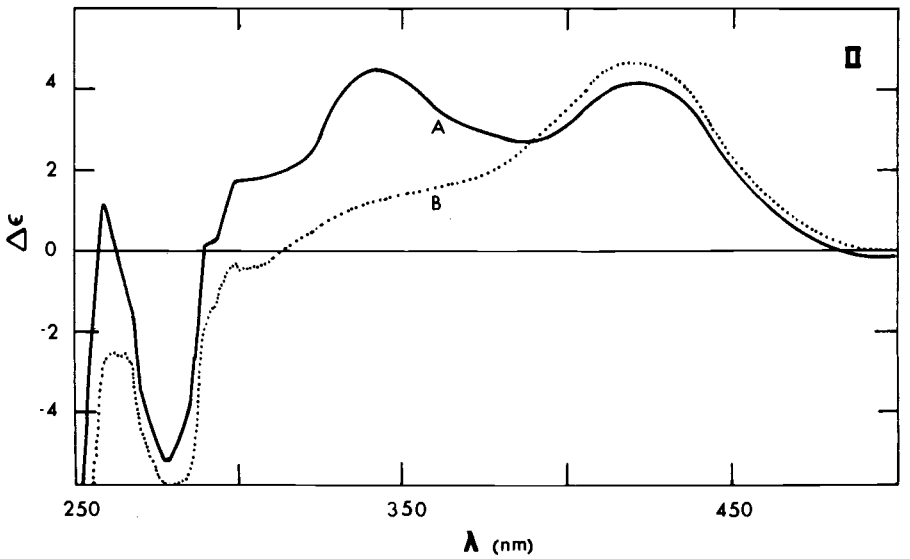
circular dichroism curve for holoenzyme in 0.1 M Na^+ decreased in the 330 nm to 480 nm range and increased slightly between 250 nm and 300 nm. The 420 nm circular dichroism maxima for holotryptophanase in Na^+ solution decreased by about 64% upon cooling from 22° to 5°, but decreased by only about 17% for K^+ and holoenzyme. Warming salt and holoenzyme from 5° to 37° very nearly duplicated the 22° Na^+ and holoenzyme circular dichroism spectrum in the 250 nm to 500 nm range. The 37° K^+ and holoenzyme spectrum was slightly lower at 340 nm and slightly higher at 420 nm than the corresponding 22° curve (Figure 7).

Rate Measurements

Inhibition of the K^+ activation of tryptophanase by Na^+ was found to be either competitive or of a noncompetitive type inhibition depending upon the method of presenting results. When enzyme reaction rates were used as measured, the ordinate intercepts on a Lineweaver-Burk reciprocal plot were slightly different (Figure 8A) indicating either a noncompetitive sort of inhibition or the presence of some activating cation in the desalted (endogenous) reaction solution. If the endogenous rate is subtracted from each measured rate value, the intercepts with the vertical axis are nearly identical (Figure 8B). Experiments of Newton and Snell (4) showed no endogenous tryptophanase activity in the absence of activating monovalent cations

Figure 7. Cation effects on the 250 nm to 500 nm range circular dichroism spectra of holotryptophanase at different temperatures in solutions containing (A), KCl and (B), NaCl. Figure (7-I), 5°; Figure (7-II), 22°; Figure (7-III), 37° (see Figure 4 for details).


I
A
B
Δε
4
2
0
-2
-4
250
350
450
λ (nm)


II
A
B
Δε
4
2
0
-2
-4
250
350
450
λ (nm)

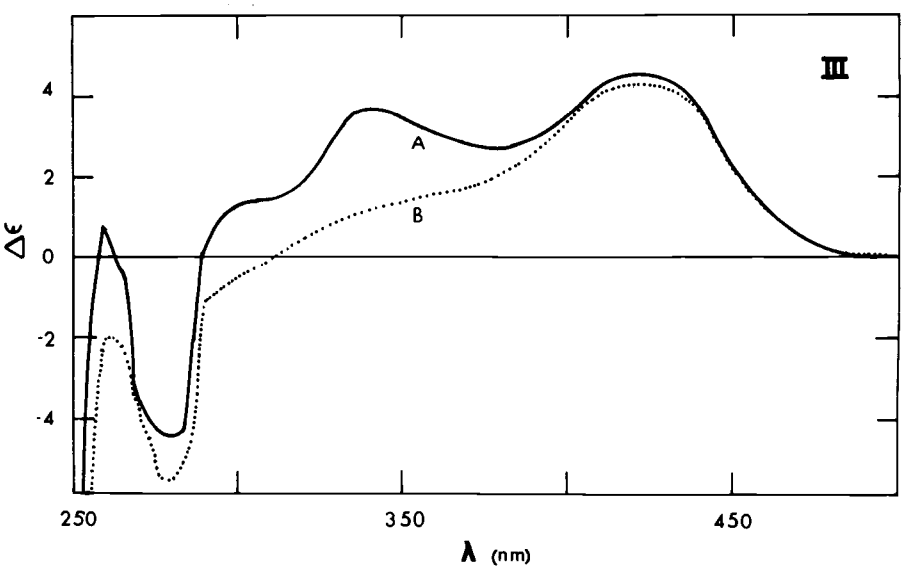
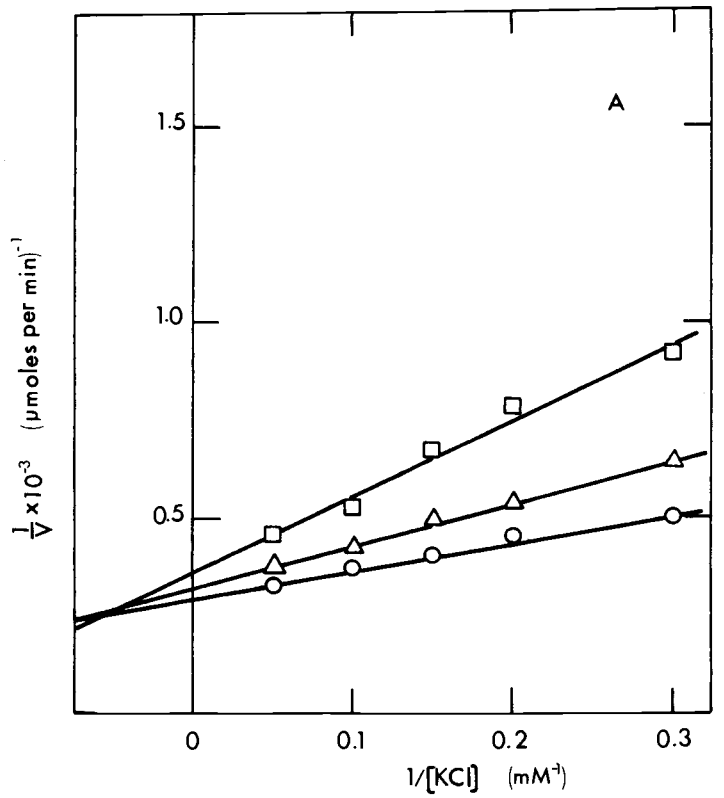
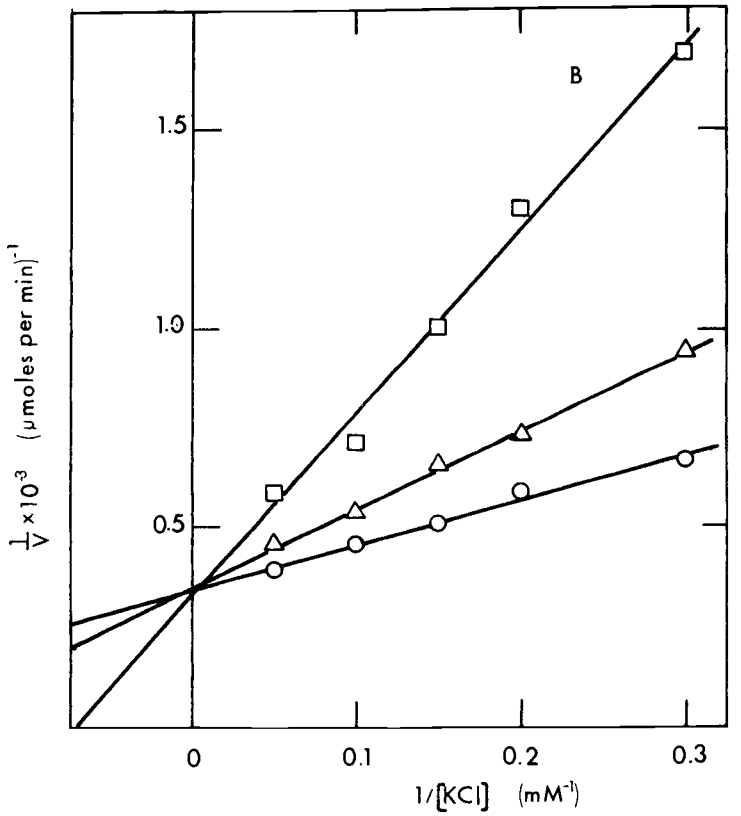

III
A
B
Δε
4
2
0
-2
-4
250
350
450
λ (nm)

Figure 8. Lineweaver-Burk double reciprocal plots of inhibition by Na^+ of the K^+ activation of tryptophanase. Assay conditions were essentially as described in "Experimental Procedures" with 4.3 nM tryptophanase. Figure 8-A, a plot using uncorrected rate values; Figure 8-B, a plot where the residual rate measured without K^+ addition was subtracted from each rate value. Concentrations of added Na^+ were (□), 140 mM; (△), 70 mM; and (○), 0.0 mM. Each value for 0 or 70 mM Na^+ represents the average of two measurements.


A
1.5
1.0
0.5
0
0.1
0.2
0.3
1/[KCl] (mM⁻¹)
1/V ×10⁻³ (μmoles per min)⁻¹


B
1.5
1.0
0.5
0
0.1
0.2
0.3
1/[KCl] (mM⁻¹)
1/V ×10⁻³ (μmoles per min)⁻¹

thus supporting the corrected results which are indicative for competitive Na^+ inhibition.

Several levels of Li^+, Cs^+, and Na^+ inhibition of K^+ activation of tryptophanase were measured (Figure 9). In the presence of 20 mM K^+, 30 mM levels of several polyatomic monovalent cations were found to strongly inhibit K^+ activation while others had almost no affect on K^+ activation of tryptophanase (Table III). Addition of 30 mM NH_4^+ or Rb^+ to a reaction mixture containing 20 mM K^+ increased the measured activity as expected from enzyme activation studies of Happold and Struyvenberg (2).

Absorbance Measurements

The absorbance spectra of 0.2 mM pyridoxal-P in 0.15 M imidazole-HCl (pH 7.8) was essentially unchanged between 260 nm and 400 nm by the addition of 30 mM $(NH_2)_2CH^+$ or $(NH_2)_2CNHOH^+$. The addition of 30 mM NH_3OH^+ results in the loss of the 385 nm absorbance maxima attributed to the aldehyde group of pyridoxal-P (8) and the appearance of a new absorbance peak at 330 nm. The addition of 30 mM $NH_2NH_3^+$ also eliminates the 385 nm peak but gives rise to an absorbance peak at 285 nm and a smaller peak at 323 nm.

As noted in earlier studies of Morino and Snell (1) and in Figure 10, various monovalent cations alter the absorbance spectrum of holotryptophanase.

