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Deuterium Oxide Exchange Reaction with Human Beta-1 Lipoproteins

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DEUTERIUM OXIDE EXCHANGE REACTION WITH HUMAN BETA-1 LIPOPROTEINS

by

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ABSTRACT

The exchange of deuterium for the amide bound hydrogen atoms of beta-lipoproteins was studied using infrared absorption as a monitoring technique. The exchange reactions were run with human lipoprotein isolated by dextran sulfate precipitation. The reaction mixture consisted of 1:1 deuterium oxide-dioxane and the isolated beta-lipoprotein. The disappearance of the amide II band was used as a measure of the rate of exchange.

A pD minimum value of 2.4 was determined with an associated rate constant of 1.20×10^{-3} sec^{-1}. These values show that the lipoproteins have an exchange rate between that of randomly-coiled molecules and proteins in a helical conformation. This data is explained on the basis of the motility theory which assumes molecules are in a dynamic conformation and on the basis of a rigid molecule with a static conformation. Factors influencing the rate of exchange are also discussed.

BIOGRAPHICAL SKETCH

Lawrence J. Crolla was born December 13, 1944, in Chicago, Illinois. In June, 1962, he graduated from Saint Patrick High School, Chicago, Illinois. He then attended Saint Mary's College, Winona, Minnesota, from which he received the degree of Bachelor of Arts in June, 1966, with a major in biology and minors in chemistry and mathematics.

In September, 1966, the author began his advanced studies in the Graduate School of Loyola University, Department of Biochemistry and Biophysics. From September, 1966, to September, 1967, he held the position of National Science Foundation Trainee. From September, 1967, to the present he has held the position of research assistant in the Department of Biochemistry and Biophysics at the Stritch School of Medicine of Loyola University, Chicago, Illinois. The position was supported financially by a grant from the Chicago Heart Association.

ACKNOWLEDGEMENT

Many people have helped the author with the preparation of this thesis. To name them all individually would take another volume. Therefore, to all of my friends I give my heartfelt thanks.

I would especially like to thank Dr. H. J. McDonald, my adviser, and my parents without whose encouragement and patience this paper would not have been written.

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CHAPTER I

INTRODUCTION TO LIPOPROTEINS

Lipoproteins can be defined simply as a combination of protein and lipids such as lecithin, cephalin and fatty acids. Lipoproteins also contain carbohydrate components normally found with glycoproteins. These are such molecules as glucosamine, mannose and sialic acid. Protein complexes are found in both plant and animal structures, occuring in milk, blood, cell nuclei, egg yolk, cell membranes, and chloroplasts of plants (25, 34).

From a functional standpoint, lipoproteins are divisible into two general categories. The first contains those molecules which serve a structural role such as in cellular and sub-cellular membranes. The second category contains molecules that serve to transport water-insoluble components, such as cholesterol, and soluble, active components, such as enzymes, in an inactive form.

The work presented in this thesis is concerned with human beta lipoproteins and the rate with which they exchange their amide bound hydrogen atoms for deuterium. The objective of this research is to shed some light upon the structure assumed by the protein-lipid complex.

BASIC CHARACTERISTICS OF BETA LIPOPROTEINS

Two different proteins are consistently isolated from plasma lipoproteins. A third has been found in one group of lipoproteins. These apoproteins are generally designated as A or α, B or β and C proteins. They differ in terminal residues, total amino acid content and immunochemical behavior. Normally, the A protein is the only protein found in the α migrating lipoproteins and the B protein in the β migrating lipoproteins. In some diseases the normal distribution of either protein is distorted.

Beta lipoproteins migrate with sharp boundaries in the zone during most types of electrophoretic separations. In the ultracentrifuge, β-lipoproteins are isolated between the densities 1.006 and 1.063 and have a mean density of about 1.03.

Using the technique of Gofman et al. (12), the pure beta lipoproteins normally are in the subclass of S_f 0-20 with a mean S_f of 6. Most are found in the subclass of S_f 0-12.

In the dry state, a beta lipoprotein consists of 20 to 25 per cent protein, 8 per cent free and 35 per cent esterified cholesterol, 22 per cent phospholipid and 10 per cent triglyceride (34). Beta lipoproteins have a molecular weight ranging anywhere from 1.3 to 3.2 x 10^6. Light scattering studies indicate the usual complex is dyssymetric and a prolate ellipsoid of about 150 x 350Å (2).

Experiments have shown that the apoprotein in the native lipoprotein consists of several identical or at least similar peptide chains. Two identical protein units of molecular weight 380,000 have been reported in lipoproteins having an S_f 7-9 flotation value. Other work suggests a protein on the order of 100,000 molecular weight as a possible repeating subunit (24).

The beta protein contains an aminoterminal glutamic acid, carboxyterminal serine and a total amino acid pattern that differs from the A protein partially in the relative contents of isoleucine, leucine, glutamic acid and alanine (23).

INTRODUCTION TO INFRARED SPECTROSCOPY

Infrared spectroscopy appears both as a fully developed technique, and as a rapidly growing and changing field. It is marked by new developments in instrumentation, technique, data handling, and application.

Infrared radiation was discovered by Herschel in 1800 by placing a thermometer at successive points in a glass prism dispersed spectrum of the sun and observing the temperature rise(31). Progress in the infrared field was painfully slow because of the difficulty of radiation detection and characterization. However, by 1900 Coblentz provided the evidence of the chemical value of infrared by obtaining absorption spectra of many organic liquids (31). In the 1930's, organic chemists began to consider infrared

as a possible tool for material identification and functional group analysis.

In answer to the interest of chemists everywhere and due to the war effort, commercial production of infrared spectrophotometers was started in 1943 and 1944. At the present time, such instruments are looked upon as a piece of standard research equipment.

BASIC INFRARED

Since the basic forms of energy in a molecule, namely, translational, rotational, vibrational, and electronic energy, can generally be treated separately, molecular vibrations can be examined selectively to determine molecular structure. It is also evident that, since atoms have different bond lengths and masses, they will vibrate at different frequencies upon absorption of electromagnetic energy of the proper frequency.

Quantitative infrared analysis is based on the Beer-Lambert law as is all other optical quantitative spectroscopy. Theoretically, the absorption obtained is proportional to the concentration and thickness of the sample. Under favorable conditions, repeatabilities and accuracies of about 0.15 per cent of the component being determined can be realized with single beam instruments. The limit of repeatability with double beam optical null instruments is close to 1 per cent, but the accuracy may be as low

as 2 or 3 per cent because of the non-linearity in the optical wedge (35).

INTRODUCTION TO DEUTERIUM EXCHANGE

Deuterium exchange reactions were first quantitatively studied by Hvidt and Linderstrøm-Lang in 1954 (16). They were qualitatively studied by Lenormant and Blout in 1953 (22). The impetus for these studies was the idea that deuterium exchange rates would in some way elucidate the macromolecular structure of proteins. The main results of these studies have shown that randomly coiled polypeptides will exchange the hydrogen atoms which are bound to oxygen, nitrogen and sulfer with solvent water or with deuterium in a few minutes at 37° C. Native proteins, however, exchange their peptide group hydrogen atoms at a much slower rate, requiring up to as much as 24 hours for completion.

Thus the difference in the rates of the exchange reactions between native and randomly coiled polypeptides give us an indication of the protein structure and in this way can be related to the latter. As Hvidt and Nielsen have said, "Quantitative measurements of the rate of hydrogen exchange in a given protein, under specified experimental conditions, therefore provide a multiparameter characterization of the protein conformation (or distribution of conformation) present under these conditions." (17)

FACTORS INFLUENCING THE EXCHANGE REACTION

Nielsen (29) and Klotz (18) have both shown that the exchange reaction is dependent on both the pH and temperature. Klotz also found the same energy of activation for N-Methylacetamide as did Nielsen for insulin and Byran (4) and Berger (1) for poly-D-L-alanine. The activation energy is, therefore, not a useful parameter in distinguishing between a free "NH group" and some structural feature such as hydrogen bonding which may cause slow exchange. Leichtling (20) has shown, on the other hand, that the pD min, (the pD at minimum exchange rate) and the minimum rate constant can be used as an effective parameter for comparing these exchange reactions. These parameters are, however, influenced by inductive, steric, and solvent effects.

As a more complex system, Blout (3) and also Byran (4) studied poly-L-glutamic acid. They found slow exchange at pD 3.4 and 4.2 in a 1:1 dioxane solvent where the compound is in a helix and instantaneous exchange at pD7 in D_2O, where the compound is in a random coil.

To account for the concept of slowly exchanging hydrogens being found in both randomly coiled and native polypeptides, Nielsen and Hvidt (17) feel that there are a number of conformations available to most regions of a protein. These conformations have varying degrees of solvent accessibility. The rate of exchange will therefore depend on the length of time a protein is in

an accessible conformation. Deuterium exchange is then a measure of dynamic conformational fluctuations rather than a static measurement such as O$^{\alpha}$D.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Solvents, Acids and Bases

Heavy water, warranted to contain a minimum of 99.75 per cent D_2O

- Isotopes Inc., Westwood, New Jersey

NaOD, 40 per cent solution and 3[illegible] per cent solution DCl in D_2O

- Isotopes Inc., Westwood, New Jersey

Dioxane, spectral grade

- Matheson, Coleman and Bell, Norwood, Ohio

2. Salts and Buffers

NaCl, analytical reagent grade

- Mallinckrodt, New York, New York

EDTA, analytical reagent grade

- Fisher Scientific Co., Fair Lawn, New Jersey

Dextran sulfate, #2000

- Pharmacia Fine Chemicals, Piscataway, New Jersey

pH standardization buffers, pH 4.1 and pH 7.0

- Beckman, Inc., Fullerton, California

Buffer for microzone electrophoresis, pH 8.6 and ionic strength of 0.075

- Scientific Products, Chicago, Illinois

2. Salts and Buffers (cont'd)

Buffer for disc electrophoresis, 100x

- Canal Industrial Corp., Rockville, Maryland

3. Resins

Sephadex G-25 and G-200, used according to data sheet

- Pharmacia Fine Chemicals, Piscataway, New Jersey

4. Unclassified Materials

Dialysis tubing

- Union Carbide Corp., Chicago, Illinois

Fresh human plasma

- Michael Reese Hospital Blood Bank, Chicago, Illinois

Thrombin, lot #975330A

- Parke Davis, New York, New York

B. Methods

1. Isolation of Human Beta Lipoproteins (6)

All isolation procedures were carried out at 4° Centigrade, the only exception being the filling of the ultracentrifuge tubes. This was done at room temperature but with the solutions at 4° Centigrade. All salt solutions used for isolation contained 0.1 g/l of EDTA and were at pH 7.0. Isolation was done on one liter of fresh human plasma per run, obtained from the Blood Bank at Michael Reese hospital.

