To the Graduate Council:

I am submitting herewith a thesis written by Donna S. Davis entitled "Temperature Effect on Affinity Chromatographic Separation of Two Protein Fractions from the Seeds of Ricinus Communis." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

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TEMPERATURE EFFECT ON AFFINITY CHROMATOGRAPHIC SEPARATION OF TWO PROTEIN FRACTIONS FROM THE SEEDS OF *RICINUS COMMUNIS*

A Thesis

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ABSTRACT

Specific adsorption capacity of Sepharose 4B as a bed material in affinity chromatography for two protein fractions from the seed of Ricinus communis (castor bean), designated as III_L and III_H, was measured from 7°C to 24°C. The adsorption coefficients for these two protein fractions as a function of temperature were also obtained. It was found that there is a characteristic transition of adsorption coefficient at 18°C for both protein fractions. Adsorption coefficients between Sepharose 4B and these two protein fractions were also expressed in terms of ΔG, ΔH, and ΔS.

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CHAPTER I

INTRODUCTION

There are numerous methods which can be used to isolate, purify, and characterize mixtures of biological macromolecules. The decision as to which technique to use is most often determined by the physical and chemical characteristics of the sample macromolecules, their mass or volume, and the degree of purification required.

Affinity chromatography, liquid-solid specific adsorption chromatography, is a liquid chromatographic method which separates molecules primarily according to selective adsorbents based on biological specificity [11, 13, 14]. Developments in this area have quickly led to the formulation of unique approaches and techniques for the isolation and purification of a variety of biological macromolecules and complex biological structures. One of the most noted characteristics of biological macromolecules is that they are capable of binding reversibly with other molecules. The characteristic of specific binding or the ability to form a specific dissociable complex serves as the basis for the purification of biological macromolecules by affinity chromatography. The effectiveness of affinity chromatography depends on the high degree of specificity, characteristic of biological interactions.

The basic principle, which is simple and rational, has been utilized in the preparation of immunoadsorbents for some years [6]. The slow progress is probably due to a lack of knowledge concerning the characteristics of the insolubilized reagent, the adsorbent or affinity matrix, which has a specific attraction for the biological macromolecules of interest. Advances and developments of various specific adsorbents during the past few years have made affinity chromatography a widely accepted technique for the separation and purification of proteins.

The first enzyme purification by affinity chromatography was reported by Lerman [7, 23] in 1953. Mushroom tyrosinase was purified 61-fold on azo dye attached to cellulose. Arsensis and McCormick [1, 2] also used cellulose derivatives (carboxymethyl cellulose) to purify flavin derivatives.

The application of affinity chromatography was greatly increased with the advent of agarose as a bed material. Cuatrecasas, Wilchek, and Anfinsen [8, 9, 10, 11, 12, 13, 14] used Sepharose 4B[1] to purify staphylococcal nuclease, α-chymotrypsin, and carboxypeptidase A. Sepharose 4B is particularly effective when activated by cyanogen bromide (CNBr) as prescribed by Axen, Porath, and Ernback [3]. Apparently, cyclic and linear imidocarbonate groups are formed which

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[1]Sepharose 4B is marketed by Pharmacia Fine Chemicals, Uppsala, Sweden.

react with primary amino groups, as well as other functional groups [20]. Ohlsson, Brodelius, and Mosback [27] simultaneously bound several proteins with similar affinities to a single column. The proteins were then eluted individually by continuously changing a process variable such as pH or ionic strength. This technique is similar to gradient elution in ion-exchange chromatography as described by Freiling [19]. The complexes are specifically dissociated at different pH, etc.

Jervis [22] utilized the differences in selectivity among various types of adsorbents. He combined three different columns in series, each to specifically retard a different protein from a single crude stream.

Hixson [21] predicted binding and elution trends from equilibrium theory based on the affinity of the macromolecules for the affinant and the degree of affinant substitution on the support matrix. Equilibrium theory has also been used by Wankat [33] to characterize the interaction of enzymes and affinants, enzymes and the elutant, and two enzymes. He also used equilibrium theory to analyze steric exclusion from the affinant sites. This phenomena was observed by Cuatrecasas, Wilchek, and Anfinsen [14]. Due to the nature of the ligand-protein interaction, it was necessary to extend the ligand into the void volume away from the solid

CHAPTER II

OBJECTIVE OF THE INVESTIGATION

The primary objective of this investigation was to determine quantitative information on the effect of temperature on the binding capacity of Sepharose 4B (a bed material) for two affinants, two protein fractions isolated and purified from the seeds of castor bean (Ricinus communis).

The absorption coefficients of the two protein fractions on Sepharose 4B were expressed in terms of Gibbs free energy ΔG, enthalpy ΔH, and entropy ΔS.

It was hoped that those findings would contribute to the scarce quantitative information for affinants and bed materials for the scale-up studies and to the design of affinity chromatographic separation techniques.

CHAPTER III

CHARACTERISTICS OF AFFINITY CHROMATOGRAPHY

Chromatography is a two-phase operation in which a mixture (the mobile phase) is passed through an adsorption or partition column (the stationary phase). The differences in equilibrium constants of the affinant between the solvents and stationary phase produce adsorption or separation based primarily on chemical and physical properties. There are several subclassifications according to their characteristics which are briefly reviewed in the following.

1. Adsorption chromatography may proceed by either physical adsorption or chemisorption through which the sorbed molecules actually react with the adsorbent. Thus the bonds retaining the chemisorbed species are much stronger than those simply physically retarding the adsorbate.

2. Ion-exchange chromatography uses pre-charged ion-exchange resins to repel components of similar charge of the feed stream and attract materials of opposite charge.

3. Gel-permeation chromatography filters mixtures based on the size and shape of the adsorbate. This is the principle used in particulate gel dialysis.

4. Partition chromatography separates molecules according to their relative solubilities in the mobile and stationary phases. Here the stationary phase may actually be a liquid-coated solid packing material.