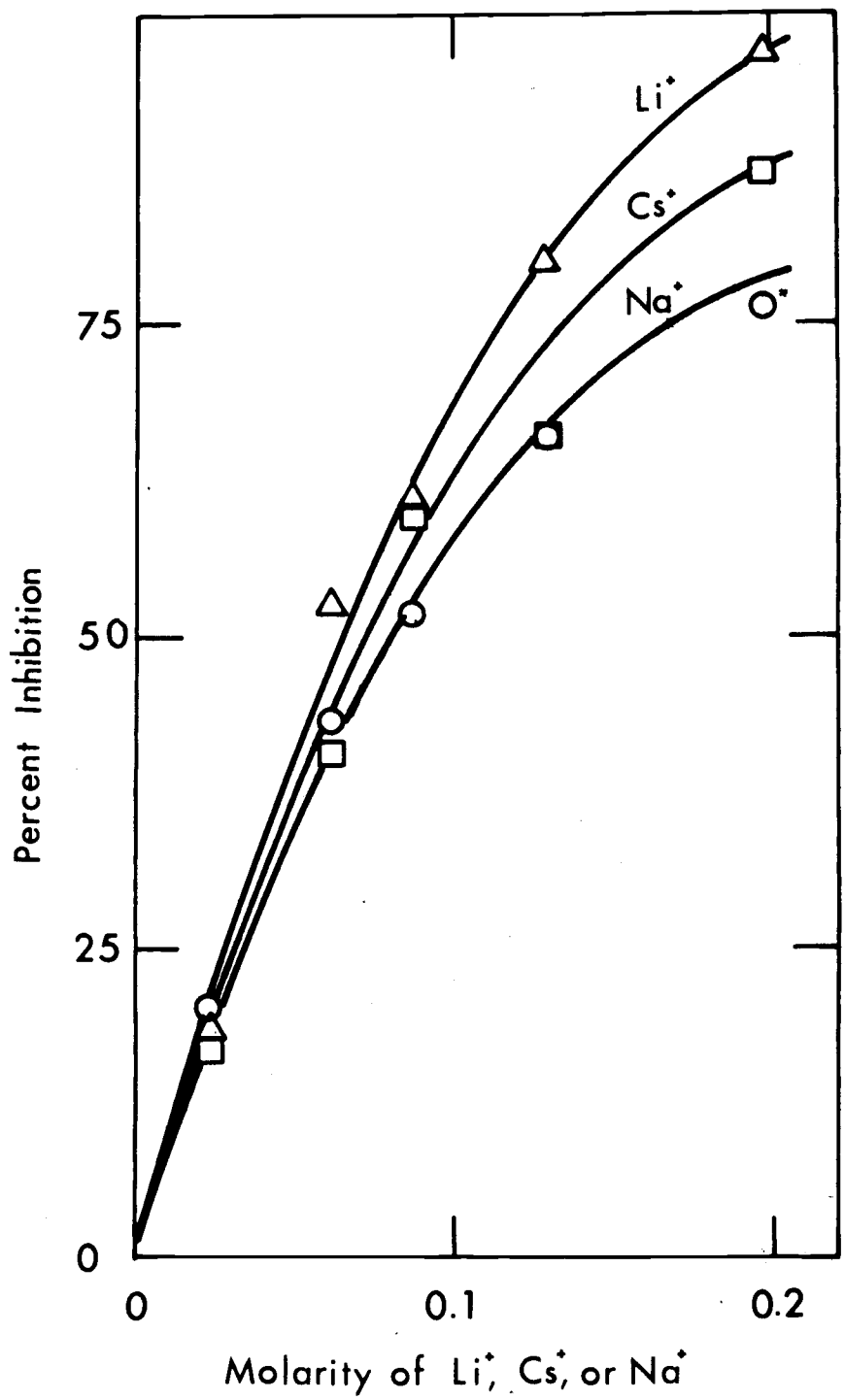


Figure 9. The inhibition of K^+ activation of tryptophanase by Li^+, Cs^+, and Na^+. Assay conditions were as described in "Experimental Procedures" with 20 mM K^+ and 4.3 nM tryptophanase.

TABLE III. RELATIVE INHIBITION OR ACTIVATION OF K^+ ACTIVATED TRYPTOPHANASE BY VARIOUS ALKALI METAL AND POLYATOMIC CATIONS

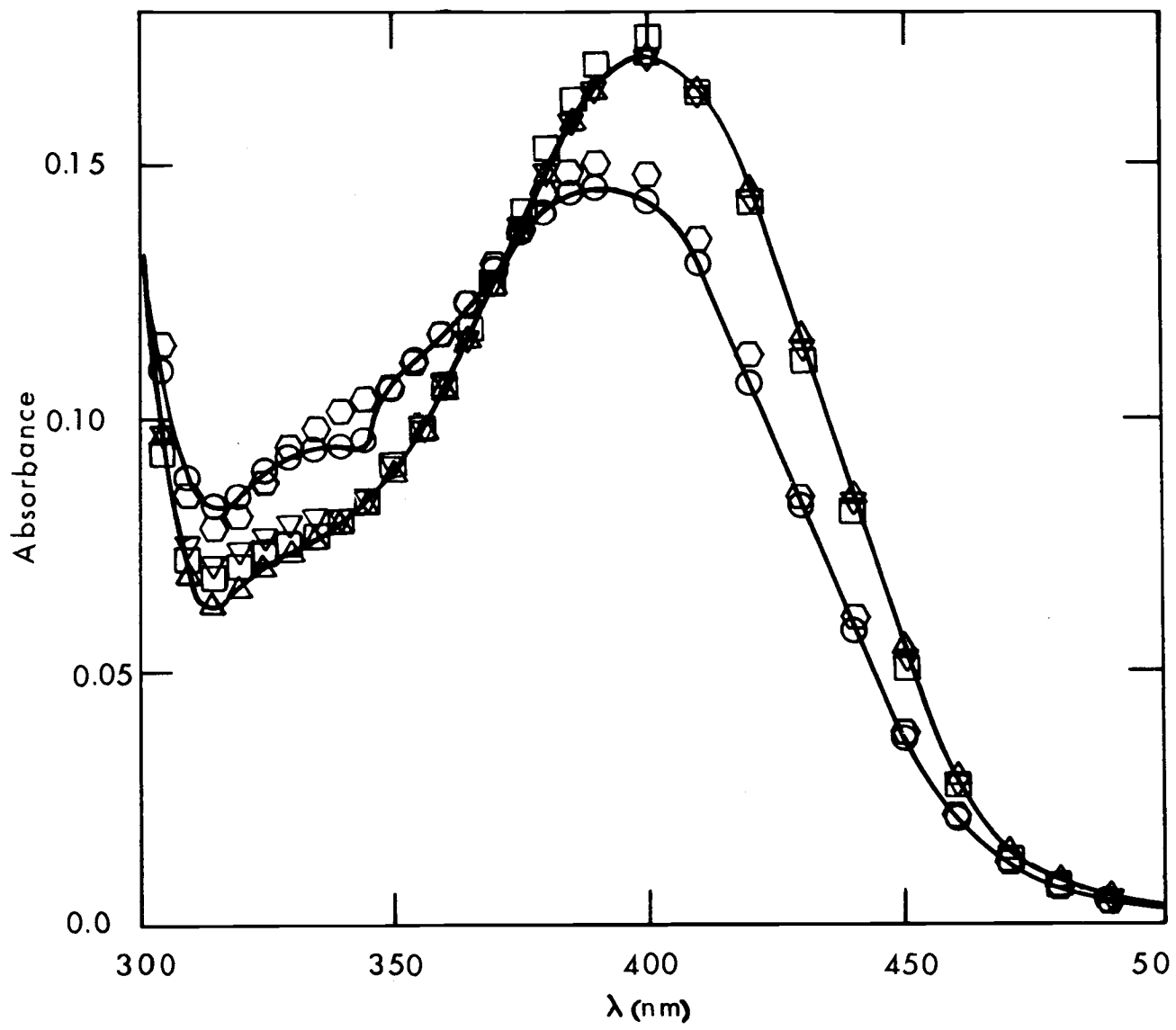
Relative activity was calculated as the rate measured with perturbing cation plus K^+ divided by the rate with K^+. Assay conditions were as for Figure 9 with 20 mM K^+ and 30 mM perturbing cation except for $C(NH_2)_3^+$ which was measured with 3 and 7 mM K^+ and extrapolated to 20 mM K^+. Relative activities for Li^+, Cs^+, and Na^+ were obtained from Figure 9.

Perturbing Cation	Relative Activity
NH_4^+	1.16
Rb^+	1.12
$(CH_3)_2NH_2^+$	1.01
$(CH_3)_4N^+$	.90
$C(NH_2)_3^+$	.82
Na^+	.78
Cs^+	.76

Perturbing Cation	Relative Activity
Li^+	.74
$CH_3NH_3^+$	.25
$(NH_2)_2CH^+$	.13
$(NH_2)_2CNHOH^+$	.06
NH_3OH^+ *	.04
$NH_2NH_3^+$ *	.01

*Yellow color of pyridoxal-P solution became colorless upon addition of perturbing cation.

Figure 10. A comparison of absorbance spectra for tryptophanase in the presence of various cations at 22° C. Samples contain 3 mg tryptophanase per 2.0 ml of 0.02 M imidazole-HCl buffer (pH 8.0), 0.2 mM dithiothreitol, 31 μM pyridoxal-P (ratio of pyridoxal-P to enzyme monomer = 1.1), and 0.1 M of the indicated chloride salt. The solid line is drawn through values for either K^+ or Li^+: (○), K^+; (△), Li^+; (⬡), Rb^+; (□), Na^+; and (▽), Cs^+.


0.15
0.10
0.05
0.0
Absorbance
300
350
400
450
500
λ (nm)

V. DISCUSSION

In order to facilitate discussion of the observed affects of monovalent cations on tryptophanase, a possible structure for the enzyme's active site is presented (Figure 11). This model is an extension of a mechanism proposed by Watanabe and Snell (3) which places an azomethine bridge between an ε-amino group of a lysine residue of the apoenzyme and pyridoxal-P with no hydrogen bonding between the Schiff base nitrogen and the phenolic group (middle structure in Figure 11).

A binding site for the activating monovalent cation is suggested on the phosphate side of the azomethine bridge where it might facilitate binding of pyridoxal-P to apoenzyme by orienting the coenzyme's phosphate group and by possibly stabilizing the azomethine bridge. The model was built with a molecular kit using the pyridoxal peptide sequence Lys-Lys(pyridoxyl group)-Asp (18) and was found to be sterically feasible. Suelter has suggested that the monovalent cation interacts with tryptophanase bound pyruvate (an α-aminoacrylate intermediate) (19). The current studies neither confirm nor contradict such a model. An interaction of monovalent cation with an azomethine bond of bound coenzyme would be consistent with either Suelter's or the current model. Selective binding of monovalent cations to many macrocyclic antibiotics and synthetic macrocyclic polyethers and polyamines is documented (20, 21). It has

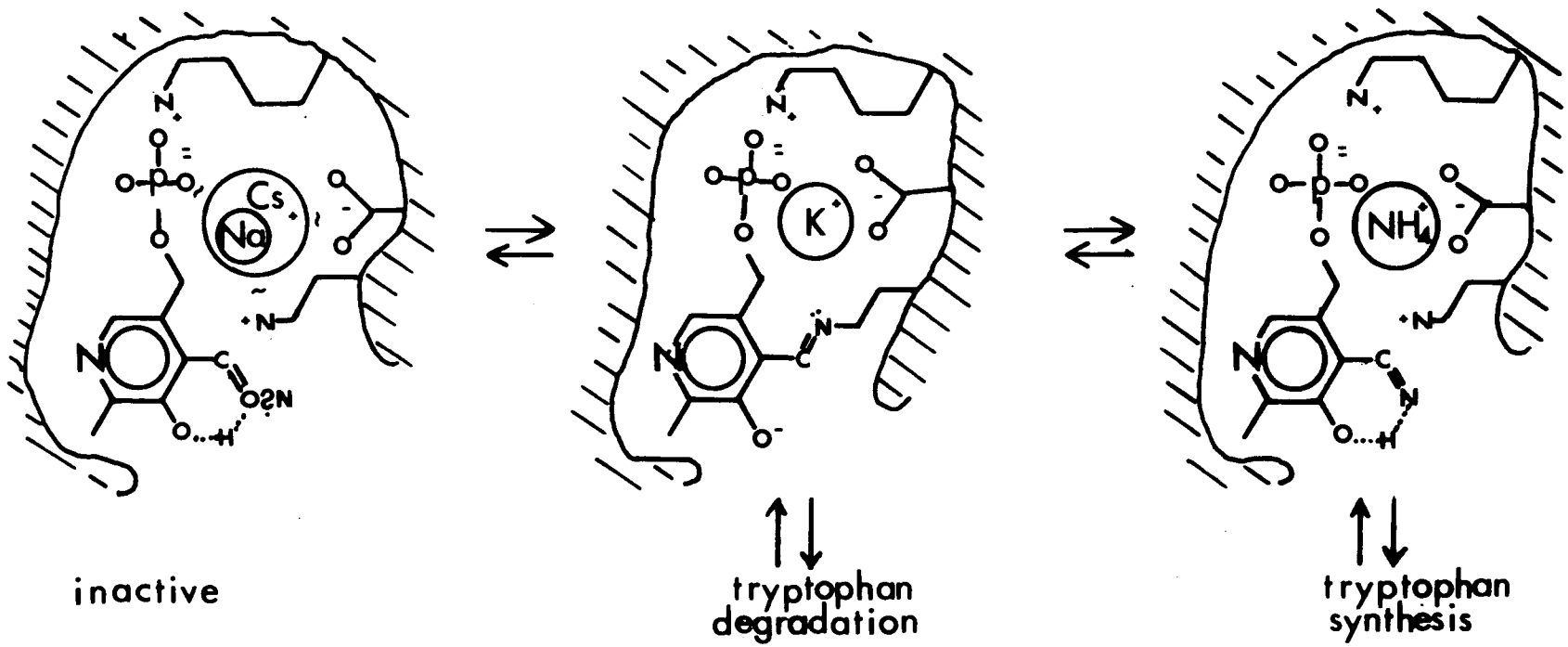


Figure 11. A possible binding site model for K^+ in holotryptophanase. The exact ionic forms are not known; the left-hand structure might contain either a carbonyl or an imine group in the coenzyme. See text for discussion.