The general procedure for isolating the lipoproteins was as follows. To the liter of plasma, thrombin was added in sufficient amount to give a concentration of 300 units per 100 ml. of plasma. The plasma and thrombin were allowed to mix in the cold. After a period of two hours, the serum was separated by filtration. A five ml. aliquot of serum was removed and titrated to maximum turbidity with 0.5 per cent dextran sulfate (30). The proper amount of dextran sulfate needed for 1 liter was then calculated and added to the serum. After mixing for two hours at 4° Centigrade the mixture was spun in a refrigerated centrifuge at 1000 x g for thirty minutes. The lipoprotein-dextran sulfate complex is found as a gelatinous pellet at the bottom of the tube. After the serum is poured off, the pellet is dissolved in 2 M NaCl. A total quantity of 25 ml. of 2 M NaCl, (5 ml. per 200 ml.

original serum) is the total amount used for dissolution. When the dissolution is complete, a density gradient tube is prepared. Six ml. of lipoprotein solution is placed in a 13.5 ml. Lusteroid tube upon which is layered 0.15 M NaCl. The tubes are spun at a temperature of 0° Centigrade, at 100,000 x g, for 20 hours. After centrifugation, the tubes are observed to contain four layers. The top layer contains the turbid S_f 10-400 or $alpha_1$ lipoproteins. The intermediate layer consists of a clear solution of the S_f 5-15 or $alpha_2$ lipoprotein, while the middle orange layer is made up of the S_f 6-10 or $beta_1$ lipoprotein. The infranate contains protein contaminants and dextran sulfate.

The layers are separated with the aid of a Beckman Spinco tube slicer. After separation, the $beta_1$ lipoproteins are dialyzed for 18 hours against twenty volumes of 2 M NaCl. An alternate procedure is to pass the lipoproteins through a Sephadex G-25 column, equilibrated with 2 M NaCl.(23) This procedure takes only about two hours. After either of the two procedures, the sample is put into a density gradient tube. The procedure is repeated as described above, that is, 6 ml. of lipoprotein solution is placed in the bottom of the Lusteroid tube, the rest of the tube being filled with 0.15 M NaCl. These tubes are again spun at 100,000 x g. After 20 hours, the tubes are removed and the purified $beta_1$ lipoproteins are found to be centered in the tube. These are removed and dialyzed for 18 hours against 0.15 M NaCl

or passed through a Sephadex G-25 column equilibrated with the same solution. The lipoproteins are now ready for use.

2. Electrophoresis

Electrophoresis was done on both cellulose acetate strips and on polyacrylamide gel. A sample of the fresh plasma was run on the microzone system as a check on the purity. Disc electrophoresis runs were made on the isolated and purified beta lipoprotein to check for homogeneity and purity (26). All materials used were reagents prepared by Canalco.

3. pH Measurements

pH determinations were done on a Beckman Zeromatic pH meter using a Sargent miniature combination electrode. Beckman standard buffers were used in the appropriate ranges required for the determinations. The pD was calculated from the formula: (11)

$$pD = pH \text{ reading} + 0.40$$

The meter was observed to have a slight drift, probably due to the protein. However, the extent of the drift was finite and repeatable. All readings were therefore taken after the drift had ceased.

4. Exchange Reactions Using Infrared

The instrument used was a Perkin and Elmer model 337 grating spectrophotometer. This instrument was used in conjunction with a Brown recorder and scale expansion attachment. The expansion ratio employed during these experiments was 10 to 1.

The procedure for a deuterium exchange measurement consisted of the following. The amount of 1:1 D_2O-dioxane needed for a set of experiments was prepared and made 0.15 M in NaCl and 0.1 g/l in EDTA. An aliquot sufficient for 3 samples was removed and the pH adjusted, roughly, with DCl and NaOD. A vial filled with 0.5 ml. of lipoprotein solution was then mixed with four mls. of the 1:1 D_2O-dioxane. The time of addition was noted as the start of the exchange reaction. An aliquot of the mixture was injected into a CaF_2 sealed cell with a path length of 0.0251 mm. The reaction was run against a blank of 1:1 D_2O-dioxane at 1449 cm^{-1}. The wave length setting was determined from the spectrum of a lyophilized sample of beta lipoprotein run as a KBr pellet (8).

5. Calculation of the Observed Rate Constants

The rate constants were calculated from a modified Kezdy plot (32). This method involves a plot of T_t vs. $T_{t+\triangle}$ where $\triangle$ is a constant time interval. T can be any value proportional to the exchange rate. In a plot of this kind, a first order reaction will yield a straight line with a rate constant, k, which is giv-

en by, ln slope/$\triangle$. The advantage of this method is that neither initial nor final concentrations need be known in order to determine the value of k. A drawback, however, can be seen since any deviation from first order cannot be compensated for.

CHAPTER III

RESULTS AND DISCUSSION

The infrared spectra of the beta lipoprotein is shown in figure I and II. As seen from the spectra, 1449 cm^{-1} is the amide II peak and 164[illegible] cm^{-1} is the absorption peak for the amide I band. The latter band is considered to be mostly a stretching vibration of a C=O group. The amide II band is thought to be a combination motion of N-H bend and C-N stretch (22, 33).

When various groups are substituted on and/or for the N-H and C O groups, a shift in the position of these peaks can be observed. Thus we can see from figure III, which is the spectra of a deuterated lipoprotein molecule, the shift in the amide II band to 1450 cm^{-1}.

This shift thus allowed us to follow the exchange reaction using the disappearance of the band at 1449 cm^{-1}. The band increase at 1450 cm^{-1} could not be used, however, since C-H stretch shows absorption in this region. The former was chosen to avoid complication.

ELECTROPHORESIS

Figure IV illustrates the pattern obtained on the plasma before the isolation procedures were carried out. It is apparent from this scan that the plasma has to be a normal distribution of protein fractions. Figure V represents a run using the technique

of disc electrophoresis. This run shows the purity and homogeneity exhibited by the isolated beta lipoproteins. These disc gel runs were done on a 3.75 per cent gel.

pD_{min}

From figure VI, it is apparent that the pD_{min} had a value of 2.4. It is of interest to note that this value coincides with the figures reported by Hartshorne (15), Klotz (18), Berger (1), Blout (3) and Kovacs (19) in showing that model amides have a minimum rate at about pD_{min} 5-6 whereas peptides have a pD_{min} of 2-3.5.

KEZDY PLOTS

Figures VII - XIII are the Kezdy plots for the reactions at the various pD's, the latter being calculated from:

$$pH = pD + 0.40$$

The time constant for these plots was chosen as 8 minutes.

MINIMUM RATE CONSTANTS

The value of pD_{min} is as seen in figure VI and chart I which lists the rate constants vs. pD values.

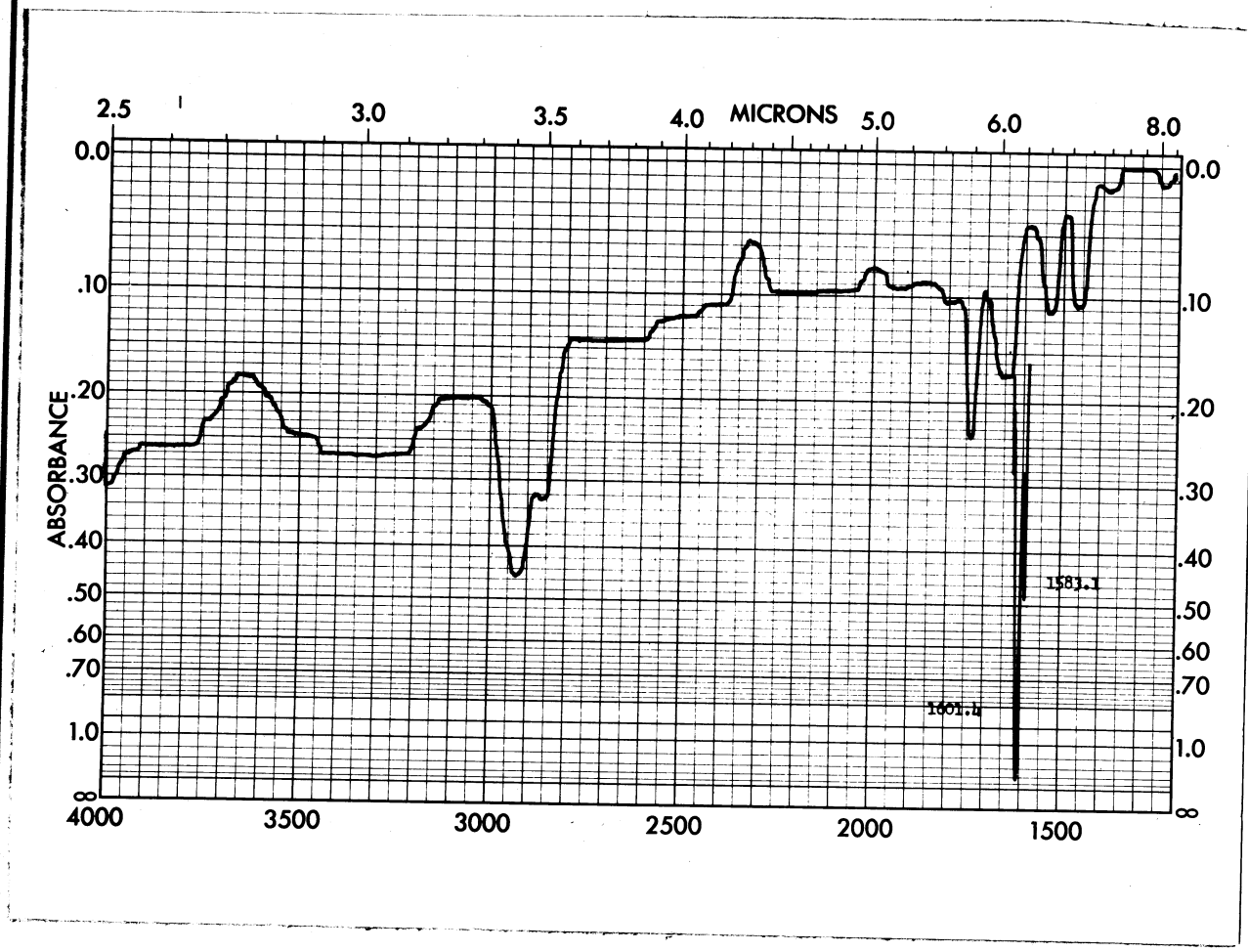


FIGURE I

KBr-Pellet of Beta Lipoprotein

(4000 cm^{-1} to 1200 cm^{-1})

