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FUNG JOU LU (Name) for the MASTER OF SCIENCE (Degree)

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Title: THE EFFECT OF COENZYME ON THE BIS(1-ANILINO-8-NAPHTHALENE-SULFONATE) INDUCED ASSOCIATION OF LACTIC DEHYDROGENASE

Abstract approved: Redacted for privacy
Dr. Sonia R. Anderson

Bis(1-anilino-8-naphthalenesulfonate) (bis(ANS)) induces the association of M_4 lactic dehydrogenase (LDH) isolated from either beef or dogfish (Squalus acanthias) muscle. The association is reversed by the addition of coenzyme (reduced 3-acetylpyridine adenine dinucleotide (AcPyADH)).

Direct binding measurements using the ultracentrifuge equipped with scanner show that the addition of 10 equivalents of AcPyADH to a solution of beef M_4 containing 14 equivalents of bis(ANS) causes the $s_{20,w}$ to decrease from 17.12 to 7.04 S and $\bar{n}$ (moles of dye bound per mole of protein) to decrease from 10.7 to 7. The concurrent release of 3.7 moles of bis(ANS) indicates that four of the bound bis(ANS) molecules are essential for association. Possibly the four essential dye binding sites overlap the four coenzyme binding sites. The seven to eight nonessential bound dye molecules are spectroscopically distinct.

Similar experiments with beef H_4 LDH show that the change in $s_{20,w}$ is negligible while $\bar{n}$ decreases from 8.78 to 7.14. Again, there appear to be at least two kinds of spectroscopically distinguishable binding sites. The value of $\bar{n}$ obtained from the scanner, 8.78, agrees well with the value predicted from fluorescence titrations, 9.2. This, in conjunction with the previous circular dichroism spectra, shows that the 12 binding sites have similar affinities for bis(ANS).

The interaction with bis(ANS) reveals that dogfish M_4 and beef M_4 are very similar to each other and that each is different from beef H_4. Apparently the binding of bis(ANS) and the reversible association reflect the same essential characteristic of M_4 which has been retained throughout the vertebrate evolutionary line.

THE EFFECT OF COENZYME ON THE BIS(1-ANILINO-8-NAPHTHALENESULFONATE) INDUCED ASSOCIATION OF LACTIC DEHYDROGENASE

by

FUNG-JOU LU

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Redacted for privacy

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Typed by Linda Knuth for Fung-Jou Lu

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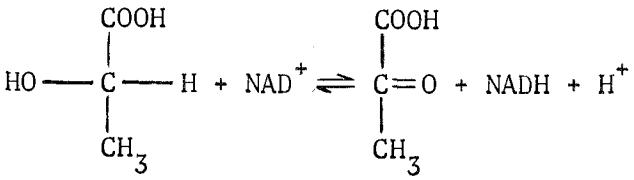
INTRODUCTION

Lactic dehydrogenase (EC 1.1.1.27) (LDH) is an NAD^+-dependent enzyme catalyzing the interconversion of lactate and pyruvate in the glycolytic pathway:



The LDH activity in many vertebrates can be separated into five electrophoretically distinct components with identical molecular weights (1, 2). Appella and Markert first reported that LDH is dissociated into four subunits of molecular weight 35,000 by reagents such as urea and guanidine hydrochloride (3). To account for the five electrophoretically distinct bands, Appella and Markert proposed that LDH is a tetramer composed of complementary proportions of two types of subunit (3). Kaplan et. al. (2) designated these subunits as M, "muscle" type, and H, "heart" type, in recognition of the characteristic distribution of the five components in various organs.

In vitro hybridization experiments supported the tetramer hypothesis. Markert obtained all five isozymes in the expected proportions 1:4:6:4:1 by freezing and thawing a one to one mixture of beef H_4 and M_4 in a solution containing sodium chloride (4). Fingerprinting (5),

amino acid analyses (5), complement fixation studies (5), and x-ray diffraction data (6) also confirmed this model.

The relationship between the subunit structure and the overall properties of LDH has attracted general interest. Anderson and Weber studied the binding of NADH to the five LDH isozymes isolated from beef (7). They found that each isozyme binds four moles of coenzyme per 140,000 g of protein and that the binding of NADH to the five isozymes varies in a characteristic fashion. Beef H_4 has four independent, equivalent binding sites whereas beef M_4 shows marked changes in the order of the binding reaction near 25% saturation. The hybrids exhibit intermediate binding characteristics.

Later, Anderson and Weber studied the reversible acid dissociation and hybridization of LDH (8). They used 1-anilino-8-naphthalenesulfonate (ANS) to measure the appearance of hydrophobic binding sites in the enzyme at low pH. ANS is nonfluorescent in water but becomes highly fluorescent in nonpolar solvents and when bound to apparently hydrophobic sites in protein (9).

Recently, Anderson has discovered that bis(1-anilino-8-naphthalenesulfonate) (bis(ANS)) is useful for studying native LDH. Bis(ANS) is a dimer with the essential fluorescence characteristics of ANS (10). Beef M_4 and H_4 both have 10 $\sim$ 12 primary bis(ANS) binding sites. The most fascinating observation is that bis(ANS) induces the association of M_4 isolated from either beef or chicken. The sedimentation coefficient of beef M_4 increases from 7 to 22 S when 12 moles of bis(ANS) is bound per mole of enzyme. This is the first

example of association beyond the tetramer in LDH. The association is reversed by the addition of NADH. The binding of bis(ANS) to H_4 results in little or no detectable association.

The purpose of this research is to answer the following questions on the interaction of bis(ANS) with LDH.

1. How does the binding of NADH affect the binding of bis(ANS)?

2. How many of the primary dye binding sites are essential for the association of M_4?

3. Are any spectroscopically distinct intermediates produced on the addition of NADH to the LDH-bis(ANS) mixtures?

4. How general is the association of M_4?

This report contains fluorescence, circular dichroism, and sedimentation measurements on the binding of bis(ANS) to both M_4 and H_4 in the presence of reduced 3-acetylpyridine adenine dinucleotide (AcPyADH). The dissociation constant for the binding of AcPyADH to LDH is 50 times smaller than the dissociation constant for NADH (Anderson, unpublished result). This tight binding is advantageous in stoichiometry determinations and in competition experiments.

MATERIALS AND METHODS

Enzymes

Beef muscle LDH was prepared by the method of Pesce et. al. (11). Dogfish (Squalus acanthias) muscle LDH was also prepared by the method of Pesce et. al. (12) with an additional step. The partially purified dogfish muscle LDH was further applied to a Bio-Gel P-200 column (60 x 2.5 cm) and eluted with 0.1 M potassium phosphate buffer at pH 7.0, 4° to obtain pure enzyme. Pure beef heart LDH was obtained from the Miles-Seravac Company as an ammonium sulfate suspension. Before the experiments, the enzyme was thoroughly dialyzed against 0.2 M potassium phosphate (pH 7.0) to give solutions containing 15 $\sim$ 20 mg/ml of protein. The published molar extinction coefficients (11, 12) were used to determine the enzyme concentrations.

Enzyme Assays

The initial reaction velocities were determined on a Cary 15 spectrophotometer. The assay of Anderson and Weber (8), based on the reduction of NAD^+ by lactate, was used.

Assay Reagents

DL-lactate and NAD^+ were obtained from Sigma Chemical Company. AcPyADH was prepared by the procedure of Rafter and Colowick (13). Bis(ANS) was donated by Professor Gregorio Weber. The dye was dissolved in glass-distilled water to give 10^{-3} M stock solutions.

An extinction coefficient of 1.7×10^4 cm^2/mole at 387 nm was used to determine the dye concentration (14).

Fluorescence Measurements

All fluorescence spectra were determined using the Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The spectra were corrected for the wavelength dependence of the grating transmission and photomultiplier response. The procedures for the fluorescence titrations were described by Anderson (10). The excitation and emission wavelengths used for the titrations were 430 and 500 nm respectively. Constant temperature (20°) was maintained by circulating water from a constant temperature bath.

Circular Dichroism

The circular dichroism spectra were recorded on the Jasco Model CD-SP circular dichroism recorder and spectrophotometer. A 0.5 ml volume cuvette with 1 cm path length was used. The scan speed was set at 1 nm per minute. The direct contribution of the protein was eliminated by measurement of the difference between the circular dichroism spectra of the LDH-dye mixture and the equivalent solution of LDH alone. This correction is significant at wavelengths below 310 nm. The values of $(\varepsilon_\ell - \varepsilon_r)$ were calculated using the total bis(ANS) concentrations.

Sedimentation Velocity

Sedimentation experiments were conducted with the Spinco Model E ultracentrifuge. The rotor speed and temperature were 48,000 rpm and 20°, respectively. The values of $s_{20,w}$ were calculated using the maximum ordinates of the gradient curves. The dye binding was measured with the Spinco Model E ultracentrifuge equipped with scanner (15).

RESULTS

Fluorescence Studies

In order to examine the effect of coenzyme binding on the fluorescence of the LDH-bis(ANS) mixture, I recorded the complete fluorescence emission spectra using an exciting wavelength of 430 nm. The reduced coenzyme does not absorb significantly at this wavelength. Thus competition for the exciting light and any coenzyme fluorescence are excluded.

The stoichiometric addition of AcPyADH to solutions containing either beef M_4 or H_4 and 14 equivalents of bis(ANS) results in a linear decrease in fluorescence with a well defined endpoint occurring on the addition of four equivalents of coenzymes (Figures 1 and 2). At saturation, the fluorescence intensity of the bis(ANS) is reduced to 31% of its initial value in the case of beef M_4 and to 60% in the case of beef H_4. In each case, the shape and position of the emission spectrum are unaltered (Figures 3 and 4).

Circular Dichroism Studies

The free bis(ANS) molecule is symmetric and therefore has no optically active absorption bands. Binding, however, induces asymmetry. The circular dichroism spectra of the bis(ANS) adsorbates of beef M_4 and H_4 shown in Figures 5 and 6 agree with those previously reported.