been suggested that carboxyl groups or backbone amide carbonyls of proteins may form selective monovalent cation binding sites (7, 22). Although the experimental results reported here can be explained by such a model, details of structure and mechanism clearly require more definitive studies and probably a determination of the enzyme three-dimensional structure.

An alternative to an active site role for monovalent cation activation is an allosteric model with salt induced conformational changes in the enzyme. Such a role could be evident by changes in the tryptophanase secondary structure or by perturbation of some aromatic amino acid residues or by structural changes invisible to the techniques used. Circular dichroism studies in the 200 nm to 250 nm range indicated no differences in holoenzyme backbone structure in K^+ versus Na^+ solutions at either 5° or 22°. Fluorescence polarization studies of 0.06 mg/ml apotryptophanase in 0.02 M imidazole-HCl (pH 8.0) showed essentially no differences in tryptophan anisotropy in 0.1 M K^+, 0.1 M Na^+, or buffered solution without added salt (see "Part B"). Tetrameric apoenzyme in either 0.02 M imidazole-HCl (pH 8.0) or 0.02 M Tris-HCl (pH 8) at 22° or 4° dissociates to dimer with either Na^+ or K^+ addition (6). Tetrameric holotryptophanase in 0.02 M Tris-HCl (pH 8) at 4° is stable in 0.1 M K^+ or NH_4^+ solutions but dissociates to the dimer in 0.1 M Na^+ (6) indicating that the

monovalent cation binding site may be close to the pyridoxal-P site in holotryptophanase.

The various monovalent cations have different affects upon the absorbance (1), fluorescence, and circular dichroism properties of holotryptophanase but not for apotryptophanase. The monovalent cation effects are most pronounced with light absorbance at 337 nm or 420 nm. The species absorbing maximally at 337 nm has been ascribed to the unprotonated Schiff base as in the center of Figure 11 (3, 1) and the 420 nm absorption band to an aldimine with an internal hydrogen bond between the coenzyme's phenolic hydroxyl group and the imine nitrogen (1, 23, 24).

The relative magnitudes of absorbance at the 337 nm maxima (1) for holotryptophanase in solutions of various cations are essentially parallel to the intensities of 385 nm fluorescence emission when the excitation wavelength is 337 nm. The 385 nm emission is strongest in solutions of activating monovalent cations (K^+, Rb^+, and NH_4^+) and weaker for the non-activating cations ($(CH_3)_2NH_2^+$, and $C(NH_2)_3^+$) and inhibiting cations (Na^+, Cs^+, and Li^+). With 420 nm absorbance (1, 3) or 420 nm fluorescence excitation and 515 nm emission, salt solutions of holotryptophanase show the greatest maxima with NH_4^+ and smaller maxima for the inhibitors and non-activators: Li^+, Cs^+, Na^+, and $C(NH_2)_3^+$. The activating cations, K^+ and Rb^+, are quite unlike NH_4^+ and give lower maxima for the 420 nm absorbance or excitation

band. In terms of the model (Figure 11), the activating cations, K^+ and Rb^+, favor the middle structure while the inhibiting cations are better depicted by the structure on the left. Solutions containing NH_4^+ are pictured as a combination of the center and right-hand structures of Figure 11. The dissimilarity in the affect of NH_4^+ and that of K^+ or Rb^+ on the 420 nm species is probably best described in terms of a dual function for NH_4^+. Watanabe and Snell have kinetic and spectral evidence that NH_4^+ also functions in the initial step of tryptophanase catalyzed tryptophan synthesis by forming a coenzyme-NH_3 complex, an imine with a 420 nm absorption maxima (3), in addition to its role as an enzyme activating cation in the degradation of tryptophan (2).

Free pyridoxal-P which absorbs strongly at 385 nm in 0.02 M imidazole-HCl pH 8 buffer is not optically active and, therefore, has no circular dichroism spectrum. Upon complexing with apotryptophanase, asymmetry is obtained as evidenced by the circular dichroism spectra between 250 nm and 500 nm. The order of magnitudes of the 340 nm and the 420 nm circular dichroism maxima of holotryptophanase in the various monovalent cation solutions is essentially the same as the sequences observed for the fluorescence spectra maxima (Table II). Only the $(CH_3)_2NH_2^+$ solution at 340 nm excitation appears out of order.

Upon cooling the holotryptophanase solutions to 5°, the circular dichroism spectrum changes similarly for Na^+ and K^+ solutions in the 200 nm to 250 nm range indicating a slight loss of α-helix structure in the enzyme backbone but no differing salt effects. The small loss of α-helix structure may be related to a lessening of hydrophobic interactions with a temperature decrease (25). At 340 nm, the circular dichroism maxima for holoenzyme in K^+ solution is not affected by a temperature drop to 5°. At 420 nm, the K^+ species does show a slight temperature dependence as noted by a decrease in the circular dichroism maxima of about 17%. The circular dichroism spectra of holotryptophanase in Na^+ solution is very temperature sensitive between 250 and 500 nm. The 420 nm maxima decreased by approximately 64% with a temperature drop from 22° to 5° indicating a considerable loss of bound coenzyme.

The apparent temperature stability of the holoenzyme as measured by the circular dichroism at 340 nm for the K^+ containing solution is consistent with the model (Figure 11) which incorporates a covalent linkage of coenzyme and apoenzyme. Cation specific stabilization of the coenzyme binding to apoenzyme might also be effected through the coenzyme's phosphate group. If hydrophobic interactions are largely responsible for holding the coenzyme to apotryptophanase, which may be the case in Na^+ containing solutions, dissociation of coenzyme and loss of circular

dichroism is expected as the temperature drops because hydrophobic bonds are less stable at lower temperatures (25). The occurrence of the 420 nm species of holotryptophanase in K^+ containing solutions may indicate some internal hydrogen bonding between the nitrogen of the imine and the phenolic oxygen as observed either in solutions of NaCl and enzyme or at low pH values (1).

Several workers have had some success substituting various multiatom cations for K^+ or Na^+ to determine the orientation of ligands at cation binding sites (7, 22, 26). Of the organic cations tried with holotryptophanase, none appeared to be an activator. The cations $(CH_3)_2NH_2^+$ and $(CH_3)_4N^+$ had little or no effect on the K^+ activated enzyme, $C(NH_2)_3^+$ was a weak inhibitor, and $CH_3NH_3^+$, $CH(NH_2)_2^+$, $C(NH_2)_2NHOH^+$, NH_3OH^+, and $NH_2NH_3^+$ were stronger inhibitors. Results are not readily interpreted since the coenzyme reacts irreversibly with some of these compounds. Inhibition observed by adding NH_3OH^+ or $NH_2NH_3^+$ is probably due to a loss of coenzyme as noted by absorbance spectra measurements (see "Results"). The absorbance spectra for pyridoxal-P and $CH(NH_2)_2^+$ or $C(NH_2)_2NHOH^+$ in imidazole buffer is essentially unchanged from that of pyridoxal-P and buffer. Therefore, these two multiatom cations may be inhibiting without removing the coenzyme from the system. Since the organic cations did not show the reversal of fluorescence and circular dichroism maxima at 340 nm and

420 nm absorbance as observed for the alkali metal cations, the effects of the multiatom cations on holotryptophanase are probably not of the same type as the effects observed with the alkali metal cations.

Though physical differences are not observed in the absence of pyridoxal-P for the various monovalent cation solutions, the fluorescence and circular dichroism spectra of holotryptophanase are dependent upon the monovalent cation present. The magnitude of the physical perturbations measured parallels approximately the cation's effectiveness as an activator or inhibitor with the exception of NH_4^+ which has a dual function and some of the organic cations which react with pyridoxal-P. Circular dichroism measurements at 5° and 22° indicate that coenzyme dissociation from apoenzyme is greater at lower temperatures in the presence of Na^+ than in K^+ solutions.

Part B:
COMPARATIVE CATION EFFECTS ON PYRUVATE KINASE AND TRYPTOPHANASE

VI. SUMMARY

The salt induced increases in the fluorescence emission anisotropy of rabbit muscle pyruvate kinase tryptophan residues were found to be similar in solutions of both activating and non-activating monovalent cations. Evidence was obtained indicating that the tryptophan residue perturbation probably results from a localized salt effect rather than a change in distances between tryptophan residues. The monovalent cation induced changes in tryptophan residue anisotropy which were observed with pyruvate kinase were not observable for several other enzymes: Escherichia coli tryptophanase, Escherichia coli β-galactosidase, yeast hexokinase, or rabbit muscle aldolase. The binding properties of the substrate analog, $1,N^6$-ethenoadenosine diphosphate (εADP)[2] to pyruvate kinase, as monitored by emission anisotropy, were found to be essentially the same in solutions of K^+, Na^+, Li^+, or tetramethylammonium cation.

Fluorescence intensity measurements were used to show that tryptophanase binds its coenzyme more tightly in the presence of the activating cations K^+ and NH_4^+ than in solutions of the non-activating cation, Na^+. No cation affects

[2] $1,N^6$-ethenoadenosine diphosphate is abbreviated εADP as suggested by Secrist et al. (35).

were observed on the binding of indole to apotryptophanase. A study of the comparative kinetics of holotryptophanase reorientation after the addition of monovalent cations indicated shorter kinetic half-times for KCl solutions than NaCl solutions. Results were consistent with a similar cation role in either pyruvate kinase or tryptophanase activation: as a bridge between either substrate or coenzyme and enzyme respectively.

VII. INTRODUCTION

At least 60 enzymes have been reported to require a monovalent cation for optimal enzyme activity (19). Rabbit muscle pyruvate kinase and tryptophanase from E. coli both require K^+, NH_4^+, or Rb^+ for enzyme activity (27, 2, 4). Na^+ weakly activates pyruvate kinase (27) but does not activate tryptophanase (2, 4). Neither enzyme is activated by Li^+ (27, 2, 4). Although these enzymes catalyze considerably different reactions it is of interest to see whether the role of activating K^+ is similar for both systems. In view of recent experiments, K^+ may exert its activating influence by binding between enzyme and substrate in the pyruvate kinase system (28, 22) and by binding between enzyme and coenzyme in the tryptophanase system. The current study supports such a bridging role for the activating cation.