Affinity chromatography relies upon specificity of the adsorbent-adsorbate interaction rather than molecular size and shape, or solubility, or other physical characteristics. It is conducted in four basic steps as follow:

1. The column is adjusted to proper pH, temperature, and ionic strength to promote complex formation by the adsorbate at the stationary phase binding site.

2. The adsorbate-containing fluid is passed through the column. In this adsorption process, only the molecules which bind specifically with the adsorbent are retained. Other materials pass through unretarded.

3. The column is "washed" to assure that any physically trapped molecules are freed and only the specific complex remains.

4. The complex is dissociated by the addition of an elutant which decreases the complex stability due to pH, ionic strength, or ligand competition. The eluted material is collected in an isolated and purified form. (At this point, the column is again ready for regeneration and the commencement of the next cycle.)

Affinity chromatography is based on biospecific interactions between interacting pairs of substances, the affinant and bed material. Discussions concerning the bed material and mobile phase carrier follow:

Matrix Choice

The matrix or backbone of the immobilizing agent should exhibit no chemical interaction with the affinant,

usually a protein. That is, it should not promote nonspecific interaction with proteins, nor should it alter the protein structure. It should be a loose porous network of rigid uniform spheres to optimize flow characteristics and reduce steric hindrance [9, 11, 12, 13, 14, 17, 29, 31]. The carrier should also be chemically stable under operating conditions, easily regenerated, and acceptable for high ligand capacities.

Ligand Choice

The ligand (which links the matrix and immobilized molecule) must be stable at the operating pH and temperature. It must readily retard and elute the macromolecule of concern under acceptable conditions [31]. The ligand-protein interaction must be specific by involving only one molecule or group of molecules [17].

Ligand Attachment

The ligand attachment is also critical. Spacers such as $NH_2(CH_2)_nNH_2$ may mean the difference in recoveries of 0 or 100 percent [9, 10, 25]. The problem is due to inaccessability of the ligand to the macromolecule. Increasing spacer length increases the availability of the ligand to the macromolecule and, consequently, the apparent affinity of the affinant for the ligand. This result is only evident to the limit at which there is no "inverse steric" inhibition.

Elution Methods

The final major consideration in affinity chromatography is the elution method. Elution of the bound

molecule may result from changes in pH, temperature, or ionic strength, or the addition of a soluble competitive ligand [4, 5, 12, 14, 17, 24]. In general, the physical conditions determining the equilibrium constant may be changed, or biospecificity may again be employed, this time to remove the attached molecule.

Applications

Affinity chromatography has many applications: enzyme purification [1, 2, 16], transport protein isolation [18], enzyme subunit isolation [18], -SH protein separation [18], antibody isolation [8, 18, 26], and nucleotide isolation [18, 30]. Macromolecular concentrations and purifications have been increased to 25,000-fold [28], and the technique may be extended to complex biological structures [17, 34].

CHAPTER IV

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials

The III_H and III_L samples were obtained from the Biology Division of Oak Ridge National Laboratory. The samples were purified by Daugherty et al. [15]. Their procedures are listed in the following:

1. The protein was extracted from defatted castor bean seed meal with 5% acetic acid.

2. Initial isolation was via chromatography on a DEAE-Sephadex A-50 column with 0.005 M sodium acetate buffer (pH 5.8).

3. The eluant was chromatographed on a CM-cellulose column using a linear gradient of 0.01-0.20 M sodium phosphate buffer (pH 6.5).

4. The three major peaks containing III_H, III_L, and V-3 were precipitated with 100% saturated ammonium sulfate.

5. The product was centrifuged for 15 minutes at 10,400 x g and redissolved in water.

6. The protein was then dialyzed against water.

7. Concentration and dialysis against 0.05 M Tris-HCl buffer containing 0.1 M sodium chloride (pH 7.6) was conducted on a PH-10 membrane-equipped Amicon ultrafiltration cell.

8. The material was chromatographed on Sephadex G-100.

9. The individual peaks were again concentrated in the Amicon cell and dialyzed against water.

Analysis of the protein fractions appears in Table I. The V-3 fraction is not studied here due to its extreme toxicity.

The washing solution was a 0.05 M sodium phosphate buffer of pH 7.2 at 20°C. The elutant was a 0.05 M sodium phosphate buffer, pH 7.2 at 20°C with the addition of 0.1 M d-galactose. Both solutions also contained 0.1% (wt/wt) sodium azide (NaN_3), a bacteriostatic agent.

It was reported that the column packing, Sepharose 4B, may be used repetitively without decreased binding capacity [6, 11, 13, 32]. It is stable indefinitely at 4°C and for short periods of time at 45°C [12]. Therefore, when not in use, the column was stored at 4°C.

Experimental Equipment

A 2-cc hypodermic syringe was packed with 0.5 cc glass beads (to eliminate end effects in flow patterns) and 1.5 cc of Sepharose 4B as a bed material for both the isothermal runs and also for the temperature effect runs. The separation runs were carried out in a 7-mm outside diameter glass tube packed to a depth of 14 cm. When not in use the columns were stored at 4°C, pH 7.2.

The runs were conducted in an ISCO Refrigerated Fraction Collector Model 328R[2] at 2C° increments between 7°C

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[2] Instrumentation Specialties Company, Lincoln, NB.

TABLE I

PROPERTIES OF III_L AND III_H PROTEIN FRACTIONS FROM THE SEEDS OF CASTOR BEANS (15)

	Protein		
	III_H	III_L	V-3
Molecular Weight	120,000	60,000	60,000
Acute Toxicity*, (µg/mouse)	28	0.42	0.4
$E^{1\%}_{1\ cm}$ (280 nm)	13.43	13.96	-
Original Sample OD at 280 nm	1.444	1.389	-
Chronic Toxicity†, (µg/mouse)	22	0.31	0.15
Hemagglutinating Activity$^{\Psi}$	1.25	40	160

*LD_{50} value for 48 hours post-injection of 0.5 ml nonpyrogenic saline containing varying concentrations of the protein fraction.