The circular dichroism spectrum of AcPyADH bound to either beef M_4 or H_4 has a strong positive band centered near 350 nm (Figure 7). Comparison of Figures 5, 6 and 7 reveals the wavelength regions useful in studying the effect of coenzyme addition on the adsorbates. At 360 nm, the bis(ANS) adsorbates have a value of 0 for $(\varepsilon_\ell - \varepsilon_r)$. Thus measurements of the circular dichroism at this wavelength should reflect directly the binding of AcPyADH. The M_4 adsorbate of bis(ANS) has a strong positive band at 420 nm while the H_4 adsorbate has negative band at 400 nm. Since AcPyADH absorbs only slightly at wavelengths above 400 nm, measurements in this region should indicate directly any changes in the binding of bis(ANS).

Figures 8 and 9 show that the addition of AcPyADH to solutions containing either beef M_4 or H_4 and 12 equivalents of bis(ANS) results in the predicted linear increase in circular dichroism at 360 nm with an endpoint corresponding to the binding of four equivalents of coenzyme. The circular dichroism measured at 420 nm decreases with an endpoint occurring on the addition of four equivalents of coenzyme to beef M_4 (Figure 8). The titration of beef H_4 gives contrasting results: the addition of AcPyADH causes the circular dichroism at 400 nm to become even more negative. However, the endpoint again corresponds to four equivalents of coenzyme.

The isodichroic points in the circular dichroism spectra recorded between 300 and 450 nm show that there are no spectrally distinct intermediates (Figure 5). I calculated the circular dichroism

spectrum of the adsorbed dye present at saturating coenzyme concentrations by subtracting the spectrum of the LDH-AcPyADH complex from the spectrum of the corresponding LDH-bis(ANS)-AcPyADH mixture (LDH-bis(ANS)-AcPyADH——LDH-AcPyADH). This difference, shown in Figure 10 and 11, indicates that there is a significant quantity of dye bound to both M_4 and H_4 at saturating coenzyme concentrations. The circular dichroism spectrum of this remaining dye differs in shape from the spectrum recorded before coenzyme addition. I also calculated the following difference spectrum: [LDH-bis(ANS)——(LDH-bis(ANS)-AcPyADH——LDH-AcPyADH)] where LDH-bis(ANS) represents the spectrum of LDH and bis(ANS) obtained in the absence of AcPyADH. The results of this calculation are also given in Figures 10 and 11.

Sedimentation Velocity Studies

I carried out the following experiments to determine the stoichiometry of the coenzyme effect on the bis(ANS) induced association.

A solution containing 8.4 mg/ml of beef M_4 and 7.2×10^{-4} M bis(ANS) in 0.1 M potassium phosphate buffer, pH 7, was sedimented in a 12 mm double sector cell at a rotor speed of 52,000 rpm (20°). The schlieren pattern of this mixture contained a single peak with $s_{20,w} = 19.5$ S. When two equivalents of AcPyADH was added to the mixture, the schlieren pattern contained two peaks--7.12 and 13.26 S.

The addition of four or more equivalents of AcPyADH caused the appearance of a single peak with $s_{20,w}$ = 7.17 S (Table 1).

Binding Measurements Using the Scanner

The Spinco Model E ultracentrifuge equipped with scanner was used to measure the amount of bound bis(ANS). The monochromator was set to 420 nm, where AcPyADH shows negligible absorbance. Solutions containing 1 mg/ml LDH and the specified concentrations of AcPyADH and bis(ANS) in 0.1 M potassium phosphate buffer, pH 7, were sedimented at a rotor speed of 48,000 rpm (20°) until the boundary was well separated from the meniscus. A standard containing bis(ANS) alone was also run to relate the observed optical density to concentration. The optical density of the supernatant solution yielded the concentration of free dye. The optical density measured in the plateau region was not used since 420 nm is 30 nm above the isosbestic point.

Table II summarizes the values of $\bar{n}$ and the sedimentation coefficients. The addition of ten equivalents of AcPyADH to a solution of beef M_4 containing 14 equivalents of bis(ANS) causes $s_{20,w}$ to decrease from 17.12 to 7.04 S and $\bar{n}$ to decrease from 10.7 to 7. Similar experiments with beef H_4 show that the change in $s_{20,w}$ is negligible while $\bar{n}$ decreases from 8.78 to 7.14. The value of $\bar{n}$ measured before coenzyme addition, 8.78, agrees well with the value calculated for 12 binding sites with $K = 10^{-5}$ M, 9.2 (10).

Dogfish Muscle Lactic Dehydrogenase

Tables 2 and 3 and the circular dichroism spectra in Figures 12 and 13 summarize comparative experiments conducted with M_4 isolated from the Pacific dogfish (Squalus acanthias).

DISCUSSION

Stoichiometric binding of AcPyADH reverses the bis(ANS) induced association of beef M_4. Sedimentation, circular dichroism, and fluorescence measurements confirm the stoichiometry of four moles of coenzyme per 140,000 g of LDH. Direct binding measurements using the ultracentrifuge equipped with scanner show that 3.7 moles of bis(ANS) are released when saturating amounts of AcPyADH are added. These results indicate that four of the 12 bound bis(ANS) molecules are essential for association. Possibly the four essential dye binding sites overlap the four coenzyme binding sites. The seven to eight nonessential bound dye molecules are spectroscopically distinct. Calculations from the fluorescence titrations (Figure 3) and the direct binding measurements show that the quantum yield of the four essential bis(ANS) molecules is four times larger than the average yield of the remaining dye. The fluorescence time decay of the beef M_4-bis(ANS) adsorbates had previously demonstrated the existence of multiple components (10).

Similar experiments with beef H_4, which is non-associating, show that saturating concentrations of AcPyADH displace 1.6 moles of bis(ANS). Again, there appear to be at least two kinds of spectroscopically distinguishable binding sites. Since LDH is a tetramer, all binding sites will occur in multiples of four. The fact that less than four moles of dye is displaced can mean either that the binding of the two ligands is noncompetitive or that the

initial bis(ANS) concentration was not large enough to saturate all of the binding sites. Distinction between these possibilities would require additional experiments using higher dye concentrations. Although the quantum yield of the susceptible dye molecules is three times larger than that of the remainder, the previous fluorescence titrations appear to be correct. The value of $\bar{n}$ obtained from the scanner, 8.78, agrees well with the value predicted from fluorescence titrations, 9.2. This, in conjunction with the previous circular dichroism spectra (10), shows that the 12 binding sites have similar affinities for bis(ANS).

Experiments with M_4 isolated from the Pacific dogfish (Squalus acanthias) are interesting since the dogfish is a primitive vertebrate which diverged from the main vertebrate evolutionary line 400 million years ago. Tables 2 and 3 show that dogfish M_4 undergoes association in solutions of bis(ANS). The addition of AcPyADH reverses the association with partial displacement of bound bis(ANS). The circular dichroism spectra of the adsorbates of dogfish M_4 and beef M_4 are also similar. Thus the interaction with bis(ANS) reveals that dogfish M_4 and beef M_4 are very similar to each other and that each is different from beef H_4. Apparently the binding of bis(ANS) and the reversible association reflect the same essential characteristic of M_4 which has been retained throughout the vertebrate evolutionary line.

Bis(ANS) and ANS have similar fluorescence characteristics. Yet the former induces the association of M_4 while the latter does

not. Bis(ANS) is a dimer of ANS probably formed by uniting the phenyl rings through their para positions. Thus bis(ANS) can serve as a bridge between two different protein molecules. The reduced binding to M-type subunits found in the hybrid LDH's was consistent with crosslinking, direct or indirect, of the M subunits.

Table 1: Sedimentation Velocity Studies on Beef M_4 LDH in Solutions Containing Bis(ANS), and AcPyADH; conditions: 0.1 M potassium phosphate, pH 7 (20°); rotor speed: 52,000 rpm; optics: schlieren, cells: 12 mm double sector

LDH	Concn (mg/ml)	[Bis(ANS)] (M)	[AcPyADH] (M)	Dyes:Protein: AcPyADH (moles:mole: moles)	$s_{20,w}$(S) Peak 1	Peak 2
Beef M_4	8.4	7.2×10^{-4}	0	12:1:0	19.50	
Beef M_4	8.4	7.2×10^{-4}	1.2×10^{-4}	12:1:2	7.12 (46%)	13.26 (54%)
Beef M_4	8.4	7.2×10^{-4}	2.4×10^{-4}	12:1:4	7.18	
Beef M_4	8.4	7.2×10^{-4}	4.8×10^{-4}	12:1:8	7.17	

Table 2: Sedimentation Velocity Studies on LDH in Solutions Containing Bis(ANS) and AcPyADH; Conditions: 0.1 M potassium phosphate pH 7.0 (20°); rotor speed: 48,000; optics: scanner; cells: 12 mm double sector.

LDH	Conc (mg/ml)	[Bis(ANS)] (M)	[AcPyADH] (M)	Dyes:Protein: AcPyADH (moles:mole: moles)	$\bar{n}$	$s_{20,w}$(S)
Beef M_4	1	10^{-4}	0	14:1:0	10.7	17.12
Beef M_4	1	10^{-4}	1.4×10^{-5}	14:1:2	9.5	--
Beef M_4	1	10^{-4}	3.5×10^{-5}	14:1:5	7.7	--
Beef M_4	1	10^{-4}	7.2×10^{-5}	14:1:10	7.0	7.04
Beef H_4	1	10^{-4}	0	14:1:0	8.78	7.31
Beef H_4	1	10^{-4}	7.2×10^{-5}	14:1:10	7.14	7.21
Dogfish M_4	1	10^{-4}	0	14:1:0	9.79	12.35
Dogfish M_4	1	10^{-4}	3.6×10^{-5}	14:1:5	7.61	7.93
Dogfish M_4	1	10^{-4}	7.2×10^{-5}	14:1:10	7.24	7.13

Table 3: Sedimentation Velocity Studies on Dogfish M_4 LDH in Solution Containing Bis(ANS); conditions: 0.1 M potassium phosphate, pH 7 (20°); rotor speed: 52,000 rpm; optics: schlieren; cells: 12 mm double sector

LDH	Concn (mg/ml)	[Bis(ANS)] (M)	Dyes:protein (moles:mole)	$s_{20,w}$(S) Peak 1	$s_{20,w}$(S) Peak 2
Dogfish M_4	8.4	0	0	6.70	
Dogfish M_4	8.4	2.4×10^{-4}	4.0	6.90(66%)	10.80(34%)
Dogfish M_4	8.4	3.88×10^{-4}	7.4	7.15(42%)	12.47(58%)
Dogfish M_4	8.4	4.98×10^{-4}	9.8	7.29(21.5%)	14.23(71.5%)

Figure 1: Fluorescence titration of beef M_4-bis(ANS) with AcPyADH.