Tentative schemes for the pyruvate kinase reaction mechanism which had no active-site role for K^+ (29, 30) have been modified by several groups to include enzyme bound K^+ at the active site. Eisenman and Krasne (7) suggest that the K^+ binding site may consist of amide carbonyls of the polypeptide backbone and a single carboxylate group. Nowak and Mildvan (31) propose that the carboxyl group of phosphoenolpyruvate coordinates with enzyme bound K^+ and that this hypothetical enzyme-K^+-carboxyl "bridge"

may have an alignment function for the correct position of the phosphoenolpyruvate phosphoryl group. Thallium and monomethylammonium cations have been shown to activate the enzyme (32, 22). The distance between these monovalent cations and the divalent cation binding site has been estimated from NMR studies (28, 22) to be less than 8 Å which is in good agreement with such a "bridge" model. The non-activating cation, trimethylammonium, was reported to be further removed from the enzyme-bound Mn (22). These interatomic distances were apparently measured by using the correlation time estimated for water protons in the complexes. Measurements made in this manner for pyruvate kinase have recently been questioned by James and Cohn (33) because only one-half water molecule of the Mn(II) first coordination sphere was found to contribute to the proton relaxation rate of water (34). Evidently Cohn's group prefers a variation of both temperature and frequency in the determination of proton relaxation rates (33).

Monovalent cations of different effective sizes than K^+ may inhibit K^+ activation through improper positioning of the phosphoryl group of phosphoenolpyruvate. Mn^{++} could coordinate between the enzyme and the phosphate groups of phosphoenolpyruvate and ADP. In such a model various monovalent cations might have little or no effect on ADP binding in the absence of a phosphoenolpyruvate bridge to the cation binding site. The development of fluorescence

analogs of ADP and ATP and the demonstration of enzyme reactivity with these analogs (35) has made available new probes for studying many enzymes. The analog, εADP, has been shown to have the same K_m and about 80% of the V_{max} of ADP in the pyruvate kinase reaction (35). Thus it is assumed in these studies that εADP plays a role similar to ADP in the pyruvate kinase reaction mechanism and that binding studies with εADP will reflect the binding behavior of ADP to pyruvate kinase. Fluorescence techniques were used in the current study to demonstrate a non-specific cation effect on εADP binding to pyruvate kinase in the absence of phosphoenolpyruvate.

Proteins containing tryptophan residues show the fluorescence of this residue to the exclusion of phenylalanine or tyrosine residue fluorescence (36). Pyruvate kinase and tryptophanase contain 12 and 8 tryptophan residues respectively (37, 11). The relative effects of various cations on tryptophan residue fluorescence emission anisotropy was investigated.

Professor Esmond E. Snell's group has carried out extensive studies on E. coli tryptophanase including the reaction mechanism (1, 3), subunit structure (6, 12), and chemical structure (18, 11, 5) of the enzyme. Though Snell's group has not suggested a mechanistic role for the monovalent cation in the tryptophanase reaction, they have reported some monovalent cation specific properties of the

holoenzyme. In solutions containing 0.1 M KCl and 0.02 M imidazole-HCl at pH 8, holotryptophanase has an absorbance maxima at 337 nm which is ascribed to a non-hydrogen bound azomethine bridge between the coenzyme and a lysine residue of the apoenzyme. In 0.1 M NaCl, the holoenzyme loses the 337 nm absorbance band and obtains a strong absorbance maxima at 420 nm which is ascribed to an imine of the bound coenzyme with H-bonding between the coenzyme's phenolic hydroxyl group and its imine nitrogen (1, 23, 24). Holotryptophanase was reported to be stable as the tetramer in 0.1 M KCl, 0.02 M Tris-HCl (pH 8) solution at 4° but dissociated to dimer in 0.1 M NaCl (6). In the absence of coenzyme, dissociation to dimers occurred in both Na^+ and K^+ solutions at 4° (6).

Cation specific effects have been observed at absorbance wavelengths between 250 nm and 500 nm for the circular dichroism spectra and the fluorescence spectra of holotryptophanase but not for apotryptophanase. The cation effect on holoenzyme seems to be closely related to the bound coenzyme. Current studies expand upon the effect that various cations have upon coenzyme binding.

VIII. EXPERIMENTAL PROCEDURE

Materials

Fluorimetric grade imidazole, pyridoxal-P, N-tris(hydroxymethyl)methyl glycine (Tricine)[3], tricyclohexylammonium phosphoenolpyruvate, lactic dehydrogenase, pyruvate kinase, rabbit muscle aldolase, and yeast hexokinase were from Sigma Chemical Company. E. coli β-galactosidase was from Boehringer-Mannheim. Apotryptophanase was prepared as described in Part A, "Experimental Procedure". The εADP was synthesized as described by Secrist et al. (35). The product, which contained small amounts of contaminating εAMP, was further purified on Eastman silica gel sheets using a solvent of isobutyric acid-ammonium hydroxide-water (66:1:33, v/v). The εADP spots were scraped off the sheets and suspended in water. The silica gel was removed by filtration and the resulting εADP solution was lyophilized to dryness. All solutions were prepared using deionized, glass distilled water. Other chemicals were of reagent grade.

Pyruvate Kinase Assay

Pyruvate kinase activities were determined before and after fluorescence measurements using a lactic

[3]N-tris(hydroxymethyl)methyl glycine is abbreviated Tricine.

dehydrogenase coupled enzyme assay (38). The reaction mixture was in 50 mM imidazole-HCl (pH 7.0) at 25°. After desalting, specific activities measured (in μmoles of pyruvate formed per mg of enzyme per min) before and after each run were typically 86.5 and 79 respectively.

Though the commercially obtained pyruvate kinase had a single sedimentation boundary (MW = 237,000) at 40,000 rpm and 23°, and a single but rather broad band on polyacrylamide gel electrophoresis (9), the measured specific activity was lower than most reported values: 110-150, Nowak and Mildvan (31); 76.9-325, McQuate and Utter (39); and 160-220, James and Cohn (33). Since these authors did not report activities after desalting, the final status of the pyruvate kinase used in their studies is not known. Cottam and Mildvan (40) have reported that the number of Mn^{++} binding sites for pyruvate kinase drops from four to two while the dissociation constant for Mn^{++} remains constant upon aging of pyruvate kinase where specific activity decreased from 280 to 100 for a 30° assay. Though our dialyzed pyruvate kinase samples may have lost some K^{+} or εADP binding sites, the comparative binding studies of εADP to enzyme in different cation solutions should be valid.

Fluorescence Anisotropy Measurements

Enzyme solutions were desalted by dialysis of 1 ml samples against 500 ml of buffer at 2-5°. The 500 ml of

buffer was replaced twice over 36 hours. Dialysis tubing was prepared as described by McPhie (41). Prior to fluorescence measurements, protein solutions were passed through a 0.45 μm Millipore "HAWP" filter. To avoid plugging the filter, the dialyzed protein solution was centrifuged (12,000 x g for 10 min) before filtering. Turbidity of solutions was monitored at 400 nm. Protein concentrations of the dialyzed and filtered solutions were obtained using the 280 nm extinction coefficients of 0.52 ml mg^{-1} cm^{-1} for pyruvate kinase (42), 0.91 ml mg^{-1} cm^{-1} for aldolase (43), 1.30 ml mg^{-1} cm^{-1} for hexokinase (44), 1.85 ml mg^{-1} cm^{-1} for β-galactosidase (45), and the 277 nm extinction coefficient of 0.795 ml mg^{-1} cm^{-1} for apotryptophanase (6). The concentration of εADP was estimated using the extinction coefficient of 11.4 ml mg^{-1} cm^{-1} at 275 nm (35).

Fluorescence anisotropy measurements were made with an instrument described by Isenberg and coworkers (46-48). The light source was a 1000 W HgXe lamp. Excitation and emission wavelengths were set at 278 nm and 348 nm respectively for tryptophan residues (49) of pyruvate kinase and at 310 nm and 440 nm respectively for εADP fluorescence. Emission anisotropy is defined (50) by the equation:

$$r = \frac{E-B}{E+2B} \qquad (1)$$

where E is the fluorescence intensity polarized parallel to the vertically polarized exciting light, and B is the

intensity of emission polarized perpendicular to the exciting light. The quantity E+2B is proportional to the total emission intensity (51). Changes in rotation time for a fluorescent transition moment are seen as changes in anisotropy. Higher values of anisotropy are correlated with less tumbling while lower values indicate a more rapid rotation.

The εADP anisotropy values were used to estimate the fraction of εADP bound to pyruvate kinase from the relation:

$$f_b = \frac{r_\varepsilon - r_2}{r_1 - r_2} \qquad (2)$$

where r_ε is the observed εADP anisotropy, r_1 is the anisotropy estimated for bound εADP, and r_2 is the anisotropy measured for free εADP. In practice, r_1 was approximated by extrapolations to the εADP anisotropy at high protein concentration. This use (equation 2) of the addition law for polarization (51) makes the assumptions that only two fluorescent species, bound and free εADP, are present and that the molar extinction coefficient and the quantum yield of each species is constant during the titration. Fluorescence intensity of εADP was essentially linear with concentrations of εADP during the titrations.

Fluorescence Intensity Measurements

Binding of pyridoxal-P to apotryptophanase in various monovalent cation solutions was measured by the change in fluorescence intensity (E+2B) of pyridoxal-P solutions with the addition of enzyme. Since the fluorescence intensity of unbound pyridoxal-P was very small, anisotropy changes with binding of coenzyme to tryptophanase were negligible after the initial increase with the first addition of enzyme. Ammonium sulfate precipitated apotryptophanase was dissolved in 0.2 mM dithiothreitol, 0.02 M imidazole-HCl (pH 8.0 at 22°) and desalted on a Sephadex G-25 column. A 1.8 ml solution containing 0.11 M KCl, NaCl or NH_4Cl, 2.1 μM pyridoxal-P, 7.1 to 8.2 μM apotryptophanase, 0.2 mM dithiothreitol, and 0.02 M imidazole-HCl (pH 8.0 at 22°) was added incrementally to an identical solution except without enzyme. The additions were made once per hour followed by 50 min incubations at 22° and 10 min intensity measurements. Enzyme activity before and after fluorescence measurements was essentially unchanged.

Pyridoxal-P solutions were excited at 365.2 nm which is an intensity peak for the 1000 W HgXe lamp and is also fairly close to the absorbance isosbestic point for the K^+ and Na^+ forms of holotryptophanase (1). Intrinsic emission was measured at the 515 nm Na^+ form fluorescence maxima (see "Part A"). Corrections were made for decay of

holoenzyme fluorescence with time. Limiting fluorescence intensities for infinite protein (all coenzyme bound) were estimated by least-squares fits of the corrected intensity values to a rectangular hyperbola. Calculations were performed on the Hewlett-Packard Model 65 and 9821-A computers using programs written in this laboratory.

IX. RESULTS

Coenzyme Binding to Tryptophanase

Fluorescence intensity of bound coenzyme increased with the ratio of apotryptophanase to pyridoxal-P in 0.11 M K^+, Na^+, and NH_4^+ chloride solutions. Fractional intensity changes were equated with fractions of total coenzyme bound to apoenzyme. Hill plots of the fluorescence data (Figure 12) yielded an approximate slope (Hill coefficient) of 1.1 for all three monovalent cations and apparent dissociation constants of 1.1 μM for coenzyme and tryptophanase monomer in NH_4^+ solution, 3.8 μM in K^+ solution, and 7.6 μM in Na^+ solution.

Preliminary experiments using changes in indole fluorescence emission intensity and anisotropy to measure binding of indole to apotryptophanase indicated essentially no change in spectral values with the addition of 0.1 M NaCl or KCl to the enzyme solutions. Comparative binding studies of indole to holotryptophanase were complicated by the overlapping, salt dependent fluorescence spectra of the holoenzyme and were therefore discontinued.

