THE INTERACTIONS OF NUCLEIC ACIDS, AMINO ACIDS AND HISTONES WITH ONE ANOTHER AND WITH ION EXCHANGE RESINS

A THESIS

PRESENTED TO

THE FACULTY OF GRADUATE STUDY AND RESEARCH

OF THE UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE DEGREE OF MASTER OF SCIENCE

by

NELSON McFARLANE

SEPTEMBER, 1958

- - - -

I am indebted to

Dr. J.H. Linford

for his guidance, encouragement, and assistance

throughout this work.

CONTENTS

		Page
INTRODUCTION		1
MATERIALS		
CHAPTER I:	Properties and Preparations	
	A. Ion Exchange Resins	6
	B. Amino Acids	16
	C. Histones	26
	D. Nucleic Acids	31
METHODS, EXPERIMENTS AND DISCUSSIONS		
CHAPTER II:	Interactions of Amino Acids with Ion Exchange Resins	38
CHAPTER III:	Interaction of Histone with Ion Exchange Resins	52
CHAPTER IV:	Interaction of Nucleic Acids with Ion Exchange Resins	57
CHAPTER V:	Section I: The Interaction of Nucleic Acids with Amino Acids Retained on Ion Exchange Resins	61
	Section II: The Interaction of Nucleic Acids with Histone Retained on Ion Exchange Resins	64
CONCLUSION		68
BIBLIOGRAPHY		72

THE INTERACTIONS OF NUCLEIC ACIDS, AMINO ACIDS, AND HISTONES WITH ONE ANOTHER AND WITH ION EXCHANGE RESINS

AN ABSTRACT OF A THESIS
by
HELEN McFARLANE

The retention of amino acids, histone, and nucleic acids by ion exchange resins under equilibrium conditions has been studied. An aggregation effect was produced when histone was brought in contact with both the weak and the strong anion exchangers. This same effect was noticed when the nucleic acids were shaken with both the weak and the strong cation exchangers. It appeared as though a positive reaction occurred with the nucleic acids and with the amino acids that were retained on the strong anion exchange resin. No such reaction occurred when the amino acids were retained on the strong cation exchanger. On the other hand, a definite reaction occurred between the nucleic acids and the histone coated weak cation exchange resin. Both $HgCl_2$ and NaCl were used to determine whether such a reaction was due to the formation of a salt linkage between the nucleic acids and the histone.

Introduction

It has been known for a long time that deoxyribonucleic acid (DNA) is located in the nucleus of the cell, and is a characteristic constituent of the chromosome. Morgan and his co-workers in 1911 (1) deduced from Mendelian genetics that the chromosome is the "gene carrier", and that DNA is very closely associated with the genetic material.

Ribonucleic acid (RNA), on the other hand, is found in the cytoplasm of the cell. Caspersson (2) and Brachet (3) independently came to the conclusion that RNA is concerned with protein synthesis.

Mirsky (32) declared that histones and nucleic acids are two of the distinctive constituents of chromatin. The work of Lucy and Butler (35) indicated that histones are associated with DNA; and Stedman and Stedman revealed that the nuclei of malignant cells differed from the normal cell nuclei in that they contained a much smaller amount of histone. Similar results have been reported by Bobov (34). The hypothesis was therefore advanced that these basic proteins function in the nucleus as gene inhibitors, thus maintaining, if not wholly determining, the character of the cells in which they are present.

Caldwell, Hecker and Hinshelwood (1950 (4) suggested that nucleic acids guide the order in which amino acid residues are laid down during the synthesis of proteins. Hultgren and Haden 1947 (5) working with B. cereus, and Dale and Gulick 1953 (6) from their work with Staphylococcus aureus pointed out that the rate of protein synthesis varied directly with the nucleic acid content of the cells. The results of Ben-Ishai from her studies with Escherichia coli indicated that only the RNA formed during protein synthesis is involved in the synthesis, whereas the pre-formed RNA was not found to

protein synthesis (6b). Brachet (7) has shown that in the organs in which there is a high protein synthesis, there is also a high RNA content, and, conversely, in tissues with a low protein synthesizing ability the proportion of RNA is relatively low. In 1952, Jonser and Jonsor (8) came to the conclusion that the rate of protein synthesis is related to the rate of nucleic acid synthesis. Davidson (9) and others (10, 11) declared that a protein poor diet is followed by a decrease in the RNA content of the liver, while the DNA content of the liver is unaffected by such conditions. More recently, Gale and Folkes 1953 (6) pointed out that protein synthesis and RNA synthesis can be inhibited by the use of certain drugs such as chloramycetin and aureamycin; also, terramycin inhibits protein synthesis but increases nucleic acid synthesis. Similar results have been reported by Levy et al (12) and by Misra et al (13). Caspersson in 1940 theorized that protein synthesis requires the presence of nucleic acids, and that the nucleus itself is a cell especially organized as the main centre for the formation of protein. However, a great deal of work has been performed on the relationship between nucleic acid and protein synthesis since 1940. Daly et al 1952 (14) and Smellie et al 1953 (15) working with formate-C^{14}, methionine-S^{35}, and glycine-N^{15}, stated that the incorporation of labelled amino acids is no higher in the nucleus than it is in the cytoplasm. Experiments by Brachet and Chantrenne 1952 (16) with radioactive CO_2 have clearly shown that the nucleus exerts only a remote control on protein synthesis, and that the incorporation of amino acids into proteins is fundamentally a cytoplasmic process. Brachet 1953 (7) went further, and postulated that there are three main theories for protein synthesis: (i) protein synthesis results from a reversal of proteolysis; (ii) protein synthesis involves the

intervention of energy-rich bonds; and (iii) protein synthesis occurs through a "template mechanism".

Linderstrom-Lang (17) pointed out that there is as yet no satisfactory evidence that protein synthesis ever occurs when proteolytic enzymes are made to act on polypeptides. The work of Borsook (18), Lipmann (19), and Spolgelman (20) demonstrated that the incorporation of labelled amino acids into proteins requires energy. This has been confirmed by Dresler and Hidayan (21). Lipmann proposed an enzyme framework upon which amino acids are assembled, phosphorylated and condensed in a head-to-tail manner whereby peptide chains are formed. A similar model has been presented by Bounce (22) in which amino acid phosphate esters are assumed to condense with a phosphorylated polynucleotide.

The "template" theory seems to have received considerable support. Haurowitz (23) and Caldwell (4) suggested that nucleic acid acts as a kind of template, on the surface of which amino acid residues and specific peptide chains are laid down. The template would act as a kind of mould or building block for the amino acids or proteins. Haurowitz (24) assumed that the nucleic acid may act only as a support for the peptide chains in an extended form while they are being "copied" by a crystallization type of process.

Gamow 1954 (25) proposed a key and lock relationship between the various amino acids and the rhomb-shaped "holes" formed by the nucleotide succession in the nucleic acid chain. He speculated that the free amino acids may get caught in these holes and subsequently be united into peptide chains.

The manner in which nucleic acid acts in protein synthesis is not definitely known; all the evidence quoted above, however, shows that a

specific relationship exists between nucleic acid and the formation of proteins.

The present work is an attempt to detect the occurrence of reactions between nucleic acids and amino acids and histone. Reactions in solution involving the destruction of chemical bonds or the creation of new ones may be detected by the changes in ultraviolet absorption spectra, providing the bonds involve structures or groups which absorb in this region. Only the amino acids which contain an aromatic groups give a characteristic U.V. absorption (26). The nucleic acids have a characteristic U.V. absorption at 2600 Å due to the purine and pyrimidine rings (27, 28). Interactions which do not involve the absorbing groups and those which do not take place by replacement of chemical bonds, such as suggested by Gamow, would not be detected by spectrophotometry. Removal of a reactant or reaction product from solution could, however, be followed by a decrease in the absorption intensity. This could be brought about if one of the reactants was held in the solid phase on a large surface.

Ion exchange resins are suitable for this purpose. The term "retention" is used as far as possible for the reactions involving ion exchange resins, since it is not quite certain whether larger molecules like amino acids, histone, and nucleic acids are actually adsorbed on the surface of the resins or whether a chemical reaction occurs between them. The interactions of ion exchange resins with amino acids have been studied by several different workers (29, 30 and 31). Since amino acids are amphoteric, they can be retained by either the anionic or the cationic ion exchangers. The carboxyl group of the amino acid is free to interact with the solution when the amino acid is retained on a cation exchanger.

On the other hand, the amino group is free to interact when the amino acid is retained by an anionic exchange resin.

The retention of histons by ion exchange resins obeys the same fundamental principles as do amino acids. Crampton et al utilized a weak acidic resin (Amerlite IRC-50) for the fractionation of calf thymus histone.

The interactions of certain amino acids, of histone, and of nucleic acids with ion-exchange resins have been studied, and the reactions between the complexes formed by the retention of the amino acids and histone on the ion exchange resins with nucleic acids have also been examined.

CHAPTER 1

A. ION EXCHANGE RESINS

Composition

The discovery by Spence (1845) that when a solution of ammonium sulphate was allowed to percolate through a column packed with soil, the effluent from the colum contained no ammonium salts but considerable amounts of calcium sulphate instead, served as an impetus to establish the study of the phenomenon of ion exchange. Later (1907), Gans began the development of artificial ion exchange materials for water-softening process (37).

An ion exchange resin particle can be visualised as an elastic three-dimensional hydrocarbon network to which is attached a large number of ionizable groups. These ionizable groups take part in double displacement reactions, i.e., they exchange ions with the surrounding solution. The nature of the hydrocarbon network affects the degree but not the specificity of the chemical reaction of an ion exchange resin.

Synthetic ion exchange resins were invented by Adams and Holmes (38) (1935). The first resin which they tried was obtained by heating various dihydric phenols with formaldehyde causing a poly-condensation reaction to occur. Since the original paper of Adams and Holmes, many modifications of the condensation resins have been proposed with a view to improving the capacity or stability of the resins to hot water or chemical reagents. The best hydrocarbon network developed to date is that formed by the copolymerization of styrene and divinylbenzene; such ion exchange resins are resistant to oxidation, reduction, mechanical wear and breakage, and are insoluble in all common solvents (36).

Preparation and Properties

The particle size and cross linkage of the resins are built into the hydro-carbon network. The ion exchange bead may be formed with an average diameter from 1.0 mm. to less than 0.04 mm. Beads much larger than 1.0 mm. in diameter tend to shatter or crack during the manufacturing process. The particle size of an ion exchange resin is usually specified by reference to U.S. standard screen sizes.

Co-polymerization of a vinyl and divinyl compound produces a resin, but in order to make an ion exchange resin, one must attach stable ionic groups to the structure; this could be accomplished either by using a monomer containing such groups, or by introducing them into the resin after polymerization (39).

The second variation which can be built into the copolymer beads is that of crosslinkage. The degree of crosslinkage in a styrene-divinyl-benzene bead indicates the fraction of divinylbenzene it contains and is directly controlled by the proportion of divinylbenzene to styrene used in the reaction mixture. Crosslinkage is a term which is also used to describe the porosity of an ion exchange particle. A highly crosslinked resin (16%) might be impermeable to ions of high molecular weight, whereas a lower crosslinked resin (4%) might be quite permeable to the same ions. The Dow Chemical Company defined the crosslinkage of a resin as the percentage of divinylbenzene which is used to prepare a particular resin. For example, the divinylbenzene content of a Dowex resin is indicated by an "X" number following the number of the particular resin; to illustrate, Dowex 50 -4 is prepared from a styrene-divinylbenzene copolymer containing 4% divinylbenzene. The crosslinkage of a resin has a definite influence on the time required for an ion to reach equilibrium. A highly crosslinked

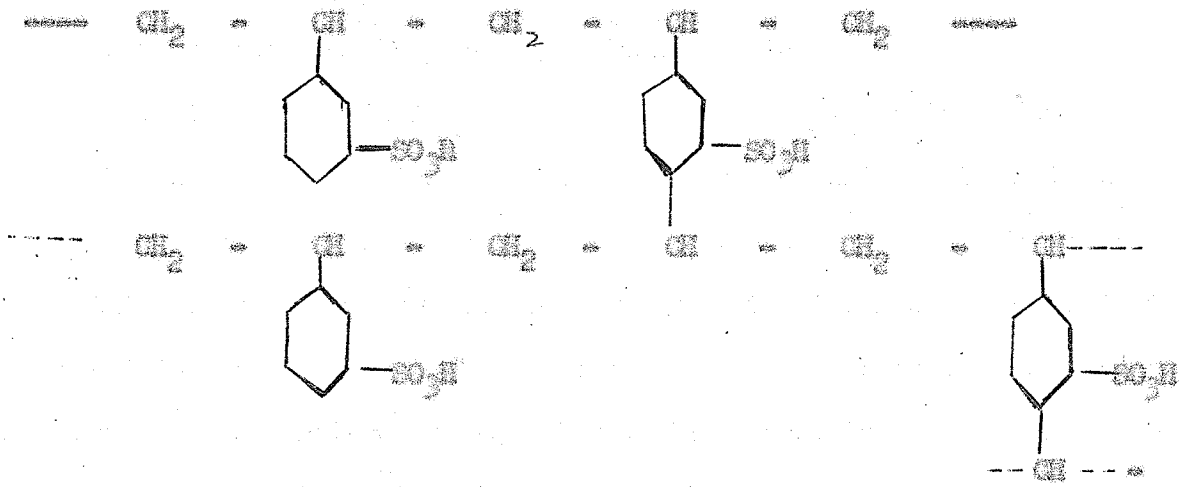
resin is quite resistant to the diffusion of various ions through it and hence the time required to reach equilibrium is much longer. In general, the larger the ion or molecule which is diffusing into an ion exchange particle, or the more highly cross-linked the polymer, the longer will be the time required to reach equilibrium conditions.

Sulphonated polystyrene resins are very stable, and possess high exchange capacity, about twice that of the phenol sulphonic acid resins. The total capacity of an ion exchange resin is defined as the total number of chemical equivalents which it has available for exchange per some unit weight or volume of resin. The capacity may be expressed in terms of milli-equivalents per ml. of wet resin, or in terms of milli-equivalents per dry gram of resin (45).

Rohm and Haas Company (U.S.A.) introduced Amberlite IRA-400 around 1940. Prior to this period, attempts to prepare strongly basic resins were not successful. The only type of strongly basic group that can be attached covalently to a polymer skeleton is the quaternary ammonium group --- $N^{+}(R,R',R'')_3$ (39). Kitchener (37) pointed out that the preparation of an anion exchanger is accomplished by first chloromethylating a cross-linked polystyrene resin, and then treating the product with a tertiary amine such as $N(CH_3)_3$; thereupon they readily undergo the quarternisation reaction, producing the quaternary ammonium chloride --- $CH_2.N^{+}(CH_3)_3Cl^{-}$. Strong base resins are not stable in hot water above 60°C.

Styrene-divinylbenzene Copolymers

Sulphonic cation exchangers are prepared by the nuclear sulfonation of these copolymers, i.e., introduction of one sulphonic acid molecule into each benzene ring (37). The structure of the resulting resin is:



The sulphonic acid groups attract water which is held inside the resin particle, and the water content of an ion exchange resin varies inversely as the crosslinkage (29). The quaternary ammonium groups of the anion resins behave in a similar manner. In the moist form, ion exchange resins are completely stable.

Most modern resins meet the following requirements:

(a) stability to hot water

(b) stability to common chemicals in solution

(c) only one type of functional group present

(d) obtainable in bead form of any desired size range

(e) range of weak and strong acidic and basic types available

(f) the degree of crosslinking controllable for special purposes

There are two types of diffusion which must be considered in an ion exchange equilibrium. The first is called film diffusion or the movement of ions from a surrounding solution to the surface of an ion exchange particle. The second is called internal diffusion and is the

movement of ions from the surface to the interior of an ion exchange particle. Film diffusion is usually the controlling reaction in dilute solutions; internal diffusion is the controlling reaction in more concentrated solutions. The particle size of an ion exchange resin affects both film diffusion and internal diffusion. A fine mesh particle presents more surface area for film diffusion and also contains less internal volume through which an ion must diffuse. A decrease in particle size thus shortens the time required for equilibration (42). The penetration of a soluble electrolyte into the resin is also governed by the Donnan theory (37).

In 1946, Freeman and Kitchener (43) found that considerable non-coulombic forces of attraction, - (van der Waals forces), play an important part between ion exchange resins and large ions in solution. The attraction is probably due to dispersion forces between the cation and the benzene rings of the resin, but ion-induced dipolar forces probably contribute. Davies has shown that these van der Waals forces also play an important role in the adsorptions of amino acids on ion exchange resins (40). This has been confirmed by Partridge and Brimley (44).

Techniques for contacting solutions with resins

The two techniques are known as the batch method and the column method.

Batch operations

The batch method consists of placing the resin and solution into a container, mixing and allowing equilibrium to be established. The extent to which the exchange takes place is limited by the selectivity of the resin under the equilibrium conditions. Thus, unless the selectivity is quite favourable, only a relatively small part of the total capacity of

the resin can be utilised. Exceptions to this are those cases in which ions are removed from the system during the exchange. Example:

$$R\text{---}SO_3^- H^+ + Na^+OH^- \longrightarrow RSO_3^- Na^+ + HOH \quad (1)$$

Another example is the reaction of the silver form of the resin with salt solution to give insoluble silver chloride:

$$RSO_3^- Ag^+ + Na^+ Cl^- \longrightarrow RSO_3^- Na^+ + AgCl \quad (2)$$

This reaction is employed for desalting of seawater in survival kits. These types of reaction will essentially go to completion, regardless of the selectivity coefficient of the resin (39).

Colum operations

The column methods may be considered as a large number of batch operations in series, and even though the exchange is limited by the selectivity coefficient, the overall effect may be much more favourable (37).

In column operations the ion exchange units consist of a vertical cylinder filled with ion exchange resin. The resin is supported on a bed of sintered glass or glass wool, and the solutions passed down through the resin.

Almost all of the work that has been done with ion exchange resins has utilised the column principle because of its practicability. For example, if calcium is to be removed from hard water by means of a sodium form resin, it would be inefficient to shake a quantity of resin with a given volume of water, as equilibrium would be set up, and some calcium would be left in solution; therefore the water is passed through a column of the resin. As the water becomes depleted of calcium by contact with the first layers, it passes to fresh resin containing little or no

calcium and so the equilibrium is constantly displaced with a sufficiently long column the effluent is entirely free from calcium.

In the present work, the column method was used only as a means of regenerating the resin; otherwise the batch operation was the chief means by which ion exchangers were used. The reason for this was that in the batch operation, the ion exchange resin was evenly coated with the solute after shaking for a specified length of time, and it was very easy to calculate the amount of solute retained by the resin.

Regeneration of Ion Exchange Resins

Pre-treatment of sulphonated polystyrene acidic resin.

Strong acidic resins supplied in the presence of free HCl were made into a slurry and poured into a column. This was then washed alternately with 2N NaCl and 1 N excess, finally with 2N NaCl, and then with distilled water to neutral pH.

For anion exchange resin containing strongly basic quaternary groups, the columns were built in water as before, but washed alternately with 0.2N NaCl and 2N NaOH (made $CO_3^{=}$ free by adding 10 gm. $Ba(OH)_2\ 8H_2O$ per litre of 2N NaOH).

When it was necessary to convert one cycle of exchange ion to another, or to regenerate a column of resin, the column was washed with 2 to 3N of the salt, acid or alkali according to the type of ions required, and then washed with de-ionized distilled water to a neutral pH. When it was necessary to cycle the anion exchange resins into the chloride form, NaCl was used, instead of HCl, to avoid the acid effect of the latter.

Strong or Salt Splitting Ion Exchange Resins

The strong or salt splitting resins are completely ionized as are strong acids, strong bases and their salts. They remain water-swollen

and completely ionized in both the salt and acid or base forms. As a result their ability to exchange ions is not dependent upon the pH of the solution, and this accounts for their so-called salt splitting capacity. Salt splitting is the formation of an acid or base from a salt of that acid or base. Such a reaction with a strong sulphonic polystyrene ion exchanger would be:

$$R - SO_3^-.H^+ + NaCl \rightleftharpoons R - SO_3^-.Na^+ + HCl \qquad (3)$$

or with a strong quaternary base:

$$R - NR_3^+.OH^- + NaCl \rightleftharpoons R - NR_3^+.Cl^- + NaOH \qquad (4)$$

The weak ion exchange resins are not ionized appreciably except in their salt form. The salt forms of these resins are water-swollen but most of their water holding ability is lost when they are converted to the free acid or free base form. As a result the exchange rates obtained with weak resins are much faster when the reaction begins with the salt or water-swollen form of the resin. The salt form of the free resin is rapidly converted to another salt or to a free acid or free base form, but the reverse reaction, in which the free acid or free base is the starting point, is a much slower reaction (3).

Example:

$$R\text{-}COOH + NaOH \xrightarrow{\text{slow}} R\text{-}COO^-Na^+ + H_2O \qquad (5)$$

$$2\ RCOO^-Na^+ + H_2SO_4 \xrightarrow{\text{fast}} 2\ RCOOH + Na_2SO_4 \qquad (6)$$

The carboxylic resins and the amino resins, being weak acids and bases, can form only very unstable salts with weak bases or acids.