A mixture of 1 mg/ml of beef M_4 and 10^{-4} M bis(ANS) in a 1 cm cuvette was titrated with 10 ul increments of 10^{-3} M AcPyADH. After each addition of AcPyADH, the fluorescence intensity was recorded. The endpoint occurred on the addition of 4 equivalents of coenzyme. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0). Excitation at 430 nm. Emission at 500 nm. One mole of protein is defined as 140,000 g (11).

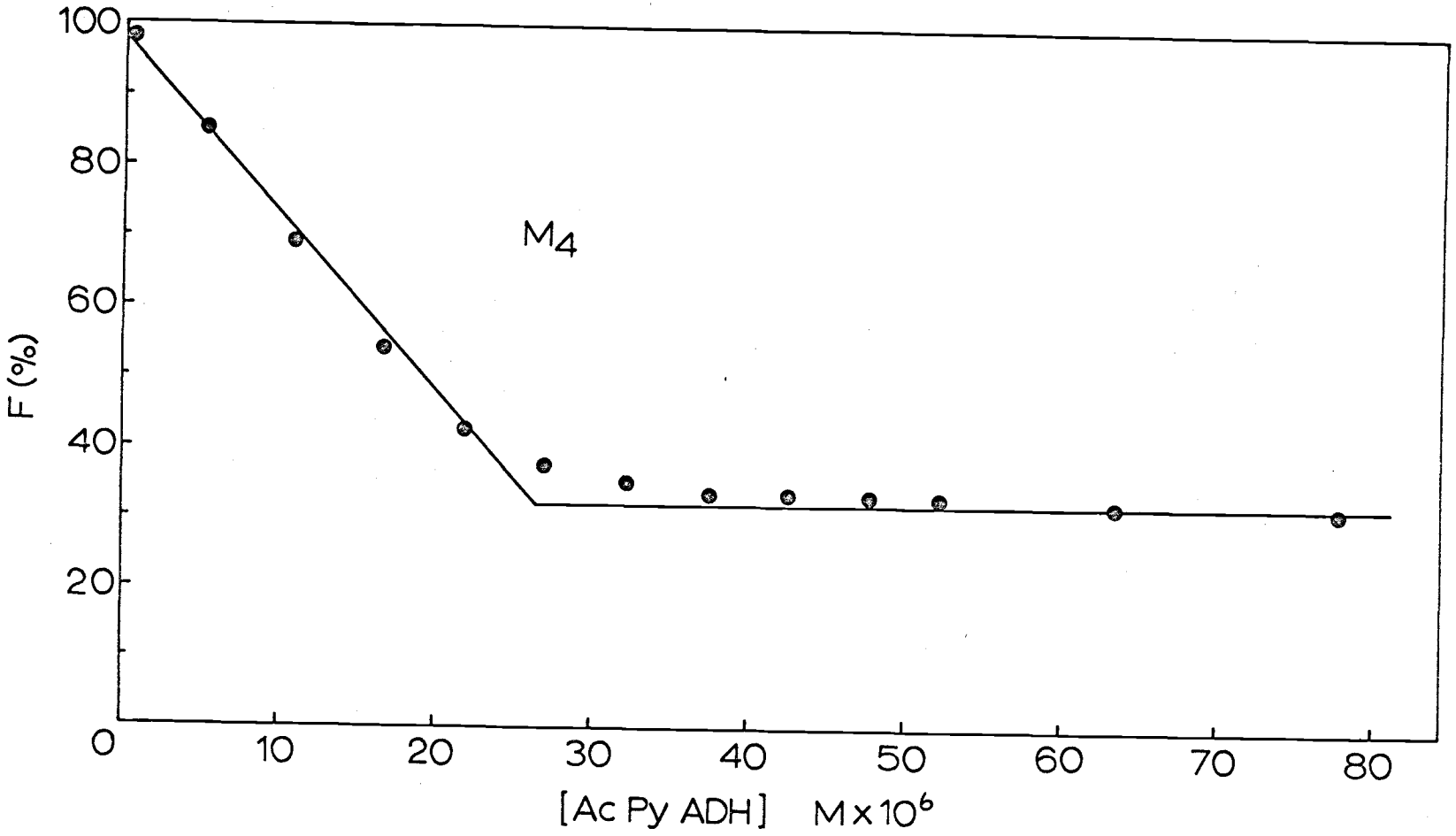

100
80
60
40
20
0
F (%)
M4
10
20
30
40
50
60
70
80
[Ac Py ADH] M x 10⁶

Figure 2: Fluorescence titration of beef H_4-bis(ANS) with AcPyADH.

A mixture of 1.25 mg per ml of beef H_4 and 10^{-4} M bis(ANS) in a 1 cm cuvette was titrated with 10 ul increments of 10^{-3} M AcPyADH. After each addition of AcPyADH, the fluorescence intensity was recorded. The endpoint occurred on the addition of 4 equivalents or coenzyme. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0). Excitation at 430 nm. Emission at 500 nm. One mole of protein is defined as 140,000 g.

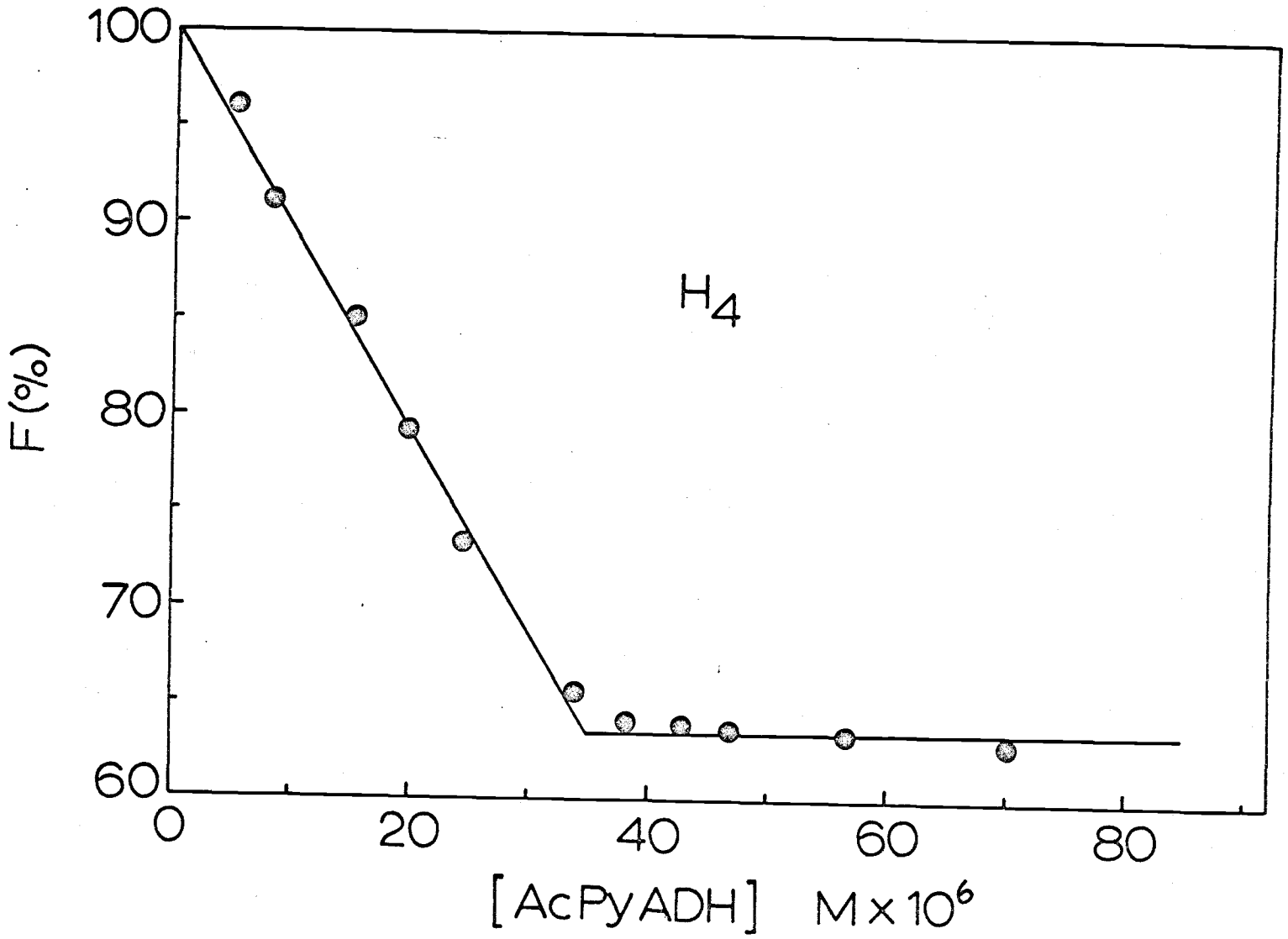

100
90
80
70
60
F(%)
H_4
0
20
40
60
80
[AcPyADH] M x 10^6

Figure 3: Fluorescence spectra of beef M_4-bis(ANS)-AcPyADH.