Kinetics of Tryptophanase Fluorescence Change

Addition of several different monovalent cations to desalted holotryptophanase solutions produced an apparent first-order increase in the measured coenzyme fluorescence

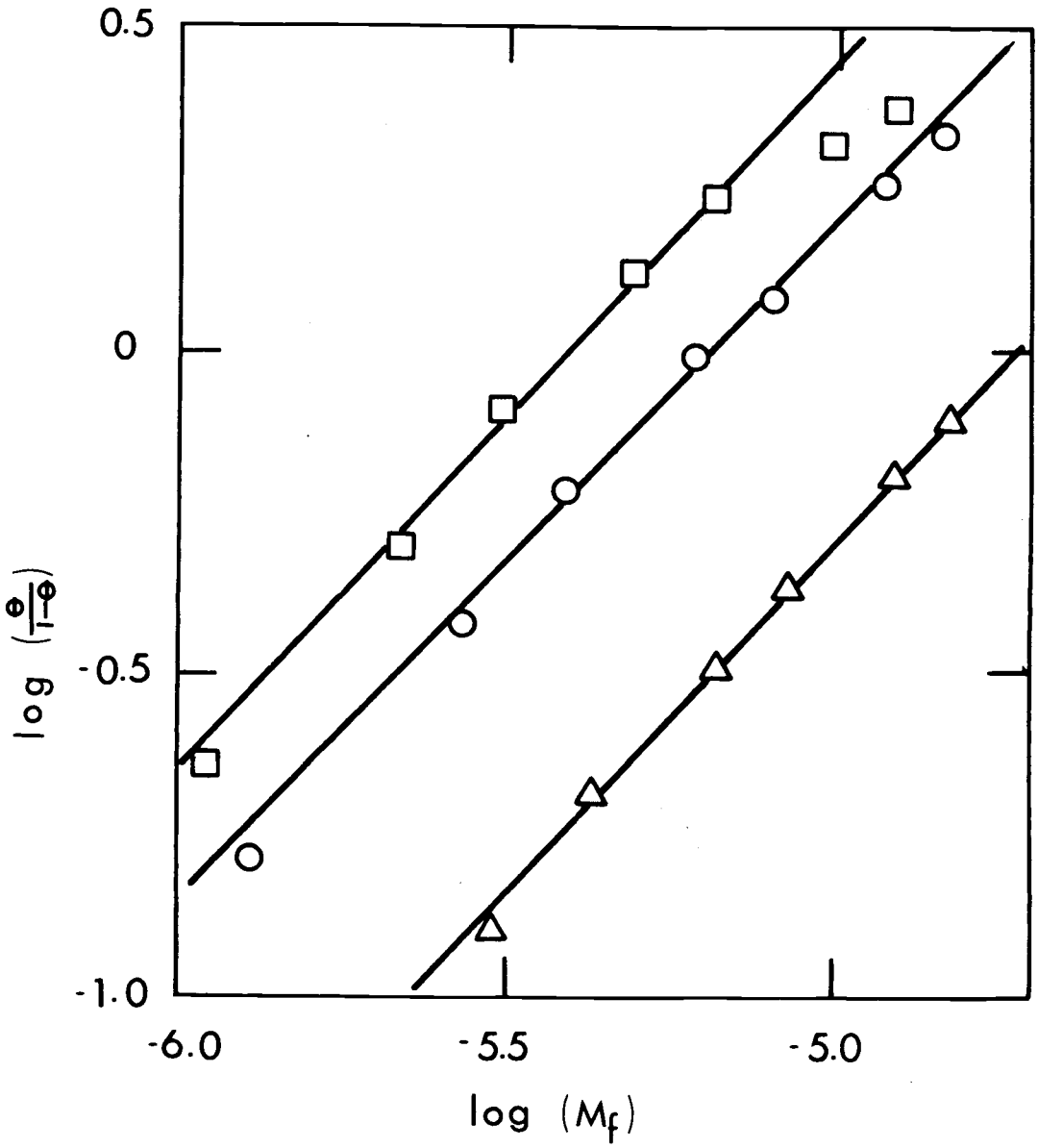


Figure 12. A Hill plot of the effect of 0.11 M Na^+, K^+ and and NH_4^+ on the binding of pyridoxal-P to apotryptophanase. "M_f" is the monomeric concentration of apotryptophanase and "θ" is the fraction of pyridoxal-P bound to enzyme. The slopes are least-squares fits to the points indicated with n = 1.1 for all three chloride salt solutions. Apparent dissociation constants for coenzyme and monomeric tryptophanase are 1.1 μM with NH_4^+, 3.8 μM with K^+, and 7.8 μM with Na^+. Symbols are (□), NH_4^+; (○), K^+; and (△), Na^+. See "Experimental Procedures" for details.

intensity. Samples were excited at 373 nm which is the approximate absorbance isosbestic point for the K^+ and Na^+ forms of holotryptophanase (1) and emission was measured at the 515 nm emission maxima. Solutions of holotryptophanase in 0.1 M KCl solution were found to have slightly shorter half-times than other alkali metal cations (Figure 13). The ammonia cation gave one of the longest half-times measured.

Similar comparative kinetic studies were made using the excitation and emission wavelengths of the K^+ form of tryptophanase, 340 nm and 385 nm respectively. Half-times for K^+ and Rb^+ were essentially unchanged from the measurements with 373 nm excitation and 515 nm emission. Rates for the other alkali metals were not calculated as the induced fluorescence change was less than 10% of the K^+ induced change.

Tryptophan Residue Anisotropy

The effect of KCl or NaCl on the tryptophan residue anisotropy of apotryptophanase was investigated at 22° and 5.5°. Samples contained 0.3 μM apoenzyme, 0.1 M KCl or NaCl (or no salt), 2.0 mM EDTA, 0.2 mM dithiothreitol, 0.02 M imidazole, and TMA-OH to pH 8.0 at 22°. Measurements were made using a 284 nm excitation and a 335 nm emission wavelength. Virtually no change in a 0.112±.001 tryptophan residue anisotropy for the apoenzyme was observed with

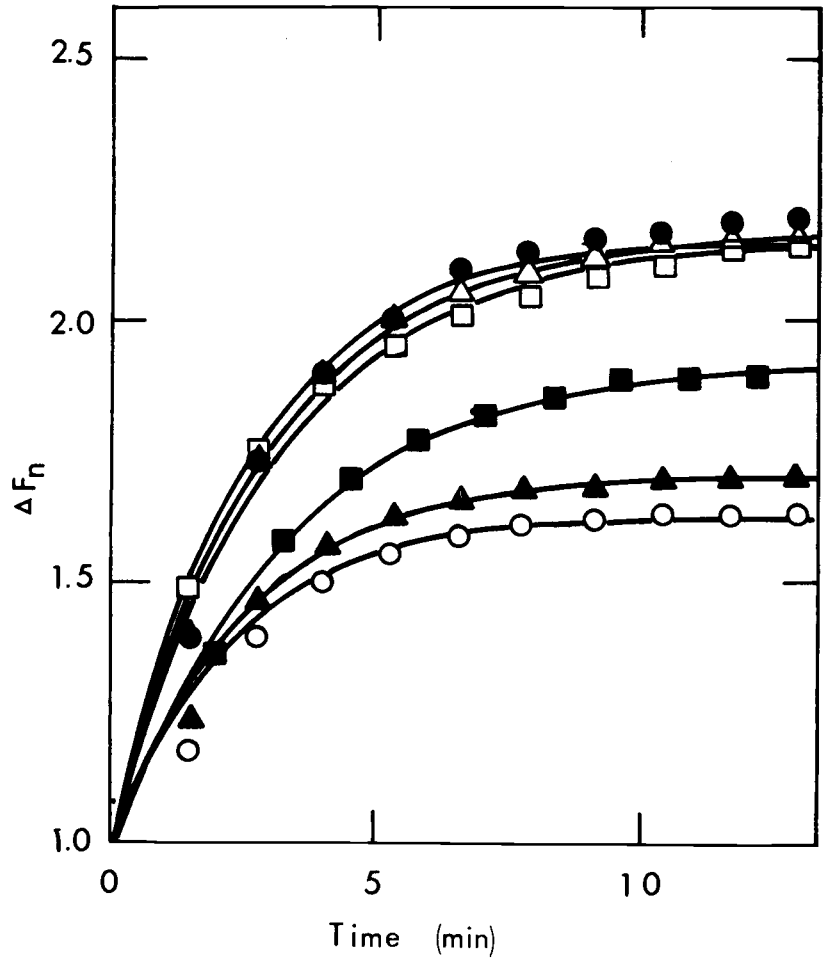


Figure 13. A kinetic comparison of the influence of various monovalent cations on holotryptophanase fluorescence. Samples of 1.8 ml volume containing 2.22 mg/ml holoenzyme (4:1 molar ratio of pyridoxal-P to apoenzyme), 0.2 mM dithiothreitol, and 0.02 M imidazole-HCl (pH 8.0 at 22°) were mixed at "zero time" with 0.2 ml of the various 1.0 M chloride salt solutions. Final concentrations were 2.0 mg/ml enzyme in 0.1 M cation solution. Dilution effects and decay of holotryptophanase fluorescence with time were corrected for by making a cation free run. Samples thermostated at 22° were excited with 373 nm light and emission was measured at 515 nm. Solid lines are least-squares fits to a first order rate equation which give the following half-times for the fluorescence change: (△), Li^+, 1.9 min; (■), Na^+, 2.2 min; (○), K^+, 1.6 min; (▲), Rb^+, 1.7 min; (●), Cs^+, 1.7 min; (□) NH_4^+, 2.1 min.

or without K^+ or Na^+ at 22° or 5.5°.

The intrinsic anisotropy of pyruvate kinase increased rapidly upon salt addition and then remained constant for at least 30 min. Without added salt, buffer dilution of the pyruvate kinase solutions produced no significant change in anisotropy. All the cations tested caused the pyruvate kinase tryptophan residue anisotropy to increase (Figure 14). Phosphoenolpyruvate and magnesium induced increases in anisotropy occur at lower salt concentrations than anisotropy increases induced by the monovalent cations Li^+, Na^+, or K^+. Aldolase, hexokinase, and β-galactosidase showed no significant changes in tryptophan anisotropy with added K^+ or Mg^{++} (Figure 15A,B).

The effect of KCl on the anisotropy of pyruvate kinase tryptophan residues was measured as a function of the exciting wavelength to determine whether or not the measured anisotropy change with salt concentration was due to a change in energy transfer (52, 53) between tryptophan residues. As observed in Figure 16, a large salt effect on tryptophan anisotropy is apparent even when exciting at the red edge of the tryptophan residue's absorbance spectrum.