Ion Exchange Reactions

A. Reactions of strongly acidic cationic resins:

This material will effect exchange of cations, including hydrogen ions, when these are associated in solution with anions of weak or strong acids:

a) with anions of strong acid:

$$R\text{-}SO_3^- \cdot H^+ + NaCl \rightleftharpoons R\text{-}SO_3^- \cdot Na^+ + H^+Cl^- \quad (7)$$

The relative position of the exchange equilibrium depends upon the relative concentration of NaCl and HCl according to the Law of Mass Action. For inorganic cations it is also governed by the hydrated radius of the ion in solution, and by the charge on the ion.

b) with anions of weak acid:

$$R\text{-}SO_3^- \cdot H^+ + Na^+Ac^- \longrightarrow R\text{-}SO_3^- \cdot Na^+ + HAc \quad (8)$$

The direction of change is to the right because H^+ ions are removed from solution.

B. Reactions of weakly acidic cationic resins: (See Equation 5).

C. Reactions of strong basic anionic resins:

These will effect exchange of anions from neutral salts and from acids:

$$R\text{-}NR_3^+ \cdot OH^- + NaCl \longrightarrow R\text{-}NR_3^+ \, Cl^- + NaOH \quad (9)$$

The direction of change depends upon the relative concentration of NaCl and NaOH according to the Law of Mass Action.

$$R\text{-}NR_3^+ \cdot OH^- + H^+Cl^- \longrightarrow R\text{-}NR_3^+ \cdot Cl^- + H_2O \quad (10)$$

The direction of change is to the right because OH^- ions are removed from solution.

The ion exchange resins used in this work were obtained from the following sources:

(1) Dowex 50-X4: 200-400 mesh, polystyrene sulphonic strong acidic resin supplied in the hydrogen (H^+) form by Fisher Scientific Co. (U.S.A.)

(2) Amberlite IRC-50: weak acid resin; (carboxylic); a product of Rohm and Haas; obtained in the (H^+) form from Fisher Scientific Co. (U.S.A.)

(3) Amberlite IRA-400: strong basic resin; quaternary amine product of Rohm and Haas; obtained in the (OH^-) form from Fisher Scientific Co. (U.S.A.)

(4) Amberlite IR-45: weak basic resin; product of Rohm and Haas; obtained in the (OH^-) form from Fisher Scientific Co. (U.S.A.)

(5) Amberlite MX and Amberlite MB): both of these are water conditioning resins and remove both anions and cations from water; obtained from the same source as above.

Throughout this work, single distilled de-ionised water was used. This was obtained by first distilling tap water in a Barnstead Water Still and then passing the distilled water through a column of mixed bed ion exchanger of either Amberlite MX or Amberlite MB). Both of these produced ion free water that gave no absorption in the ultraviolet.

All pH measurements were made, using the glass electrode of a Beckman Model H2 pH meter.

An amount of 10 grams of the desired ion exchanger was placed in a column and cycled as outlined on Page 12. Regardless of the form in which the ion exchange resins were obtained from the manufacturer, they were regenerated before use, because several authors have proclaimed that most commercially available resins contain impurities as a result of the process of manufacturing.

The regenerated resins were stored in the moist form, and all experiments were carried out at room temperature, and unless otherwise stated 0.50 gm. of resin was used in each case.

2. AMINO ACIDS

Vickery and Schmidt (16) pointed out that the principles on which the identity of an amino acid should be based are: isolation of the amino acid from protein hydrolysates by an individual other than the discoverer, establishment of its constitution by synthesis, and proof of its identity with the corresponding synthetic compound. The proteins vary considerably in their content of the twenty-two accepted amino acids. In certain proteins, some of the amino acids are absent, or present only in traces; in other proteins, the proportion of particular amino acids is relatively high.

According to recent theories, proteins are composed of amino acid residues which are held in firm chemical union by covalent bonds. An individual chain of this nature is known as a peptide.

CLASSIFICATION OF AMINO ACIDS

Amino acids have been classified in various ways. One system classifies them according to the number of unbound carboxylic groups present in the molecule as mono-amino-monocarboxylic acids and mono-amino-dicarboxylic acids. Another system designates them as aliphatic, aromatic and heterocyclic amino acids, depending upon the presence of chain or ring structures. Again they are classified according to reaction in solution as neutral, acidic and basic amino acids. Classification often utilizes two or more of the above systems.

In the present study, seven amino acids were used by the author, and they were chosen in such a way as to be representatives of the entire classification of amino acids.

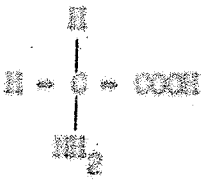
1. ALIPHATIC AMINO ACIDS (CHAIN COMPOUNDS)

a) Monoamino-monocarboxylic acids

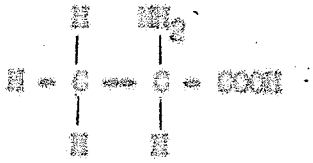
Neutral in reaction

(i) glycine or glycocoll (Amino acetic acid obtained from the British Drug Houses Ltd.),

IpH = 5.97



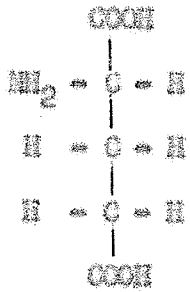
(ii) Alanine (α-amino propionic acid obtained as DL Alanine from Nutritional Biochemical Co., (N.B.C.)) IpH = 6.0



b) Monoamino-dicarboxylic acid

Acid in reaction

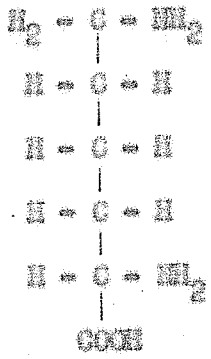
(iii) Glutamic acid (α-aminoglutaric acid obtained as l-glutamic acid from N.B.C.) IpH = 3.22



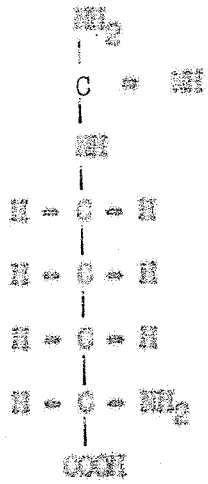
c) Diamino-monocarboxylic acids

Basic in reaction

(iv) Lysine (α -ε Diamino-n-caproic acid obtained as L-lysine monohydrochloride from N.B.C. IpH = 9.7



(v) Arginine (α -amino- δ guanidino -n-valeric acid obtained as L-Arginine monohydrochloride from Eastman Kodak Co. Res. Lab.) IpH = 10.8



d) Aromatic amino acids

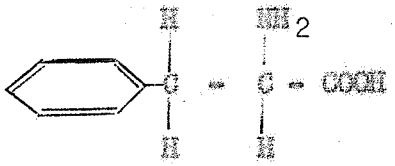
Monoamino-monocarboxylic acids

Neutral in reaction

(vi) Phenylalanine (phenyl derivative of alanine)

(α -amino-β-phenyl propionic acid obtained as dl-β-phenylalanine from Eastman Kodak Co.)

IpH = 5.48



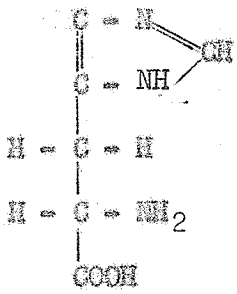
e) Heterocyclic amino acids

Monoamino-monocarboxylic acids

Neutral in reaction

(vii) Histidine is very slightly basic

Histidine (α-amino-β-imidazolepropionic acid obtained as L-histidine monohydrochloride from N.B.C. and from Eastman Kodak Research Laboratory) IpH = 7.59



DIPOLAR IONS AND ACID-BASE EQUILIBRIA OF AMINO ACIDS

Many substances contain both acidic and basic groups and react with both acids and alkalies to form salts. Such substances are AMPHOTERIC and are called AMPHOLYTES. Notable among such substances are the amino acids of which proteins are composed.

The -COOH group is the acidic group or proton donor, and the NH_2 group is the basic group or proton acceptor. It has been found that such amino acids in the crystalline state exist as Zwitterions (= hybrid ions = dipolar ions).

Dipolar ions may be formed by intramolecular proton shifts from uncharged isomers of lower dipole moment (47). Thus glycine may exist either as the dipole ion $H_3^+N.CH_2.COO^-$; or as the uncharged "molecule" $H_2N.CH_2.COOH$, and either of these forms may go over into the other through interchange of a proton between the carboxyl and the amino group (48).

The position of the equilibrium between the dipolar ion and the uncharged molecule depends on the acidic and basic properties of the groups involved. According to Bronsted's conception, an acid is any substance capable of giving up a proton, such as CH_3COOH, the NH_4^+ ion, or the ethylene diammonium ion, $H_3^+N.CH_2^-.NH_3^+$.

A base is any substance capable of taking up a proton, such as the CH_3COO^- ion, ammonia, or the hydroxyl ion.

To be capable of forming a dipolar ion, a substance must contain at least two acidic groups in strongly acid solution; at least one group must be of the uncharged type and at least one of the cationic type; that is, the molecule must be an amphoteric substance or ampholyte. Furthermore, if the substance is to exist in the isoelectric state as a dipolar ion, not as an uncharged molecule, the acidity of the uncharged acid group must be greater than that of the cationic group (47).

These general considerations may be illustrated by the particular case of glycine, the simplest of the aliphatic amino acids. In strongly acid solutions, glycine exists in the form of the cation: $H_3^+N.CH_2COOH$. On addition of a base, such as hydroxyl ion, both the carboxyl and the ammonium group tend to give up a proton to the base. The first dissociation of glycine K_1 is found to be:

$$K_1 = \frac{(H^+)(\pm)}{(\overset{+}{H})} = 10^{-2.3}$$

where (R^+) stands for the concentration of glycine cation, and (R) for that of the isoelectric glycine. This value of $10^{-2.3}$ represents the ionization of the carboxyl group, strengthened by the positive charge on the adjoining ammonium group.

In water, amino acids exist as the dipolar ion (R), to an extent depending upon the groups involved:

$$NH_2 - CH_2 - COOH$$

$$NH_3^+ - CH_2 - COOH \rightleftharpoons R^+ + NH_3^+ - CH_2 - COO^- \rightleftharpoons NH_2 - CH_2COO^- + R^+$$

(R^+) (R) (R^-)

In strong acid, the equilibria are displaced to the left. The dissociation constant of the COOH group in the equilibrium (R) $\rightleftharpoons$ (R^+) is given by:

$$k_1 = \frac{(R^+)\ (R)}{(R^+)}$$

In the presence of strong base, the equilibria are displaced to the right. The dissociation constant of the NH_3 group in the equilibrium, (R) $\rightleftharpoons$ (R^-) is given by:

$$k_2 = \frac{(R^+)\ (R^-)}{(R)}$$

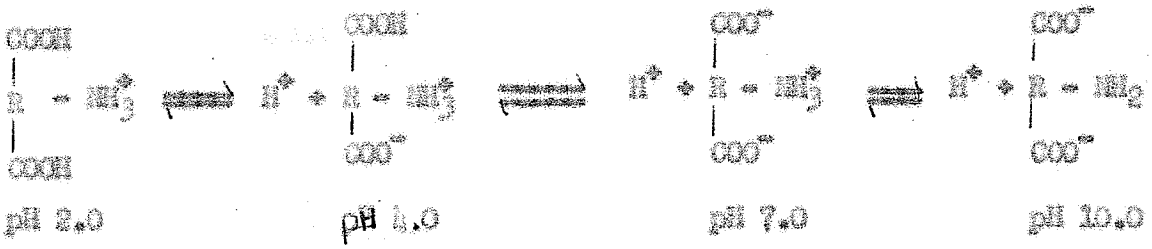
In strongly acid solution, all amino acids and peptides are positively charged and migrate as cations in an electric field; in strongly alkaline solution, all are negatively charged and migrate as anions. For any such substance, therefore, some intermediate pH value may be found where the average net charge on the ampholyte molecules is zero (R) and no net movement toward either pole will occur. This pH is known as the isoelectric point (IpH) of the ampholyte.

The condition for the isoelectric state is that the average net charge per molecule of ampholyte in solution should be zero, that is, the number of cations (R^+) should be equal to the number of anions (R^-).

$(R^+) = (R^-)$.

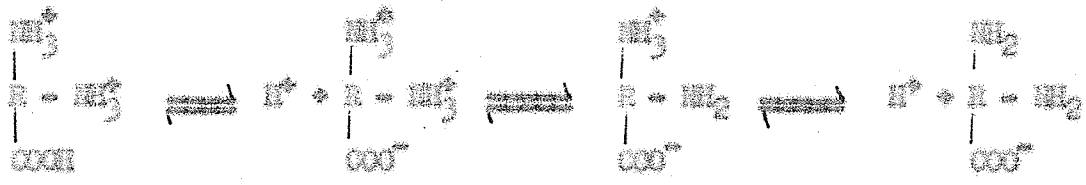
For any simple ampholytes, pK_1 and pK_2 are far apart, and a broad zone of pH values exists in which the ampholyte is practically isoelectric: (R^+) and (R^-) is less than 1% of the $(R^{\pm})$ state. The total charge on a glycine molecule is a maximum (±2) at its isoelectric point, while its net charge (zero) is minimum at this point. Simple amino acids have isoelectric points near pH 6, while those containing an additional amino or carboxyl group are isoelectric at more alkaline or more acid reactions. The classical theory is that the isoelectric point of a simple amino acid corresponds to minimum dissociation, while on the amphion theory it corresponds to maximal charges, since the amphion is bivalent, and the anion and cation are univalent. Cohn (50) has pointed out that on the basis of the amphion theory the di-amino acids and di-carboxylic acids have maximal charges, not at their isoelectric points, but when the univalent cation or anion respectively, is present in maximal amount.

Dicarboxylic amino acids will be in the isoelectric state at pH values lower than 7, i.e., at pH7 they will be doubly ionized. Dibasic amino acids will be in the isoelectric zone at values greater than pH 7.



For amino acids of the type $R(NH_2)(COOH)_2$ the pH of the maximal charge is given by:

$$pH = \frac{pK_2 + pK_1}{2}$$



pH 2.0 pH 4.0 pH 7.0 pH 10.0

For amino acids of the type $R(NH_2)_2COOH$ the relation is:

$$pH = \frac{pK_1 + pK_2}{2}$$

PRINCIPLES GOVERNING THE INTERACTION OF AMINO ACIDS WITH ION EXCHANGE RESINS

The interaction of amino acid, in solution, with ion exchange resin is governed by the pH of the solution with respect to the isoelectric pH (IpH) of the individual amino acid.

In the Zwitterion (dipolar form) where pH = IpH, the amino acid is in form II and at higher and lower pH of the medium the amino acid takes form I and form III below:

$H_2N - R - COO^-$	$H_3^+N - R - COO^-$	$H_3^+N - R - COOH$
$pH > IpH$	$pH = IpH$	$pH < IpH$
I	II	III

REACTION OF AMINO ACIDS WITH CATIONIC RESINS

In order that exchange adsorption take place on a cation exchanger the amino acid must be in form III, i.e., the cationic form: $H^+H_3 - R - COOH$ ($pH < IpH$).

Strong cationic exchange resin in the (H^+) form is completely ionised $R.SO_3^-$. H^+ and is therefore capable of producing excess H^+ ions, that is, the hydrogen ion concentration at the resin surface is high.

(i) Acidic Amino Acids

In water, acidic amino acids like glutamic and aspartic are in form I (NH_2-R- COO^-). However, since there are excess hydrogen ions at the resin interface the amino acids are converted to the cationic form III. NH_3^+ - R - COOH and then adsorption would take place.

$$R - SO_3^-.H^+ + H^+ + NH_2 - R - COO^- \rightleftharpoons NSO_3^- . H^+H_3 - R - COOH \quad (1)$$

But if the sulphonic acid cation exchanger is in the salt or sodium form, retention of an acidic amino acid would not take place unless the amino acid is first converted to the cationic form. This is quite apparent because of the lack of excess hydrogen ions as shown above:

$$R - SO_3^-.Na^+ + H^+H_3 - R - COOH \rightleftharpoons R - SO_3^-.H^+H_3 - R - COOH + Na^+ \quad (2)$$

(ii) Basic Amino Acids

Basic amino acids, e.g., arginine and lysine in water, are in the cationic form H^+H_3 - R - COOH and will react directly with strong cationic exchange either in the acid or salt form.

(iii) Neutral Amino Acids

Neutral amino acids will be in the dipolar form II in water, but will be converted to the cationic form III by the excess (H^+) ions of the sulphonic acid exchanger or else retention would not occur.

Elution of amino acids from strong cationic resins by acid is possible because the (H^+) ions compete for the resin. Elution by means of acid buffers of controlled pH, say sodium buffers, supplies (Na^+) ions

to compete with the amino acids for the resin and suppresses the cationic form of the amino acid by increase of the pH relative to the IpH.

WEAK ACID EXCHANGERS

Winters and Kunin 1949 have shown that weak acid exchangers can be buffered to almost any pH. All basic amino acids and proteins are adsorbed on weak acid exchangers. Neutral amino acids are also retained if first converted to the cationic form III, but the more acidic amino acids, acetic and glutamic acid, are not retained on weak acid exchanger.

Dilute mineral acid or a sodium acetate buffer of pH 4 will elute the adsorbed amino acids. The hydrogen ion serves to control the formation of weakly ionized amino acid and therefore the position of the equilibrium in equation (). The anion of the acid or buffer replaces the amino acid in the resin.

WEAK BASIC ANIONIC EXCHANGE

If a mixture of amino acids is dissolved in water, only the dicarboxylic amino acids would be retained on a weak basic anion exchanger (49, 50). Neutral amino acids will be retained on a weak basic anion exchanger if they are dissolved in an alkaline medium of low ionic strength. That is, they must be converted to the anionic form to allow the following equation to take place:

$$R\text{-}NR_3^+.\bar{O}H + H^+ + \bar{O}OC\text{-}R\text{-}NH_2 \rightleftharpoons R\text{-}NR_3^+.OOC\text{-}R\text{-}NH_2 + H_2O$$

Moore and Stein declared that better separation of amino acids is achieved when a 4% crosslinked resin is used instead of 8% crosslinking (51).

C. HISTONES

The histones are basic proteins and yield a large proportion of basic amino acids upon hydrolysis. On account of their basicity they usually occur in tissue, in salt combination with acidic substances such as haem of haemoglobin and nucleic acids. Two types of basic proteins have been distinguished: protamines and histones, the former occurring in the sperm heads of certain fishes. For example, salmine and clupeine are protamines which occur in the sperm heads of the salmon and herring respectively.

The protamines are distinguished from the histones in three respects: their salts form true solutions in water, no aromatic amino acid enters into their composition, and they possess considerably stronger basic properties due to a higher content of basic amino acids.

Prior to 1940 the amount of work done on histones was very limited. From 1955 onwards, however, considerable attention was paid to the nucleoprotein of calf thymus, and particularly to the histones which can be isolated from it.

HETEROGENEITY OF HISTONE PREPARATIONS

The histones extracted from cell nuclei are individual proteins, and every histone so obtained has now proved to be composite, consisting of two or three components. All the components are basic proteins. Bailey et al (52) referred to these as the "fast" and "slow" components according to their electrophoretic mobility; whereas Crampton and associates (53) referred to them as Histone Fraction A, B, and C, Stedman and Stedman (54) called them the "main" and "subsidiary" histones, and Luck et al (55) described them as the "light" and "heavy" histones. Electrophoretic analysis

by Kruft et al (56) indicated that histone extracted from isolated nuclei consists of three components: α, β and γ histone, in order of descending mobility. The so-called main histone consists essentially of the β - component contaminated with a small proportion of the γ - component. Stedman declared that the β - histone from liver cells differs from the β - histone from thymocytes, and that the basic proteins of many cell nuclei are cell specific. On the other hand, Crampton et al (53) declared that calf thymus, liver and kidney yield histone fractions that are similar in terms of chromatographic behaviour and amino acid composition; furthermore, that the histone fractions A and B, prepared from guinea pig testes by similar procedures, show barely detectable differences from the calf thymus proteins. Khouvine and Baron (57) and Khouvine and Gregoric (57b) have reported rather striking differences in the amino acid composition of histone prepared from calcium deoxypentose nucleoprotein and that prepared from sodium deoxypentose nucleoprotein.

Davison and Butler (58, 59) have shown that calf thymus histone mixtures are improved if conditions which inhibit cathepsins are used during the preparation of the samples. Crampton has subsequently shown that if, as a preliminary step, dialysis is avoided in the preparation of crude histone accompanied by more rapid procedures, this permits the isolation of more nearly native histone with histone fraction B containing a higher percentage of arginine than in the earlier preparations.

The aggregation of protein micelles of histone takes place much more rapidly with increase in pH than is usual with other proteins; the particle weight at pH 14 is approximately 100 times that at pH 7 (60). Davison and Butler (58) pointed out that the amount of aggregation increases with the age of the solution, and that at pH 4.5 aggregation is slow at 4°C,

but is considerably accelerated by raising the temperature of the solution. Furthermore, rapid aggregation occurs even at 4°C at high pH's. Luck and associates, without mentioning the pH of the buffer used, indicated that histone aggregated strongly in buffer solutions.

Mirsky and Pollister (61) showed that a solution of liver histone became opalescent at pH 10.72 and precipitation occurred at pH 10.83. They suggested that the IpH of liver histone is probably in the region of 10.8.

-histone from ox liver and ox thymus possesses IpH of 11.0 and 10.8 respectively (56).

Histones are not precipitated by divalent mercury in the presence of strong acid. Consequently when a solution of any protein is added to an equal volume of 0.34 M $HgSO_4$ in 1.66 N H_2SO_4 and the mixture is warmed at 60°, the protein precipitates, but if the protein in solution is a histone no precipitation occurs. The non-precipitability of histone by $HgSO_4$-H_2SO_4 makes it possible to tell when other proteins are present with a histone.