Curve 1: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS)

Curve 2: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 4.9 x 10^{-6} M AcPyADH

Curve 3: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 9.9 x 10^{-6} M AcPyADH

Curve 4: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 14.8 x 10^{-6} M AcPyADH

Curve 5: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 19.6 x 10^{-6} M AcPyADH

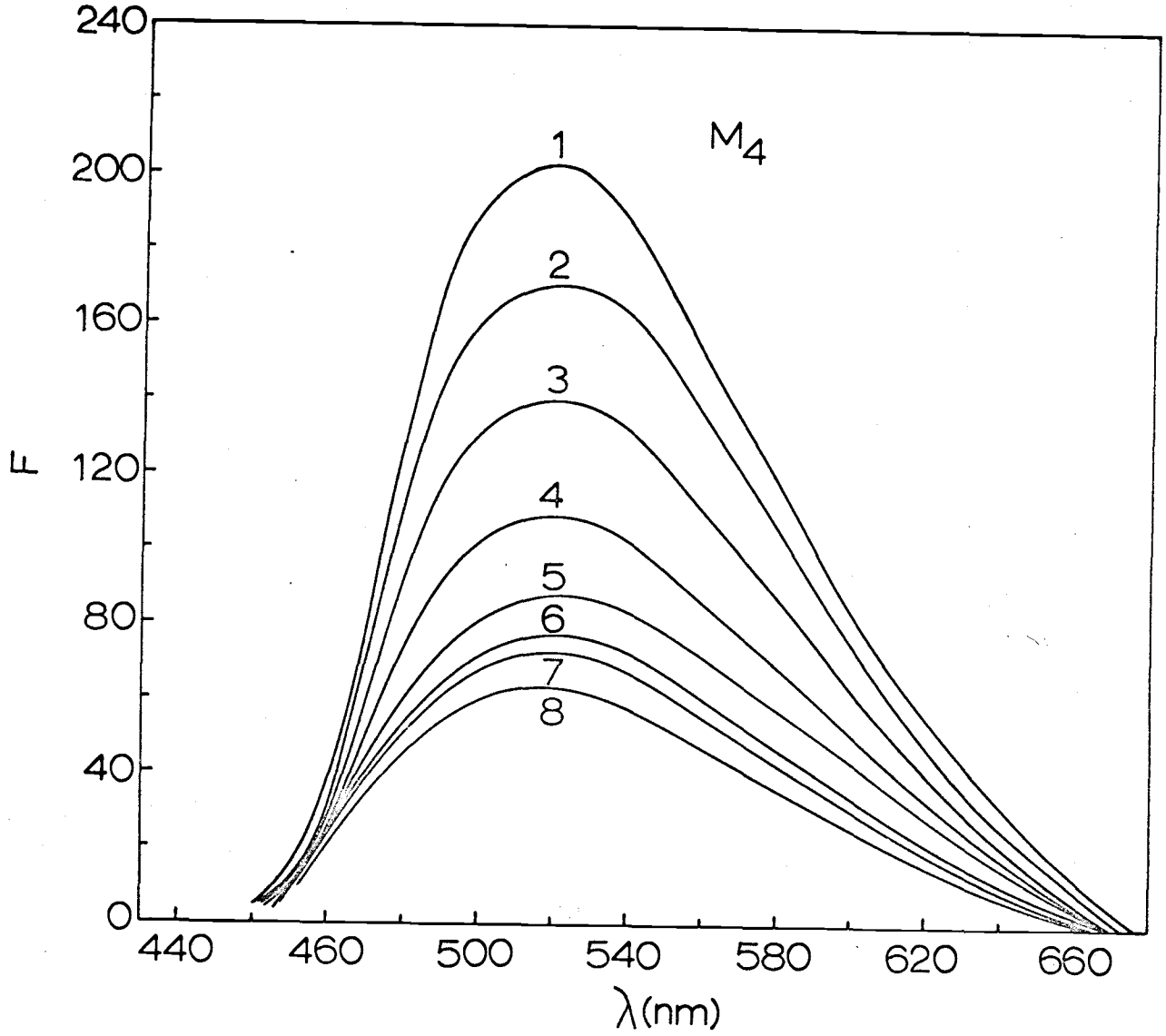
Curve 6: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 24.3 x 10^{-6} M AcPyADH

Curve 7: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 29.1 x 10^{-6} M AcPyADH

Curve 8: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 70.1 x 10^{-6} M AcPyADH

Conditions: 20°, 0.1 M potassium phosphate, pH 7.0

Excitation: 430 nm. Emission: 430 - 600 nm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80
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M4
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2
3
4
5
6
7
8
440
460
500
540
580
620
660
λ(nm)

Figure 4: Fluorescence spectra of beef H_4-bis(ANS)-AcPyADH

Curve 1: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS)

Curve 2: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 4.9 x 10^{-6} M AcPyADH

Curve 3: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 9.9 x 10^{-6} M AcPyADH

Curve 4: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 14.8 x 10^{-6} M AcPyADH

Curve 5: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 19.6 x 10^{-6} M AcPyADH

Curve 6: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 24.3 x 10^{-6} M AcPyADH

Curve 7: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 29.1 x 10^{-6} M AcPyADH

Curve 8: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 33.8 x 10^{-6} M AcPyADH

Curve 9: 1.25 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 70 x 10^{-6} M AcPyADH

Conditions: 20°, 0.1 M potassium phosphate, pH 7.0

Excitation: 430 nm. Emission: 430 - 600 nm

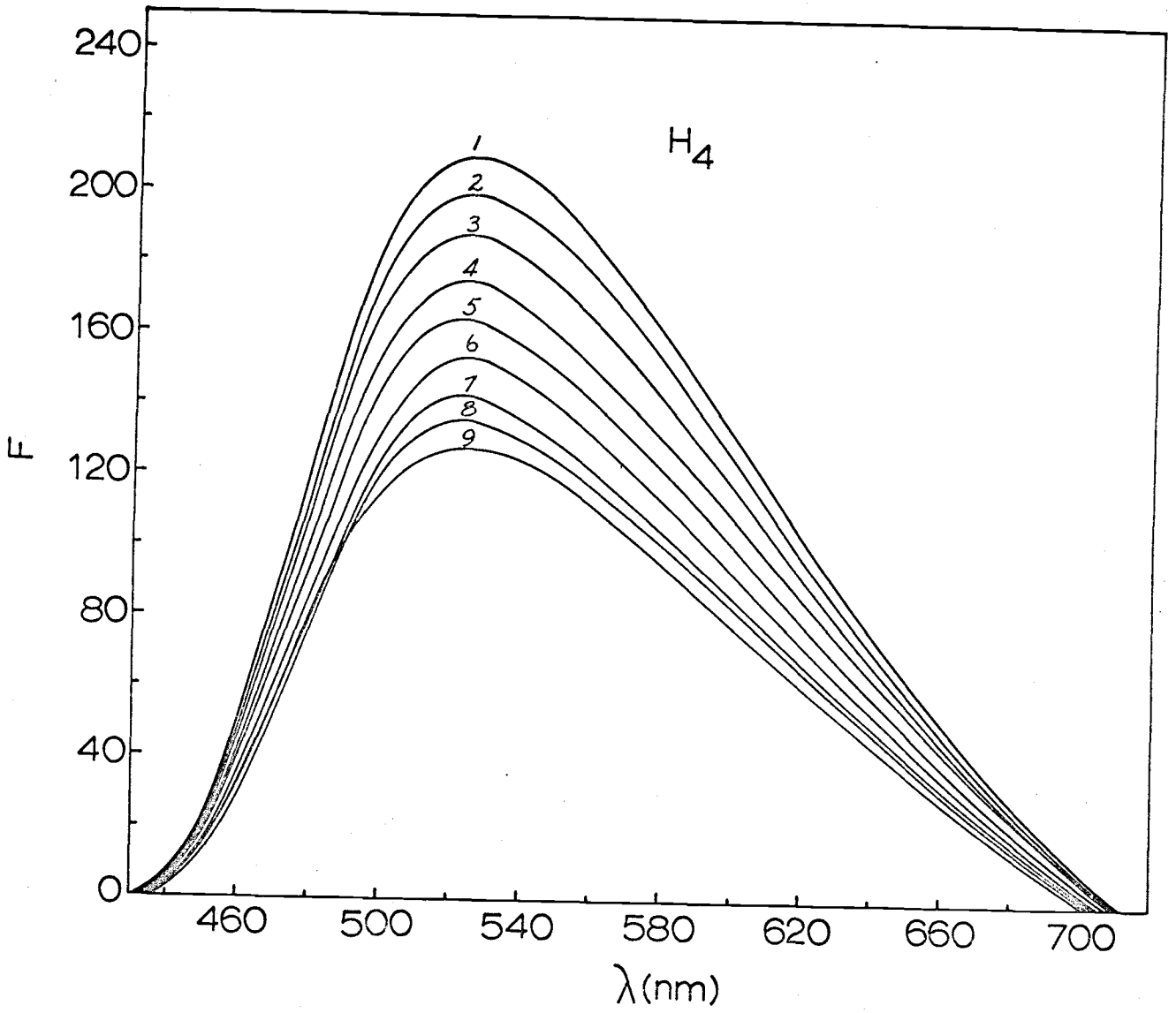

240
200
160
120
80
40
0
F
460
500
540
580
620
660
700
λ(nm)
H4
1
2
3
4
5
6
7
8
9

Figure 5: The circular dichroism spectra of beef M_4-bis(ANS)-AcPyADH.

Curve 1: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS)
Curve 2: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 3.98 x 10^{-6} M AcPyADH
Curve 3: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 7.93 x 10^{-6} M AcPyADH
Curve 4: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 11.85 x 10^{-6} M AcPyADH
Curve 5: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 15.74 x 10^{-6} M AcPyADH
Curve 6: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 19.6 x 10^{-6} M AcPyADH
Curve 7: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 23.4 x 10^{-6} M AcPyADH
Curve 8: 1 mg/ml beef M_4 + 10^{-4} M bis(ANS) + 38.5 x 10^{-6} M AcPyADH

The values of $(\varepsilon_\ell - \varepsilon_r)$ (cm^{-1} M^{-1}) were calculated using the total concentration of dye. Conditions: 20°, 0.1 M potassium phosphate, pH 7.0