†LD_{50} value for one week post-injection of 0.5 ml nonpyrogenic saline containing varying concentrations of the protein fraction.

$^{\Psi}$Minimum concentration of protein in µg/ml necessary to cause agglutination of a 2% suspension of sheep red blood cells.

and 24°C (± 1°C). The elutant was pumped with a Monostat Cassette Pump[3].

The UV absorbance of the eluant was monitored at 280 nm with an ISCO Model UA-4 Absorbance Monitor (± 0.0002 OD at sensitivity of 0.02) and was recorded simultaneously on a Heath Servo-Recorder Model EU-20B[4].

Experimental Procedures

Isothermal and Temperature Effect Runs. The column conditions were preset by rinsing the packing with the washing solution. A sample of the individual protein solution was then layered on top of the packing and washed through at the maximum naturally occuring flow rate (about 0.5 cc/min.) After the absorbance peak representing excess (hence unattached) protein had leveled, the d-galactose-containing elutant was added to dissociate the complex. The exit stream was UV-monitored and recorded throughout the process. After the eluant peak had also leveled, the column was again rinsed with washing solution in regeneration for the following run. For isothermal runs the sample size was changed in each run while for temperature effect runs, a constant sample size was maintained. The experimental conditions for each run are presented in Tables II and III.

Separation Runs. The separation runs were conducted in a manner similar to the isothermal and temperature

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[3]Monostat, New York, NY.

[4]Heath-Schlumberger Company, Benton Harbor, MI.

TABLE II

EXPERIMENTAL CONDITIONS FOR THERMODYNAMIC CHARACTERIZATION RUNS FOR III_H

Temperature (°C)	Elutant Flow Rate, (ml/min)	Sample Size, (ml)†	Absorbance Sensitivity at 280 nm, (OD)	Chart Speed (min/in)
7	0.4667	0.5	0.05	10
8	0.4667	0.5	0.02	10
10	0.4823	0.4	0.02	10
12	0.4965	0.4	0.05	10
14	0.4823	0.3	0.05	10
16	0.4965	0.3	0.02	10
18	0.4628	0.3	0.02	10
20	0.5057	0.2	0.02	10
22	0.5057	0.2	0.02	10
24	0.5135	0.2	0.02	10

†1 ml = 1075 µg.

TABLE III

EXPERIMENTAL CONDITIONS FOR THERMODYNAMIC CHARACTERIZATION RUNS FOR III_L

Temperature (°C)	Elutant Flow Rate, (ml/min)	Sample Size, (ml)†	Absorbance Sensitivity at 280 nm, (OD)	Chart Speed (min/in)
7	0.4628	0.5	0.02	10
8	0.4823	0.5	0.02	10
10	0.4667	0.5	0.05	10
12	0.5135	0.4	0.02	10
14	0.5057	0.3	0.02	10
16	0.5057	0.3	0.02	10
18	0.5291	0.3	0.02	10
20	0.4965	0.2	0.05	5
22	0.5057	0.2	0.02	10
24	0.5603	0.2	0.02	10

†1 ml = 995 µg.

effect runs. The column conditions were set by rinsing with washing solution. In these runs, the sample layered on the packing was a mixture of the two protein fractions: 1.924 mg III_L and 2.078 mg III_H per ml. The column was washed; and after the peak or peaks had leveled, the elutant was added. Again, the column was regenerated by rinsing with washing solution.

Experimental conditions are presented in Table IV.

TABLE IV

EXPERIMENTAL CONDITIONS FOR SEPARATION RUNS

Temperature (°C)	Elutant Flow Rate, (ml/min)	Sample Size (µg) III_L	Sample Size (µg) III_H	Absorbance Sensitivity at 280 nm, (OD)	Chart Speed (min/in)
0	0.0356	1039	962	0.02	20
4	0.0356	1039	962	0.02	20
14	0.0356	312	289	0.10	20
24	0.0432	312	289	0.02	20
34	0.0536	312	289	0.02	20

CHAPTER V

RESULTS, ANALYSIS, AND DISCUSSION

Results

Binding isotherms between III_H and Sepharose 4B and between III_L and Sepharose 4B were made at 8°C and 24°C. The results are presented in Table V and Figure I.

Affinity chromatograms for III_L at 14°C and 24°C and for III_H at 7°C and 20°C are presented in Figures II and III. Masses of the eluted proteins and the attached proteins were obtained from the integration for the area under the optical density-versus-volume of elution curves together with the values of $E_{1\ cm}^{1\%}$ (280 nm), 13.96 and 13.43 for III_L and III_H respectively. Elution velocities were sufficiently slow so that equilibrium was reached between the proteins and the bed material in each run. Material balances of the sample proteins in each run were made by comparing the masses of the eluted and attached proteins to the initial charge. Agreement in each run was within 3%.

The attached protein masses were converted into a per unit bed material basis in each run. The results obtained for each temperature are shown in Table VI. Using these results, the bed capacities[5] for III_L and III_H were correlated

[5]The bed capacity is defined as μg of protein attached to 1 cc of bed material.

TABLE V

RESULTS OF ISOTHERMAL RUNS

Temperature (°C)	Material	Sample Size (ml x OD)	C_{P-S} (µg/cc)	C_L (µg/cc)
8	III_L	0.6945	213.1	120.9
8	III_L	0.5764	212.4	61.0
8	III_L	0.4584	212.0	6.6
8	III_H	0.6498	312.8	10.2
8	III_H	0.6722	313.2	19.9
8	III_H	0.6945	322.2	23.2
8	III_H	0.7842	319.5	68.1
8	III_H	0.8945	315.7	131.4
24	III_L	0.2310	91.0	19.9
24	III_L	0.2778	95.0	37.2
24	III_L	0.4478	94.2	122.4
24	III_H	0.2311	108.7	6.1
24	III_H	0.2888	110.9	32.0
24	III_H	0.4888	112.3	133.8