Luck and associates (55) have recently found that the molecular weights of various histone preparations appear to be 10,000 or less when measured in the ultracentrifuge, and experiments by Frautman and Crampton (72) have led to similar conclusions. A fraction of similar composition and molecular weight of 11,000 to 10,000 has been reported by Butler and Davison (58, 59). Raby et al (62) estimated that the "fast" histone yielded a molecular weight of 11,000 to 16,000.

Daly and Mirsky (63) in 1955, as well as Davison and Shooter (58) in 1956, reported that lysine-rich histone gives twice as much ninhydrin colour as arginine-rich histone, but that the non-fractionated histone gives less colour than either of these sub-fractions.

The above results certainly show that histone as isolated from the cell nuclei is not a homogeneous substance but rather a mixture of several components.

THE PREPARATION OF HISTONE

The preparation of histone falls into two main classes: (i) extraction of the histone salt by treatment of the source material with dilute acid (64) (66); or (ii) dissociation of the precursor, deoxypentose-nucleoprotein in neutral salt solutionof high ionic strength (66). The former method introduced by Kossel has been subjected to many modifications, such as the use of dilute HCl or H_2SO_4, dilute citric acid (68) and 1.88 N H_2SO_4 containing 0.3% N $MgSO_4$. Histone chloride or sulphate is usually isolated from the acid extracts by salting out, or the protein is obtained by precipitation with ammonia.

The two histone samples used in this work were prepared, and generously supplied by Dr. J.A.V. Butler of the Chester Beatty Research Institute, London, England. Briefly, the method of preparation was as follows: Fresh calf thymus was homogenised in a Waring Blender with 0.9% NaCl solution and then washed and centrifuged several times until the supernatant was clear. The nucleoprotein thus obtained was dissolved in 10% NaCl solution in which the DNA and protein were largely dissociated. The DNA was removed from solution by precipitation with acetone or ethanol. The histone which remained in solution was isolated by salting out with ammonium sulphate and freeze-dried. This produced a white powder which dissolved quite easily in water. Dr. Butler also used hydrochloric, phosphoric and citric acids to extract the histone from the nucleoproteins, as well as the neutral salt technique described above. However, he has stated that both the salt and the acid methods yielded similar histones.

THE INTERACTION OF HISTONE AND ION EXCHANGE RESINS

Since the pH has such an important effect on histones, most of the experimental work with ion exchange resins and histones has been performed with a weak acid resin. Crampton et al (67) (68, 69) studied the behaviour of histone on weak resin-Amberlite IRC-50 in both the Na^{+} and Ba^{++} forms. Brown and Watson (70) utilised a Kieselguhr column for the adsorption of histones. Hyln Cook adopted the method of Kirs Moore and Stein for fractionation of histone on Amerlite IRC-50 (Na^{+}) column.

Daly and Mirsky (63) and Crampton et al (68) (69), working seperately, found that there was a very intense adsorption of histone on Amberlite IRC-50 (Na^{+}) which was eluted only by very strong alkali. Rasmussen working with Luck et al (55) succeeded in eluting histone from a colum of IRC-50 (Na^{+}) with guanidinium chloride.

The behaviour of histone in contact with an ion exchange resin is due partly to its high molecular weight and to the contribution of van der Waals forces towards the net adsorption affinity (71). These workers, Doscream and Partridge, postulated that these forces may be large enough to account for the high adsorption of proteins at low pH's and the electrostatic attraction between protein and resin is dependent on the net charge on the protein molecule.

There is no evidence in the literature that any work has been performed with histone and ion exchange resins by the batch technique. In the

present work, the batch method was used for all operations with histone and ion exchange resins.

D. NUCLEIC ACIDS

Our present knowledge of nucleic acids dates back to 1870 when Friedrich Miescher isolated nuclear components from pus cells by digesting them with pepsin-hydrochloric acid. Later, Altman (73) referred to this nuclear component as nucleic acid, and Kossel (74) established the presence of the purine and pyrimidine bases in nucleic acids. The work of Hammarsten (75) and Levene (76) revealed that the nucleic acids from yeast, on hydrolysis, yielded adenine, guanine, cytosine, uracil and ribose; while the nucleic acid from thymus yielded adenine, guanine, cytosine, thymine, phosphoric acid and deoxyribose. These two nucleic acids came to be called ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) respectively.

Both RNA and DNA are essentially high molecular weight polynucleotides in which individual nucleotides are joined by 3', 5' phosphodiester linkages (77). The X-ray studies of Watson and Crick (78) indicated that DNA is actually a double molecule, with one chain twined around the other in helical fashion, the bases of one chain fitting neatly onto the bases of the other; thus guanine pairs with cytosine and adenine with thymine. The RNA isolated so far gives rather poor X-ray pictures, and the evidence about its structure is not conclusive.

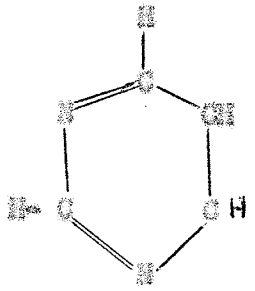
COMPOSITION OF NUCLEIC ACIDS

Condensation of a purine or pyrimidine base with a pentose sugar yields a nucleoside. When a nucleoside condenses with phosphoric acid a

nucleotide is formed; further condensation of two nucleotides with the elimination of water yields a dinucleotide. This process may go on indefinitely until a long chain of nucleotides has been produced, forming a polynucleotide.

Pyrimidine Bases

The pyrimidine bases are all derivatives of the parent compound pyrimidine which is structurally a six membered ring with 2 nitrogen and 4 carbon atoms.



Pyrimidine

The pyrimidine bases found in nucleic acids are:

(i) cytosine or 2-hydroxy-6-amino pyrimidine

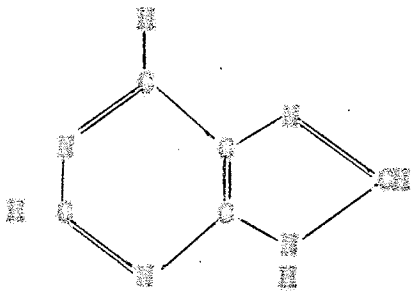
(ii) uracil or 2:6-dihydroxy pyrimidine

(iii) thymine or 5-methyl uracil

(iv) 5-methyl cytosine

Purine Bases

The purine bases are derivatives of the parent compound purine which contains a pyrimidine ring and an imidazole ring fused together:



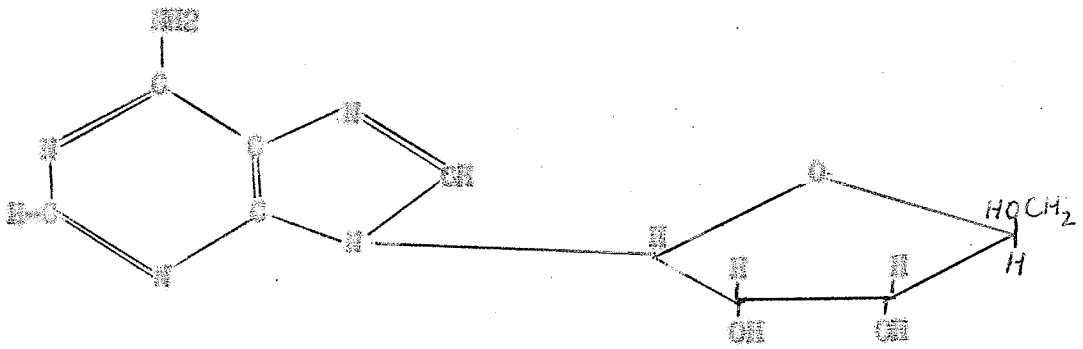
Purine

The purine bases which occur in nucleic acids are adenine and guanine.

adenine or 6-amino purine

guanine or 2-amino-6-hydroxypurine

When a purine or pyrimidine base is condensed with a sugar radical a nucleoside is formed; thus the condensation product of adenine with the pentose ribose is the nucleoside adenosine:

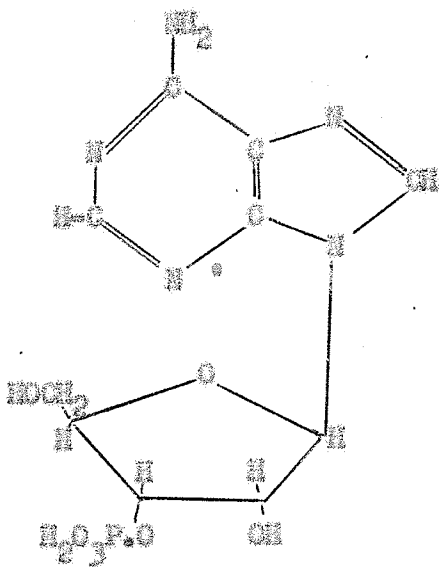


adenosine (9-β ribofuranosidoadenine)

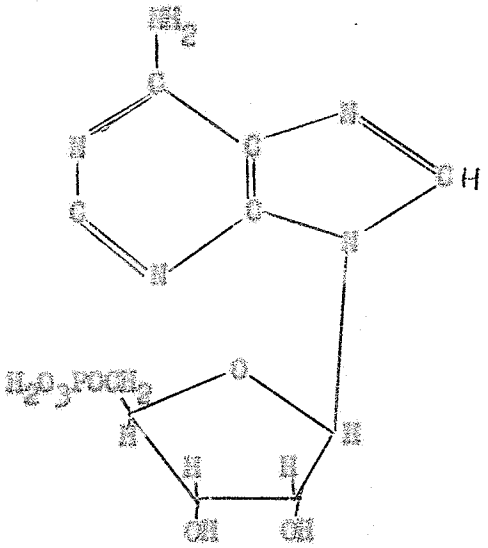
Similarly, guanine on condensation with ribose gives the nucleoside guanosine, thymine gives thymidine, and so on.

Nucleotides

When a sugar residue in a nucleoside is condensed with phosphoric acid, a nucleotide is formed. A nucleotide consists of a base, a pentose sugar and phosphoric acid coupled together. For example, adenosine can condense with phosphoric acid to give the nucleotide adenylic acid or adenosine monophosphate: adenine-ribose-phosphoric acid. The phosphoric acid may be attached in the positions 2', 3', or 5' in the ribose ring to form three possible adenine nucleotides. In nucleic acids the 3' and 5' linkages are the rule:

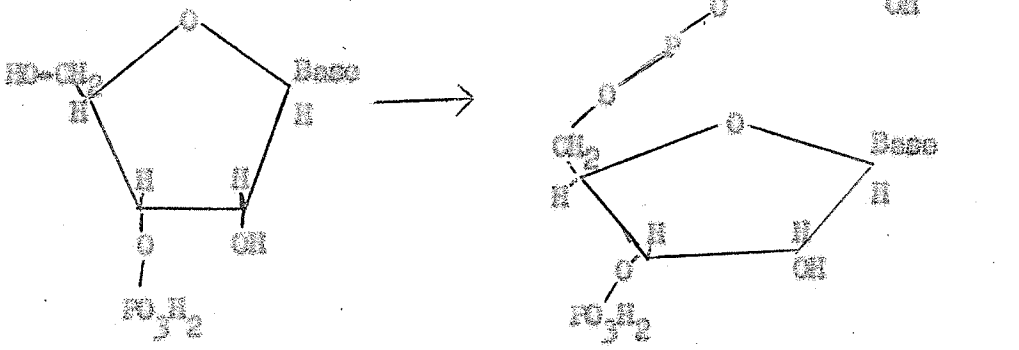


Adenylic acid (Adenosine 3' (P))



Adenylic Acid (Adenosine 5' (P))

Two nucleotides could condense together with the elimination of water to form a dinucleotide in which a phosphate residue links carbon 3' in one sugar residue with carbon 5' in the other:



By this process polynucleotides are formed and finally they give rise to nucleic acids.

The Interaction of Nucleic Acids with Ion Exchange Resins

Most of the previous studies with nucleic acids on ion exchange resins have been mainly concerned with the fractionation and resolution of nucleic acid hydrolysates into their component parts (79, 80, 81). Leland (82) used an Amberlite IR-120 (H^+) resin to hydrolyse RNA, and Volkin and associates (83) utilised an anion exchange resin for the separation of mixtures of nucleotides from an enzyme hydrolysate of nucleic acid.

There is no evidence in the literature that any one has actually studied the behaviour of nucleic acids as such in contact with ion exchange resins either by the column technique or by the batch technique.

The Preparation and Heterogeneity of Nucleic Acids

One of the major difficulties facing the student working with nucleic acids is the degree of homogeneity of his sample, and whether such an isolated sample may still be regarded as representative of the state in which it occurred in the living cell. Kirby and Pollister (84) concluded that traces of protein remain in the best preparation of "protein-free" nucleic acid, and that the best criterion for the purity of a nucleic acid preparation is its elementary composition and especially the nitrogen: phosphorous ratio.

Several methods are now known for the preparation of nucleic acids. They are based on: (a) the extraction with solutions of low ionic strength (85, 86); and (b) extractions with strong salt solutions (84, 87, 88). Extraction of nucleic acids with solutions of low ionic strength has the advantage of avoiding the exposure of the nucleic acid to high salt concentrations and therefore to dissociating conditions (89). In this method the degradation of the nucleic acid by enzyme is encountered, though this could be offset by the use of enzyme inhibitors like arsenate, citrate

or sodium dodecyl sulphate.

Extraction with strong salt solution has been employed by Kirsby and Pollister and by several others (90). This method is advantageous in that it suppresses the action of nucleases, and a partial separation of the nucleic acid from the proteins is achieved.

Chargaff (69) and Butler (91) declared that the nucleic acid content of different nucleoproteins is around 5% of the dry weight. However, no one has adopted a foolproof method for the estimation of how much degradation has occurred, or whether the isolated nucleic acid is entirely free from protein or from other contaminating cellular material. The colour reactions used for nucleic acids are not adequately specific for either DNA or RNA (69). Furthermore, since the absorption spectra in the ultraviolet for both RNA and DNA are identical, the U.V. cannot be used as a method for establishing the purity of a nucleic acid sample. Though limited, the Sendak colour test for both cytosine and uracil is very useful. Since cytosine can be removed by a zeolite resin, the presence or absence of RNA in a sample of nucleic acid can be determined (91).

The DNA used in this study was prepared, and courteously supplied by Dr. R. H. Fein and Dr. J. A. V. Butler of the Chester Beatty Research Institute (92). Briefly, the method of preparation was as follows: Freshly chilled rat livers were minced and homogenized for 30 seconds in 0.14 M NaCl in a Waring Blendor. The nuclei and cell debris were removed by centrifugation and the crude nucleoprotein was obtained as a white precipitate by acidifying to pH 4.5 with N HCl. A partial separation of the protein and nucleic acid was obtained by heating the acid precipitate in neutral salt solution. After heating and cooling again to room temperature, the flocculated precipitate was removed by centrifugation. This was

resuspended in 95% ethanol and isotonic saline, then digested with chymotrypsin. The purified product was then freeze-dried. This gave samples of RNA which, on hydrolysis and paper chromatography, were shown to contain: ribose, adenine, guanine, uracil and cytosine, with only a trace of thymine, corresponding to less than 2% of DNA and less than 1% of protein. Fain and Butler declared that RNA appeared to be quite stable after heating at 60° for 10 minutes.

The RNA used in this study was prepared and kindly supplied by Dr. J.A.V. Butler of the Chester Beatty Research Institute (93). The method of preparation was as follows: 500 gm. of fresh calf thymus in 0.14M saline was mixed in a Waring Blendor for 30 seconds and left to stand overnight. The residue was removed by centrifugation and digested with 200 mg. of chymotrypsin and the nucleic acid was precipitated with 70% alcohol. The precipitate was dialysed for one week against distilled water, filtered and washed again with saturated NaCl and 70% alcohol. The fibrous precipitate was dried in a desiccator over phosphorous pentoxide.

SECTION II

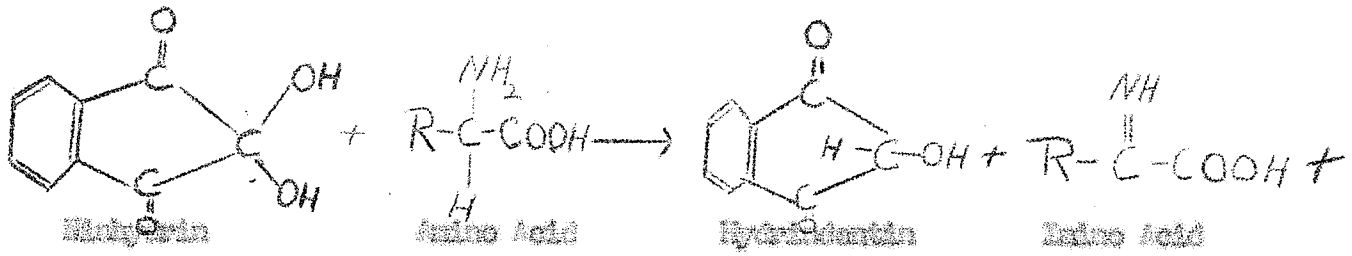
DETERMINATION OF AMINO ACIDS WITH SOY BEAN TISSUES

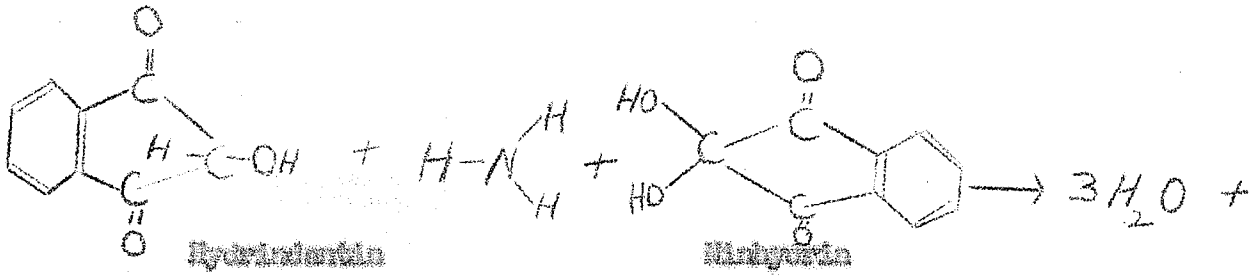
Quantitative Determination of Amino Acids.

Several methods are now in use for the quantitative estimation of amino acids. In this study, the ninhydrin method was used.

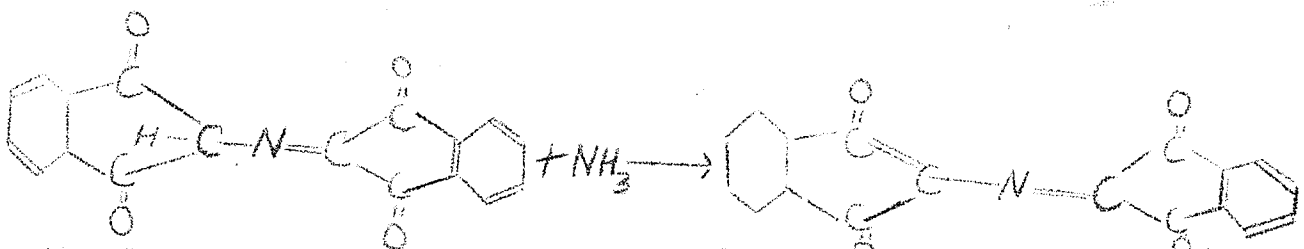
The Ruhemann Reaction

When amino acids which contain a free amino group in the position are allowed to react with triketohydrindenehydrate (ninhydrin)  a blue coloured compound is formed. β and γ amino acids and α amino acids in which the amino group is substituted do not react. A free amino group and a free carboxyl must be present in the molecule in order to obtain the colour (9). So far as is known, all the amino acids except proline and hydroxyproline, which have been obtained from protein hydrolysate give a positive ninhydrin reaction. The sequence of the reaction is as follows:





Hydrindantin Ninhydrin



The blue coloured compound, dihydroxydihydrodiketohydrindylidene-diketohydrindamine (DYDA) is formed whenever ninhydrin is heated with α amino acids or with peptides and proteins that contain the α amino acid group.

Moore and Stein (95, 96) have developed the reaction into a photometric method for the quantitative estimation of amino acids. However, it was disadvantageous to use the modified photometric method of Moore and Stein because their ninhydrin blank gave very high readings and the colour formed in their procedure slowly faded on standing at room temperature over a period of a few hours. This was also pointed out by Troll and Cannan (97). Consequently, in searching for a new method, the modified photometric method of Troll and Cannan (97) was adopted. The method used was as follows:

Reagents: (1) Ninhydrin solution: 0.500 gm. of ninhydrin were dissolved in 10 ml. of absolute alcohol.

(2) 80% phenol solution: 80 gm. of reagent grade phenol were dissolved in 20 ml. of absolute alcohol, with gentle heating. The solution was shaken with 1 gm. of Permutit for 20 minutes to remove traces of ammonia and then decanted.

(3) KCN-pyridine reagent: 2 ml. of 0.01 M solution of KCN were diluted to 100 ml. of ammonia free pyridine, prepared by shaking 100 ml. of pyridine with 1 gm. of Permutit for about 20 minutes.

(4) 50% alcohol by volume.