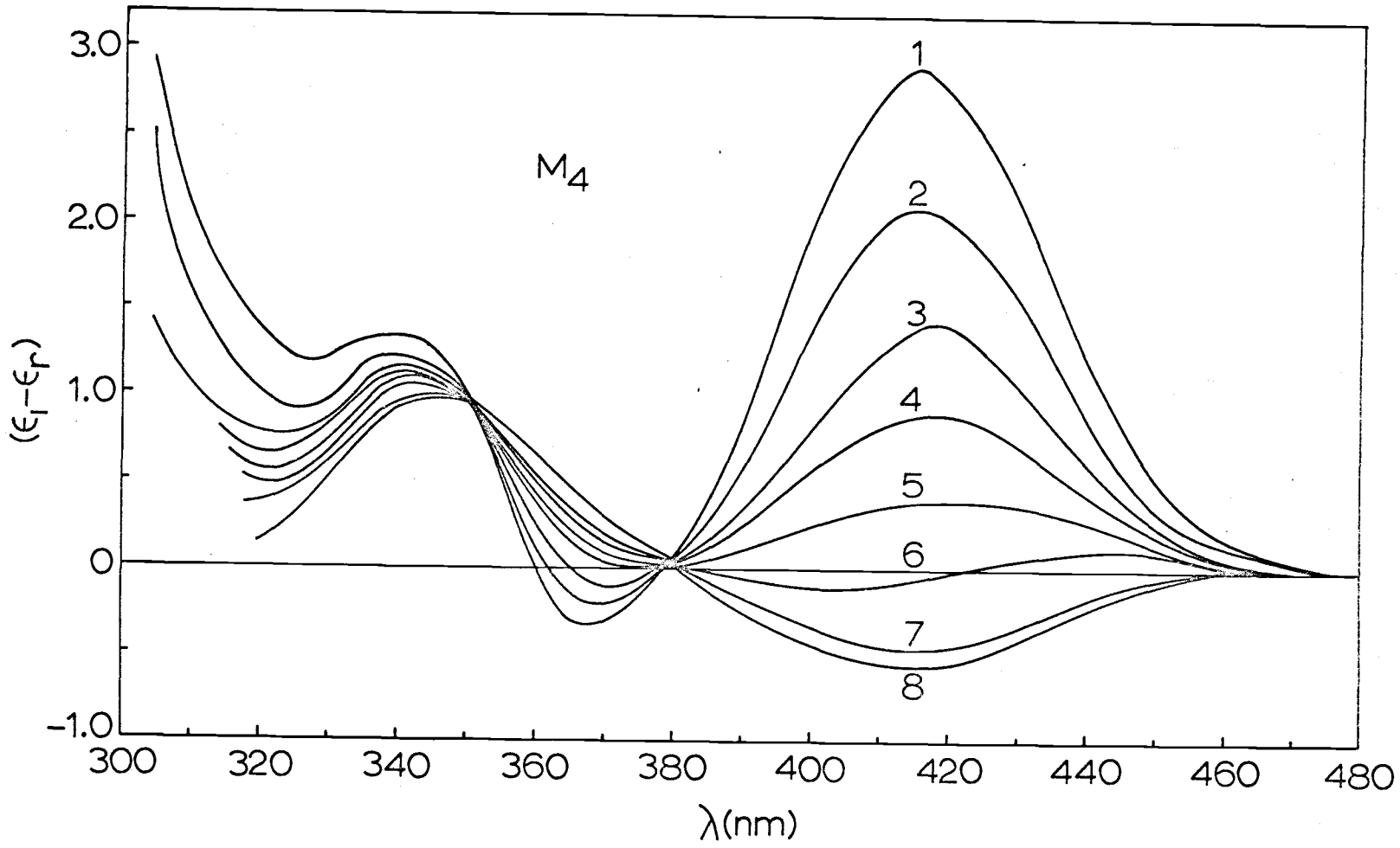

3.0
2.0
1.0
0
-1.0
$(\epsilon_l - \epsilon_r)$
M_4
1
2
3
4
5
6
7
8
300
320
340
360
380
400
420
440
460
480
λ(nm)

Figure 6: The circular dichroism spectra of beef H_4-bis(ANS)-AcPyADH

Curve 1: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS)
Curve 2: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 3.98 x 10^{-6} M AcPyADH
Curve 3: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 7.93 x 10^{-6} M AcPyADH
Curve 4: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 11.85 x 10^{-6} M AcPyADH
Curve 5: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 15.74 x 10^{-6} M AcPyADH
Curve 6: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 23.4 x 10^{-6} M AcPyADH
Curve 7: 1 mg/ml beef H_4 + 10^{-4} M bis(ANS) + 38.5 x 10^{-6} M AcPyADH

The values of $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1} M^{-1})$ were calculated using the total concentration of dye. Conditions: 20°, 0.1 M potassium phosphate, pH 7.0

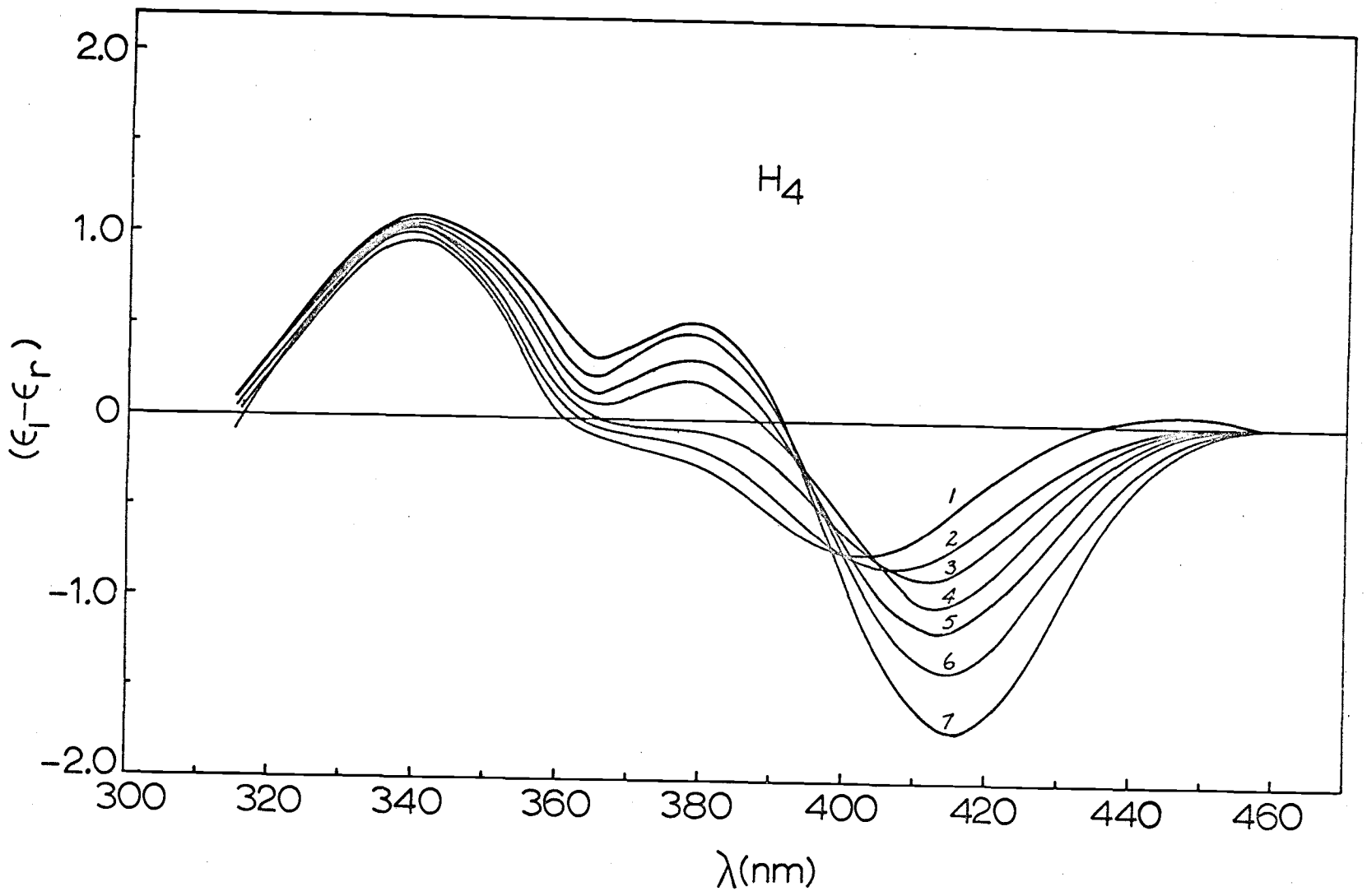

2.0
1.0
0
-1.0
-2.0
$(\epsilon_l - \epsilon_r)$
H_4
1
2
3
4
5
6
7
300
320
340
360
380
400
420
440
460
λ(nm)

Figure 7: The circular dichroism spectra of solutions containing beef M_4 and AcPyADH.

Curve 1: 1 mg/ml beef M_4 + 1.5 x 10^{-5} M AcPyADH
Curve 2: 1 mg/ml beef M_4 + 3.0 x 10^{-5} M AcPyADH
Curve 3: 1 mg/ml beef M_4 + 4.5 x 10^{-5} M AcPyADH
Curve 4: 1 mg/ml beef M_4 + 6.0 x 10^{-5} M AcPyADH
Curve 5: 1 mg/ml beef M_4 + 12 x 10^{-5} M AcPyADH
Curve 6: 10^{-4} M AcPyADH

The total AcPyADH concentration of each curve was used to calculate $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1}\ M^{-1})$. Conditions: 20°, 0.1 M potassium phosphate, pH 7.0