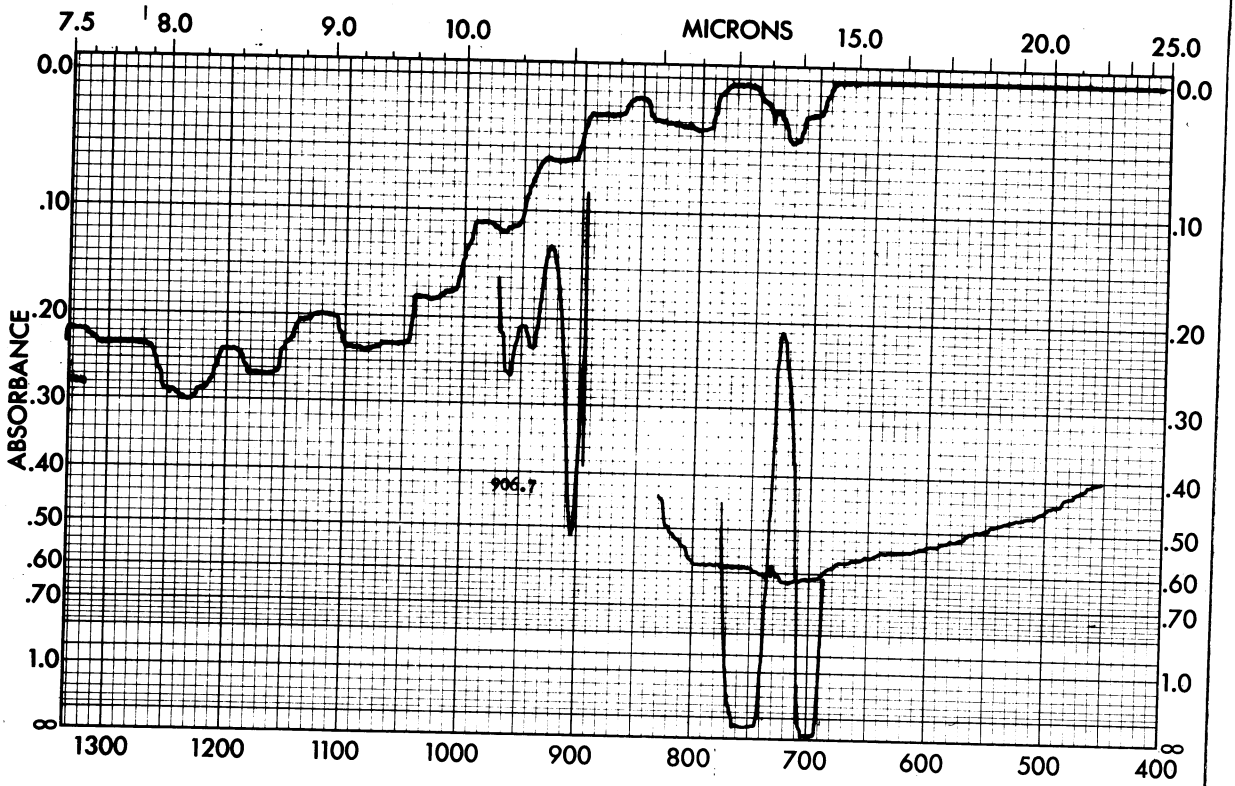


FIGURE II

KBr-Pellet of Beta Lipoprotein

(1200 cm^{-1} to 400 cm^{-1})

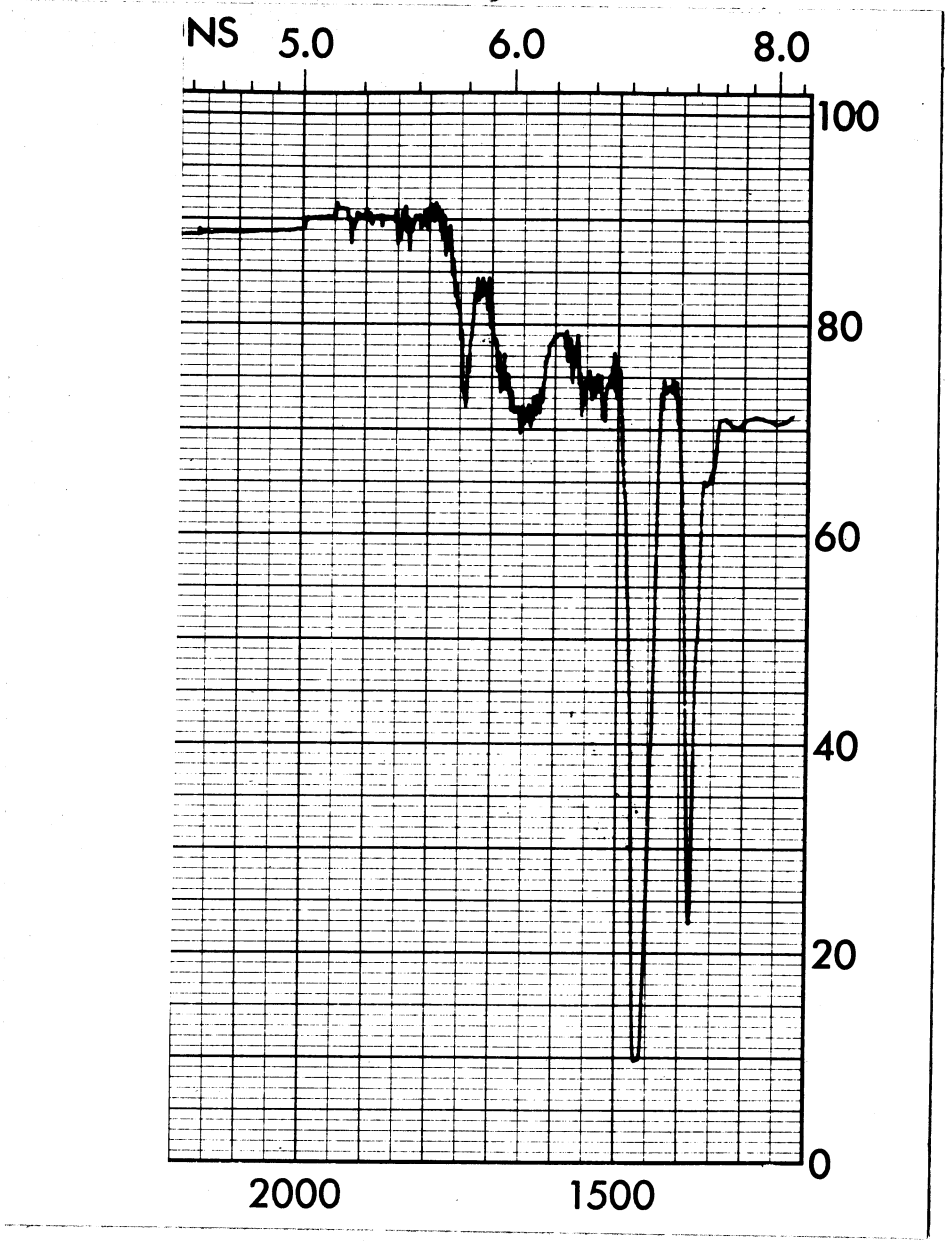


FIGURE III

An infrared scan of a deuterated lipoprotein molecule showing the shift in the amide II band to 1450 cm^{-1}