All reagents were stable for at least one month at room temperature. Procedure: 0.5 ml. of aqueous solution of any pH between 1 and 8 containing the amino acid, was heated with 1 ml. of KCN-pyridine reagent and 1 ml. of 80% phenol reagent in a boiling water bath to about 100°C. 0.2 ml. of the ninhydrin solution was then added and the reaction allowed to proceed for five minutes. The solution was cooled and made up to 10 ml. with 60% alcohol. The optical density between wave numbers 15000 cm^{-1} and 21000 cm^{-1} was determined. 0.5 ml. of water was subjected to the same procedure, and used as reagent blank.

In the figures that follow, the optical density is plotted on the ordinate, and the abscissae are plotted in wave numbers. Wave numbers, instead of wave lengths, were used because the spectrophotometric instruments were calibrated in this unit. A wave number is the number of waves in a centimeter, whereas a wave length is the length of the wave measured in $\mathring{A}$ngstrom. Since 1 $\mathring{A}$ngstrom = 10^{-8} centimeters, then a wave number could be converted to a wave length by the formula: wave length $\mathring{A} = \frac{10^8}{\text{wave number}}$.

Fig. I shows the photometric absorption spectrum of phenylalanine ninhydrin complex. The absorption for the ninhydrin complex with the other amino acids was found to be the same as that for phenylalanine, and their maximal optical densities occurred at wave number 17000 cm^{-1}. The same concentration of different amino acids gave different optical densities, and Fig. II shows that unless individual standard curves are made for each amino acid the ninhydrin method is not applicable to the quantitative determination of amino acids. Thus if alanine is used as a standard, small amounts of alanine can be determined accurately. If, however, the attempt is made to determine glycine or phenylalanine by means of the alanine standard, the values would be incorrect. Since the ninhydrin reaction obeys Beer's Law, it was then easy to calculate the quantity of amino acids remaining in solution after the latter were shaken with the resin.

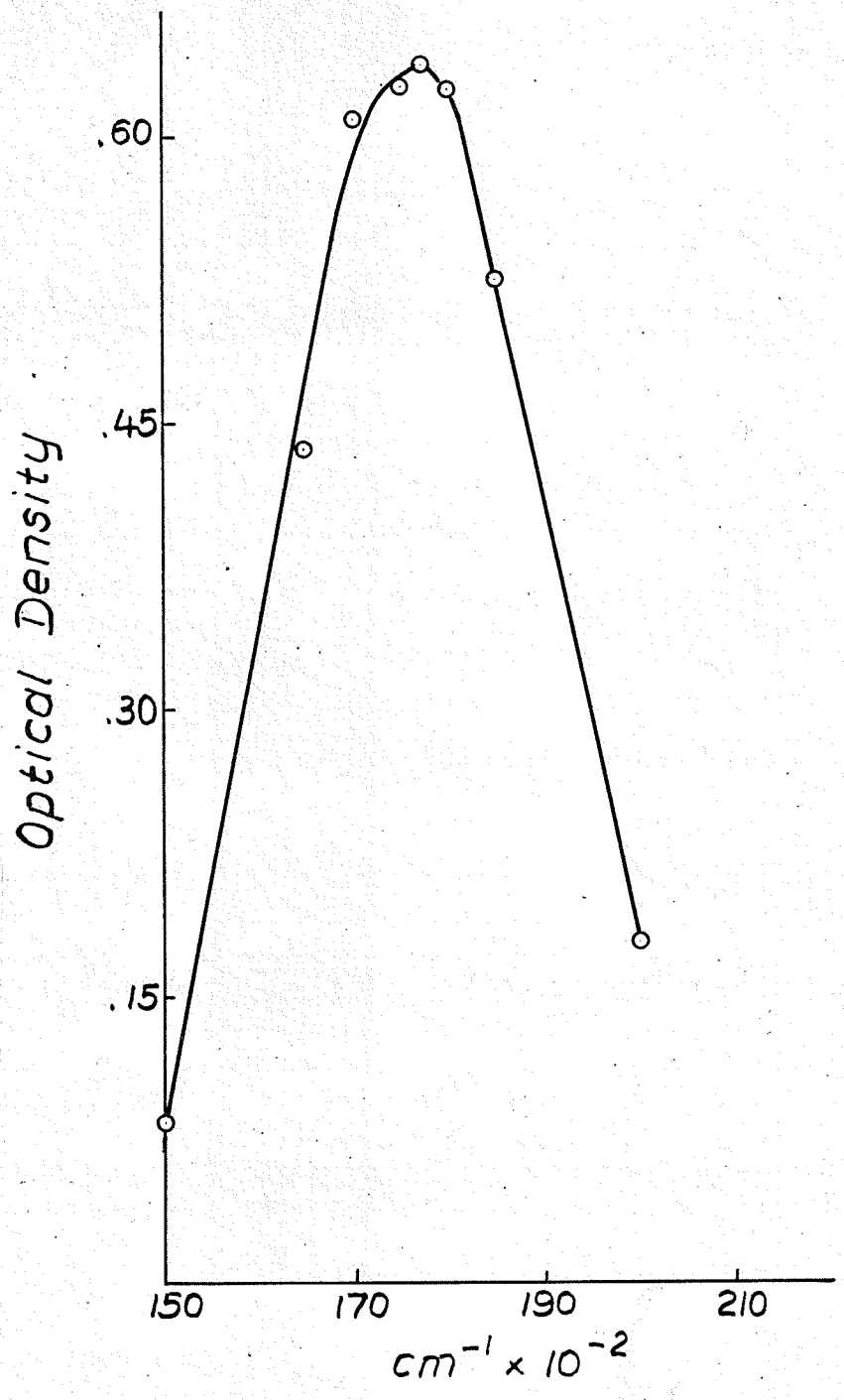


FIGURE I: Photometric absorption spectrum of phenylalanine-ninhydrin compound.

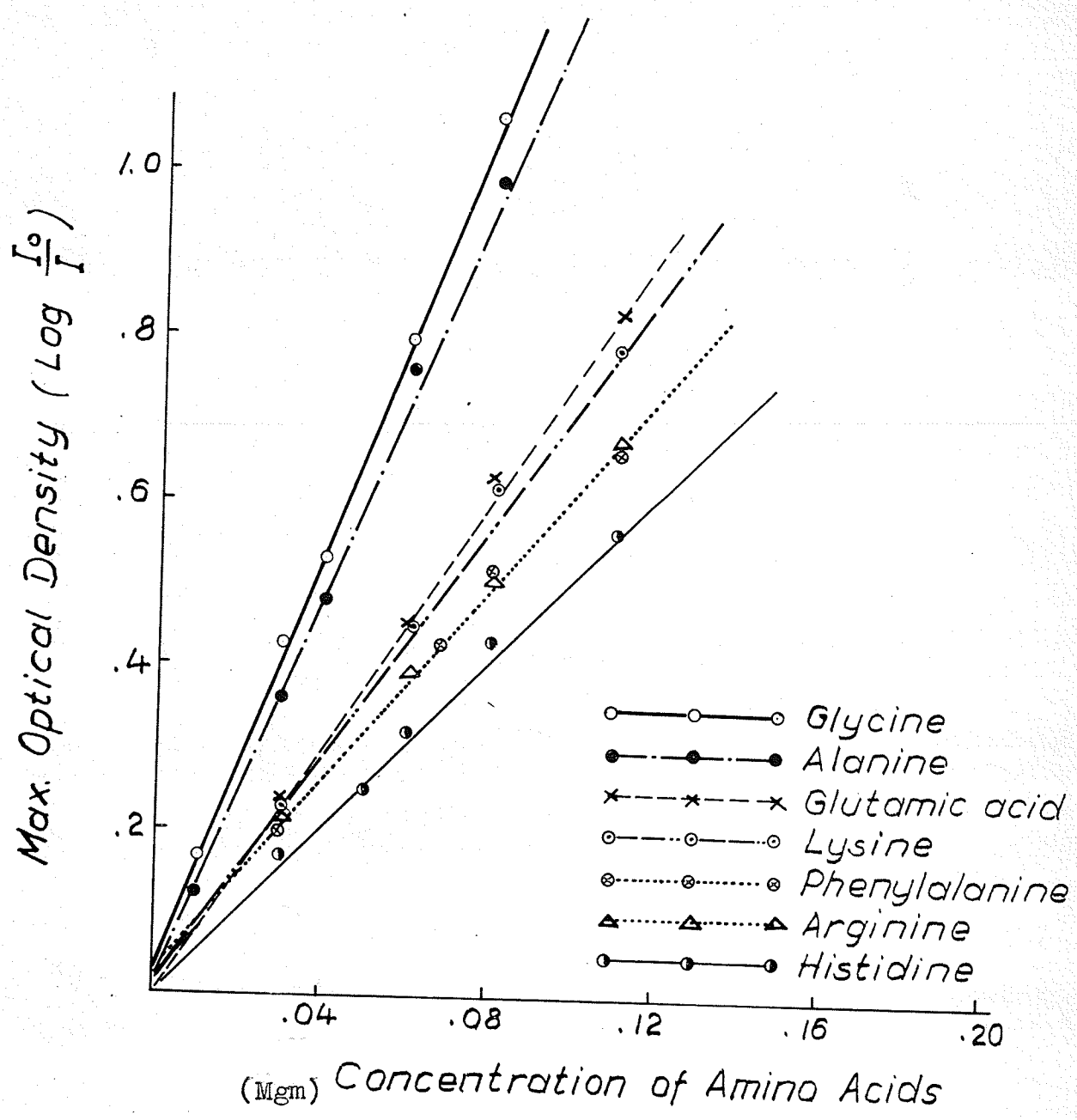


FIGURE II: Photometric ninhydrin standards of the amino acids. Ordinate: maximal optical densities at wave number 18000 cm^{-1}. Abscissa: Increasing concentration of the amino acids.

Interaction of Amino Acids with Ion Exchange Resins

Up to the time of writing this thesis, there has been no evidence of any work performed dealing with the retention of amino acids by ion exchange resins under the equilibrium conditions, i.e., by the batch technique. Consequently, the following experiments were performed to study the conditions under which amino acids would be retained by resins, using the batch method.

Reactions with Strong Acidic Resins (H^+) and (Na^+) forms

To 0.50 gm. of Amex 50-44 in each of 7 centrifuge tubes were added 4.0 mg. of glutamic acid, glycine, alanine, phenylalanine, histidine, lysine, and arginine respectively. Each amino acid was dissolved in 5.0 ml. of distilled deionized water before addition to the resin. The centrifuge tubes were shaken by hand for 10 minutes, then centrifuged, and 0.50 ml. of the supernatant removed and tested with the ninhydrin reagents. It was not necessary to shake the centrifuge tubes longer than 10 minutes, because equilibrium between the amino acids and the ion exchange resins was achieved in less than 10 minutes.

Since standard curves were plotted for each amino acid, and since the initial concentration of each amino acid was known, it was then easy to calculate the amount of amino acid retained by the resin. Table I (A) shows that regardless of the pH of the amino acid solution in water, all the amino acids so far tested were easily retained on a strong acidic resin in the (H^+) form. The equilibrium pH of the amino acids in contact with the hydrogen form of the strong cation exchanger was 2.5. On the other hand, no retention occurred on the same resin in the (Na^+) form, (Table I (B)) unless the pH of the amino acid solution was adjusted to pH 3.0 by adding 3 drops of 4 N HCl to the latter. This was especially true of the neutral and acidic amino acids. The final pH at which phenylalanine, arginine, histidine and lysine were retained on the sodium form of the resin was 4.0, but glutamic acid, alanine and glycine were not retained at this pH.

TABLE I

A. Retention of amino acids from water solution by strong acid cation exchange resin in (H^+) form.

B. Retention of amino acids from water solution by strong acid cation exchange resin in (Na^+) form.

Amino Acid	Pkl	A			B		
		pH of Solution	eq. added	% Retained on (H^+)	pH of Solution	eq. added	% Retained on (Na^+)
Glutamic	3.22	3.5	4.00	95.00	3.0	4.00	nil
Phenylalanine	5.9	4.5	4.00	97.00	3.2	4.00	95.00
Glycine	5.97	5.5	4.00	92.00	3.3	4.00	nil
Alanine	6.0	5.5	4.00	90.00	3.2	4.00	nil
Histidine	7.6	4.5	4.00	97.00	3.1	4.00	97.00
Lysine	9.7	5.5	4.00	98.00	3.0	4.00	99.00
Arginine	10.8	6.0	4.00	98.50	3.0	4.00	98.00

TABLE II

A. Retention of amino acids from 5.00 ml. water solution by strong basic anion exchange resin in (OH^-) form.

B. Retention of amino acids from 5.0 ml. water solution by strong basic anion exchange resin in (Cl^-) form.

Amino Acid	Ref.	A			B		
		pH of solution	mg. added	% Retained on (OH^-)	pH of solution	mg. added	% Retained on (Cl^-)
Glutamic	3.22	3.5	1.00	96.5	10.0	1.00	96.3
Phenylalanine	5.9	4.5	1.00	94.0	10.5	1.00	65.4
Glycine	5.97	5.5	1.00	95.0	11.0	1.00	nil
Alanine	6.00	5.0	1.00	93.0	11.5	1.00	nil
Histidine	7.6	4.5	1.00	96.1	11.5	1.00	60.7
Lysine	9.7	5.5	1.00	90.1	11.5	1.00	92.4
Arginine	10.8	5.0	1.00	nil	11.5	1.00	nil

Since the pH of the solution was greater than the IpH of glutamic acid (IpH = 3.22), it was easy to understand why glutamic acid was not retained by the strong sodium resin. However, 93.0% of the glutamic acid was retained by the resin when the pH of the solution was reduced to 1.5, by adding 3 drops of 5 N HCl. This kept the final pH between the glutamic acid and the sodium resin at pH 2.95.

Alanine and glycine were not retained by the strong acidic resin in the (Na^+) form even when the final pH of their solutions was reduced to less than 2.00. The reason for this could not be due to their iso-electric pH, since the IpH of alanine is 6.00 and that of glycine is 5.97.

Reactions with Strong Basic Resins (OH^-) and (Cl^-) Forms

With the exception of arginine all the amino acids listed in Table I were retained by the (OH^-) form of the strong basic resin from a neutral solution. When the same resin was used in the (Cl^-) form, alanine, glycine and arginine were not retained by the resin, regardless of the pH of the solution. The percentage of phenylalanine, histidine and glutamic acid retained by the (Cl^-) form of the resin are shown in Table II (B).

Reaction with Weak Acidic and Weak Basic Resins

Reaction with weak acidic resin (H^+). The method of adding the amino acid to the resin was the same as with the strong acidic and basic resins. 4.00 mg. of the amino acid were dissolved in 5.0 ml. distilled deionized water. In experiments involving the weak acidic resins the pH of the amino acid solution was adjusted to 3.0 with 2 drops of 1N HCl, and the equilibrium pH of the amino acids and this resin remained at pH 3.0. 16% of arginine, 30% of lysine, and 23.5% of histidine were retained by the resin. Phenyl-alanine, glycine and alanine were not retained by the weak acidic resin.

Reaction with Weak Basic Resin (OH^-)

Lysine and arginine were not retained by the weak basic resins but 30% of histidine, 65% of phenylalanine, 30% of glycine and 50% of alanine were retained by the weak basic resins.

Displacement of Amino Acids from Ion Exchange Resins

The following experiments were performed to study the conditions under which the retained amino acids were released by the resins.

The resins with the retained amino acids were washed three times with distilled deionized water to remove all traces of free amino acids. With the exception of the first washing, all elutions gave negative ninhydrin tests.

An amount of 5.0 ml. of increasing concentration of HCl, starting from 0.001 N, was added to the resin with the retained amino acid, and then shaken by hand for 30 minutes. 0.5 ml. of the supernatant was removed and tested by the ninhydrin reagent.

When the strong cation exchanger was used in either the (H^+) or the (Na^+) form, 0.05 N HCl displaced both arginine and lysine, phenylalanine and histidine were displaced by 0.03 N HCl, whereas 0.01 N HCl was strong enough to remove glutamic acid. Glycine and alanine were not retained by the strong cation exchanger in the (Na^+) form; however, 0.01 N HCl was able to displace both of these amino acids from the (H^+) form of the cation exchanger.

With the exception of glutamic acid, 0.03 N HCl was strong enough to displace any one of the amino acids studied from either the (OH^-) or the (Cl^-) form of the strong anion exchanger. The glutamic acid was only displaced by 0.05 N HCl.

Saturation of the Ion Exchanger with Amino Acids

20.0 mg. of the amino acid dissolved in 5.0 ml. distilled deionized

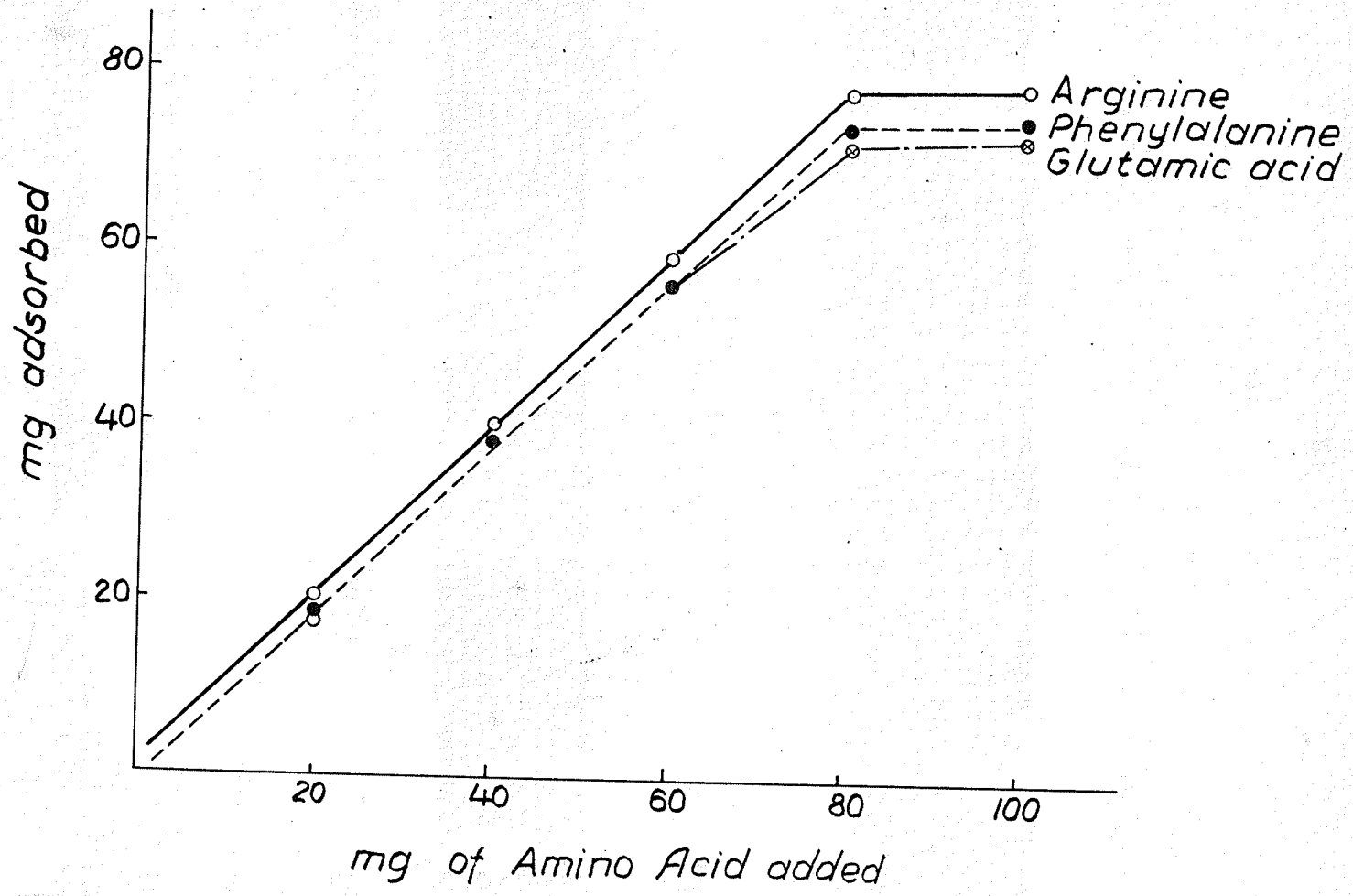


FIG. III: Saturation of 0.50 gm. of strong cation exchanger (H^+) with the amino acids.