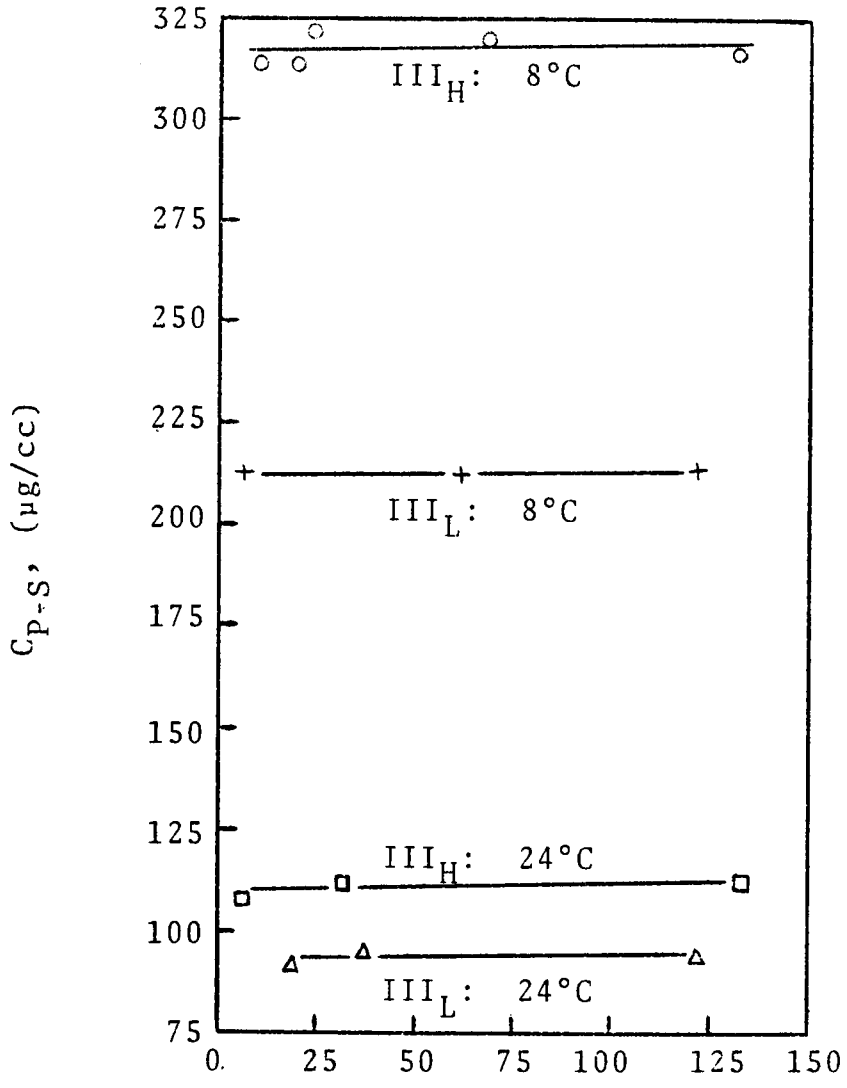


FIGURE I: BINDING ISOTHERMS FOR III_H AND III_L AT 8°C AND 24°C.

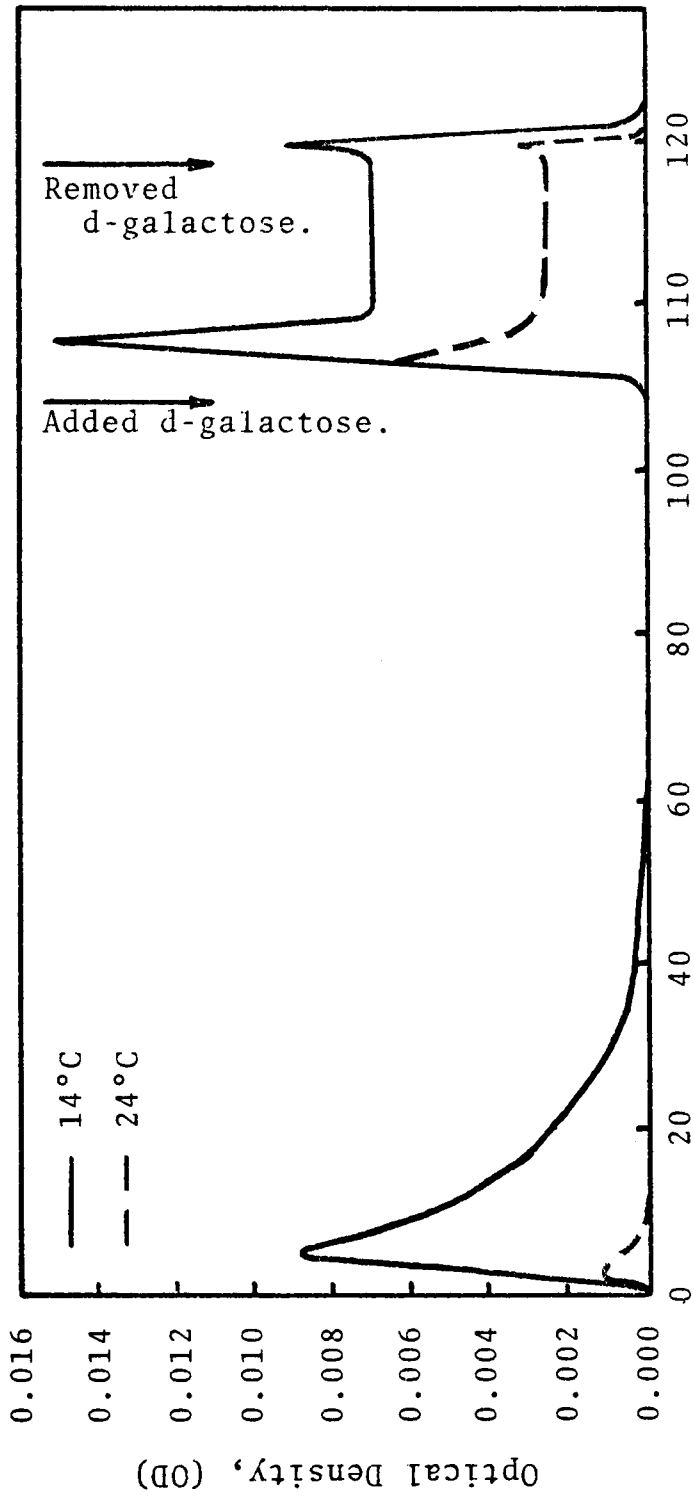


FIGURE II: SAMPLE AFFINITY CHROMATOGRAMS FOR III_L AT 14 AND 24°C.