Binding of εADP to Pyruvate Kinase

Experiments similar in design to the tryptophan anisotropy studies were conducted to observe the effect of increased ionic strength of the various cations on εADP

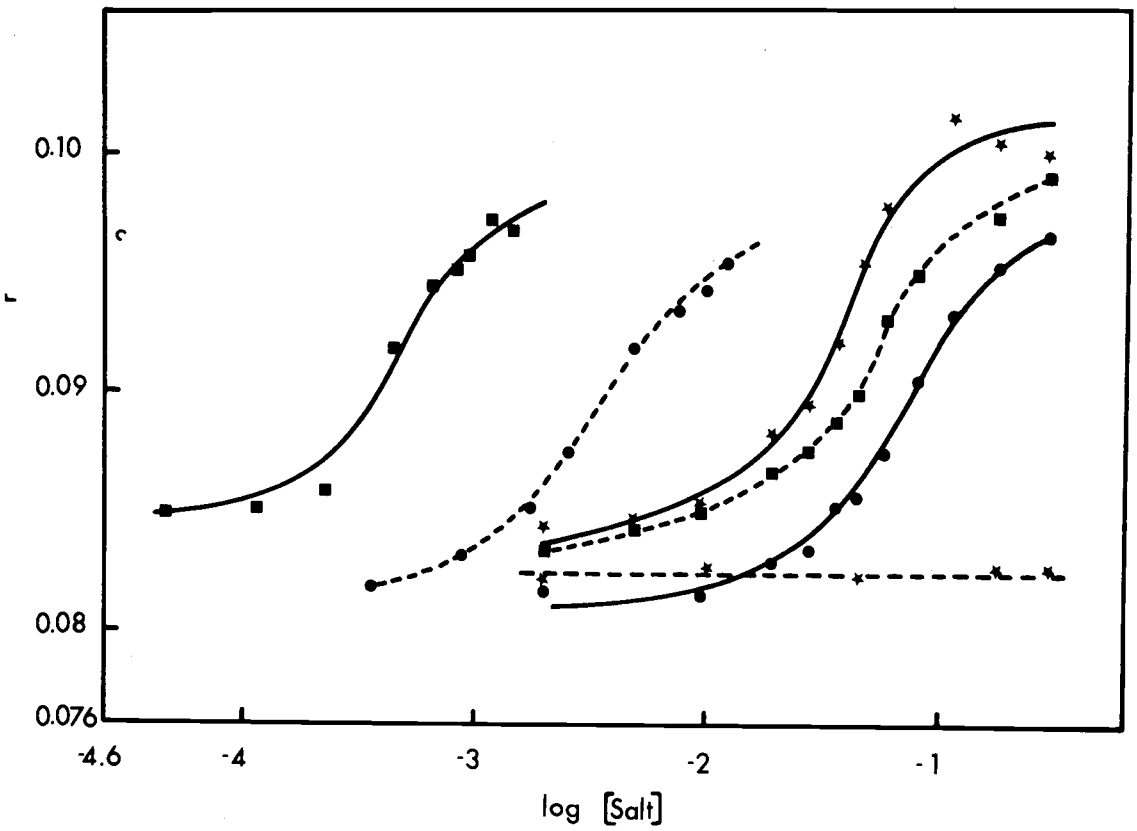
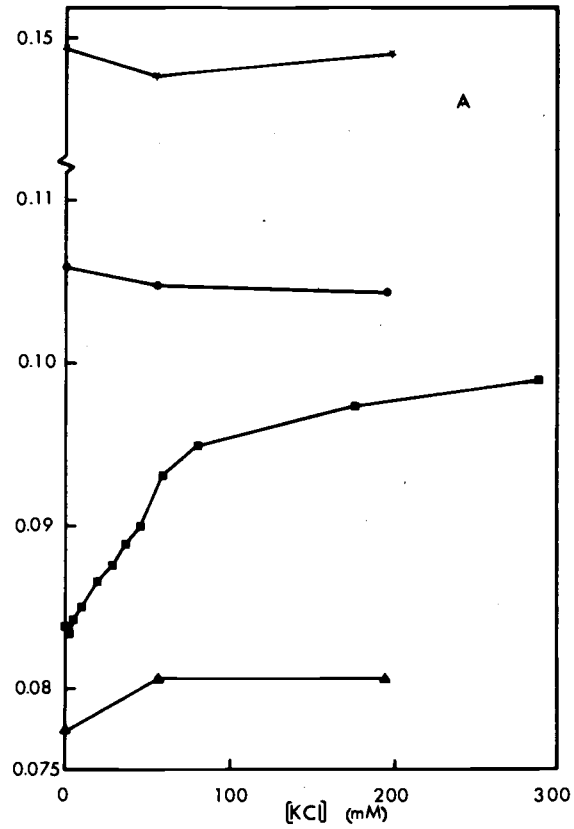
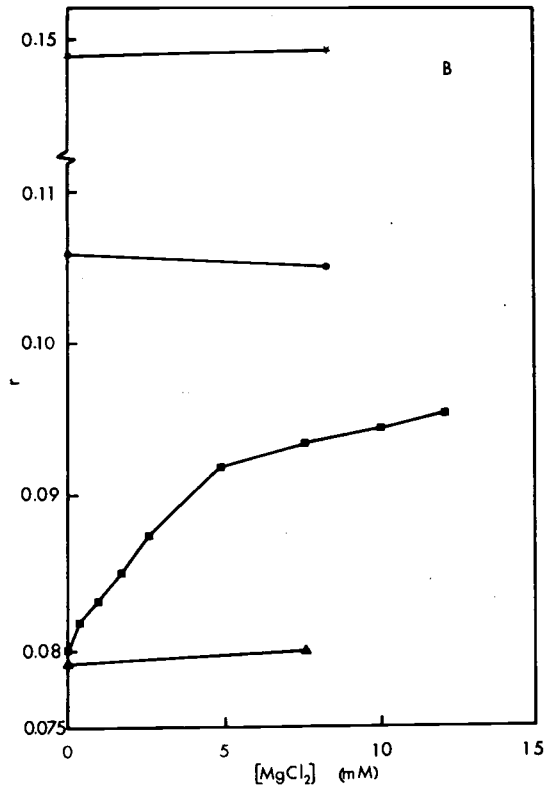


Figure 14. The effect of various salts on the anisotropy of pyruvate kinase tryptophan residues. Anisotropy, r, was measured with excitation at 278 nm and emission at 348 nm as the salt concentration was increased. A total of 0.7 ml of salt in 10 mM Tricine, 1 mM TMA-Cl, and TMA-OH (pH 7.6) at 22° was added incrementally to 2.5 ml of 0.41 μM enzyme in buffer. (●—●), NaCl; (■—■), KCl; (★—★), LiCl; (●—●), $MgCl_2$; (■—■), tricyclohexylammonium phosphoenolpyruvate; (★—★), blank.

Figure 15. Comparison of tryptophan residue anisotropies for several different enzymes as a function of salt concentration; Figure 15-A, KCl and Figure 15-B, $MgCl_2$. Buffered chloride salt solutions were added to buffered enzyme solutions as in Figure 14. Initial enzyme concentrations were 98 µg/ml pyruvate kinase, ■; 55 µg/ml aldolase, ★; 40 µg/ml hexokinase, ● ; and 31 µg/ml β-galactosidase, ▲ , for both (A), K^+ and (B), Mg^{++} solutions.


0.15
0.11
0.10
0.09
0.08
0.075
r
A
0
100
200
300
[KCl] (mM)


0.15
0.11
0.10
0.09
0.08
0.075
r
B
0
5
10
15
[MgCl2] (mM)

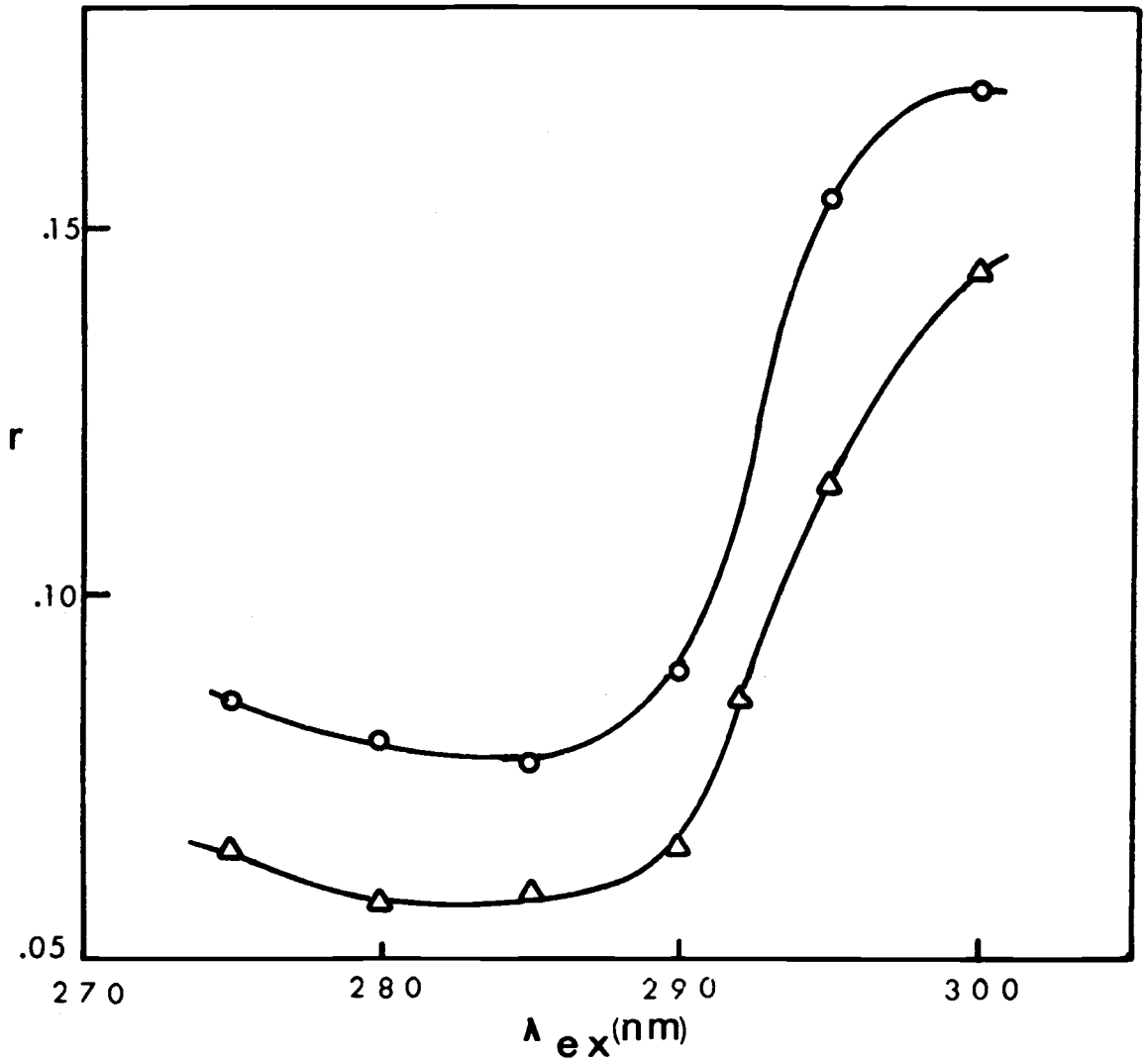


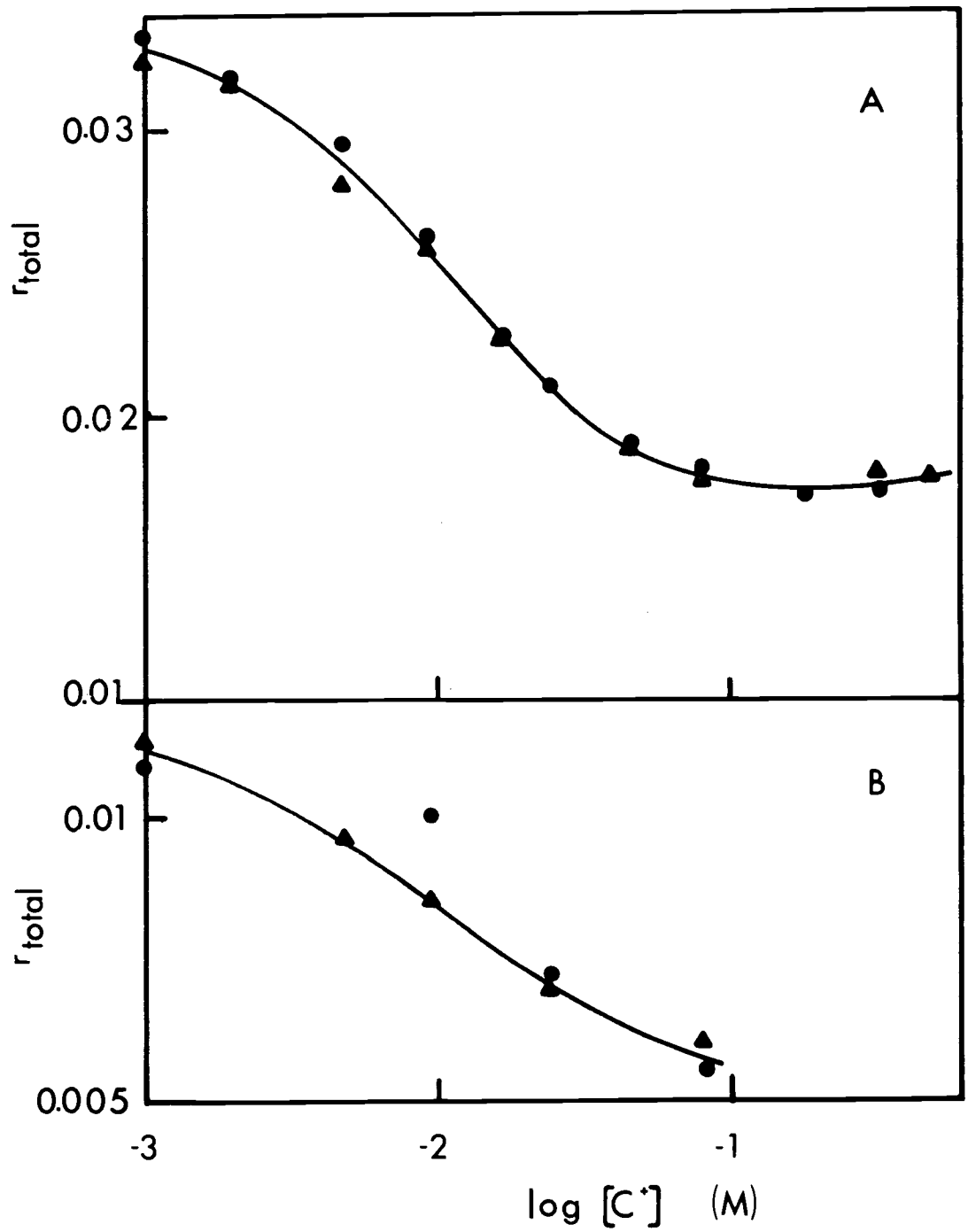
Figure 16. The effect of potassium chloride on energy transfer between tryptophan residues. Light scatter problems at longer excitation wavelengths were essentially elliminated by increasing the emission wavelength to 365 nm for all measurements. Solutions contained 0.1 mg/ml pyruvate kinase in 10 mM Tricine-TMA-OH (pH 7.6) buffer at 22°. Salt concentrations were (○), 0.3 M KCl and (△), 1.0 mM KCl.