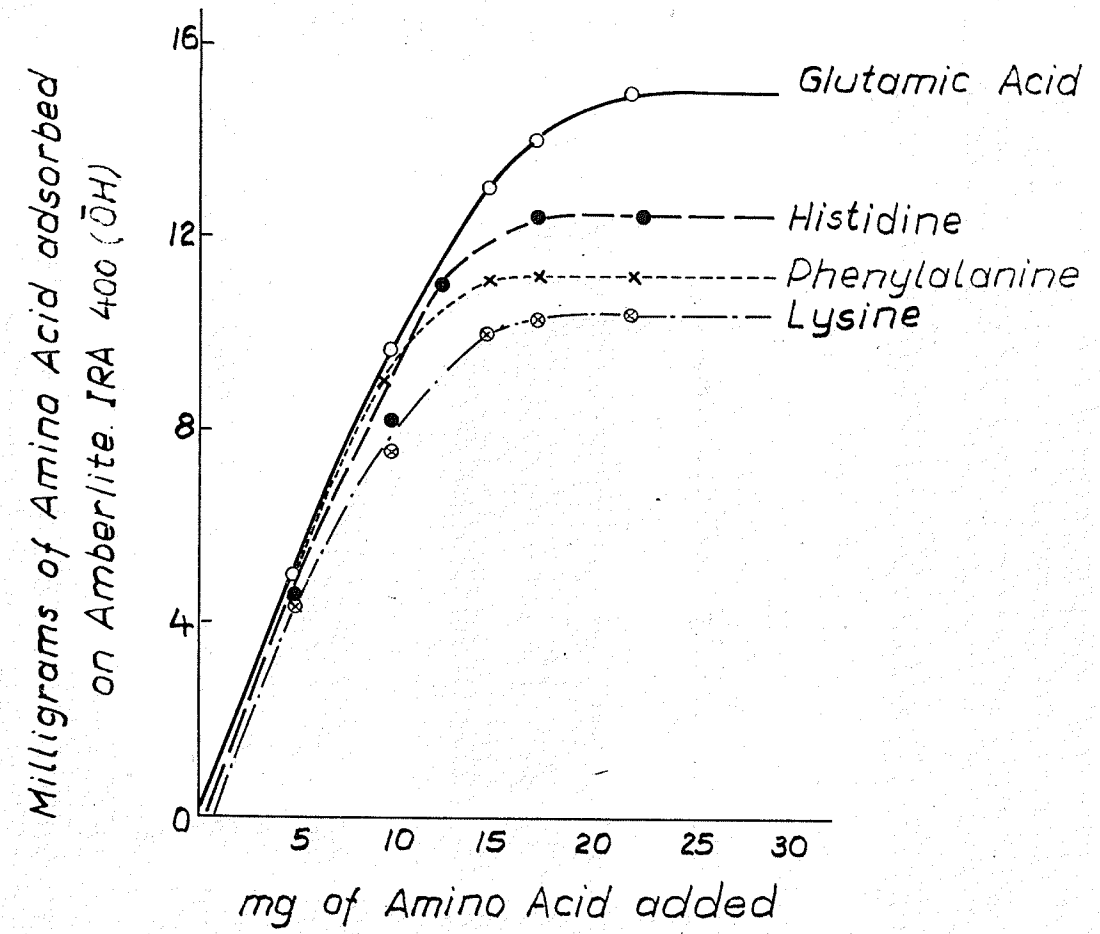


FIG: IV: Saturation of 0.50 gm. of strong anion exchanger (OH^-) with the amino acids.

water was added to 0.50 gm. of the resin, in a centrifuge tube, shaken for 10 minutes and 0.50 ml. of the supernatant removed and tested by the photometric ninhydrin method. The amount of amino acid retained by the resin was then calculated. This process was repeated until no more amino acid was retained by the resins. The pH of the amino acid solutions was regulated with a few drops of ½ N HCl or ½ N NaOH before addition to the resin so that optimum conditions existed for the retention of maximum amounts of amino acids by the resins.

Table III shows the final pH's of the amino acid solution in contact with the different resins and the total amounts of amino acids retained by the acidic and basic resins respectively. Figs. III and IV show that each amino acid was added to the solution in contact with the resin until no more retention occurred.

Discussion

These results show that the basic amino acids were more readily retained than the acidic amino acids on the (H^+) form of the strong cation exchanger. Similarly the acidic amino acids were more readily retained than the basic amino acids on the (OH^-) form of the strong basic resin. The weak ion exchange resins had a smaller capacity for the retention of amino acids than the strong cation exchange resins, and for this reason the strong cation exchange resins were saturated with amino acids and used for further studies with nucleic acids.

TABLE III

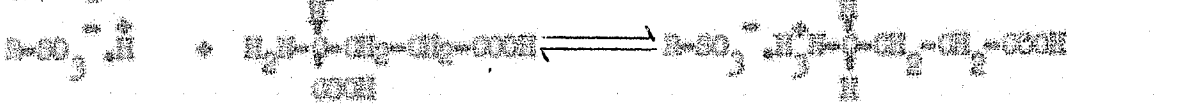
Maximum weight (in mgs) of amino acid retained by 0.50 gm. of Ion Exchange Resins of Type strom.

Amino Acid	IpH	Cation Exchange Resin: pH of Soln.	Strong (H) mgs	Acidic (Na) mgs	Weak Acidic (H) mgs	Anion Exchange Resin: pH of Soln.	Strong (OH) mgs	Basic (Cl) mgs	Weak (OH) mgs
Glutamic Acid	3.22	2.00	70.00	50.30	21.20	10.00	20.00	16.00	12.20
Phenylalanine	5.8	3.00	78.30	62.30	"	10.5	11.50	10.0	5.0
Methionine	7.6	2.30	75.20	65.80	"	11.5	13.20	10.00	"
Arginine	10.8	3.30	77.00	76.00	43.60	12.0	"	"	"

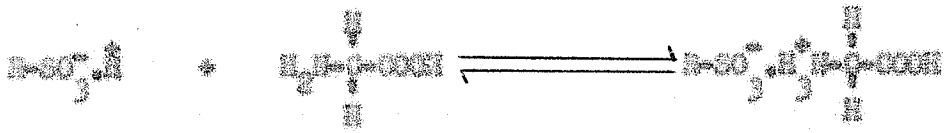
The following equations represent the different reactions between the various amino acids dissolved in water and ion exchange resins studied.

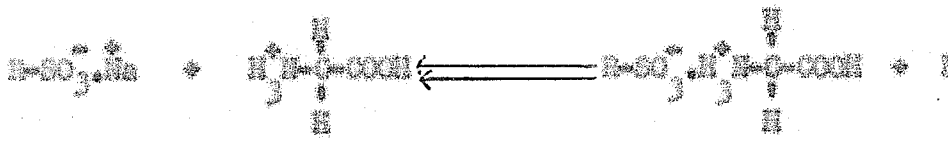
Cation Exchange Resins

(i) glutamic acid (pH 3.22)

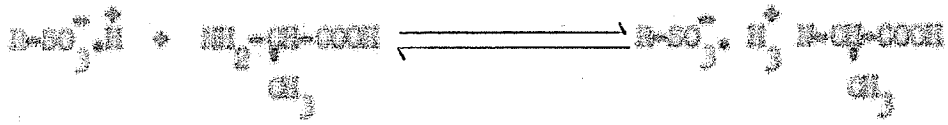


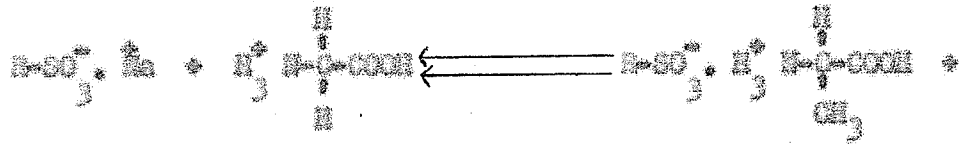
(ii) glycine (pH 5.97)



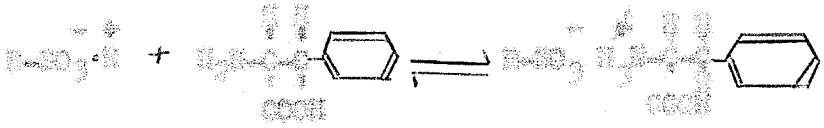


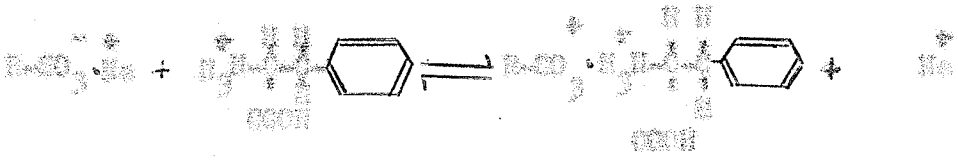
(iii) alanine (pH 6.0)



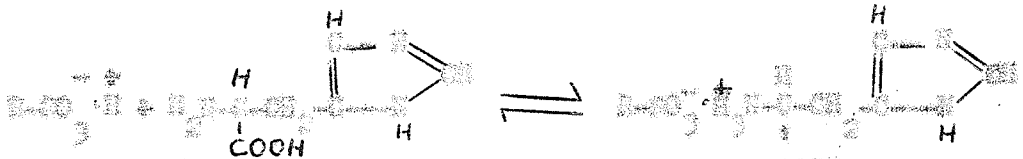


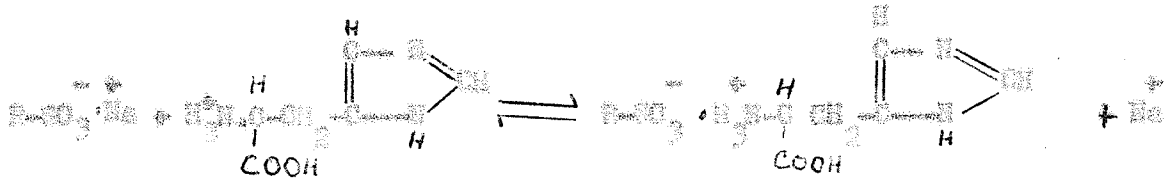
(iv) phenylalanine (I.P. 5.9)



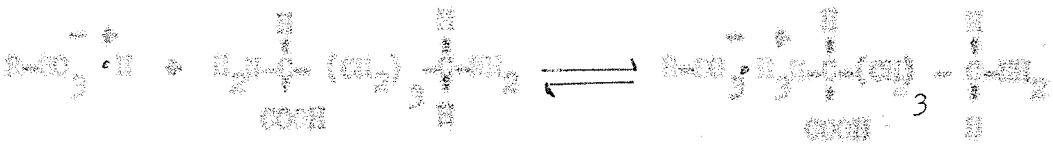


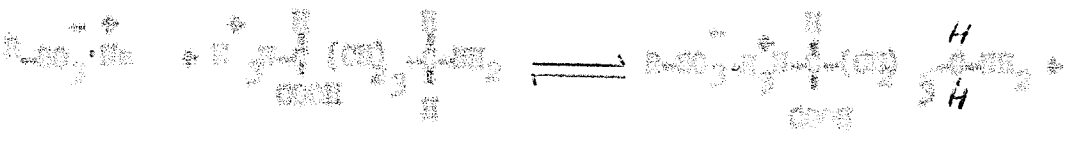
(v) histidine (I.P. 7.6)



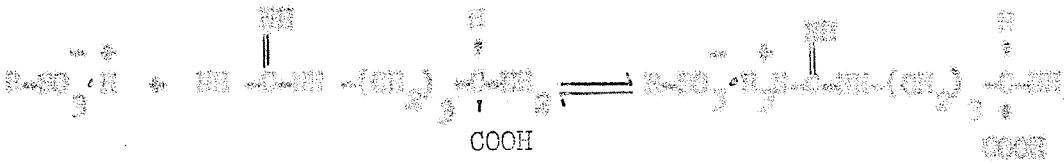


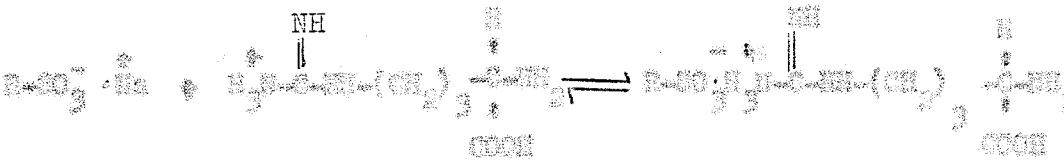
(vi) Lysine (Eph 9.7)





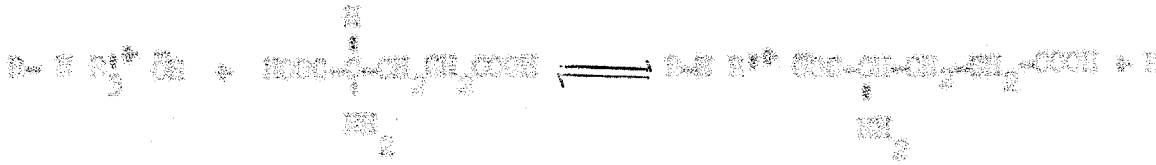
(vii) Arginine (Eph 10.3)

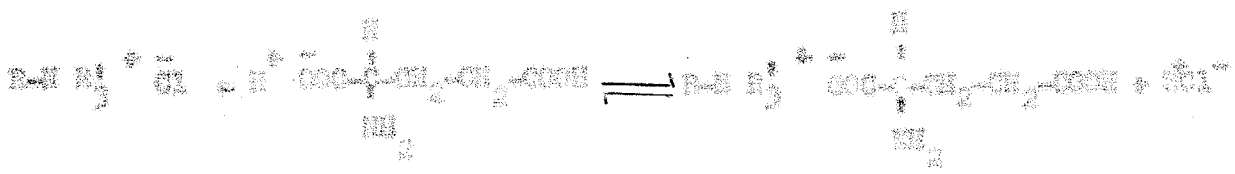




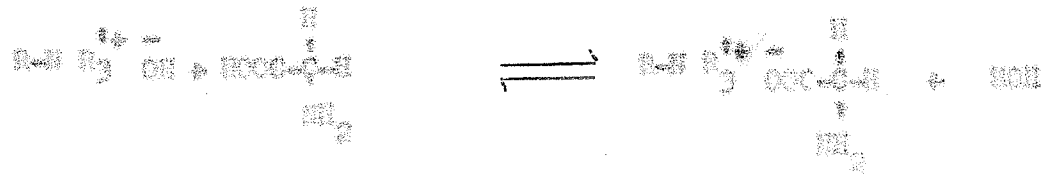
Anion Exchange Resins.

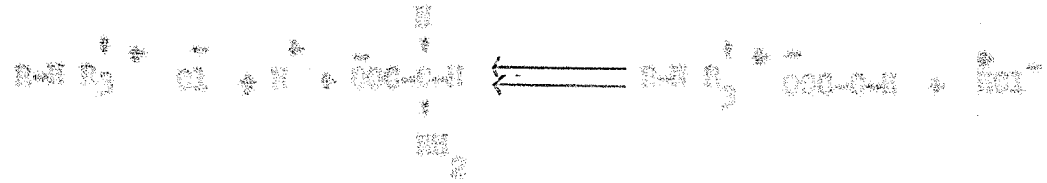
(i) Cystine acid (I.E.P. 5.02)



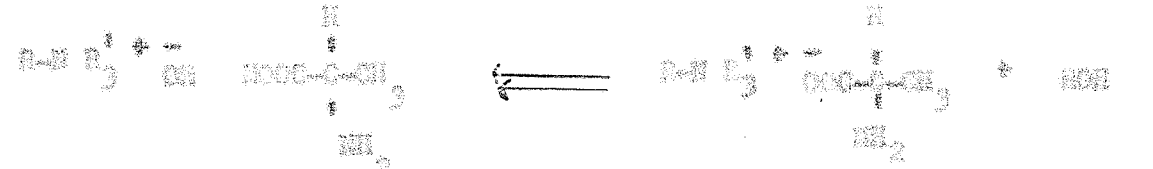


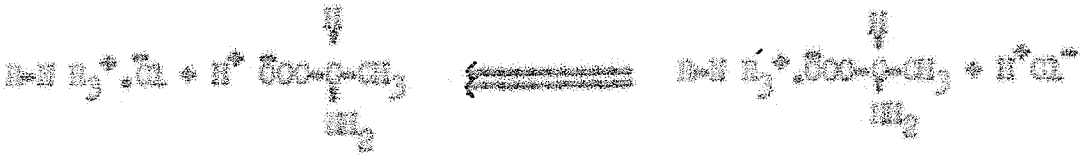
(ii) Glycine (I.E.P. 5.97)



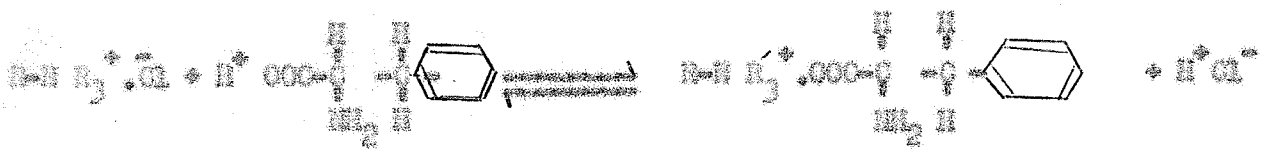


(iii) Alanine (I.E.P. 6.0)

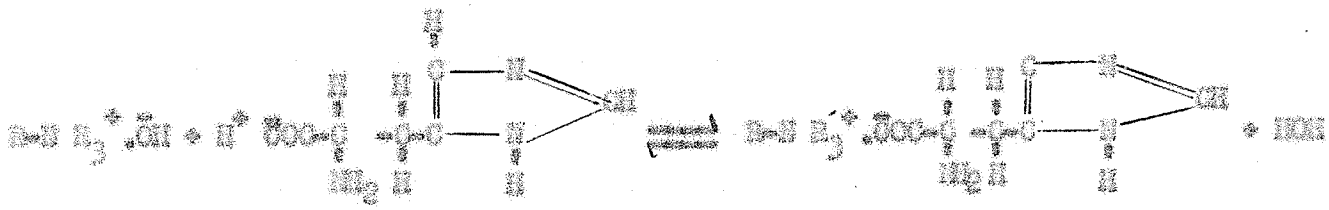


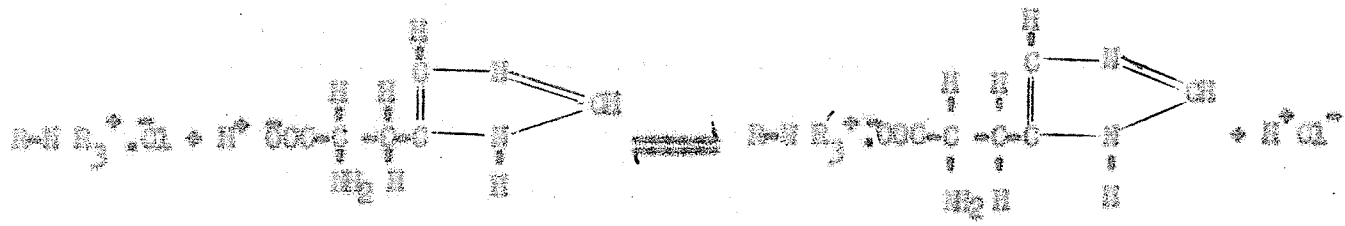


(iv) phenylalanine (IpH 5.9)

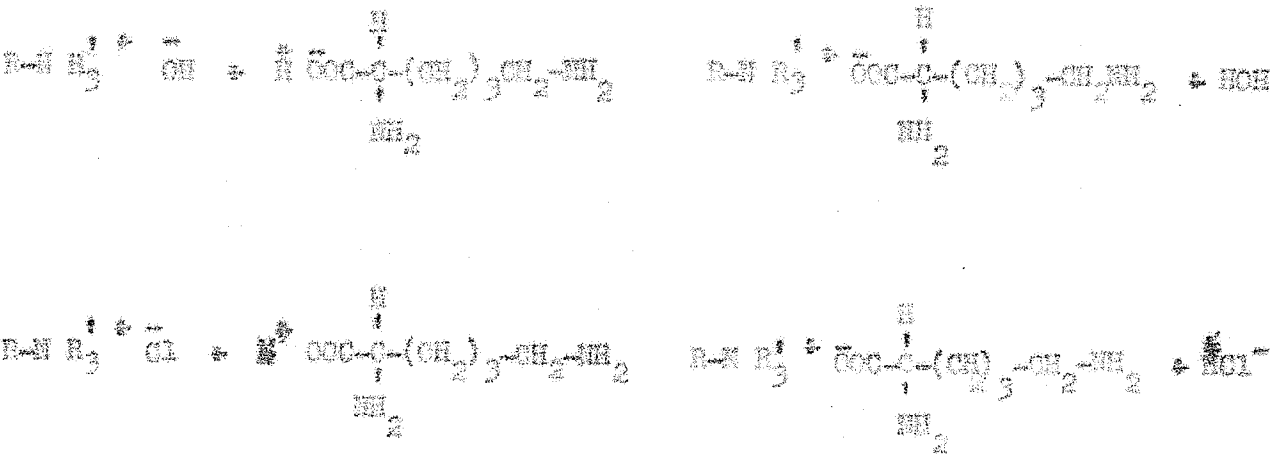


(v) histidine (IpH 7.6)



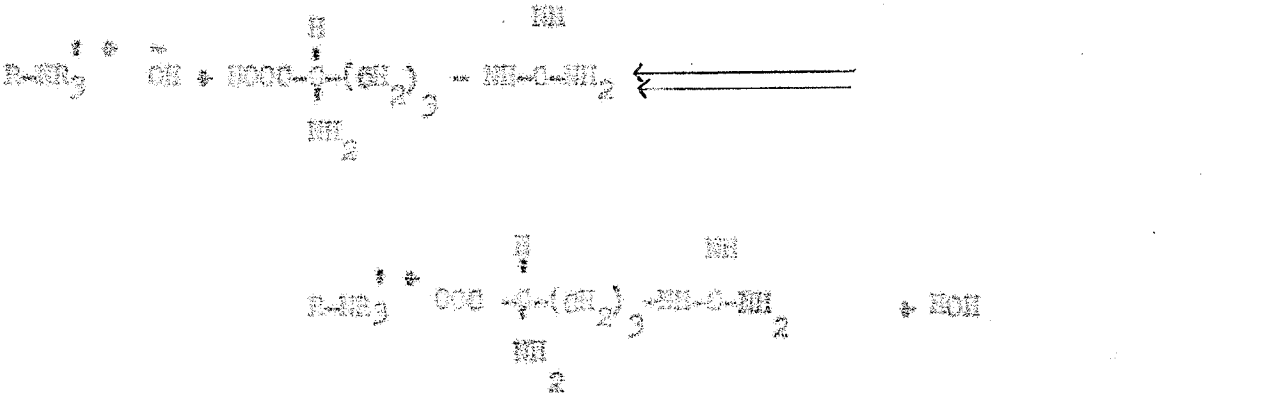


(vi) Lysine (I.E. 9.7)



(vii) Arginine (I.E. 10.8)

Arginine was not retained on any form of the cation exchanger



CHAPTER III

THE INTERACTION OF HISTONE WITH ION EXCHANGE RESINS

The Quantitative Estimation of Histone

Histone gives a positive ninhydrin reaction, but in this work use was made of the ultraviolet absorption spectrum as the means of measuring the amount of histone retained by the ion exchanger. The U.V. was more advantageous than the ninhydrin method because (a) any change in the histone molecule was easily detected by a change of the U.V. absorption spectra; (b) if the ninhydrin test was carried out on a sample of histone solution, this histone sample could not be used again, whereas when the U.V. was used, the same sample could be returned with the resin, and subsequent measurements made on the same sample, thus keeping the concentration constant.