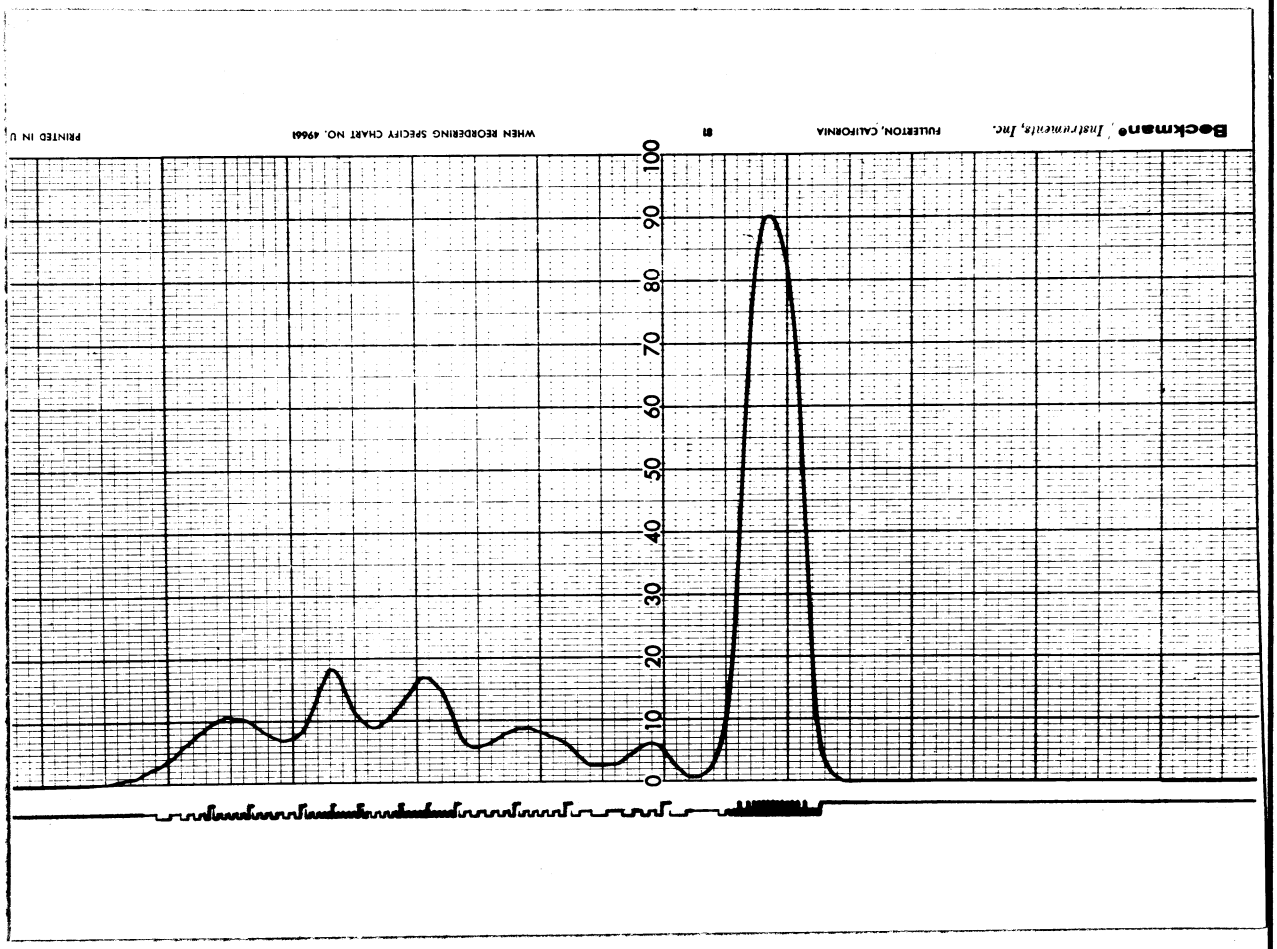


FIGURE IV

A scan of the microzone electrophoresis of fresh human plasma

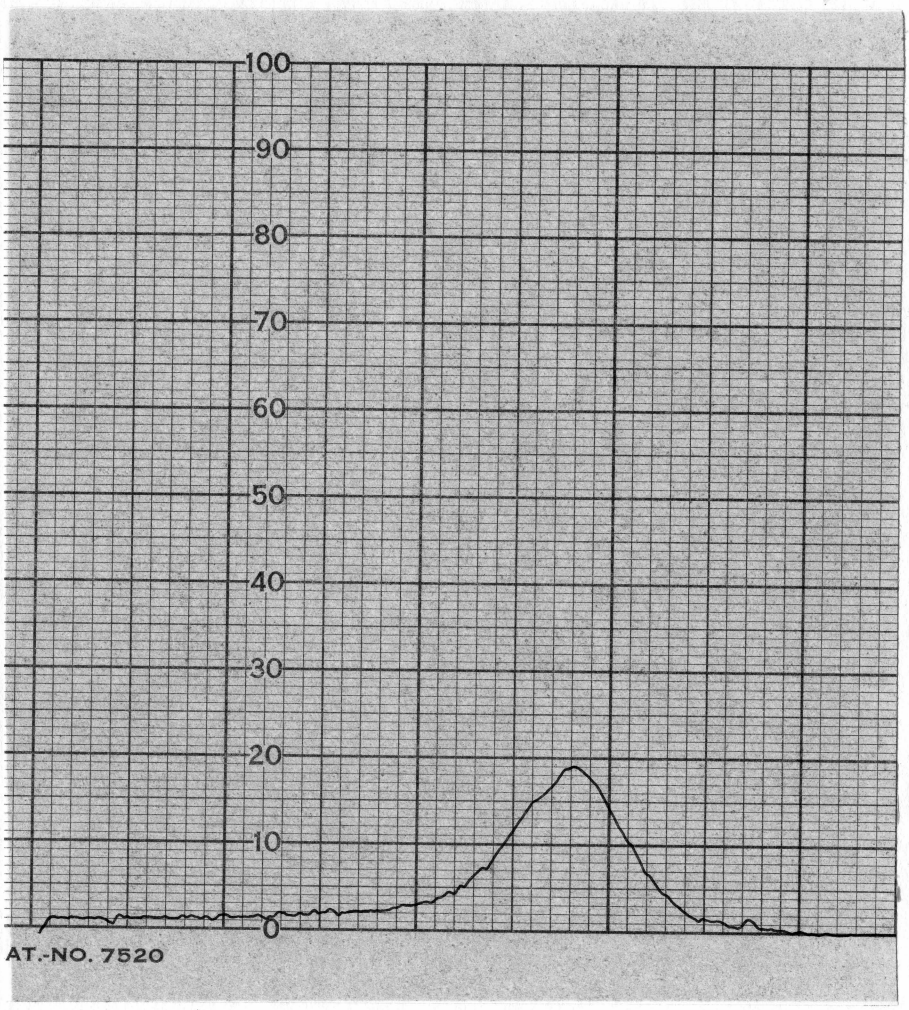


FIGURE V

A scan of the disc electrophoresis pattern of the isolated beta lipoprotein showing the homogeneity of the sample