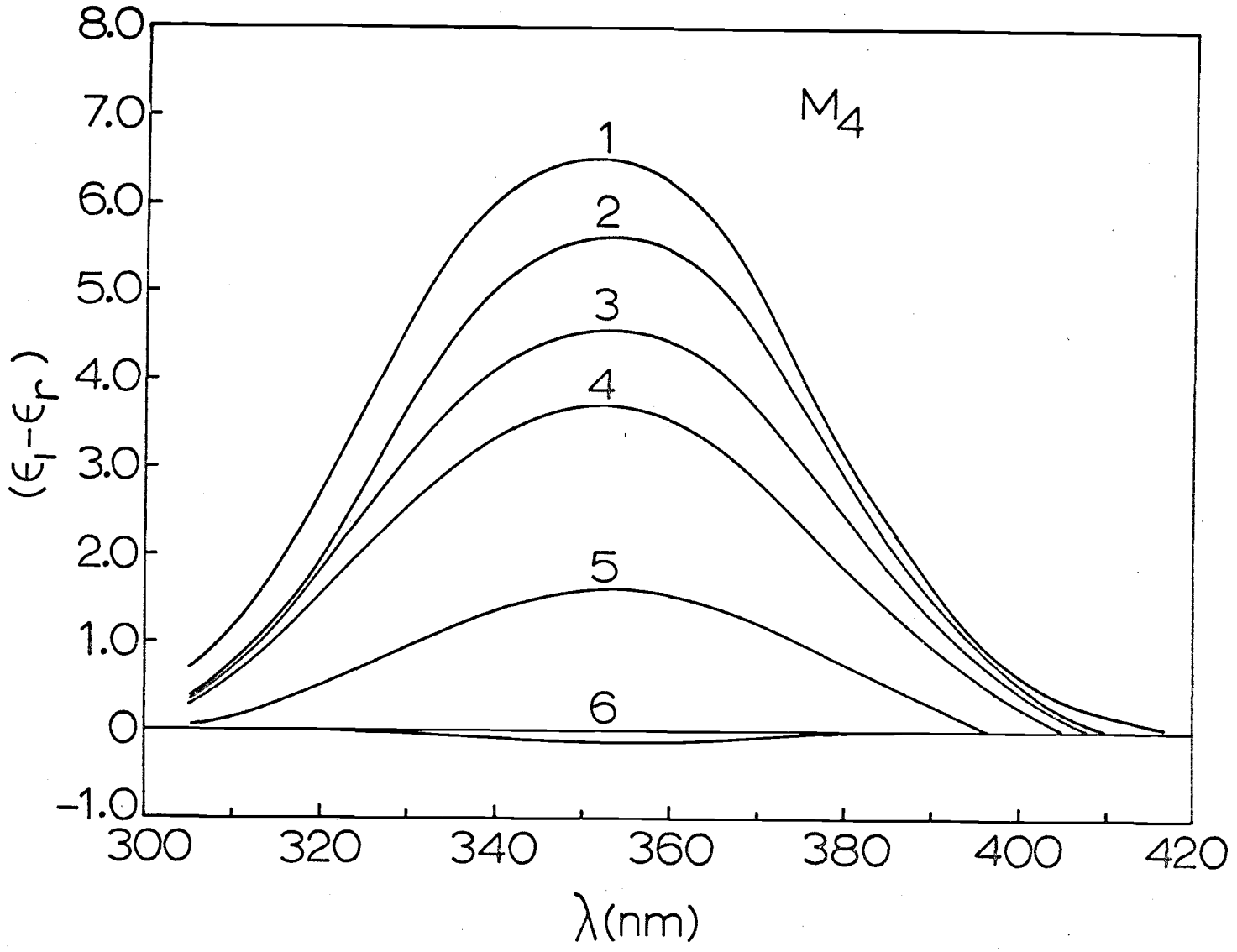

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
-1.0
$(\epsilon_l - \epsilon_r)$
M_4
1
2
3
4
5
6
300
320
340
360
380
400
420
λ(nm)

Figure 8: The circular dichroism titration of beef M_4-bis(ANS) with AcPyADH.

A mixture of 1 mg/ml beef M_4 and 8.6×10^{-5} M bis(ANS) was titrated with 2 ul increments of 10^{-3} M AcPyADH at 360 nm and 420 nm, respectively. The values of $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1}\ M^{-1})$ were calculated using the concentration of dye of 8.6×10^{-5} M. An endpoint occurred on the addition of four equivalents of coenzyme. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

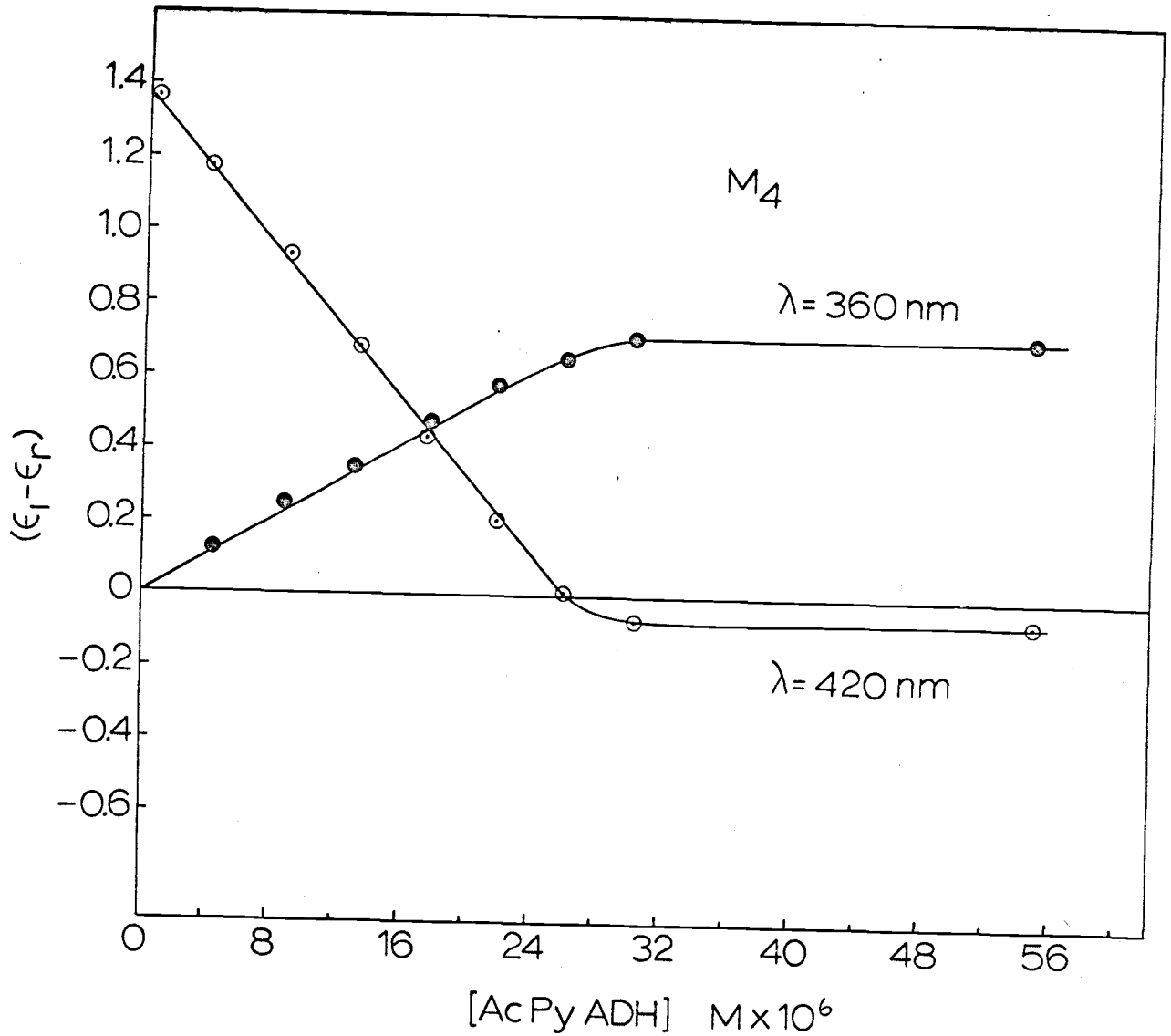

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
$(\epsilon_I - \epsilon_R)$
M_4
λ= 360 nm
λ= 420 nm
0
8
16
24
32
40
48
56
[Ac Py ADH] M x 10^6

Figure 9: The circular dichroism titration of beef H_4-bis(ANS) with AcPyADH.

A mixture of 1 mg/ml beef H_4 and 10^{-4} M bis(ANS) was titrated with 2 ul increments of 10^{-3} M AcPyADH at 366 nm and 420 nm respectively. The values of $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1}\ M^{-1})$ were calculated using the concentration of dye of 10^{-4} M. An end-point occurred on the addition of four equivalents of coenzyme. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

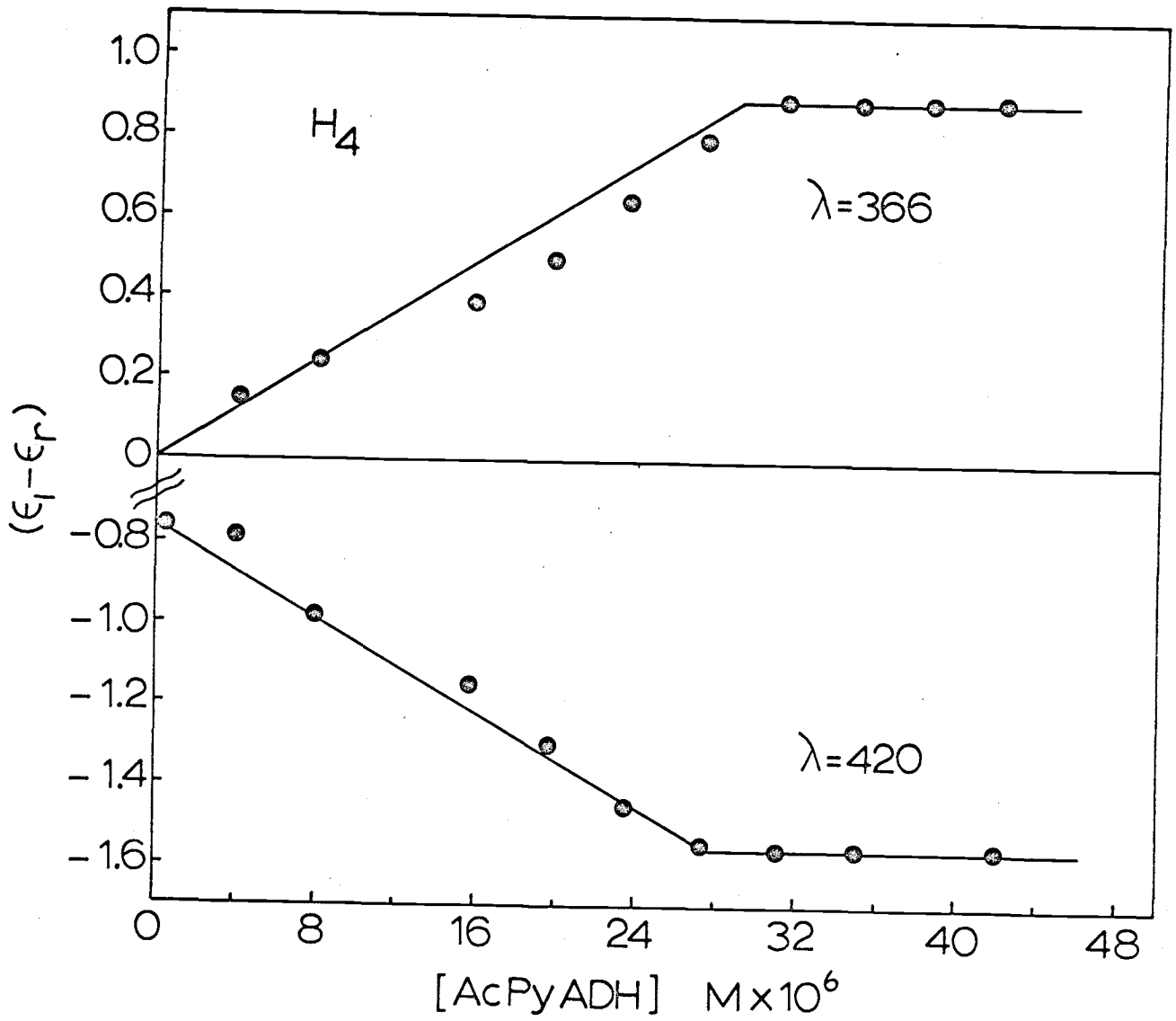

1.0
0.8
0.6
0.4
0.2
0
-0.8
-1.0
-1.2
-1.4
-1.6
$(\epsilon_l - \epsilon_r)$
H_4
λ=366
λ=420
0
8
16
24
32
40
48
[AcPyADH] M x 10^6