All measurements were made on a photoelectric spectrophotometer with quartz optics and light source suitable for the ultraviolet region. 2.2 ml. of a known concentration of histone solution was placed in quartz absorption cells of 10 mm. thickness and the absorption spectrum was determined between wave numbers 3200 cm^{-1} and 4400 cm^{-1} relative to quartz plate blank. The optical density was proportional to the concentration of histone to the limit of measurement of the instrument, and knowing the concentration of histone, it was then easy to calculate the amount that was retained when the histone was shaken with the resin.

Two samples of calf thymus histone dissolved in distilled deionized water were used. The U.V. absorption spectra were determined on a concentration of 1.00 mg/ml for both samples. Fig. V shows that the shape of the U.V. spectra for both samples was the same, but the optical densities

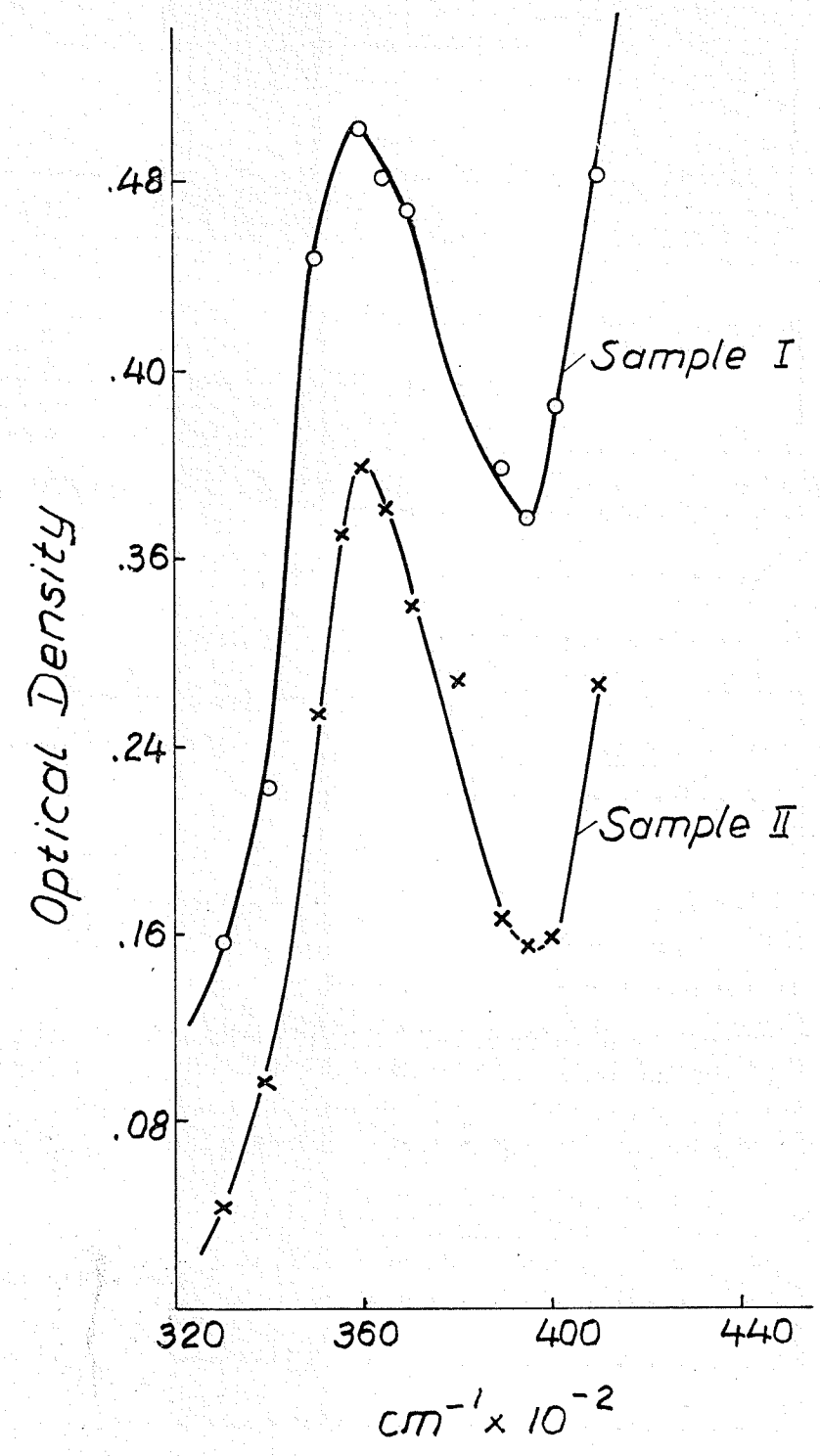


FIG. V: U. V. absorption spectra of the same concentration (1.00 mg/ml) of the two different samples of pure histone in water.

for the pure solutions of the two samples at a concentration of 1.00 mg/ml each was .50 and .38 respectively at 3600 cm^{-1}. This difference in optical densities was probably due to a concentration of aromatic amino acids in the two samples, and not to the amount of salt present in the two preparations, since the silver nitrate tests for chlorides were negative. Soret, as early as 1865 (26), pointed out that the U.V. absorption spectra of proteins are due to the presence of aromatic amino acids in the protein molecule; and Hirsly and Pelliter, in 1942 (28), showed that the absorption spectra of purified histones cannot be distinguished from those of more complex proteins, as, for example, egg albumin.

Interaction with Anion Exchangers

When histone was dissolved in an alkaline medium (pH 11.0), in attempts to convert it into the anionic form necessary for retention on an anionic resin, the histone solution became very cloudy, and thus a very great increase of the U.V. spectrum occurred, (Fig. VI). The result in Fig. VI also shows that in an alkaline medium, there was a shift in the peak of the histone curve from 36500 cm^{-1} at pH 3.0 to 34000 cm^{-1} at pH 11.0. The cloudiness which occurred when histone was dissolved in an alkaline medium, appeared to be similar to that observed when histone was shaken with the anion exchangers (see below). This effect was reversible, because when 2 drops of 1N HCl were added to the cloudy histone solution that was either in contact with the anion exchanger or just simply dissolved in an alkaline solution, the cloudiness disappeared immediately and the U.V. absorption spectra returned to normal.

Fig. VII (b) and (c) are U.V. absorption spectra obtained when 1.0 mg/ml of histone dissolved in distilled deionized water was shaken for 10

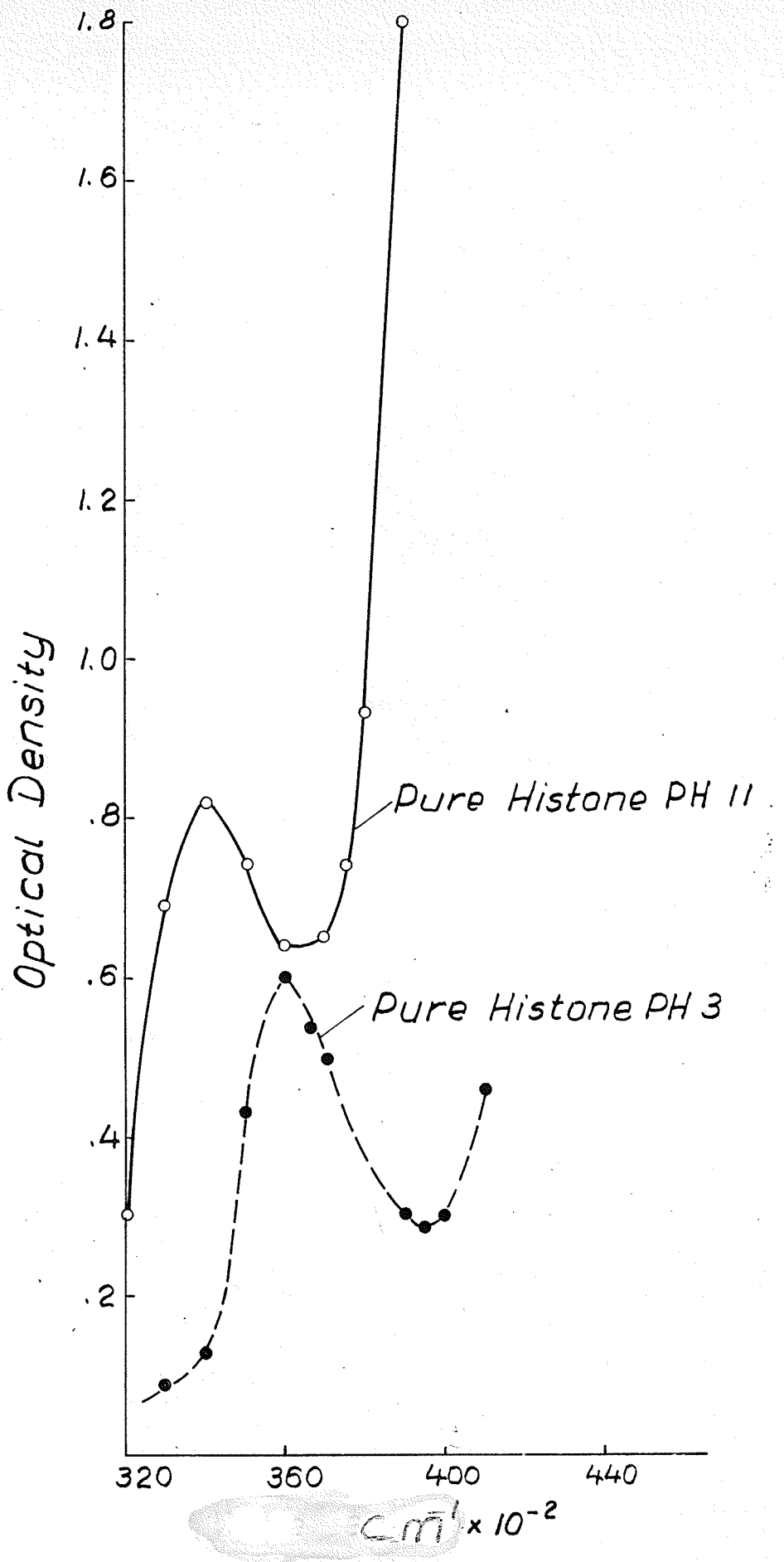


FIG. VI: Effect of pH on the U.V. absorption spectrum of pure histone solution

FIG. VII: U.V. absorption curves of 1.00 mgm/ml of histone in water (a) before, (b) and (c) after shaking for 10 mins. with 0.50 gm. of the weak (Cl^-) form and the strong (OH^-) form of the anion exchangers respectively.

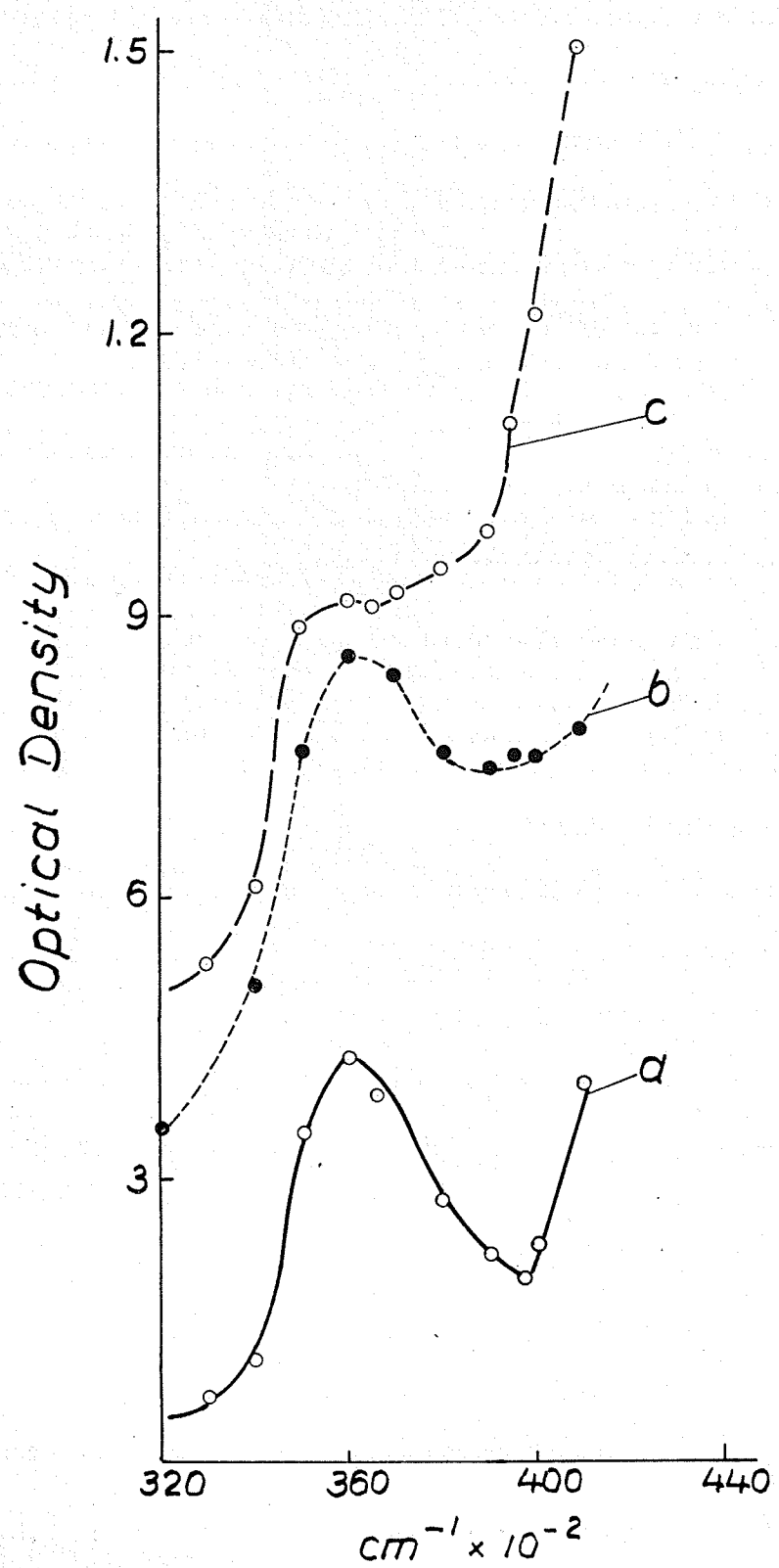
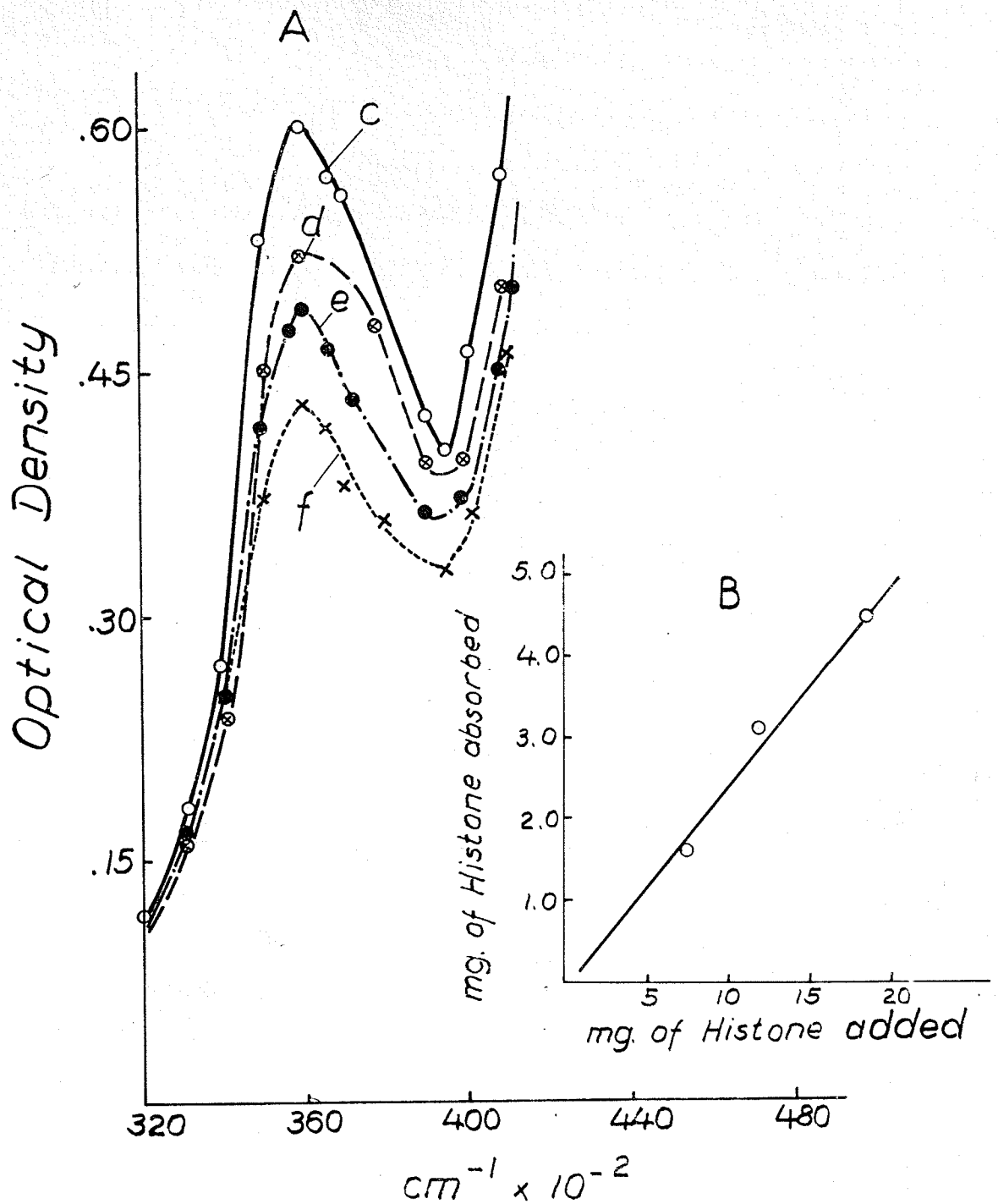


FIG. VIII: A. U.V. absorption curve of (c) 10.0 mgm of pure histone in 5.00 ml. of 0.01 M potassium phosphate buffer pH 5.5 (d) (e) and (f) after this histone was shaken with IRC-50 (Na^+) for 1, 2 and 4 hours respectively.