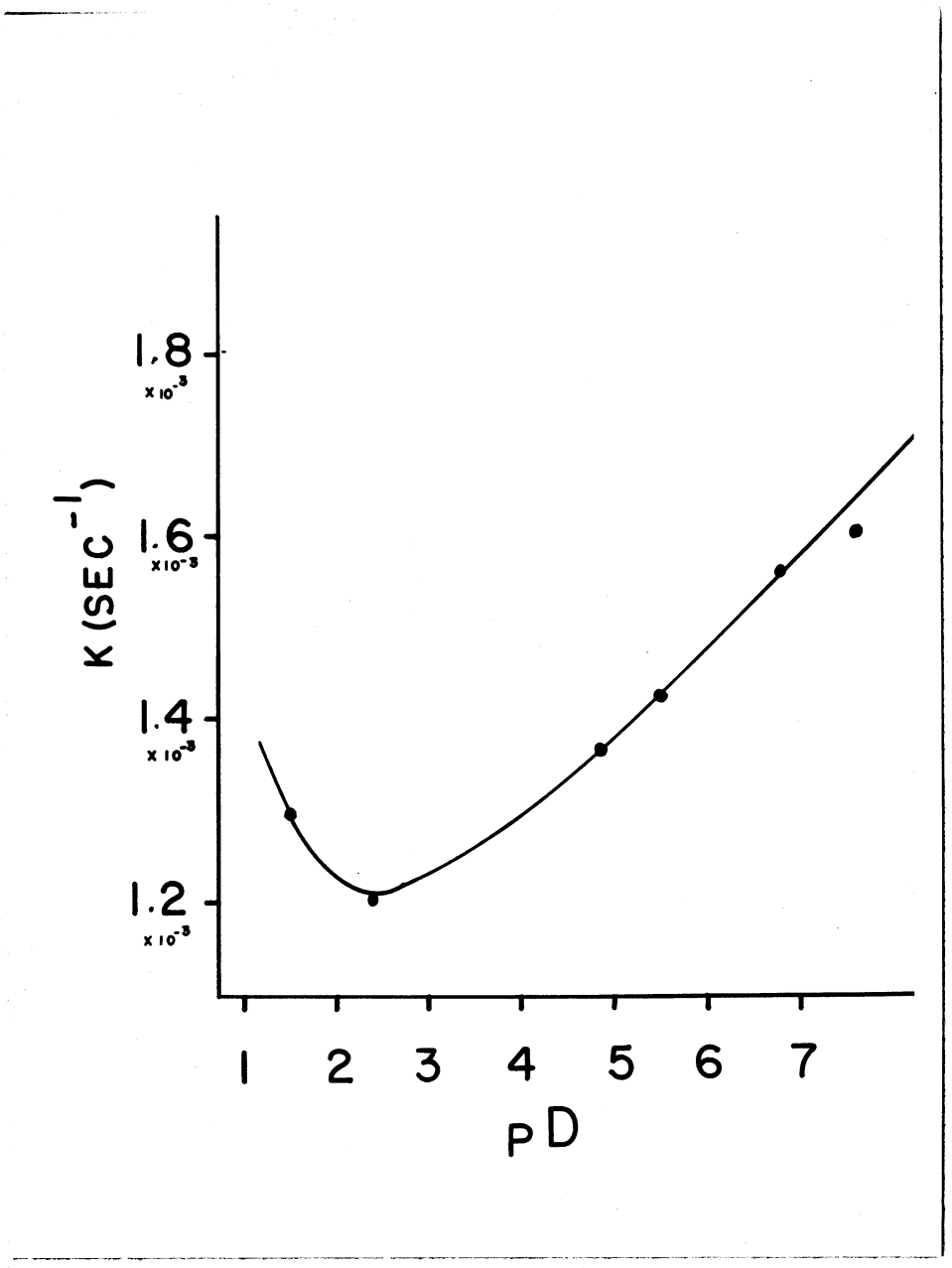


FIGURE VI

A plot of pD vs. k

showing the pD minimum value of 2.4

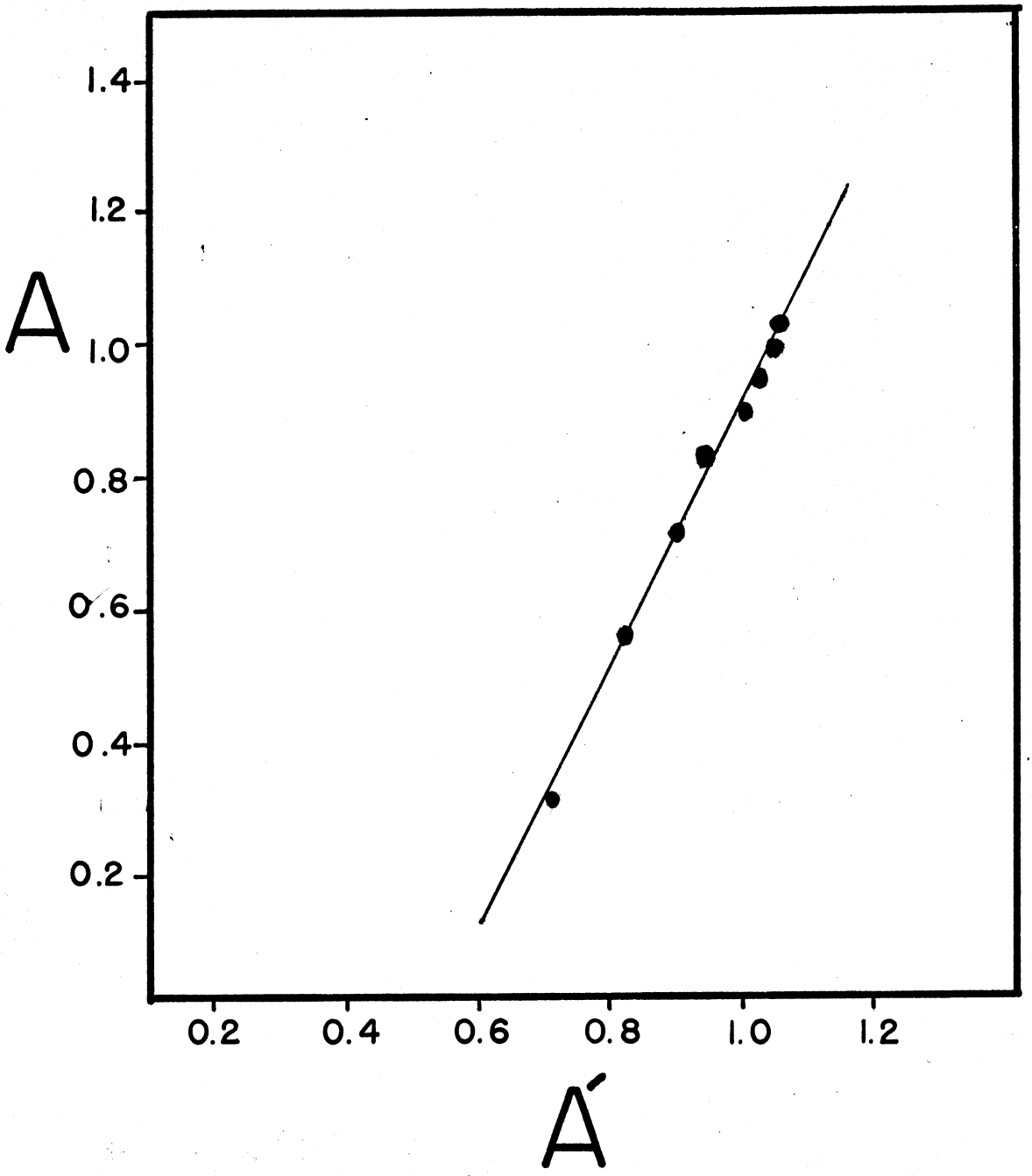


FIGURE VII

A Kezdy Plot at pD 1.5

(A and A' are numbers proportional to absorbance)

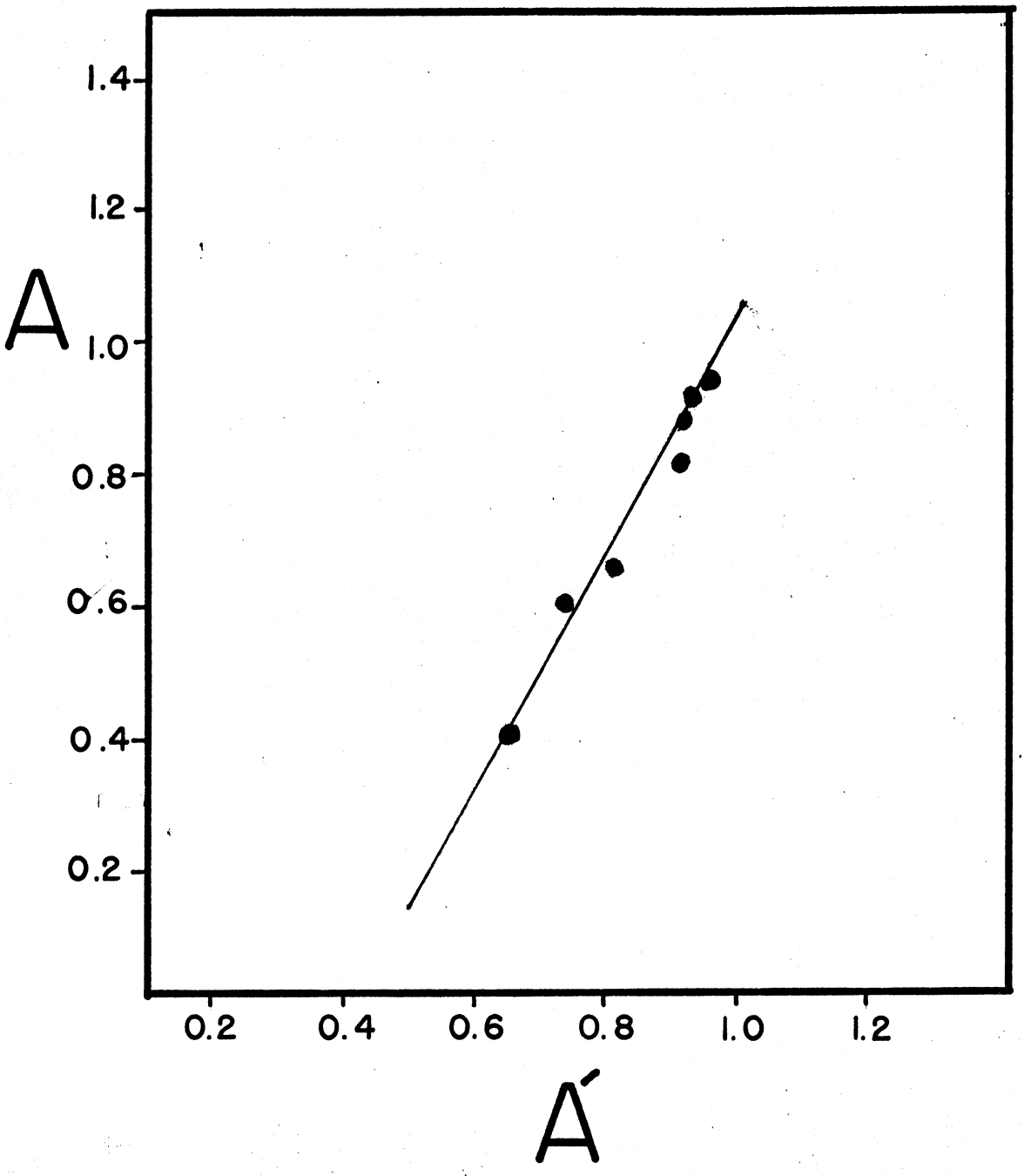


FIGURE VIII

A Kezdy Plot at pD 2.4

(A and A' are numbers proportional to absorbance)

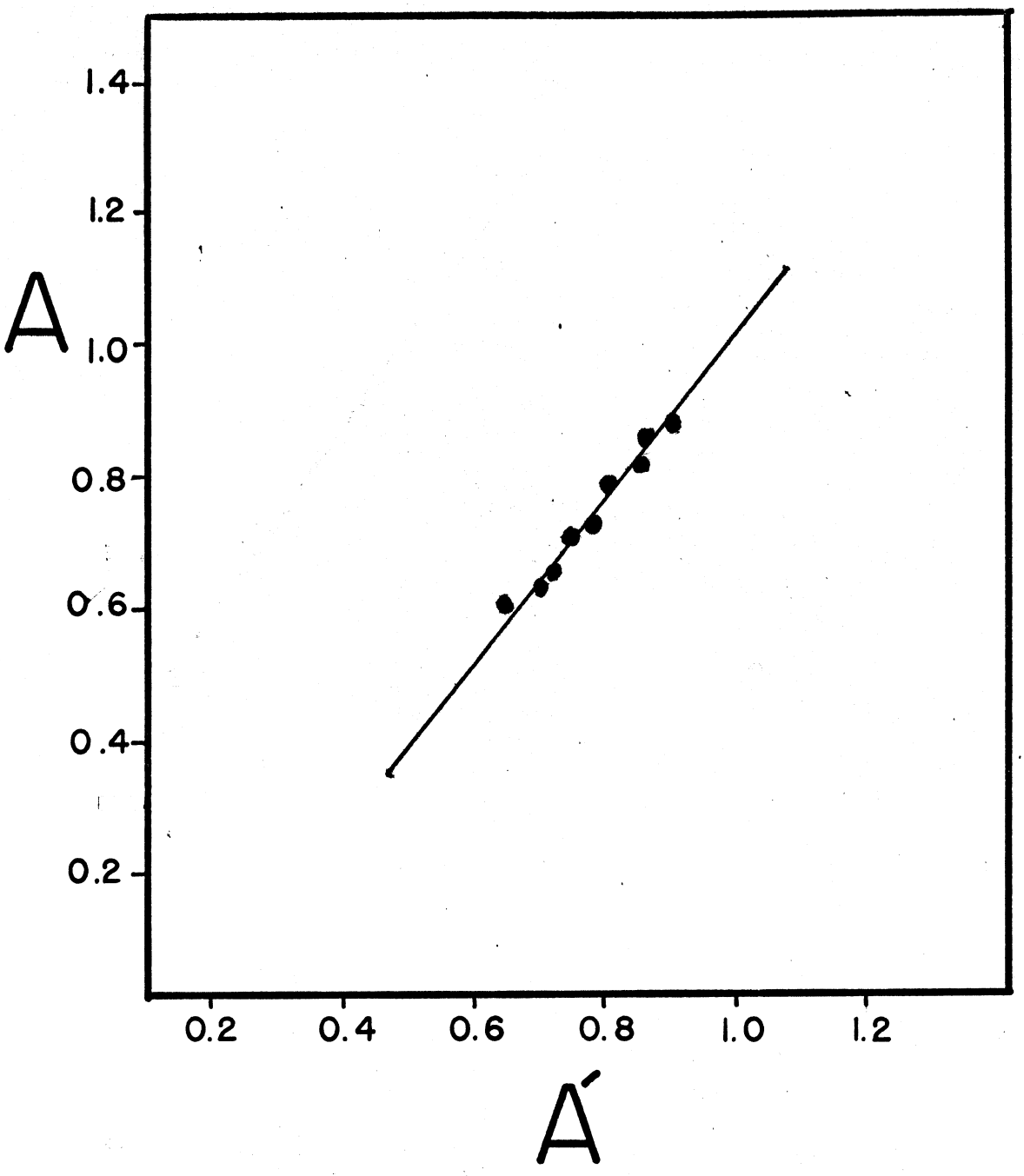


FIGURE IX

A Kezdy Plot at pD 3.7

(A and A' are numbers proportional to absorbance)