Figure 10: The difference circular dichroism spectra of solutions containing 1 mg/ml beef M_4, 10^{-4} M bis(ANS) and 3.85×10^{-5} M AcPyADH.

Curve 1: [LDH-bis(ANS)——(LDH-bis(ANS)-AcPyADH——LDH-AcPyADH)]

Curve 2: (LDH-bis(ANS)-AcPyADH——LDH-AcPyADH)

LDH-bis(ANS): the spectrum of LDH and bis(ANS) obtained in the absence of AcPyADH. The bis(ANS) concentration of 10^{-4} M was used to calculate $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1}\ M^{-1})$. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

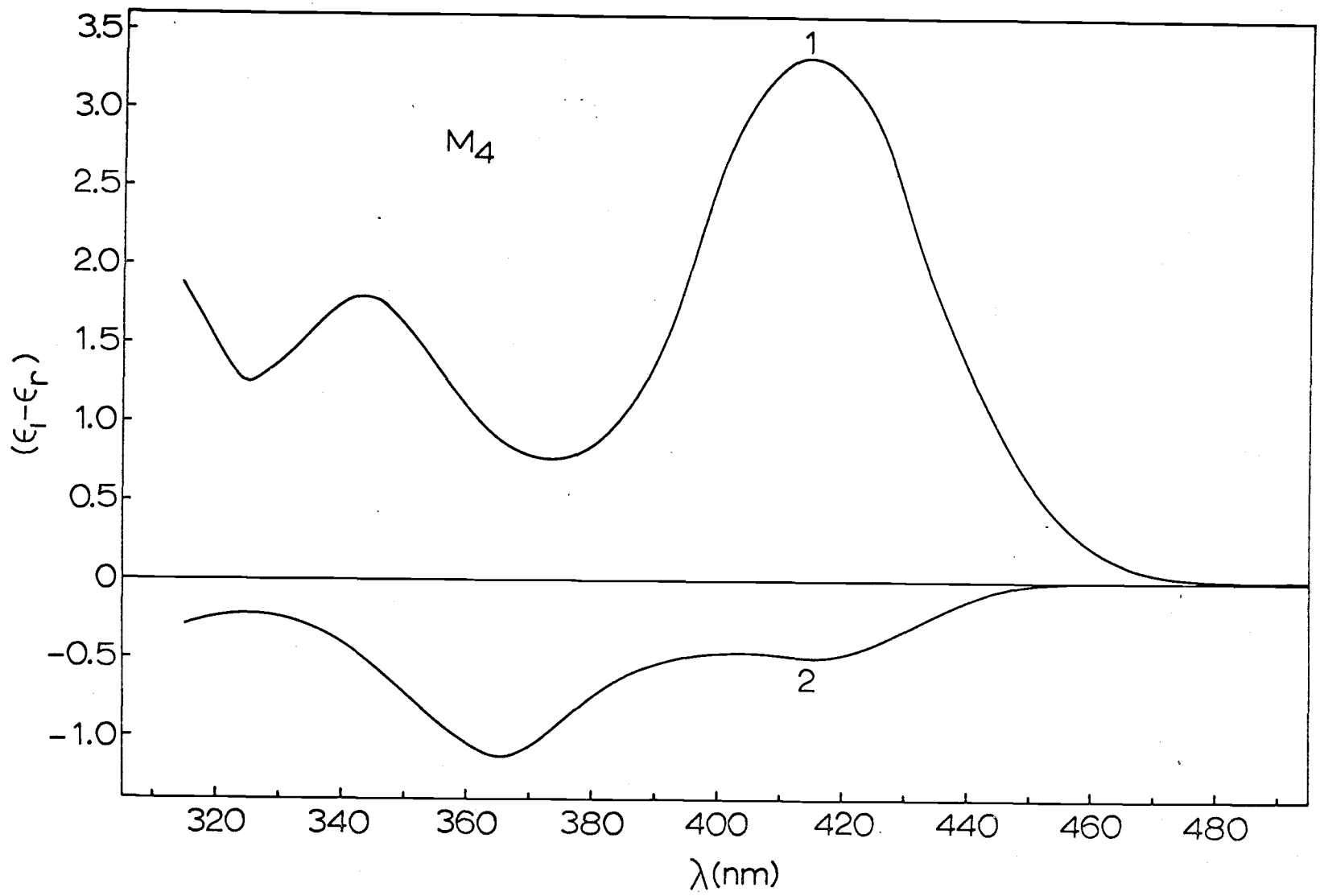

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
-1.0
$(\epsilon_l - \epsilon_r)$
M_4
1
2
320
340
360
380
400
420
440
460
480
λ(nm)

Figure 11: The difference circular dichroism spectra of solutions containing 1 mg/ml beef H_4, 10^{-4} M bis(ANS) and 3.85×10^{-5} M AcPyADH.

Curve 1: [LDH-bis(ANS)——(LDH-bis(ANS)-AcPyADH——LDH-AcPyADH)]

Curve 2: (LDH-bis(ANS)-AcPyADH——LDH-AcPyADH)

LDH-bis(ANS): the spectrum of LDH and bis(ANS) obtained in the absence of AcPyADH. The total bis(ANS) concentration of 10^{-4} M was used to calculate $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1}\ M^{-1})$. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

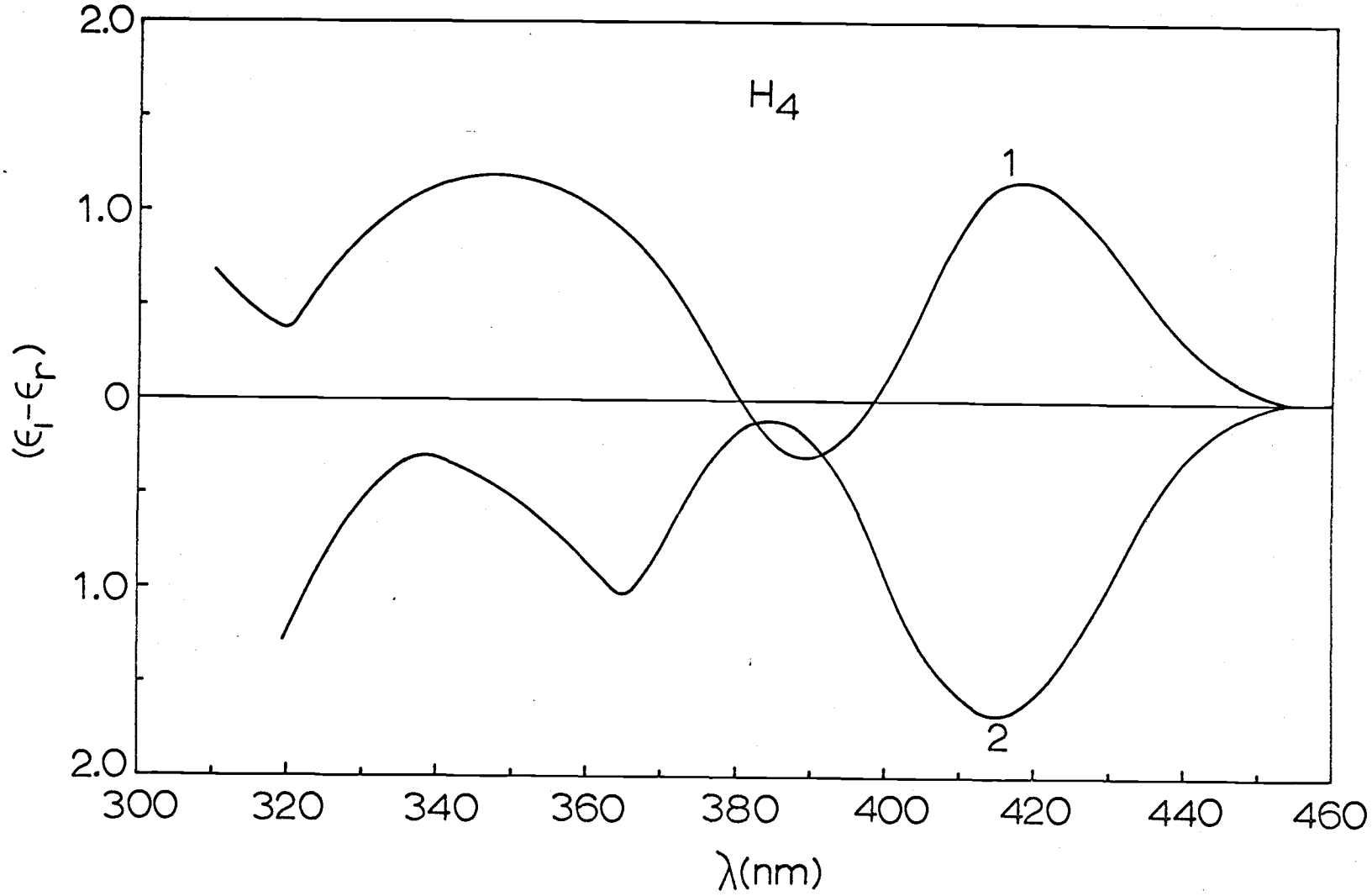

2.0
1.0
0
1.0
2.0
$(\epsilon_l - \epsilon_r)$
H_4
1
2
300
320
340
360
380
400
420
440
460
λ(nm)

Figure 12: The circular dichroism spectra of solutions containing dogfish M_4, bis(ANS) and AcPyADH.