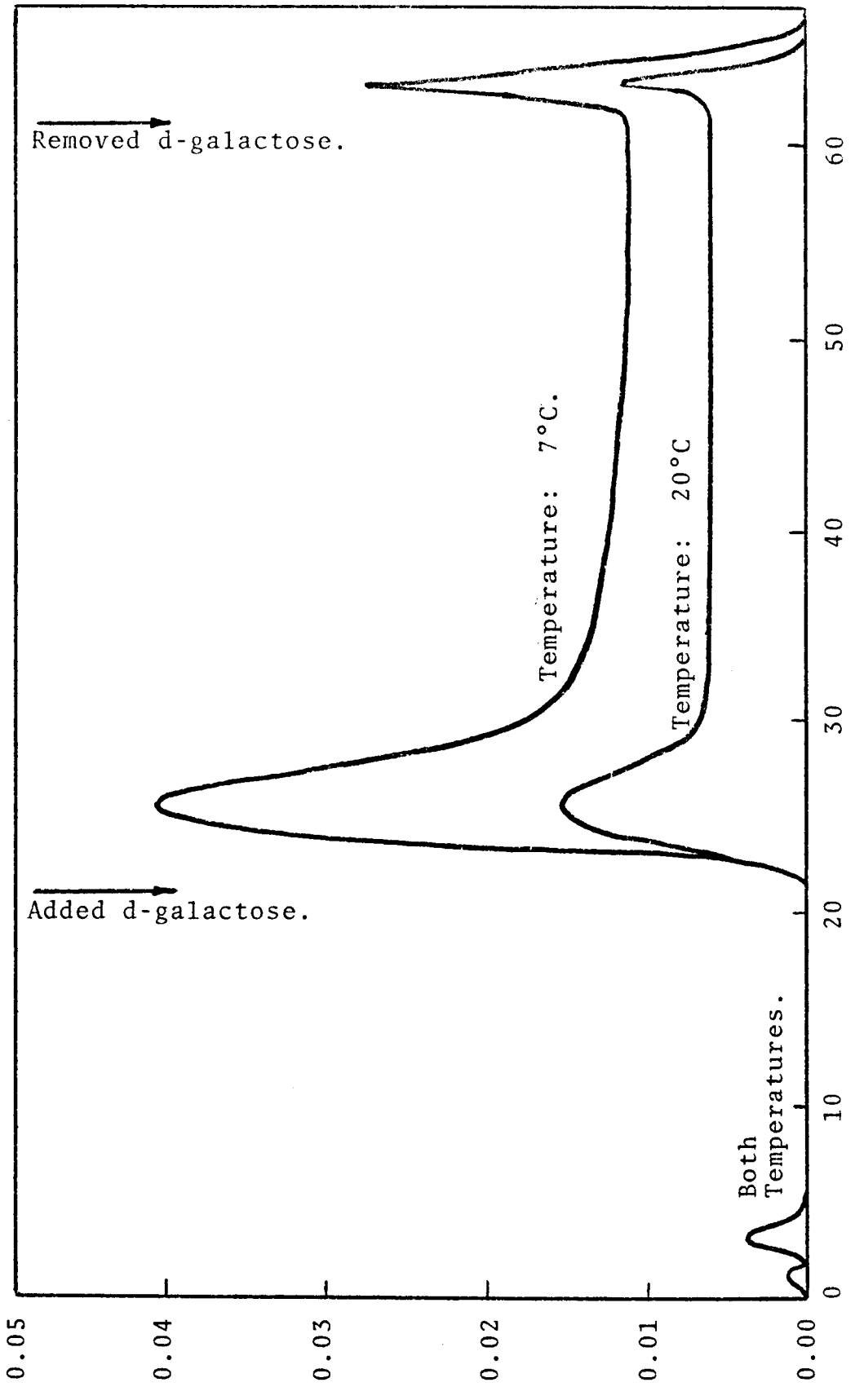


FIGURE III: SAMPLE AFFINITY CHROMATOGRAMS FOR III_H AT 7 AND 20°C.

TABLE VI

ABSORPTION COEFFICIENTS FOR III_L AND III_H ON SEPHAROSE 4B

Temperature (°C)	III_L		III_H	
	ml x OD	$C_{P-S'}$ $\left(\frac{\mu g\ III_L}{cc\ Sepharose\ 4B}\right)$	ml x OD	$C_{P-S'}$ $\left(\frac{\mu g\ III_H}{cc\ Sepharose\ 4B}\right)$
7	0.4831	230.7	0.7148	354.8
8	-	-	0.6492	322.2
10	0.3677	175.6	0.5584	277.2
12	0.3360	160.5	0.4270	212.0
14	0.3024	144.4	0.3683	182.8
16	0.2442	116.6	0.3293	163.5
18	0.2265	108.2	0.2805	139.2
20	0.2091	99.8	0.2544	126.3
22	0.2053	98.0	-	-
24	0.1989	95.0	0.2234	110.9

and presented in Figure IV together with the measured bed capacity data.

Analysis

The bed capacity C_{P-S} is the saturated capacity at a given temperature; so that it is the adsorption coefficient between the protein and the bed material. From the isotherms as shown in Figure I, the bed capacity is constant at a given temperature and independent of the sample size. Therefore, the binding energy between the protein and the bed material may be related temperature by

$$\ln C_{P-S} = -\frac{\Delta G}{R'T} \qquad (1)$$

where

ΔG = Gibbs free energy of adsorption of protein on Sepharose 4B;

R' = 1.987/(molecular weight), cal/μg °K.