anisotropy. Low concentrations of pyruvate kinase were necessary with the LiCl titrations in order to avoid enzyme precipitation. While 10 mM monovalent cation reduced the εADP anisotropy of solutions of 2 μM εADP and 2.7 μM pyruvate kinase to about 50% of their salt free levels (Figure 17), 2 mM Mg^{++} was sufficient to reduce the anisotropy to less than 5% of that observed when Mg^{++} was omitted.

A comparative binding study of εADP to pyruvate kinase in different cation salt solutions was conducted. Concentrations of εADP in the titrations were sufficient for 0.15 to 0.8 saturation of the available enzyme binding sites as suggested by Deranleau (54). A 10 mM cation salt concentration was chosen in order to run parallel experiments with different cations yet avoid the precipitation of pyruvate kinase observed in the presence of 0.1 M LiCl and 2.3 μM pyruvate kinase. Since the K_m for K^+ is about 11.4 mM (27), the 10 mM cation concentration used in the titrations is sufficient to measure activity related effects of salt on εADP binding to enzyme.

Measurements of εADP anisotropy at low εADP concentrations in solutions of pyruvate kinase showed interference from the overlapping fluorescence of enzyme bound tryptophan. To correct the εADP anisotropy for this interference, the following expression, derived from the

Figure 17. The relative effect of cations on εADP binding. Solutions of pyruvate kinase and εADP in 2.5 ml of 10 mM Tricine, 1 mM TMA-Cl, and TMA-OH (pH 7.6) buffer were titrated with solutions of the chloride salts of K^+, Na^+, or Li^+ in buffer. Excitation and emission wavelengths were set at 310 and 440 nm respectively; r_t is the uncorrected total anisotropy measured. Initial concentrations of enzyme and εADP were (A) 2.7 μM and 2.0 μM; (B) 0.54 μM and 2.0 μM respectively. Final volumes after titrations were (A) 3.5 ml; and (B) 2.6 ml. (A): ●, K^+; ▲, Na^+; and (B): ●, K^+; ▲, Li^+.


A
B
0.03
0.02
0.01
0.01
0.005
r_{total}
r_{total}
-3
-2
-1
log [C⁺] (M)

addition law for polarization (51) was used:

$$r_{\varepsilon} = \frac{rI - r_{pk}I_{pk}}{I - I_{pk}} \qquad (3)$$

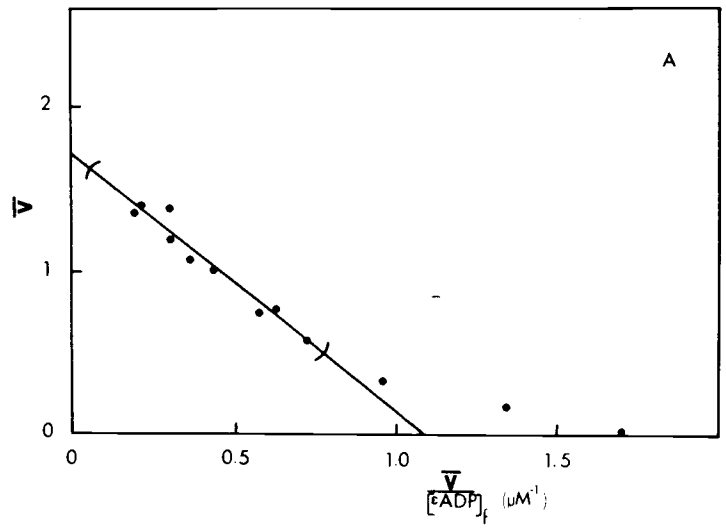
where r_{ε} is the corrected εADP anisotropy, r and I are total measured anisotropy and intensity, and r_{pk} and I_{pk} are anisotropy and intensity of the enzyme solution prior to the addition of εADP. The absence of significant perturbation of tryptophan anisotropy by 10 μM εADP was assumed on the basis of a control experiment with pyruvate kinase where 10 μM 5'-ADP produced negligible changes in tryptophan anisotropy.

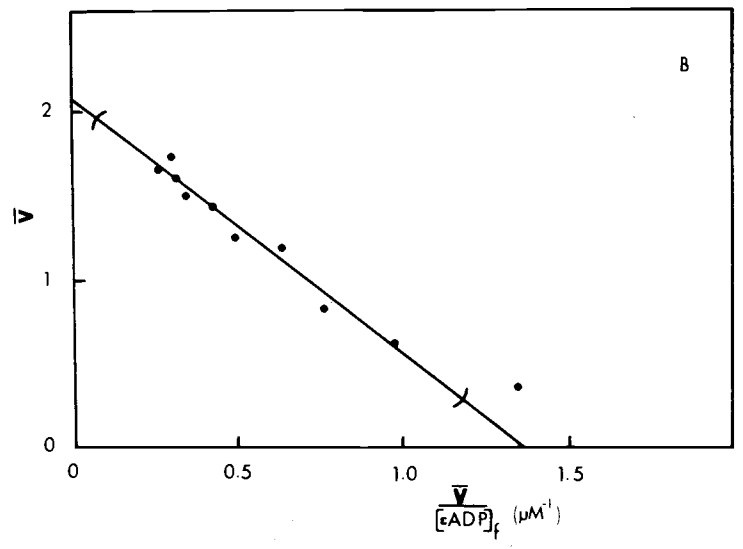
The εADP titrations were designed so that salt and enzyme concentrations remained constant as the concentration of εADP in the cuvette increased. Enzyme solutions containing the different salts were prepared in duplicate and diluted with equal volumes of either buffer or εADP solution. The buffer-diluted solution was placed in a cuvette and the εADP-diluted solution was placed in a micrometer syringe. Anisotropy measurements were made at 22° after the additions of εADP to the cuvette. No significant εADP anisotropy change was observed within 30 minutes of the additions to the cuvette. Limiting εADP anisotropy values were taken as r_2 (Equation 2) = 0.0028 for free εADP and r_1 (Equation 1) = 0.0239 for εADP bound to enzyme. The value for free εADP anisotropy was measured for εADP in buffer solution and the value for bound εADP was estimated

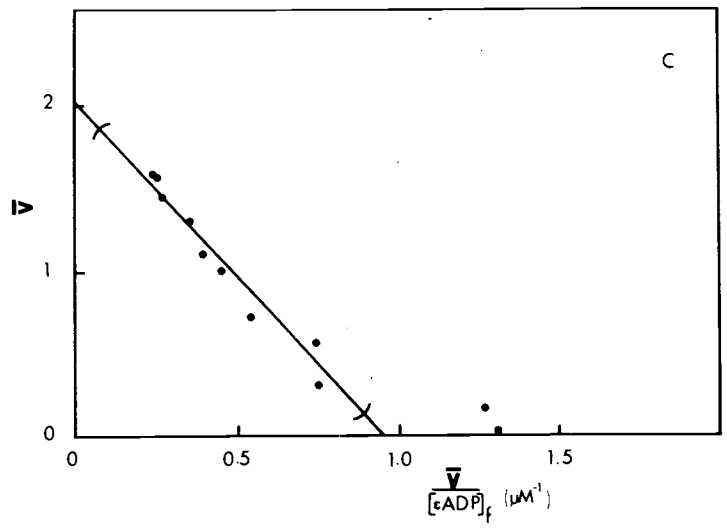
graphically from protein titrations where concentrations of εADP and salt remained constant.

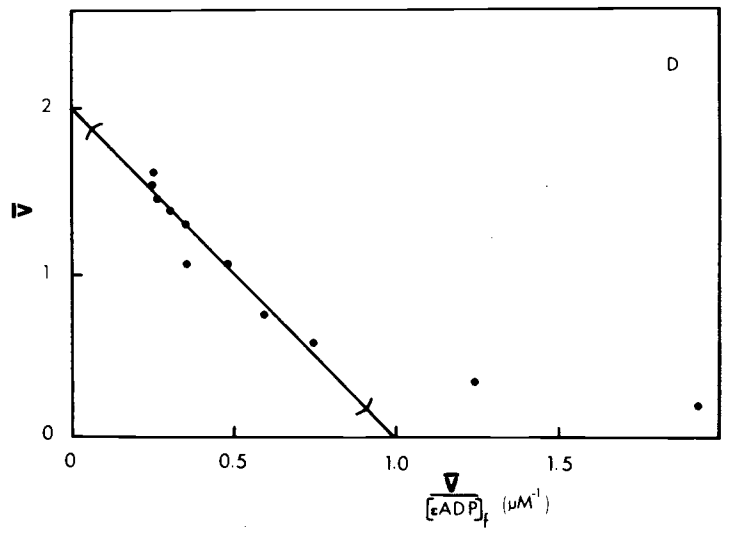
Changes in εADP anisotropy in the presnece of 2.3 μM pyruvate kinase and 10 mM K^+, Na^+, Li^+, or TMA^+ were alike within experimental error. An apparent dissociation constant, K_d' = 1.8 (±0.3) μM (four experiments), and an apparent number of binding sites, two, were obtained from least-square fits of Scatchard (55) plots with each of these monovalent cations (Figure 18A-D). These results should not be interpreted on a stoichiometric basis since enzyme activity was low and the limiting εADP value was estimated (see Pyruvate Kinase Assay in "Experimental Procedure"). Either partially denatured binding sites or a low estimate for the limiting bound εADP anisotropy could cause the apparent number of εADP sites to be less than the four sites observed for phosphoenolpyruvate (32, 56), Tl^+ (32), or Mn^{++} (40).

Figure 18. Scatchard plots of the comparative binding of εADP to pyruvate kinase in various 10 mM chloride cation solutions. Lines are linear least-squares fits to the indicated data points. (A) K^+, (B) TMA^+, (C) Li^+, (D) Na^+. See text for details.