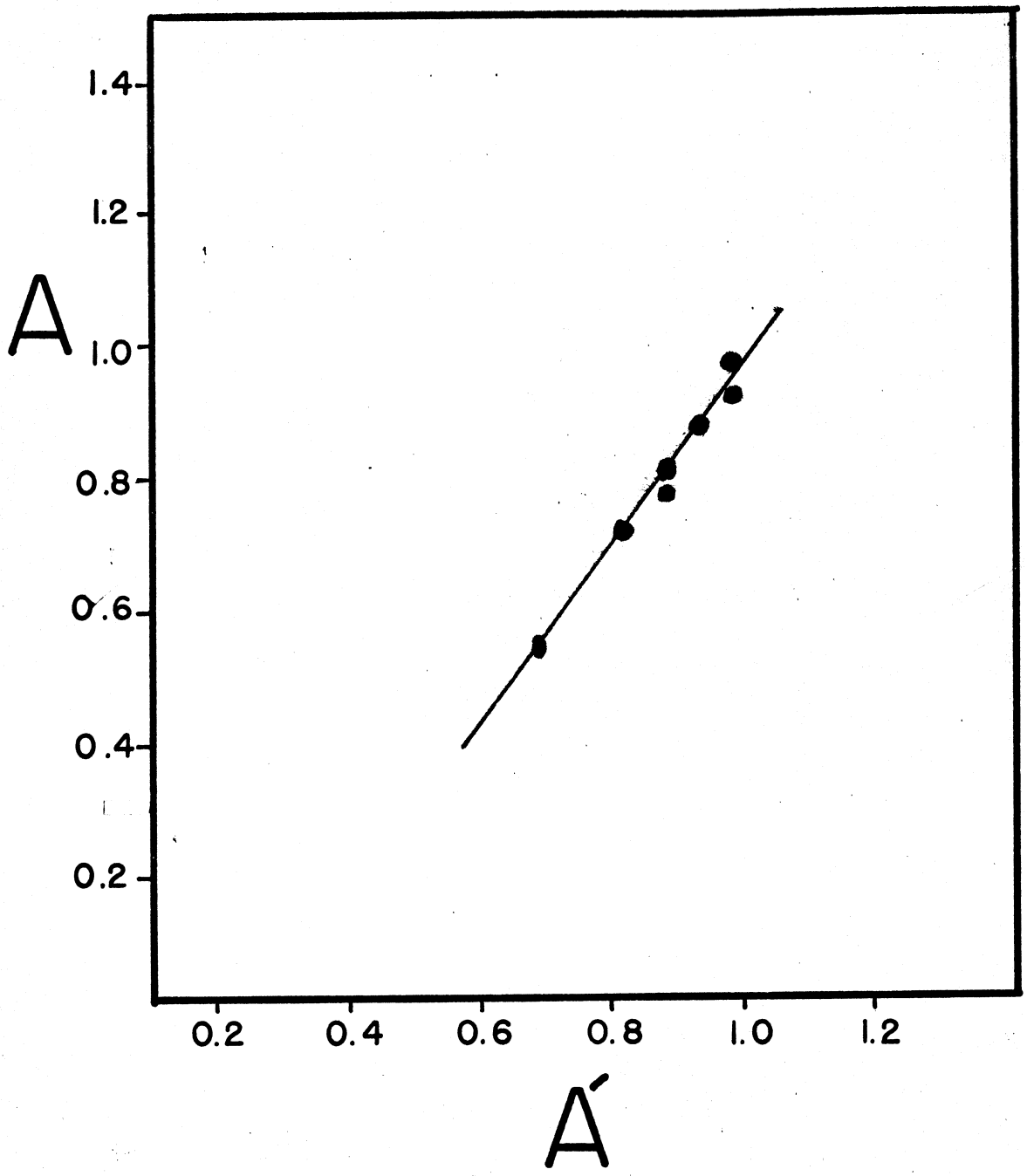


FIGURE X

A Kezdy Plot at pD 4.8

(A and A' are numbers proportional to absorbance)

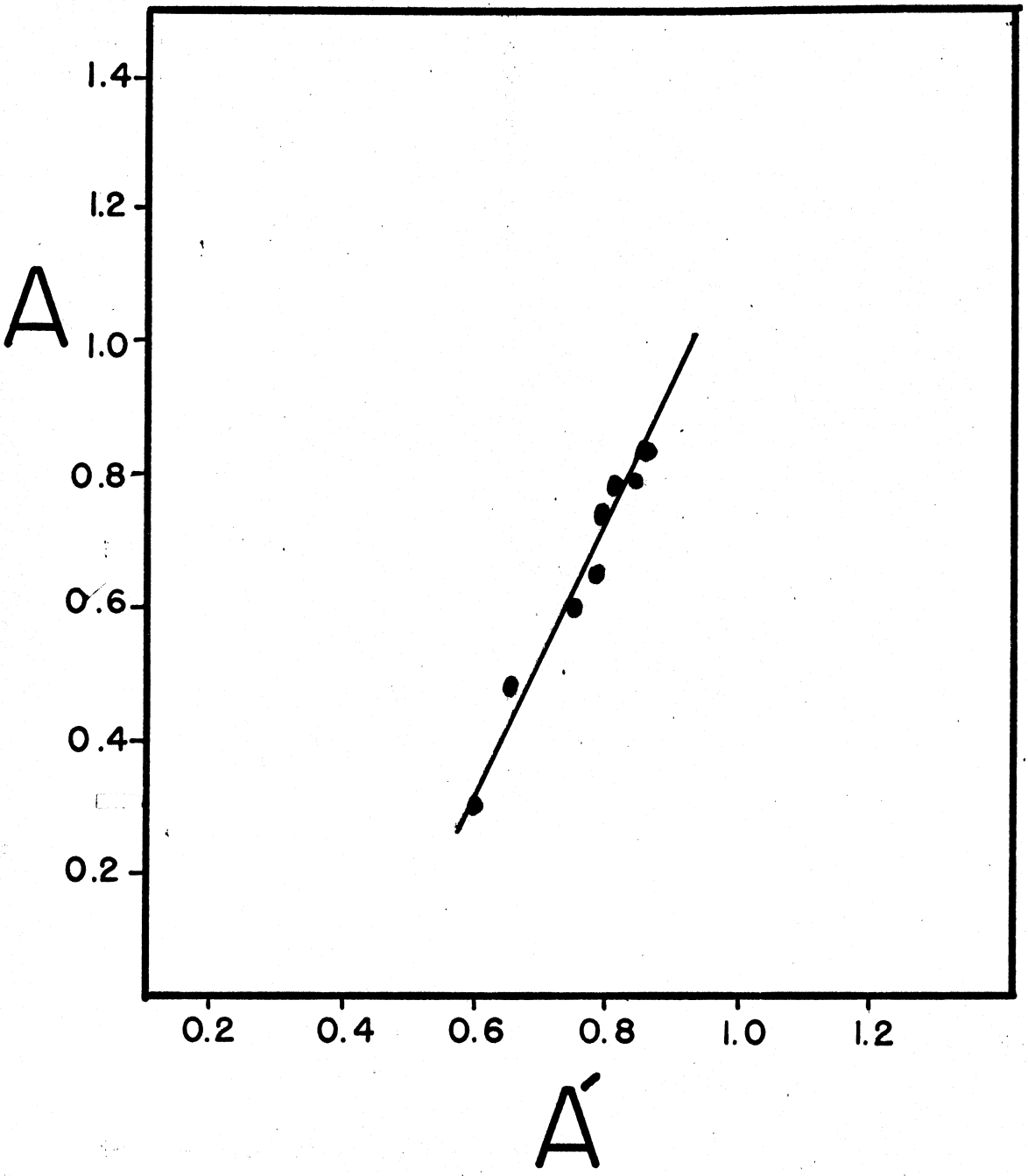


FIGURE XI

A Kezdy Plot at pD 5.5

(A and A' are numbers proportional to absorbance)