B. milligrams of histone retained on 3 separate lots of 0.50 gm. of IRC-50 (Na^+) resin.



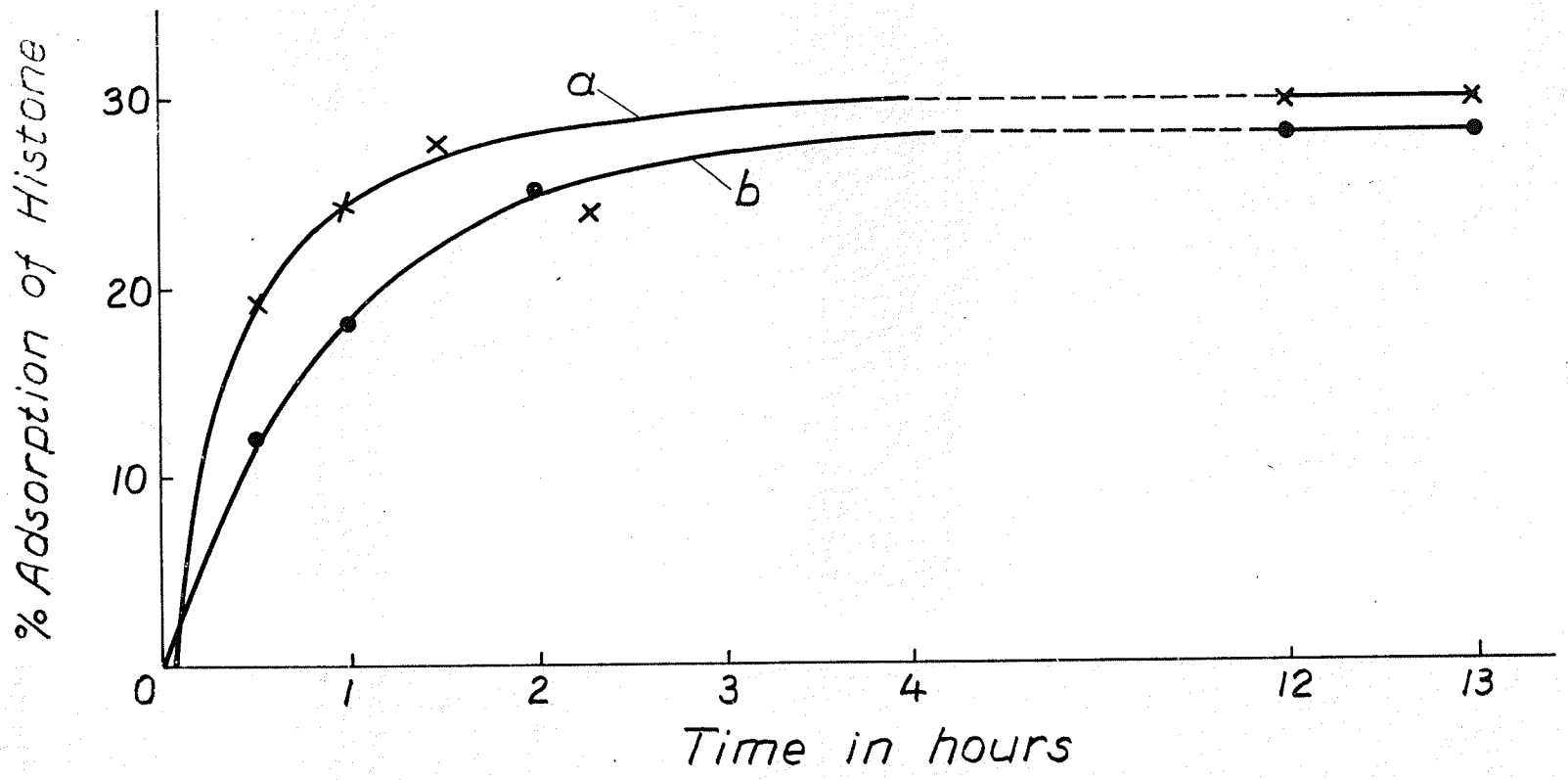


FIG. IX: Equilibrium curves of (a) 2.00 mg/ml, and (b) 1.5 mg/ml of histone in .01 M potassium phosphate pH 5.5 after shaking with 0.50 gm. of IRC-50 (Na^+) for 12 hours.

minutes with 0.50 gm. of weak anion exchanger in the (Cl^-) form and the strong anion exchanger in the (OH^-) form respectively. The increased optical density that occurred when histone was shaken with either the strong or weak anion exchanger, masked any indication that histone was retained by anion exchangers. This increased optical density was greater when the histone was shaken longer than 10 minutes with the anion exchanger. Fig. VII (a) is the U.V. spectrum of the pure histone before it was shaken with either form of the resins, and was used as the control.

Interaction with Cation Exchanger

(a) Strong Cation Exchanger. Table IV shows the amount of histone that was retained by separate batches of 0.5 gm. strong cation exchanger. The shape of the U.V. curve remained unchanged after histone was shaken with the strong cation exchanger. However, when the nucleic acid was added to the histone resin complex, the optical density of the nucleic acid increased considerably. For this reason the weak cation exchanger was used instead.

(b) Weak Cation Exchanger. (IRC-50 Na^+): 0.50 gm. of this resin was placed in a 50 ml. Erlenmeyer flask and to this was added 5 ml. of .01M potassium phosphate buffer pH 5.5 containing 10.0 mg. of histone. The mixture was shaken for 4 hours on a mechanical shaker. During this time, the U.V. absorption spectra was followed at $\frac{1}{2}$ hour intervals. Fig. VIII A (d), (e) and (f) show the decrease of the optical density which occurred when histone was shaken with the cation exchanger for a number of hours. Fig. VIII A (c) is the U.V. absorption spectrum of the pure histone before it was shaken with the resin. The percentage of histone retained by the resin during every $\frac{1}{2}$ hour was calculated, and is shown in Fig. IX. The

FIG. X: U. V. absorption curve (a) histone solution in equilibrium with 0.50 gm. of IRC-50 ($Na^{\oplus}$), (b) the same solution taken immediately after addition of 1.50 mgm of solid histone, (c) (d) and (e) the same solution after the 1.50 mgm of histone was shaken with the equilibrium mixture for 3, 5, and 12 hours respectively.

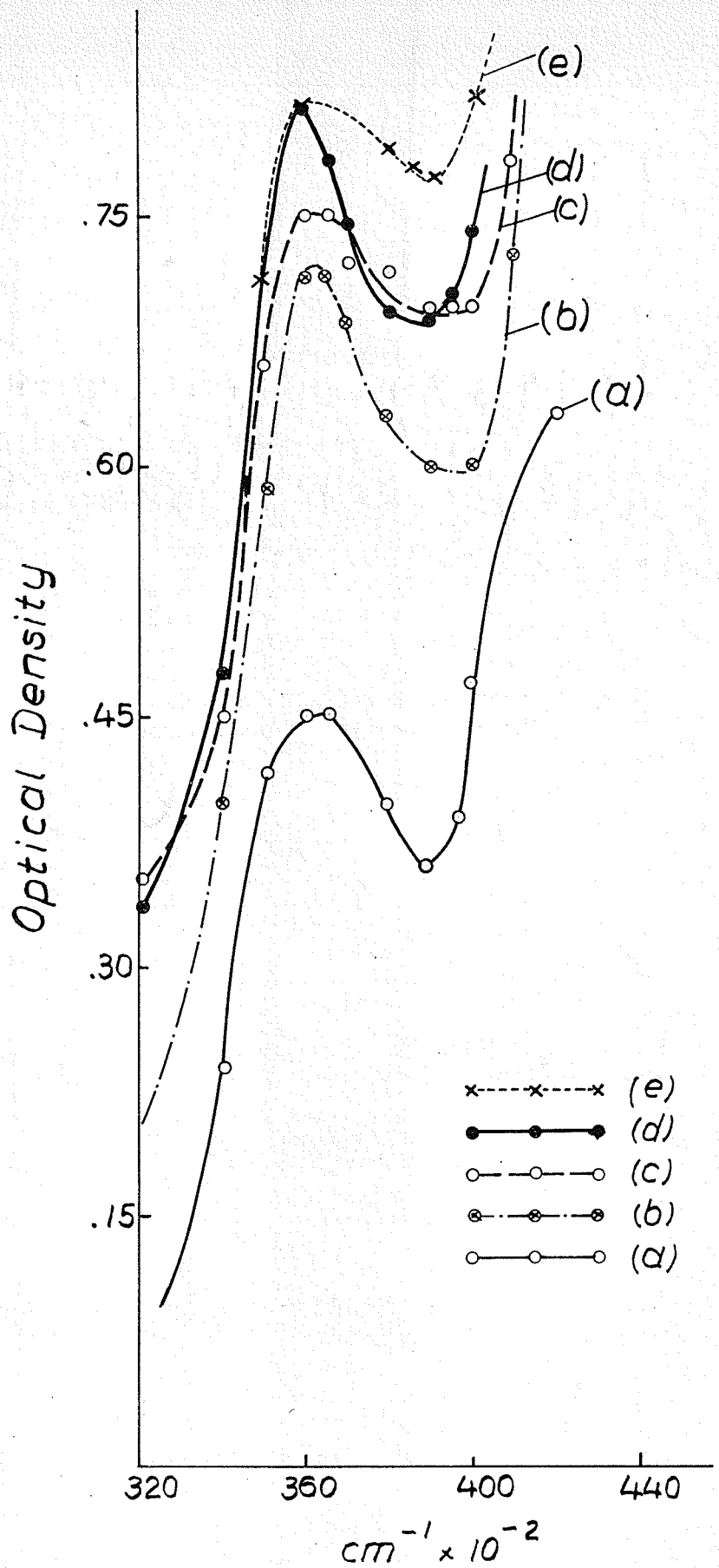

(e)
(d)
(c)
(b)
(a)
.75
.60
.45
.30
.15
Optical Density
320
360
400
440
cm⁻¹ x 10⁻²
(e)
(d)
(c)
(b)
(a)

FIG. X

histone solution was left in contact with the resin overnight to make certain that equilibrium had been achieved; Fig. IX shows that equilibrium had been achieved in 3 hours; Fig. IX (a) shows that 27% or 2.7 mg. of the 10.0 mg. of histone was retained by the resin, and Fig. IX (b) shows that 25% or 1.5 mg. of 6.0 mg. of histone was retained by another batch of 0.50 gm. of IRC-50 (Na^+). Table IV shows the amount of histone that was retained on separate lots of 0.5 gm. of the weak acid resin - IRC-50.

When solid histone was added to, and shaken with, a solution of histone - IRC-50 complex in equilibrium, a considerable amount of cloudiness occurred. This cloudiness did not occur when histone was added to the pure IRC-50 (Na^+) only (Fig. VIII A).

Fig. X (a) is the U.V. absorption curve of the histone solution in equilibrium with the histone - IRC (Na^+) complex before the solid histone was added, i.e., equivalent to Fig. VIII (f). Fig. X (b) is the U.V. curve taken immediately after 1.50 mg. of solid histone were added to the equilibrium mixture. Fig. X (c), (d) and (e) are the U.V. curves after the solid histone was shaken with the equilibrium mixture of histone - IRC-50 (Na^+) complex for 3, 5, and 12 hours respectively. This result indicated that once histone has been initially retained on IRC-50 (Na^+), any attempts to add solid histone to this would result in cloudiness of the histone. Fig. VIII B shows that more than 4.15 mg. of histone can be retained by 0.50 gm. of IRC - resin.

Discussion

Histone was retained by both the weak and strong cation exchangers. The strong cation exchanger was not used to study the interaction between

TABLE IV

Amount of Histone retained on 0.50 gm. of different types of Ion Exchange Resins at equilibrium. The histone was dissolved in 5.0 ml. of 0.01 M potassium phosphate buffer.

		Type of Ion Exchange Resins			
Amount of Histone added	pH of Solution	Strong Acidic (H)	Weak Acidic (Na)	pH of Solution	Strong and Weak Base
5.0 mg.	5.5	1.70 mg.	1.50 mg.	7.0-11.0	Nil
12.0		5.50	3.15		Nil
10.0		7.00	4.50		Nil

nucleic acids and histone because even when this resin was coated with the histone, it produced an increase of the optical density of the nucleic acid. The weak cation exchanger (IRC-50 H^+) did not have this effect upon the nucleic acids and was used to study the reactions between nucleic acids and histone.

After the histone was retained on the IRC-50 H^+ resin, the histone-resin complex was washed four times with distilled deionized water, pressed against filter paper to remove the excess water. This complex was then ready to be treated with nucleic acids as described in Chapter V.

Fig. 11: U.V. absorption curves of (a) 0.0312 mg/ml of pure BSA in water, (c) of the same concentration of BSA after shaking for 30 min. with 0.50 gm. of strong cation exchanger (H+), then transferred to fresh resin and after shaking for 30 min. (e) and 60 min. (g) respectively. (b), (d), (f) and (h) are the U.V. curves of the same type of experiment with 0.0312 mg/ml of BSA in water.

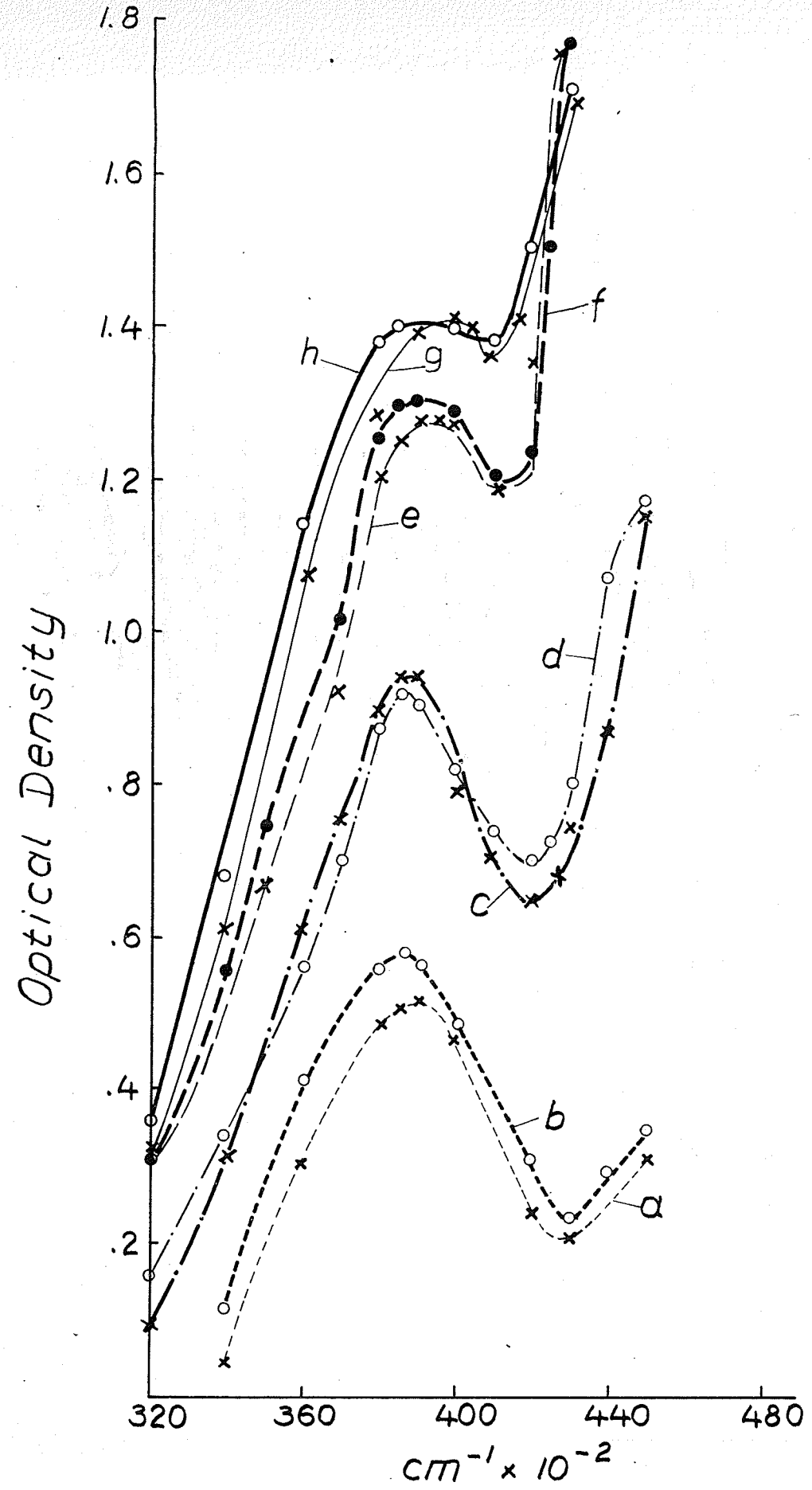

1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
Optical Density
320
360
400
440
480
cm⁻¹ x 10⁻²
a
b
c
d
e
f
g
h

FIG. XI

CHAPTER IV

THE INTERACTION OF NUCLEIC ACIDS WITH ION EXCHANGE RESINS

The Quantitative Estimation of Nucleic Acids

The ultraviolet absorption spectrophotometer was used for the quantitative estimation of both RNA and DNA. The method adopted was the same as that described for histone. A known concentration of RNA or DNA dissolved in distilled deionised water was placed in a 10 mm. quartz cell and the optical density determined between wave numbers 32000 cm^{-1} and 45000 cm^{-1}, relative to a quartz plate blank. The maximum U.V. absorption of pure nucleic acid solutions fell between 38000 cm^{-1} and 38500 cm^{-1}.

Fig. XI (a) and (b) represents the normal U.V. spectra of pure solutions of 0.0322 mg/ml of RNA and 0.0366 mg/ml of DNA respectively. The U.V. curves of both RNA and DNA dissolved in distilled deionised water were the same as when both nucleic acids were dissolved in 0.01 M potassium phosphate buffer, pH 5.5. The curves are in agreement with those obtained by Peiche (98) and Elson (99).

In the present study with nucleic acids and ion exchange resins, no attempt was made to saturate the resins with nucleic acids. The experiments were carried out to determine whether nucleic acids were retained by ion exchange resins or not.

Reaction with Strong Cation Exchanger (H^+) and (Na^+) forms

Into two separate centrifuge tubes containing 0.5 gm. of Dowex 50-X4 (H^+) was added 0.161 mg. of RNA and 0.161 mg. of DNA. Both nucleic acids were dissolved in 5.0 ml. distilled deionised water. The centrifuge

Fig. III: U.V. absorption curves of 0.0122 mg/ml of MM in water after shaking for 30 min.(a) and 60 min. (b) with the (H+) form of the strong cation exchanger. Curves (c) and (d) are the results of the same type of experiment with 0.0366 mgm/ml of MM in water.

FIG. XXX

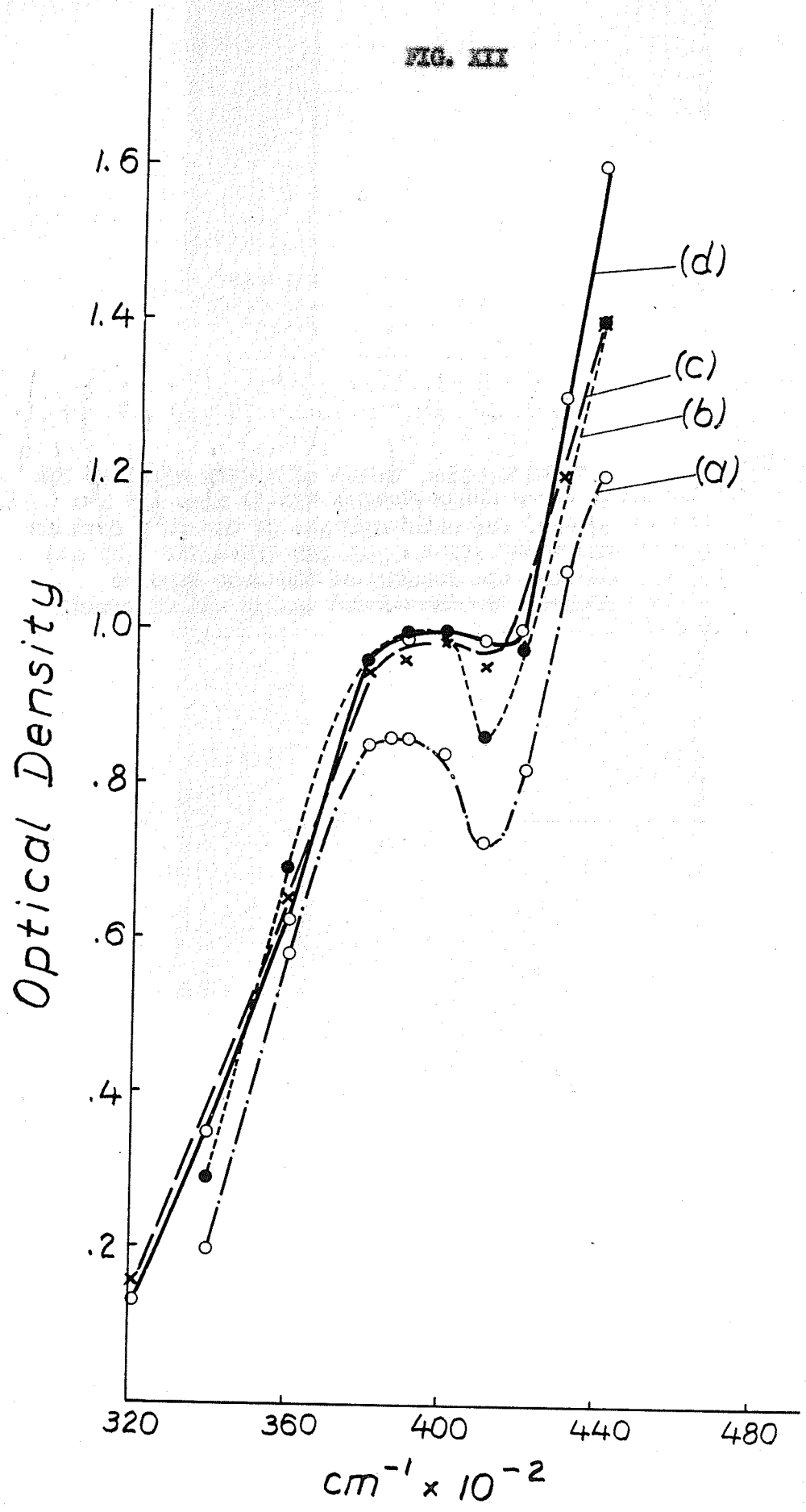
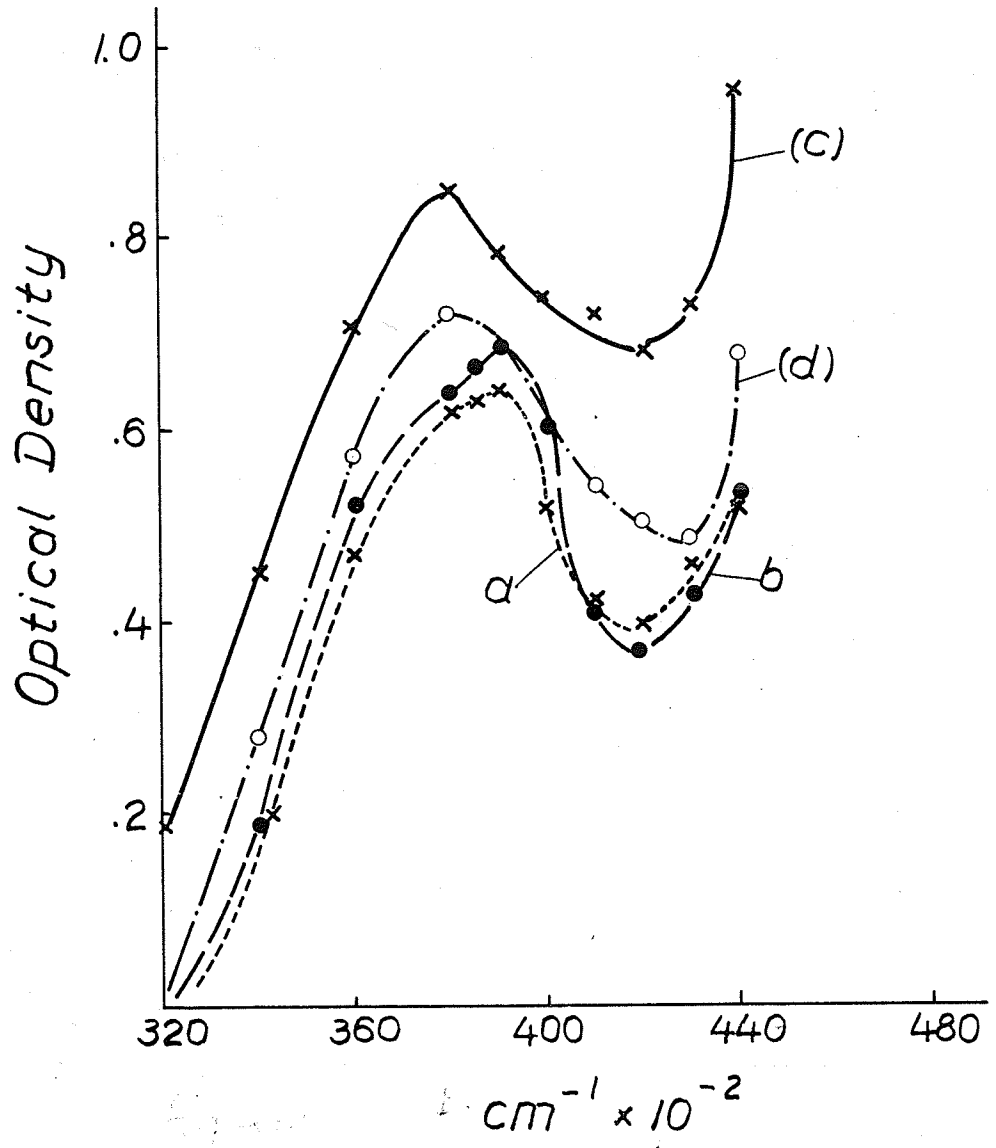

1.6
1.4
1.2
1.0
.8
.6
.4
.2
Optical Density
320
360
400
440
480
cm⁻¹ x 10⁻²
(d)
(c)
(b)
(a)

FIG. XXII: U.V. absorption curves of 0.0322 mg/ml. of EMA in water after shaking for 30 min. (a) and 60 min. (c) with 0.50 gm. of the (H^+) form of the weak cation exchanger (IRC-50). (b) and (d) are the results of the same type of experiment with 0.0366 mgm of EMA in water.