Curve 1: 1 mg/ml dogfish M_4 + 8.6×10^{-5} M bis(ANS)
Curve 2: 1 mg/ml dogfish M_4 + 8.6×10^{-5} M bis(ANS) + 3.9×10^{-6} M AcPyADH
Curve 3: 1 mg/ml dogfish M_4 + 8.6×10^{-5} M bis(ANS) + 7.9×10^{-6} M AcPyADH
Curve 4: 1 mg/ml dogfish M_4 + 8.6×10^{-5} M bis(ANS) + 11.8×10^{-6} M AcPyADH
Curve 5: 1 mg/ml dogfish M_4 + 8.6×10^{-5} M bis(ANS) + 15.7×10^{-6} M AcPyADH
Curve 6: 1 mg/ml dogfish M_4 + 8.6×10^{-5} M bis(ANS) + 23.4×10^{-6} M AcPyADH

The total concentration of 8.6×10^{-5} M bis(ANS) was used to calculate the values of $(\varepsilon_\ell - \varepsilon_r)$ (cm^{-1} M^{-1}). Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

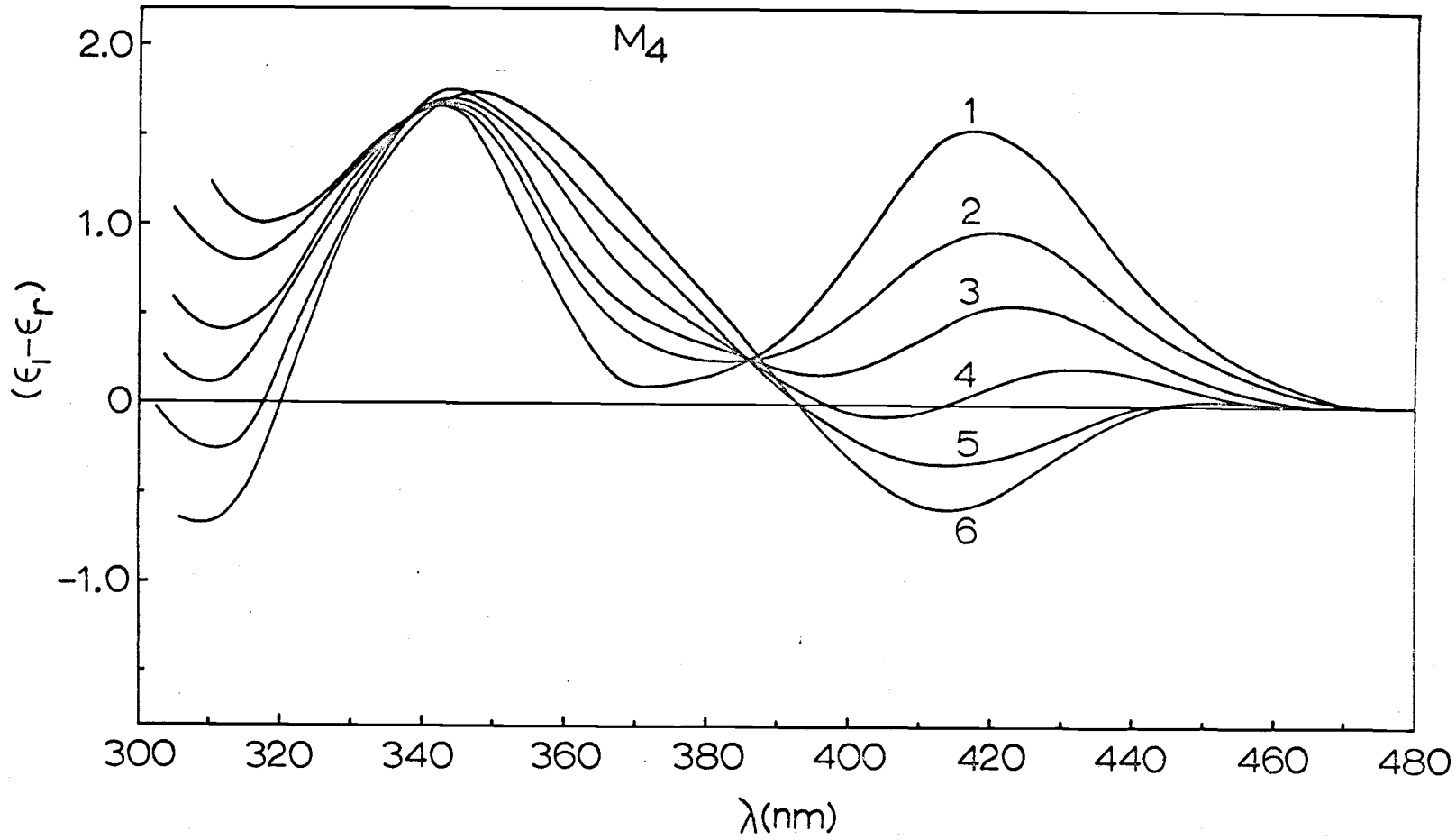
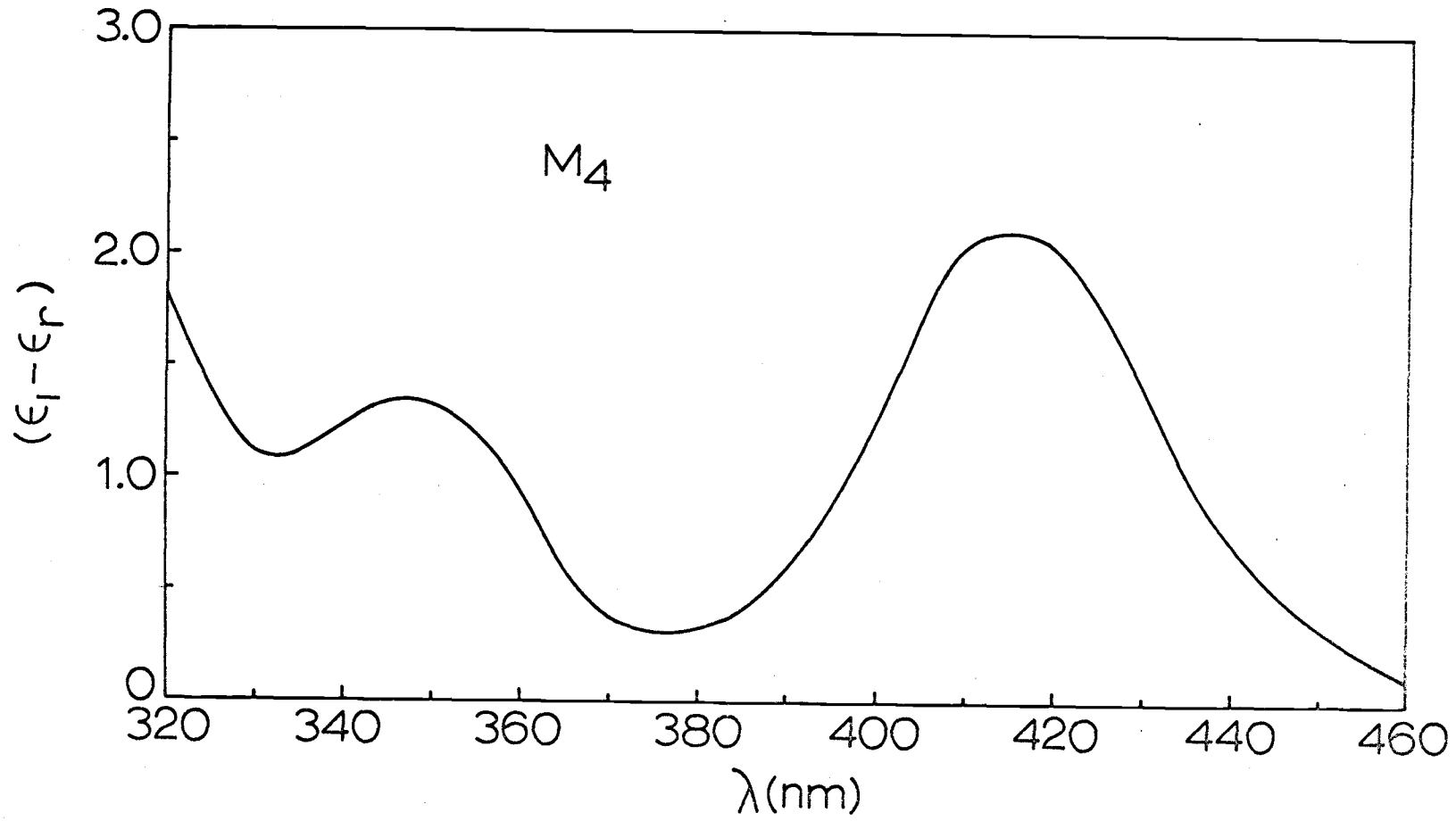

M4
2.0
1.0
0
-1.0
(ε_l−ε_r)
1
2
3
4
5
6
300
320
340
360
380
400
420
440
460
480
λ(nm)

Figure 13: The difference circular dichroism spectra of solutions containing 1 mg/ml dogfish M_4, 8.6×10^{-5} M bis(ANS) and 2.72×10^{-5} M AcPyADH.

The curve represents the difference spectrum of [LDH-bis(ANS)—— (LDH-bis(ANS)-AcPyADH——LDH-AcPyADH)].
The total bis(ANS) concentration was used to calculate the values of $(\varepsilon_\ell - \varepsilon_r)$ $(cm^{-1}\ M^{-1})$. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).


3.0
2.0
1.0
0
M_4
$(\epsilon_l - \epsilon_r)$
320
340
360
380
400
420
440
460
λ(nm)

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