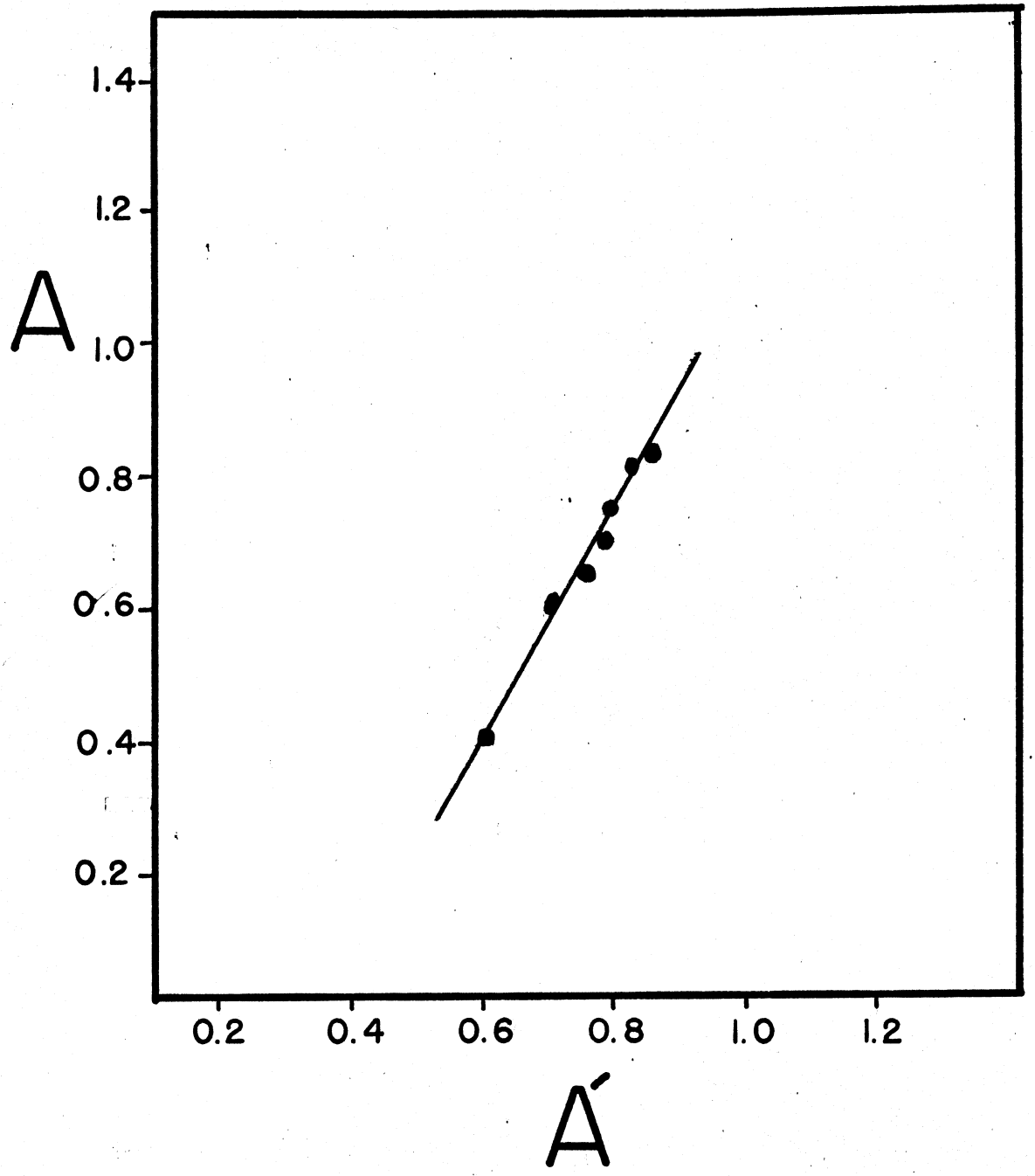


FIGURE XII

A Kezdy Plot at pD 6.5

(A and A' are numbers proportional to absorbance)

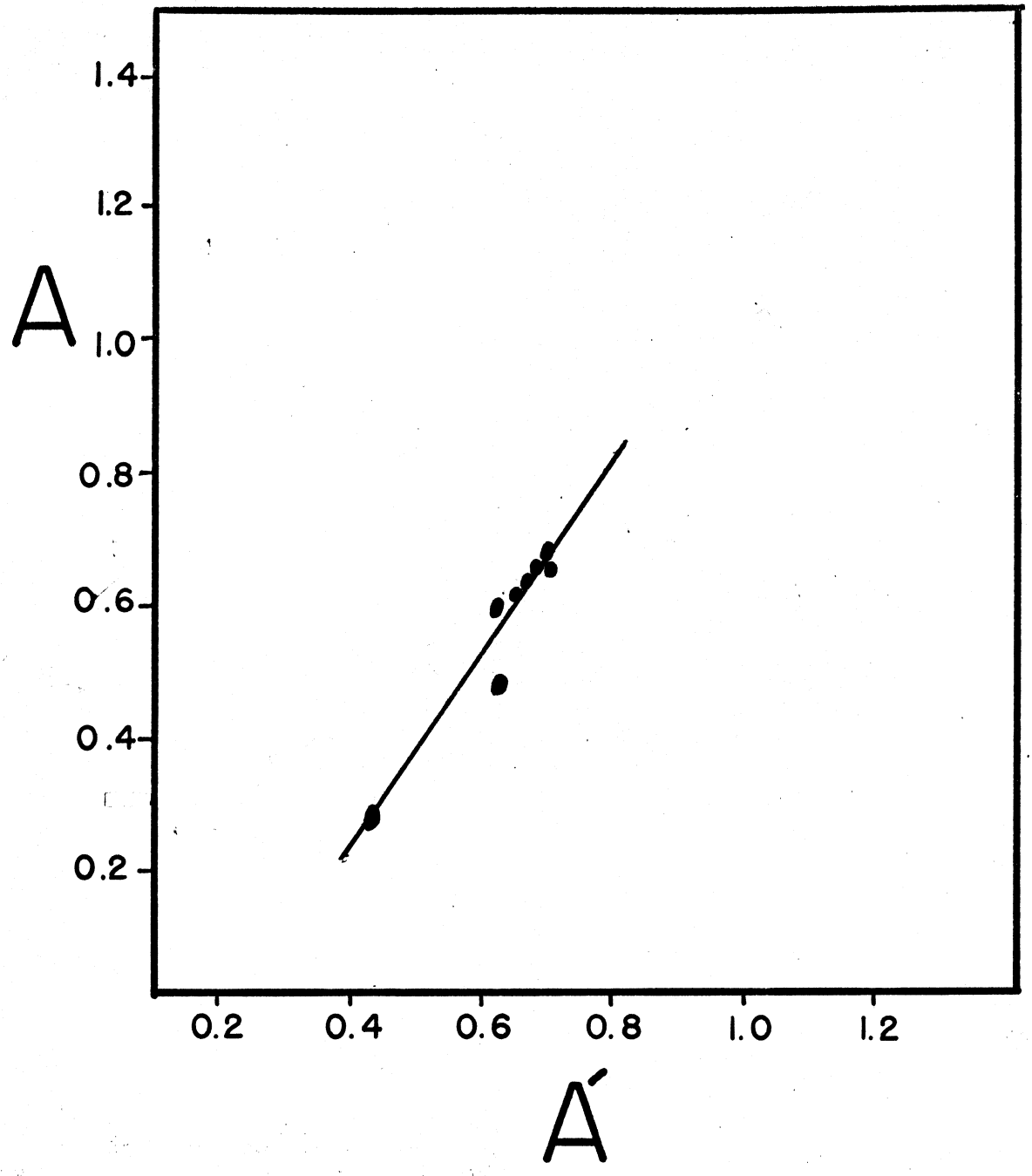


FIGURE XIII

A Kezdy Plot at pD 7.1

(A and A' are numbers proportional to absorbance)

CHART I

(pD vs. k)

pD	Rate Constant k x 10^{-3} sec^{-1}
1.5	1.30
2.4	1.20
3.7	1.30
4.8	1.37
5.5	1.42
6.5	1.60
7.1	1.56

PROPOSED MECHANISMS OF EXCHANGE

Before considering the exchange reaction with beta lipoprotein, it will be of value to review the semi-quantitative treatment of the factors effecting exchange reaction as given by Leichtling and Klotz (21).

The rate equation for a pseudo-first order reaction.

$$>NH + D_2O \xrightarrow{k} >ND + HOD \qquad (1)$$

is

$$\frac{-d\,(NH)}{dt} = \frac{d\,(OH)}{OH} = k\,(NH) \qquad (2)$$

Now k, as has been shown, is acid-base catalyzed and can be broken up into the following components:

$$k = k_o + k_d(\overset{+}{D}) + k_{od}\,(OD^-) \qquad (3)$$

AND

$$k = k_o + k_d(\overset{+}{D}) + k_{od}\,\frac{k_w}{(D)} \qquad (4)$$

where k_o = spontaneous rate constant

k_d = rate constant for acid catalyzed Rx

k_{od} = rate constant for base catalyzed Rx

k_w = self dissociation constant for water in the mixed solvent being used.

From equation 4 it is evident that k must go through a minimum at some $(\overset{+}{D})$ or pD.

By differentiation:

$$\frac{k}{(D^+)} = k_d - k_{od}\frac{k_w}{(D^+_{min})^2} \qquad (5)$$

and

$$(D^+_{min})^2 = \frac{k_{od}}{k_d}(k_w) \qquad (6)$$

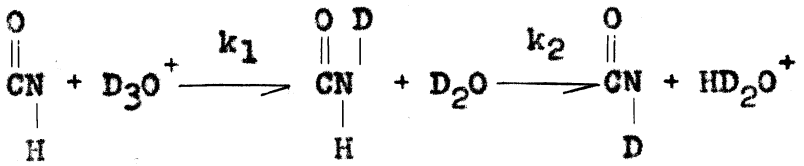
now

$$k_{min} = k_o + k_d(D^+_{min}) + \frac{k_{od}k_w}{(D^+_{min})} \qquad (7)$$

or by substitution with equation 6,

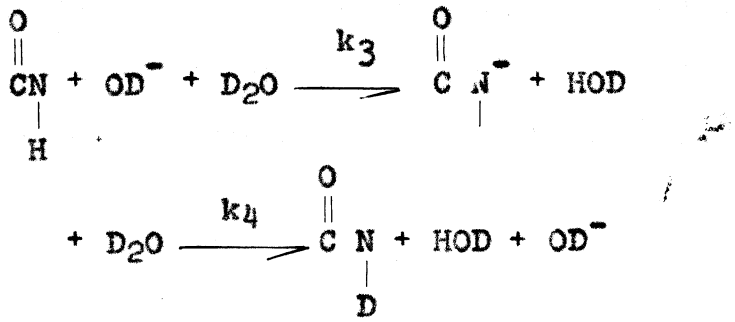
$$k_{min} = k_o + 2k_d(D^+_{min}) \qquad (8)$$

Now, the exchange reaction acid solution may be depicted as:



(9)

or in basic solution as:



(10)

Thus k_1 would be the rate controlling step in the acid catalyzed reaction and k_3 in the base catalyzed reaction.

Therefore: $k_1 = k_d$ (11)

$k_3 = k_{od}$ (12)

Substituting into equation 6, the proper values from the above two equations, we get:

$$pD_{min} = \tfrac{1}{2} pk_w - \tfrac{1}{2} \log \frac{k_3}{k_1} \qquad (13)$$

Now we can see how inductive effects influence the pD_{min}. "An electron withdrawing substituent attached to the CONH group will weaken the basicity of the nitrogen and hence decrease k_1, the rate of protonation. The same substituent, being electron withdrawing, should increase the acidity of the NH group and hence increase k_3. The net result, as is apparent from equation 13, would be a decrease in pD_{min}, i.e., a shift of the minimum in the rate -pH profile to lower pD." (21)

SOLVENT AFFECTS

From the above presentation, it is obvious that the addition of an apolar substance to water does not affect k_3/k_1 but does affect k_w and therefore must shift pD_{min}. Thus if pk_w rises, pD_{min} will be shifted to higher values.