T = absolute temperature, °K.

Since the individual runs were conducted at constant temperature, Gibbs free energy may be written as

$$\Delta G = \Delta H - T\Delta S \qquad (2)$$

where

ΔH = enthalpy of adsorption of protein on Sepharose;

ΔS = entropy of adsorption of protein on Sepharose.

Combination of equations (1) and (2) yields

$$\ln C_{P-S} = \frac{\Delta S}{R'} - \frac{\Delta H}{R'T}. \qquad (3)$$

Based on equation (3), the data was plotted as $\ln C_{P-S}$ versus inverse temperature as shown in Figure IV and

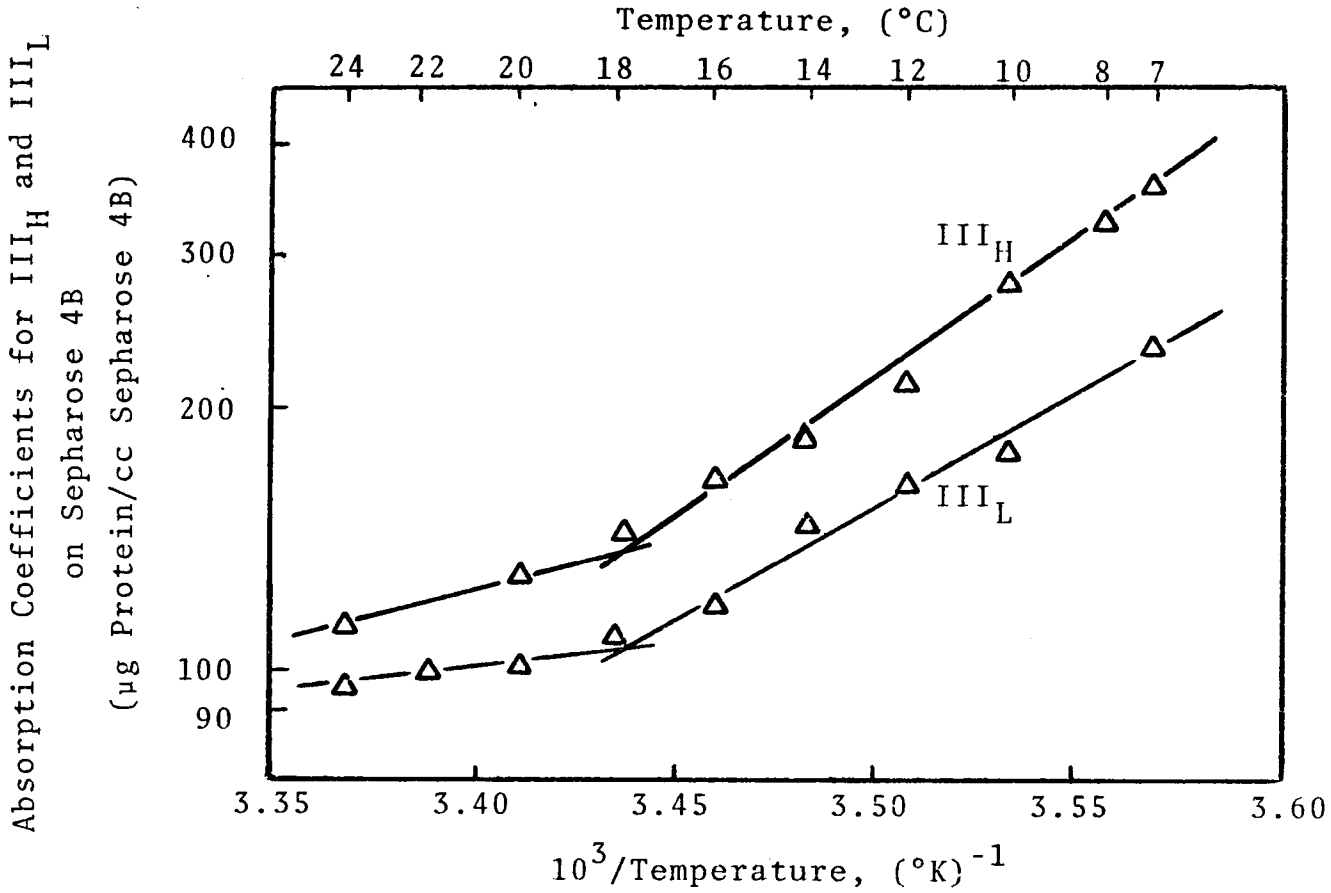


FIGURE IV: ABSORPTION COEFFICIENTS FOR III$_L$ AND III$_H$ ON SEPHAROSE 4B AS A FUNCTION OF INVERSE TEMPERATURE.

fitted with a least squares analysis. As the data indicated a breakpoint at 18°C for both proteins, each protein was fitted with two line segments. They are:

for III_L

$$\ln C_{P-S} = \frac{5857}{T} - 15.47, \begin{cases} T < 18°C \\ \sigma = 0.0358 \end{cases} \quad (4)$$

$$\ln C_{P-S} = \frac{1255}{T} + 0.3412, \begin{cases} T > 18°C \\ \sigma = 0.0067 \end{cases} \quad (5)$$

and for III_H

$$\ln C_{P-S} = \frac{7261}{T} - 20.03, \begin{cases} T < 18°C \\ \sigma = 0.0427 \end{cases} \quad (6)$$

$$\ln C_{P-S} = \frac{2745}{T} - 4.514, \begin{cases} T > 18°C \\ \sigma = 0.0238 \end{cases} \quad (7)$$

where σ is the standard deviation[6] for each fit.

Binding energies between the protein and Sepharose 4B (Gibbs free energy, ΔG; enthalpy, ΔH; and entropy, ΔS) were calculated using equations (1), (2), and (3). They are tabulated in Table VII.