A
$\bar{v}$
2
1
0
0
0.5
1.0
1.5
$\frac{\bar{v}}{[\varepsilon ADP]_f}$ (μM^{-1})


B
$\bar{v}$
2
1
0
0
0.5
1.0
1.5
$\frac{\bar{v}}{[\varepsilon ADP]_f}$ (μM^{-1})


C
$\bar{v}$
2
1
0
0
0.5
1.0
1.5
$\frac{\bar{v}}{[\varepsilon ADP]_f}$ (μM^{-1})


D
$\bar{v}$
2
1
0
0
0.5
1.0
1.5
$\frac{\bar{v}}{[\varepsilon ADP]_f}$ (μM^{-1})

X. DISCUSSION

Pyruvate kinase fluorescence intensity measurements (57) and difference spectra (58, 59) have been reported to be functions of K^+ concentration. Some of these studies (57, 58) have neglected to mention whether similar measurements are obtained in the presence of the inhibitor, Li^+. If these parameters are to be interpreted as reflecting K^+ activation, they should also be observable when induced by other activating cations such as Rb^+ or NH_4^+ and the changes should be different for the weak activator, Na^+, the non-activator, TMA^+, and the inhibitor, Li^+.

Of the monovalent cations used, all were found to have essentially similar effects on the tryptophan residue anisotropy and εADP binding for pyruvate kinase. In general, salt additions increased the tryptophan residue anisotropy and appeared to increase the dissociation of εADP from enzyme. Similarly, no differential effects of the monovalent cations have been observed on dissociation studies (6), apotryptophanase, or tryptophan residue anisotropy measurements. Although thallium has been used as an NMR sensitive, monovalent, cation probe in pyruvate kinase studies (28, 32), only immunoelectrophoretic behavior (60) and activity (27) have been reported to clearly distinguish between activating K^+ and non-activating Li^+. Nowak recently measured NMR differences between pyruvate

kinase solutions containing either activating or non-activating organic monovalent cations (22). If a parallel is drawn to tryptophanase, where spectral measurements of the holoenzyme indicate a specific cation function near the coenzyme binding site, monovalent cations might also activate pyruvate kinase by forming a "bridge" between the carboxyl of phosphoenolpyruvate and the enzyme as suggested by Nowak and Mildvan (31). The systhesis of an enzymatically active fluorescent analog of phosphoenolpyruvate would be most useful for checking such a model.

Of the enzymes investigated, pyruvate kinase was unique in giving a large increase in tryptophan anisotropy with increased ionic strength. The nature of this anisotropy change was investigated by altering the wavelength of exciting light. According to Weber and Shinitzky, identical aromatic molecules fail to transfer energy when excited at the long wavelength edge of their absorption spectrum (52). If the observed anisotropy increase were due to an increased distance between tryptophan residues (a decrease in energy transfer), then the anisotropy change with added salt should be less as the exciting wavelength becomes longer. If tryptophan freedom becomes more restricted or the fluorescent lifetime is decreased with the salt addition, tryptophan anisotropy changes might be expected to be essentially independent of the exciting wavelength. The observed increases were nearly parallel for the 0.1 mM and

300 mM KCl solutions (Figure 16) indicating that the salt effect is probably related to tryptophan residue freedom or lifetime of fluorescence rather than energy transfer.

The apparent holotryptophanase dissociation constant of 3.8 μM obtained in this paper for coenzyme to apoenzyme in 0.1 M K^+ at 22° is consistent with the 2 μM K_m obtained by Snell et al. (61) at 37° considering probable temperature dependent differences in the binding forces (25). The observation that the Na^+ solution K_d is larger than the K^+ solution K_d agrees with spectral evidence (1) that the K^+ species of coenzyme is covalently bound to apotryptophanase. This binding difference is also in accord with the temperature effects on holotryptophanase circular dichroism spectra which show the coenzyme dissociating from the apoenzyme at low temperatures more readily in Na^+ solution than in K^+ solution. Ammonia effects on coenzyme binding to apoenzyme are more difficult to interpret since this cation has a dual function as activator for the tryptophan degradation reaction (2, 4) and substrate in the tryptophan synthesis reaction of tryptophanase (3). The apparently tighter binding of coenzyme to enzyme in NH_4^+ solution may in part be due to the charge orientation of the NH_4^+ cation relative to the binding site ligands in accord with "finger printing" studies of Eisenman and Krasne (7).

The order of magnitudes for the half-times of rates for binding of pyridoxal-P to apotryptophanase of

approximately 0.5 min with K^+ and 2.5 min with Na^+ at 23° (1) are in agreement with the order of the magnitudes of the half-times obtained when holotryptophanase is perturbed by the addition of K^+ or Na^+, about 1.6 and 2.2 min respectively. Both rate studies indicate that the Na^+ species is slower to form which is consistent with a model where K^+ may facilitate positioning and stabilizing of the coenzyme on the enzyme. Interpretation of the intermediate rate values measured for the other cations might also be feasible in terms of their respective interactions with enzyme and pyridoxal-P.

The current study suggests that tryptophan perturbation by monovalent cations is not necessarily related to enzyme activation since Li^+, Na^+, and K^+ have similar restricting effects on pyruvate kinase tryptophan rotation and no apparent effect on apotryptophanase tryptophan anisotropy. The distance between pyruvate kinase tryptophan residues is apparently unaffected by salt additions. Binding of εADP to pyruvate kinase is altered to essentially the same extent by activating or non-activating monovalent cations. Holotryptophanase tends to bind coenzyme more tightly in the presence of the activating cations, K^+ or NH_4^+, than in the presence of the non-activator, Na^+. All the observations are consistent with but do not prove a

similar K^+ role for either binding between phosphoenolpyruvate and pyruvate kinase or between coenzyme and tryptophanase.

BIBLIOGRAPHY

1. Morino, Y. and Snell E. E. (1967) J. Biol. Chem. 242, 2800-2809

2. Happold, F. C. and Struyvenberg, A. (1954) Biochem. J. 58, 379-382

3. Watanabe, T. and Snell, E. E. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 1086-1090

4. Newton, W. A. and Snell, E. E. (1964) Proc. Nat. Acad. Sci. U.S.A. 51, 382-389

5. Kagamiyama, H., Matsubara, H. and Snell, E. E. (1972) J. Biol. Chem. 247, 1576-1586

6. Morino, Y. and Snell E. E. (1967) J. Biol. Chem. 242, 5591-5601

7. Eisenman, G. and Krasne, S. J. (in press) in MTP International Review of Sciences, Biochemistry Series (Fox, C. F., ed) Vol. 2, Butterworths, London

8. Peterson, E. A. and Sober, H. A. (1954) J. Amer. Chem. Soc. 76, 169-175

9. Howard, R. L. and Becker, R. R. (1970) J. Biol. Chem. 245, 3186-3194

10. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427

11. Kagamiyama, H., Wada, H., Matsubara, H., and Snell, E. E. (1972) J. Biol. Chem. 247, 1571-1575

12. Morino, Y. and Snell, E. E. (1967) J. Biol. Chem. 242, 5602-5610

13. Morino, Y. and Snell, E. E. (1967) J. Biol. Chem. 242, 2793-2799

14. Morino, Y. and Snell, E. E. (1967) Proc. Nat. Acad. Sci. U.S.A. 57, 1692-1699

15. Newton, W. A. and Snell, E. E. (1962) Proc. Nat. Acad. Sci. U.S.A. 48, 1431-1439

16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275

17. Greenfield, N. and Fasman, G. D. (1969) Biochemistry 10, 4108-4116

18. Kagamiyama, H., Morino, Y., and Snell, E. E. (1970) J. Biol. Chem. 245, 2819-2824

19. Suelter, C. H. (1970) Science 168, 789-795

20. Mueller, P. and Rudin, D. O. (1967) Biochem. Biophys. Res. Commun. 26, 398-404

21. Christensen, J. J., Hill, J. O., and Izatt, R. M. (1971) Science 174, 459-467

22. Nowak, T. (1974) Fed. Proc. 33, 496

23. Metzler, D. (1957) J. Amer. Chem. Soc. 79, 485-490

24. Honikel, K. O. and Madsen, N. B. (1972) J. Biol. Chem. 247, 1057-1064

25. Kauzmann, W. (1959) in Advances in Protein Chemistry (Anfinsen, C. B., Jr., Anson, M. L., Bailey, K., and Edsell, J. T., eds) Vol. XIV, pp. 1-63, Academic Press, New York and London

26. Hille, B. (1971) J. Gen. Physiol. 58, 599-619

27. Kachmar, J. F. and Boyer, P. D. (1953) J. Biol. Chem. 200, 669-682

28. Kayne, F. J. and Reuben, J. (1970) J. Amer. Chem. Soc. 92, 220-222

29. Mildvan, A. S. and Cohn, M. (1966) J. Biol. Chem. 241, 1178-1193

30. Wilson, R. H. and Evans, H. J. (1968) in The Role of Potassium in Agriculture, pp. 189-202, 677 South Segoe Road, Madison, Wisconsin 53711

31. Nowak, T. and Mildvan, A. S. (1972) Biochemistry 11, 2819-2828

32. Kayne, F. J. (1971) Arch. Biochem. Biophys. 143, 232-239

33. James, T. L. and Cohn, M. (1974) J. Biol. Chem. 249, 3519-3526

34. James, T. L., Reuben, J., and Cohn, M. (1973) J. Biol. Chem. 248, 6443-6449

35. Secrist, J. A., III, Bario, J. R., Leonard, N. J., and Weber, G. (1972) Biochemistry 11, 3499-3506

36. Teale, F. W. J. (1960) Biochem. J. 76, 381-388

37. Cottam, G. L., Hollenberg, P. F., and Coon, M. J. (1969) J. Biol. Chem. 244, 1481-1486

38. Bucher, T. and Pfleiderer, G. (1955) in Methods in Enzymology, Vol. 1, pp. 435-440, Academic Press, New York

39. McQuate, J. T. and Utter, M. F. (1959) J. Biol. Chem. 234, 2151-2157

40. Cottam, G. L. and Mildvan, A. S. (1971) J. Biol. Chem. 246, 4363-5365

41. McPhie, P. (1971) in Methods in Enzymology (Jakoby, W. B., ed) Vol. 22, pp. 23-32, Academic Press, New York

42. Beisenherz, V. G., Boltze, H. J., Bucher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G. (1953) Z. Naturforsch. 8b, 555-577

43. Handbook of Biochemistry (Sober, H. A., ed) 2nd edn, p. C-71, The Chemical Rubber Co., Cleveland, Ohio

44. Handbook of Biochemistry (Sober, H. A., ed) 2nd edn, p. C-79, The Chemical Rubber Co., Cleveland, Ohio

45. Hu, A. S. L., Wolfe, R. G., and Reithel, F. J. (1959) Arch. Biochem. Biophys. 81, 500-507

46. Evett, J. and Isenberg, I. (1969) Ann. N. Y. Acad. Sci. 158, 210-222

47. Evett, J., McKenzie, R. L., and Isenberg, I. (1970) Biochemistry 9, 4513-4519

48. Ayres, W. A., Small, E. W., and Isenberg, I. (1974) Anal. Biochem. 58, 361-367

49. Teale, F. W. J. and Weber, G. (1957) Biochem. J. 65, 476-482

50. Jablonski, A. (1960) Bull. Acad. Polon. Sci. Phys. 8, 259-264

51. Weber, G. (1952) Biochem. J. 51, 145-155

52. Weber, G. and Shinitzky, M. (1970) Proc. Nat. Acad. Sci. U.S.A. 65, 823-830

53. Burstein, E. A., Vedenkina, N. S., and Ivkova, M. N. (1973) Photochem. Photobiol. 18, 263-279

54. Deranleau, D. A. (1969) J. Amer. Chem. Soc. 91, 4044-4049

55. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672

56. Cardenas, J. M., Dyson, R. D., and Strandholm, J. J. (1973) J. Biol. Chem. 248, 6931-6937

57. Suelter, C. H. (1967) Biochemistry 6, 418-423

58. Suelter, C. H., Singleton, R., Jr., Kayne, F. J., Arrington, S., Glass, J., and Mildvan, A. S. (1966) Biochemistry 5, 131-139

59. Wilson, R. H., Evans, H. J., and Becker, R. R. (1967) J. Biol. Chem. 242, 3825-3832

60. Sorger, G. J., Ford, R. E., and Evans, H. J. (1965) Proc. Nat. Acad. Sci. U.S.A. 54, 1614-1621

61. Newton, W. A., Morino, Y., and Snell, E. E. (1965) J. Biol. Chem. 240, 1211-1218