THE MINIMUM EXCHANGE RATE

The inductive effect has been shown to affect the acid-base constants k_d and k_{od}. This relationship is reciprocal.

For: $$k'_d = (1/z)k_d \quad (14)$$

$$k'_{od} = (z)k_{od} \quad (15)$$

where k' is the rate constant affected by inductive effects.

Combining with equation 6 we get:

$$(D^+{}'_{min})^2 = \frac{(z)k_{od}k_w}{1/z\ k_d} \quad (16)$$

or $$(D'^+_{min}) = z\ (D^+_{min}) \quad (17)$$

From equation 8 we have:

$$k'_{min} = k_o + 2k'_d(D^+{}'_{min})$$

Now, by substitution involving equations 14 through 17, we get:

$$k'_{min} = k_o + 2(1/z)k_d \frac{(zk_{od}k_w)^{\frac{1}{2}}}{(1/z\ k_d)}$$

$\therefore$ by cancellation: $k'_{min} = k_{min}$

and inductive effects should not influence k_{min}.

"Although inductive effects do not affect k_{min}, changes in the nature of the solvent should and do affect the minimum rate." (21) Equations 6 and 8 show this point, for when k_w becomes smaller, D^+_{min} must drop and therefore k_{min} must become smaller.

STERIC EFFECTS

"If k_o, k_d and k_{od} are affected in the same direction by steric factors so that each one is changed by the same factor z, then it follows from equation 6 that the new pD_{min} would not be different from the original. On the other hand, equation 8 gives for the new minimum rate constant

$$k'_{min} = z\ k_{min}$$

Thus if $z < 1$, as would be expected for steric interference, k_{min} will decrease but pD_{min} will remain unchanged." (21)

GENERAL DISCUSSION

The solvent system employed for the study of beta lipoprotein D_2O exchange reactions contained dioxane. The latter was used in order to slow down the exchange rate to a point where it became measurable by standard laboratory equipment. However, the addition of dioxane caused some precipitation of the lipoprotein. This latter reaction was, in all probability, caused by the delipidization of the lipoprotein molecule. The exchange data presented is, therefore, representative of the apoprotein rather than of the intact beta lipoprotein.

The rates of exchange found for the beta lipoproteins place these molecules in between the fast-exchanging randomly-coiled proteins and the slowly-exchanging helical molecules. This phenomena can be explained in two different ways. The first concerns the motility theory of protein structure. (17) This theory states that molecules tend to fold and unfold in a rhythmic manner. The folding and unfolding exposes or "hides" the peptide bonds of the molecule, thus causing them to become "solvent accessible" or "solvent inaccessible". This phenomena has been termed molecular "breathing". The exchange mechanism of any labile slowly- exchanging protein hydrogen atom can be written according to Hvidt and Nielsen as: (17)

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} \text{exchange}$$

where N represents the native conformation, in which the hydrogen atom is buried or hidden from the aqueous solvent, and I, represents an unstable intermediate, in which the slowly-exchanging hydrogen atom is fully exposed to aqueous solvent. The constants, k_1 and k_2 refer to the "breathing" of the molecule. The constant k_3 describes the rate of the solvent-accessible hydrogen atoms and is considered to be the same as the exchange constant of free-NH groups under the same conditions of the exchange reaction.

The exchange data observed with lipoproteins can now be explained on the basis of the motility theory. The rate observed, being between randomly-coiled molecules with full solvent accessibility and molecules with buried peptide hydrogens, would seem to indicate that the molecule is, in part, in a helical conformation. This seems to be contrary to the physical evidence that the lipoprotein was denatured by the addition of dioxane. This denaturation should normally give an exchange rate indicative of a randomly-coiled molecule. It can also be proposed that the slower exchange rates observed were caused by part of the molecule being out of solution. Haggis has shown (14), however, that crystals of insulin, when exposed to D_2O humidity can come to complete exchange, thus indicating that exchange takes place as long as the molecule remains in contact with an aqueous environment. Another indication that the molecule is in a partially helical conformation is the fact that the pD_{min} was 2.4 and this

is in the range found for most helical proteins. It thus appears, from using the motility theory as a mechanism, that the beta lipoprotein apoprotein exists in a semi-helical conformation. This phenomena may come about from the joining of the lipid binding sites onto one another after the lipid has been separated by the dioxane.

The second explanation for the intermediate exchange rates observed comes from a mechanism which views the molecule as being essentially rigid and therefore having no dynamic changes of "breathing" occuring in the molecule.

This mechanism can be written as: (20)

$$N \xrightarrow{k_4} \text{exchange}$$

where N again represents the native or folded conformation with buried-NH groups and k_4 represents the exchange rate constant of these buried groups. According to Leichtling, this k_4 can be broken into components. "k_4 can be thought of as a product of a k_3-like term and a term denoting the relative inaccessibility of the peptide. The inaccessibility may be due to physical-steric factors or to an alteration of k_w which can decrease the rate markedly." (20)

Using this mechanism of the rigid molecule, the rates of exchange for beta lipoprotein indicates that possibly steric hindrance was the cause of the rates being slower than in randomly-

coiled molecules. This can be understood in view of the fact that the molecule under consideration has a weight of approximately 380,000. Also the fact that the bound lipids were removed, causing the molecule to rearrange itself, would lead one to the conclusion that a molecule of this size would have to be involved in some type of steric interaction.

Having proposed an explanation for the data observed on the exchange of beta lipoproteins with deuterium oxide, it would be in order to review the factors affecting this exchange reaction.

The factors which account for the slowly exchanging-NH groups are seen by the proponents of the motility theory as constraints, such as disulfide bridges and hydrophobic bonds, which prevent the opening of the molecule to expose the NH group to the solvent. The second mechanism presented which assumes the protein molecule to be a rigid structure, uses the same kind of factors to show that a particular region of the molecule is not accessible to the aqueous solvent. This latter mechanism does not state, however, that the slowly or non-exchanging hydrogen be in a region devoid of any solvent but rather that the local OD concentration is not in great excess of the local NH concentration (20). In all cases it appears that the local environment is the main factor in determining the observed rates with any protein-D_2O exchange reaction.

The factors that in turn influence the local environment can be named as: inductive effects, D_2O concentration, k_w change, steric effects, side chain effects, pH and temperature. Taking a closer look, we can see how the lipoprotein molecule is affected.

Previously it has been shown that the inductive effects can cause a decrease in the pD_{min} without causing a change in the k_{min}. With lipoproteins, it can be assumed that inductive effects are certainly prevalent, again owing to the size of the molecule. Examining D_2O concentration, we see that the latter must be in excess of local NH concentration but that according to Leichtling, "...for the comparison of pD-rate profiles in different solvents, the correct procedure involves no compensation for the D_2O concentration. The lack of a special dioxane effect lends a measure of confidence to analogies between the dioxane mixed solvent systems and pure D_2O." Examining the k_w we can see from previous discussion that the k_{min} will go down accordingly with a lower k_w. This was observed to be the case with the lipoproteins whose rate was immeasurably fast in pure D_2O solvent.

Steric effects and side chain effects can be looked at together. Steric effects, as shown previously, tend to lower the k_{min} rate. "By steric factors are meant any bulky groups or ordering of water which limits the approach of (D^+) and (OD^-) or limits the number of possible favorable collisions." (20) In lipoproteins there are definitely a large number of bulky groups

that would tend to favor a decreased k_{min}. Also, side chains that can cause an increase in the amount of steric effect for a local segment of the molecule, can be noted to be present in lipoproteins.

The next predominant factor is the effect of pH. As shown by the exchange data, pH has a great influence on the reaction. The parabolic curve of pD versus rates, can be somewhat accounted for by viewing the two mechanisms (equations 9 and 10) previously presented. Here we see both acid and base catalysis for the exchange reaction.

Finally the last effect to be examined is that of temperature. Kinetic reactions in general exhibit an increased rate with rise in temperature and so do deuterium exchange reactions (18, 21). With the data obtained from beta lipoprotein, this fact is again borne out. In the sample runs, it was observed that the infrared cells had to come to thermal equilibrium before any conclusive data could be taken. Before this equilibrium was reached the rate was rapidly increasing, thus giving a profile of the temperature gradient of the system. However, after reaching equilibrium, a normal rate curve could be observed.

In summary, it can be concluded that the data observed on beta lipoproteins is influenced by a variety of factors. Since the local environment is the most important, it is much harder to reach a conclusion on the rates obtained. Although using both the

"breathing" and rigid models of protein conformation, some explanation of the data on the inaccessibility of the NH groups could be presented.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Beta lipoproteins have been isolated from fresh human plasma by dextran sulfate precipitation. The method followed for the isolation was that adopted by Cornwell and Kruger (6) with the modification of Margolis and Langdon (23). Purity of the preparation was checked by the technique of disc electrophoresis. Following the isolation procedures, KBr pellets of the purified lipoprotein were made and run on a grating infrared spectrophotometer. This was done in order to identify the amide II band in the beta lipoprotein. This band was found to occur at 1449 cm^{-1}.

After identifying the amide II region, deuterium exchange reactions were monitored using this band. These runs were carried out in a 1:1 D_2O-dioxane solvent. The effect of pD versus the rate was studied; the minimum rate was determined to occur at a pD of 2.4. A rate constant of 1.20×10^{-3} sec^{-1} was also calculated.

Two mechanisms were proposed and an explanation of the exchange reaction was attempted using these mechanisms of both a "breathing" and a "rigid" molecule. Also, the various factors influencing the reaction were discussed and their possible influence on the rate were considered. These factors, as pointed out by Leichtling, are inductive effects, steric effects, k_w change, D_2O concentration, side chain effects, pH and finally tempera-

ture. These factors all influence the rate but the controlling influence is the local environment of the NH groups.

In conclusion, the complexities of deuterium exchange reactions, using a molecule as complicated as that of beta lipoproteins, are readily evident. The difficulty of interpretation, however, will probably decrease in the next few years as more data becomes available and the full impact of deuterium exchange, as a measure of the dynamic conformational fluctuations of a molecule, will be realized.

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APPROVAL

The final copies of the thesis, submitted by Lawrence J. Crolla, have been examined and approved by the director of the thesis. The signature which appears below verifies the fact that any necessary changes have been incorporated, and that it is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 24, 1968
Date

Hugh J. McDonald
Signature of Adviser