Discussion

The Gibbs free energy of adsorption was negative for both III_L and III_H. Therefore, it was thermodynamically very favorable for both proteins to be attached to the Sepharose 4B as protein-Sepharose 4B complexes. Furthermore, at temperatures above 18°C, the positive and very small entropies indicated a tendency toward spontaneous elution without the addition of d-galactose.

In fact, the above predicted elution trends were seen in the separation runs. The results were summarized in

TABLE VII

THERMODYNAMIC PROPERTIES OF ABSORPTION OF III_L AND III_H ON SEPHAROSE 4B

Protein	Temperature (°C)	$\Delta H \times 10^8$ $\left(\frac{\text{cal}}{\text{cc Sepharose 4B}}\right)$	$\Delta S \times 10^{11}$ $\left(\frac{\text{cal}}{°K \text{ cc Sepharose 4B}}\right)$	$\Delta G \times 10^8$ $\left(\frac{\text{cal}}{\text{cc Sepharose 4B}}\right)$
III_L	7	-19.40	-51.23	-5.047
	10	-19.40	-51.23	-4.894
	12	-19.40	-51.23	-4.791
	14	-19.40	-51.23	-4.689
	16	-19.40	-51.23	-4.586
	20	- 4.16	1.13	-4.491
	22	- 4.16	1.13	-4.494
	24	- 4.16	1.13	-4.496
III_H	7	-12.02	-35.12	-2.181
	8	-12.02	-35.12	-2.146
	10	-12.02	-35.12	-2.075
	12	-12.02	-35.12	-2.005
	14	-12.02	-35.12	-1.935
	16	-12.02	-35.12	-1.860
	20	- 4.55	- 7.47	-2.360
	24	- 4.55	- 7.47	-2.330

Table VIII. At temperatures greater than 18°C (Figures V and VI), elution and separation was achieved without the addition of d-galactose. The III_L was eluted first as expected from its greater complex entropy and hence lower stability.

No elution nor separation was seen at temperatures below 18°C without the addition of d-galactose. In those cases, elution can be achieved only with the addition of d-galactose. However, the competition resulted in complete and immediate dissociation of both proteins so that no separation was observed.

The separation at 24°C was quite complete with the peaks almost totally distinct (no overlap). Conversely, the 34°C separation was not complete, exhibiting a large overlap of the peaks. According to the adsorption coefficient-temperature correlation, the ratio of the adsorption coefficients $C_{P\text{-}S}(III_H)/C_{P\text{-}S}(III_L)$ is 1.172 at 24°C and 1.004 at 34°C. Thus, more complete separation would be expected at temperatures just above 18°C with decreasing peak distinction with increasing temperature.

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[6] $$\sigma^2 = \frac{\sum[(\ln C_{P\text{-}S})_{calc.} - (\ln C_{P\text{-}S})_{obs.}]^2}{(\text{number of data points} - 1)}.$$

TABLE VIII

RESULTS OF SEPARATION ATTEMPTS FOR III_L AND III_H ON SEPHAROSE 4B AT VARIOUS TEMPERATURES

Temperature, (°C)	Peak Distinction	Peak Size
0	None	-
4	None	-
14	None	-
24	Good	III_L: Medium III_H: Medium
34	Fair	III_L: Large III_H: Small

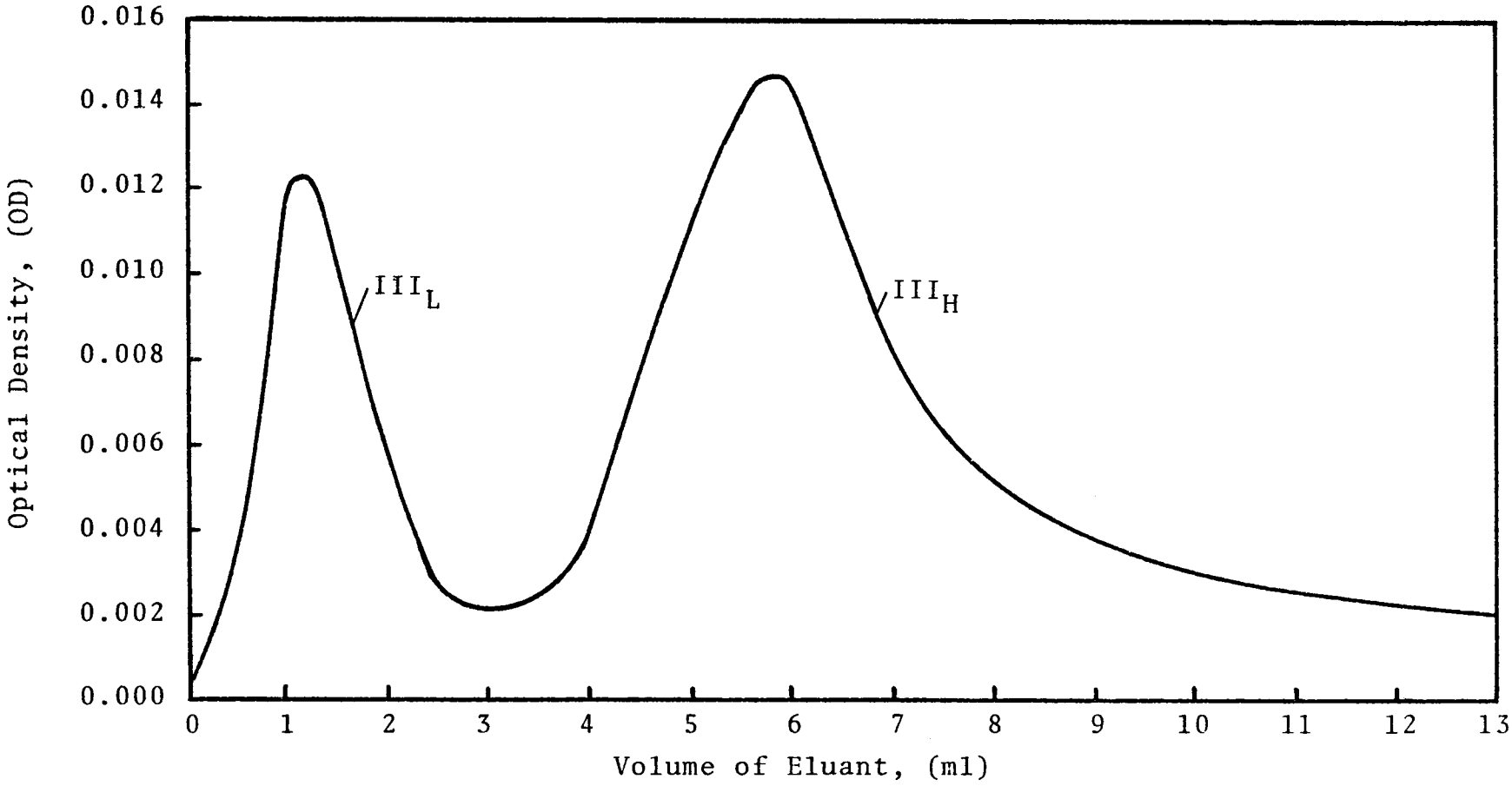


FIGURE V: AFFINITY CHROMATOGRAM OF III_L AND III_H MIXTURE AT 24°C.

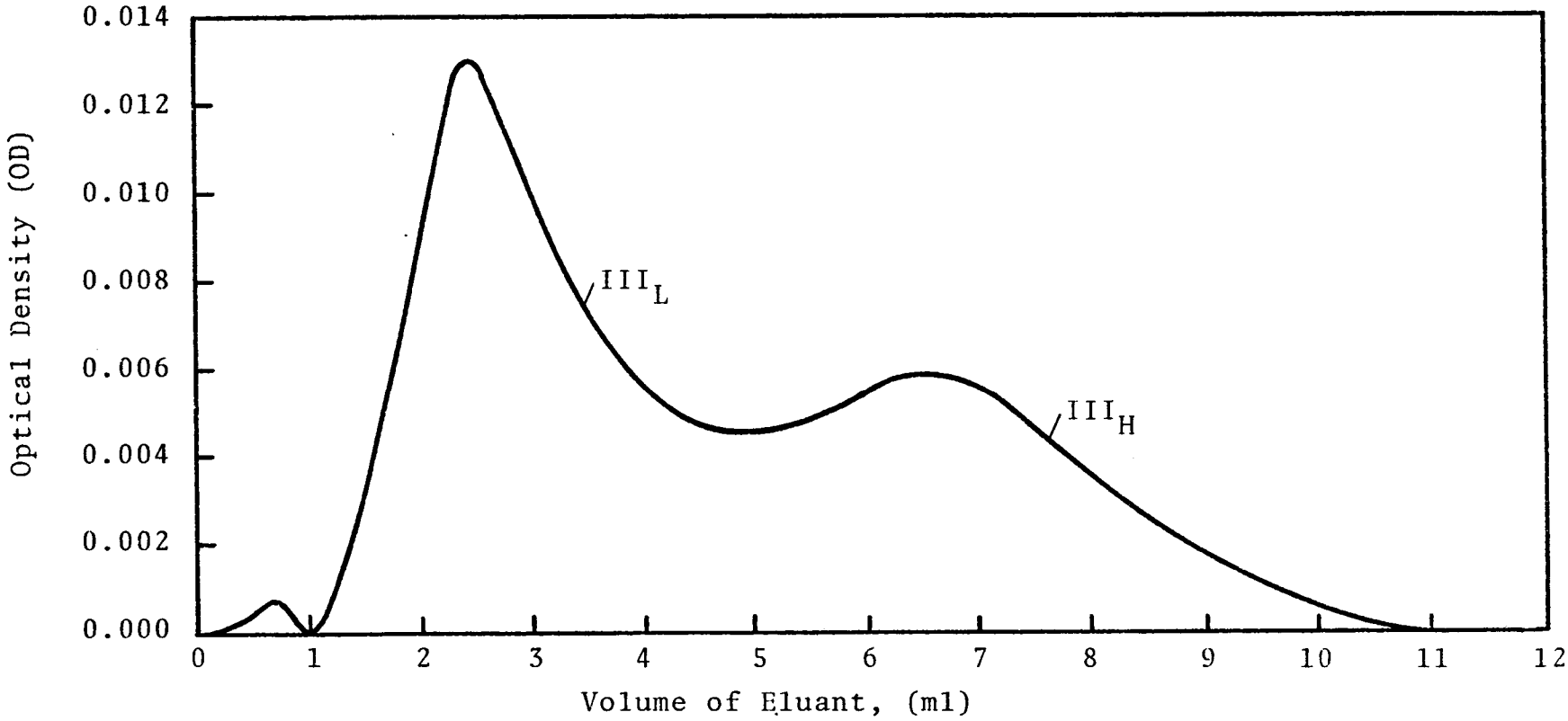


FIGURE VI: AFFINITY CHROMATOGRAM OF III_L AND III_H MIXTURE AT 34°C.

CHAPTER VI

CONCLUSIONS

The investigation established relationships of absorption coefficient of III_L and III_H on Sepharose 4B as a function of temperature. From these affinity chromatographic characterizations, the following conclusions were drawn:

1. Both III_L and III_H exhibit absorption mechanism changes at 18°C as indicated by abrupt changes in the absorption coefficient as a function of temperature.

2. Thermodynamic characterization predicts separation at temperatures above 18°C only, but decreasing peak distinction with increasing temperature to 34°C.

3. Good peak distinction is achieved at 24°C. Less complete distinction is observed at 34°C.

4. Separation attempts at 14°C and below require the addition of d-galactose for elution. This results in immediate elution of both peaks simultaneously and hence no separation.

5. Absorption rates should be studied by a similar technique in which sample size is held constant and flow rate is varied.

6. Further absorption characterization should include the effect of pH and ionic strength on the affinity chromatographic separation.

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