FIG. XIII



tubes were shaken by mechanical shaker for 30 minutes. Fig. XI (c) and (d) are the U.V. curves of RNA and DNA after they were shaken with the resin. Both of the nucleic acid solutions were then transferred to separate lots of the Dowex 50 resin (H^+) and again shaken for 30 minutes and the U.V. readings estimated on the supernatant (Fig. XI (e) and (f)). The samples were left overnight in contact with the resin and shaken for another 30 minutes on the following morning. The U.V. results of the supernatant can be seen in Fig. XI (g) and (h). The U.V. curves of the pure RNA and DNA solutions before they were placed in contact with the strong acidic resin are shown in Fig. XI (a) and (b).

The U.V. curves obtained after RNA and DNA were shaken with the acidic form of the strong acidic resin for 30 minutes and 60 minutes are shown in Fig. XII.

Reaction with Weak Cation Exchanger (H^+) and (Na^+) forms

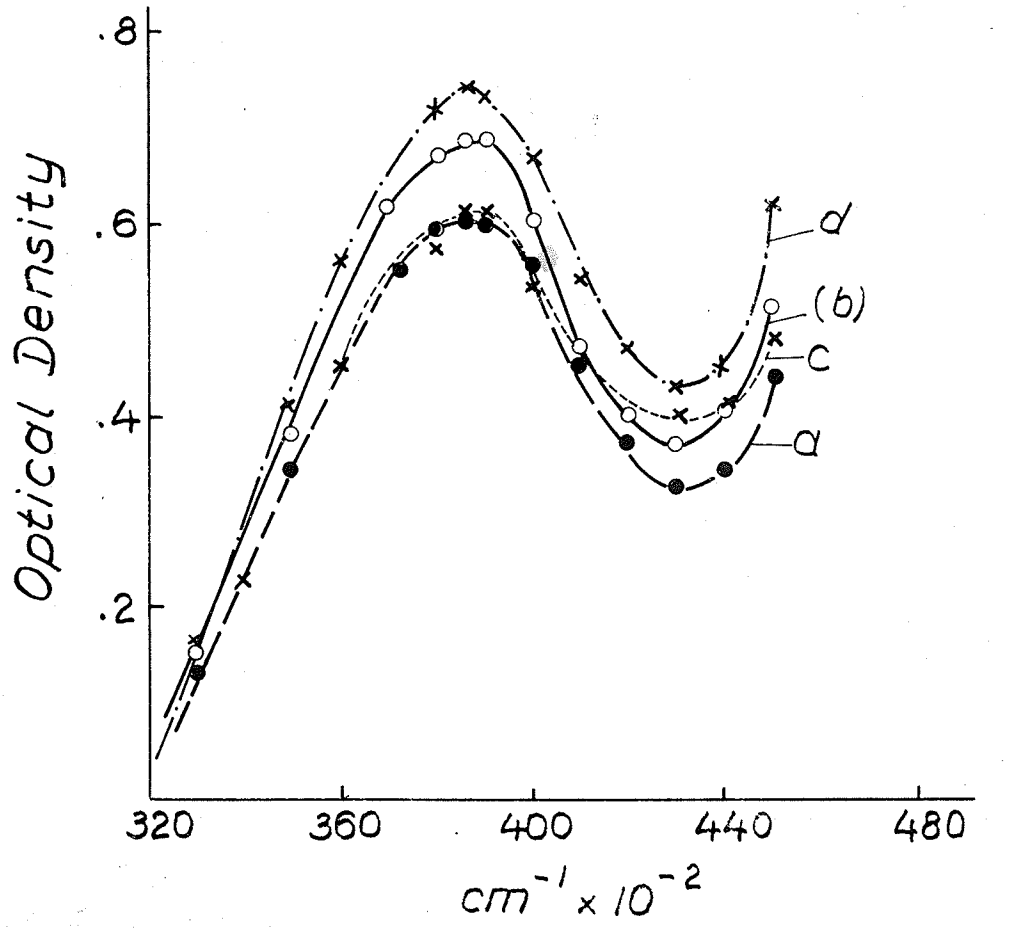
Fig. XIII represents the U.V. curves of RNA and DNA after they were shaken with the weak acidic resin IRC-50 (H^+) for 30 and 60 minutes. The results with the (Na^+) form of the weak acidic resin are shown in Fig. XIV.

Reaction with Strong Anion Exchanger (OH^-) and (Cl^-) forms

The same concentrations of nucleic acids were used here as described for the experiments with the cation exchanger. In this experiment the nucleic acids were shaken for only 10 minutes with the anion exchangers. When RNA was shaken with the (OH^-) form of the strong anionic resin, a decrease was noticed in the optical density from 0.52 for the pure RNA to 0.12 after it was shaken with the resin for 10 minutes (Fig. XVIII (a) and (b)). On the other hand, when DNA was shaken with the (OH^-) form of the resin the U.V. curves showed a decrease from 0.54 for the pure DNA to

Fig. XIV. U.V. absorption curves of 0.0322 mgm/ml. of fish in water after shaking for 30 min. (a), and 60 min. (c) with 0.50 gm. of the (H$^+$) form of the weak cation exchanger. (b) and (d) are the results of the same type of experiments with 0.0366 mgm/ml. of fish in water.

FIG. XIV



0.46 for the DNP after it was eluted with the resin (Fig. XVII (a) and (b)). This decrease in optical density was interpreted to signify that the nucleic acids were retained by the anion exchangers.

Discussion

The increased optical density which occurred after nucleic acids were eluted with any form of the cation exchangers, made it impossible to determine whether the nucleic acids were retained by this type of ion exchangers. Since the increased optical density produced by the (H^+) form of the weak acid exchanger was negligible (Fig. XXII and XIV), this type of resin was used to study the interaction between the histone and the nucleic acids.

In Chapter V it is shown that nucleic acids were retained by the anion exchange resins.

Fig.XV: U.V.absorption curves of (a) 0.0366 mgm/ml of pure DNA in water,(b) DNA after shaking for 10min with the strong cation exchanger (H^+) only, (c)DNA after shaking for 10 min.with glutamic acid-saturated cation exchange complex, and (d)DNA after shaking with arginine-saturated cation exchange complex. The same concentration of DNA was used throughout.

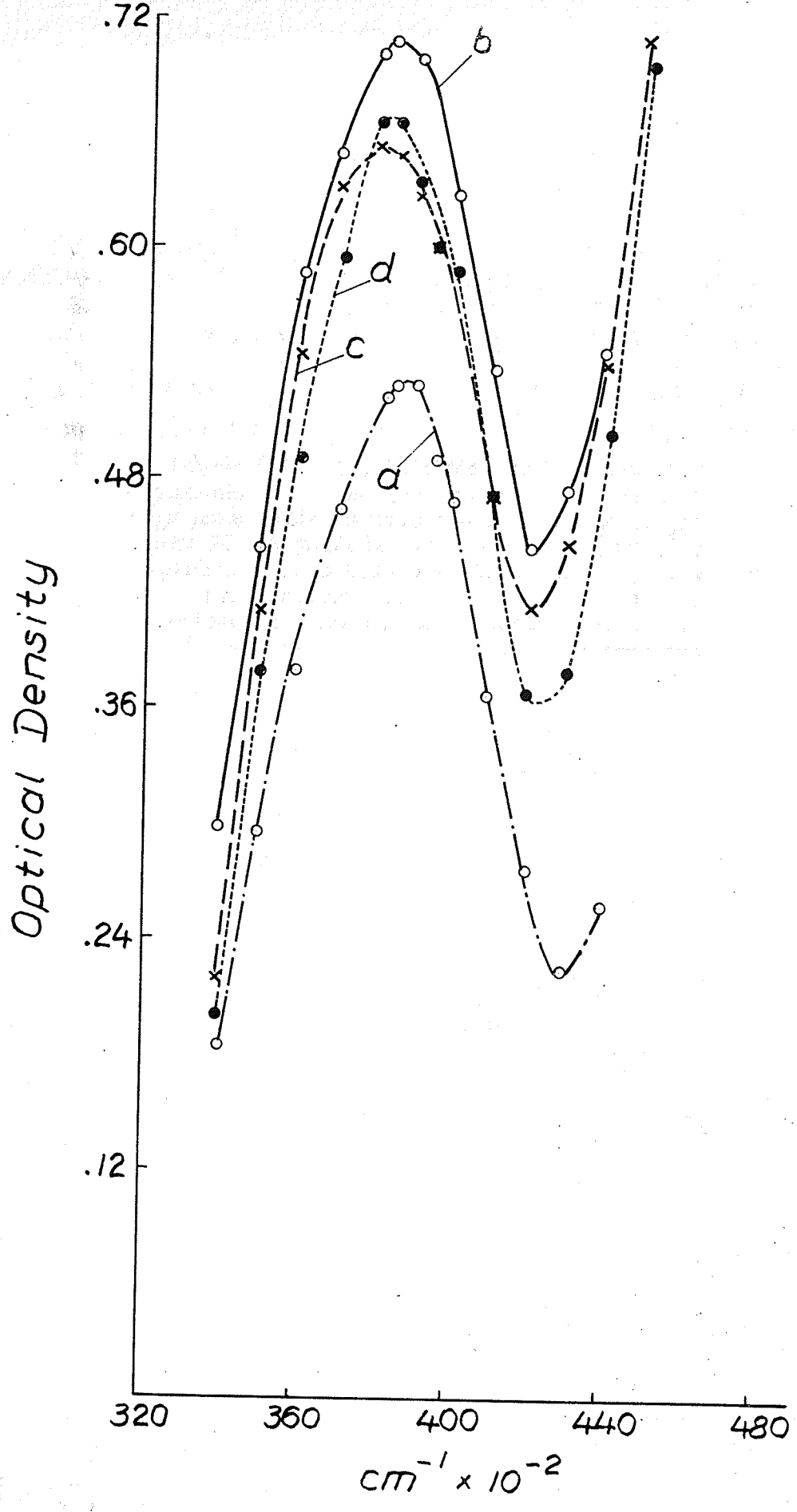

.72
.60
.48
.36
.24
.12
Optical Density
320
360
400
440
480
cm⁻¹ x 10⁻²
a
b
c
d

FIG. XV

FIG. XVI: U.V. absorption curves of (a) 0.322 mgm/ml of pure RNA in water, (b) RNA after shaking for 10 mins. with the strong cation exchanger (H^+) only, (c) RNA after shaking for 10 mins. with glutamic acid-saturated cation exchange complex, and (d) RNA after shaking with arginine-saturated cation exchange complex. The same concentration of RNA was used throughout.

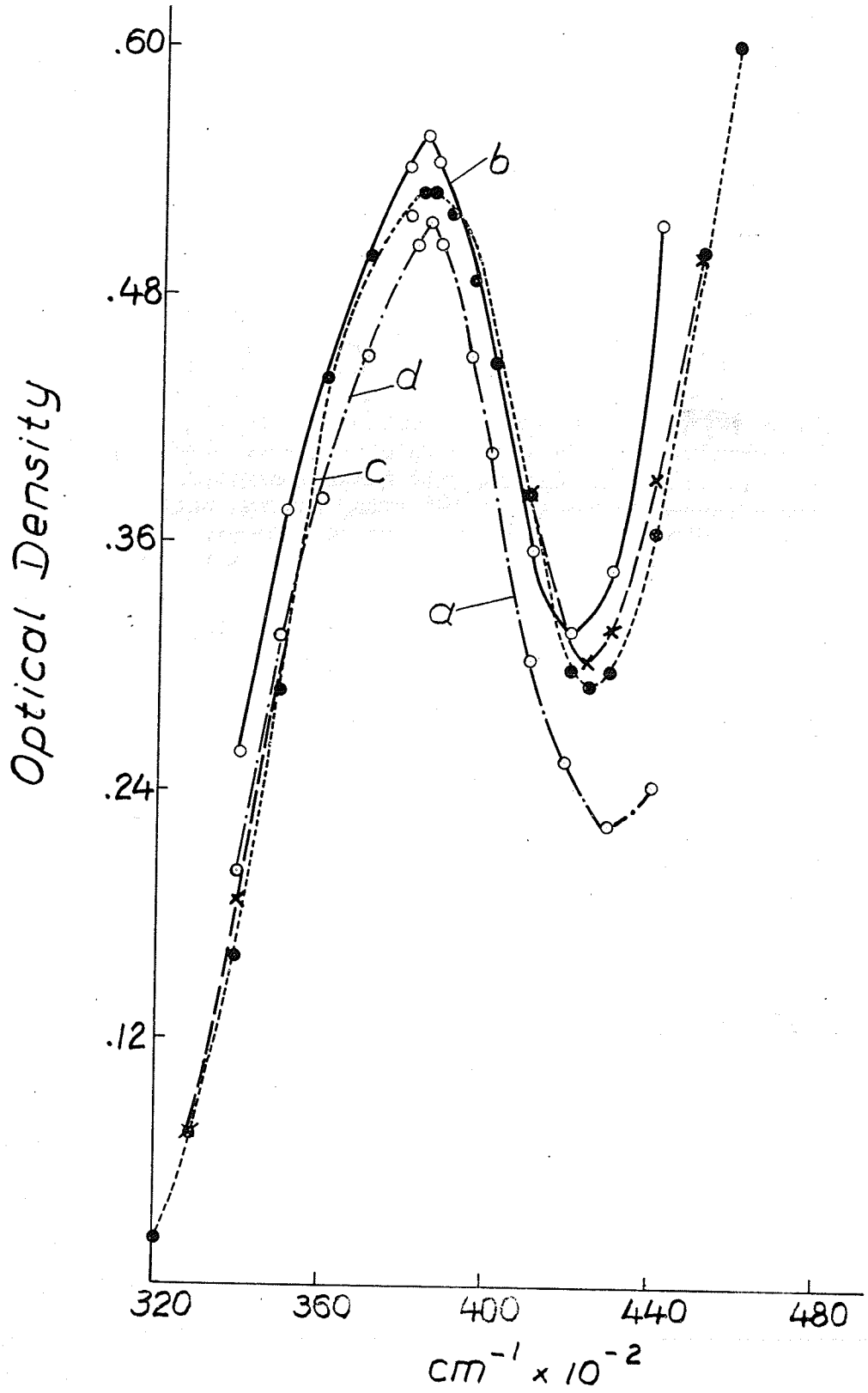

.60
.48
.36
.24
.12
Optical Density
b
d
c
a
320
360
400
440
480
cm⁻¹ x 10⁻²

FIG. XVI

FIG. XVII: U.V. absorption curves of (a) pure DNA, (b) DNA after shaking with the strong anion exchanger (OH^-) only, (c) DNA after shaking with histidine-saturated anion exchange complex, (d) DNA after shaking with glutamic acid-saturated anion exchange complex. The same concentration of DNA used here as stated in Fig. XV.

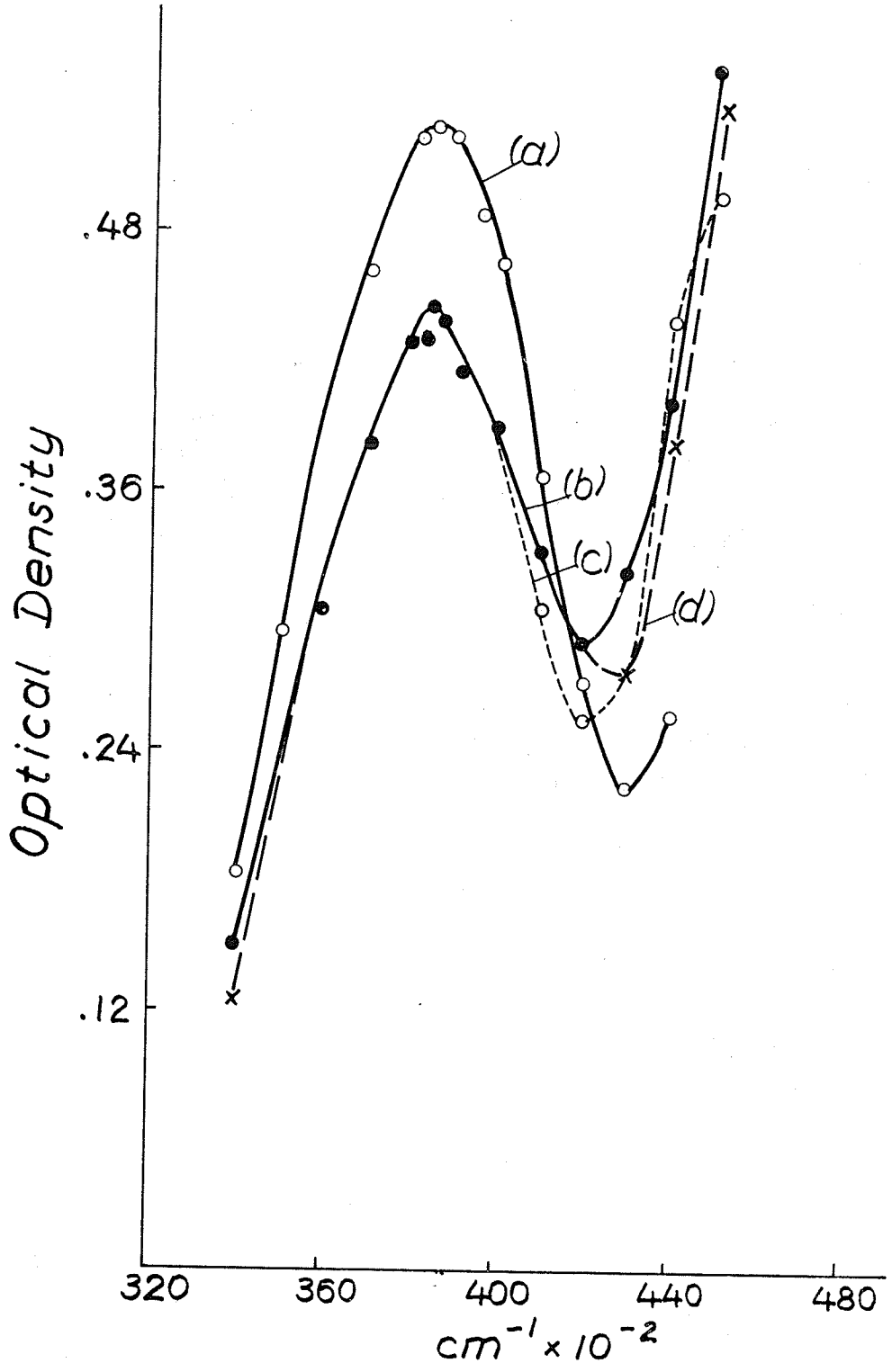


FIG. XVII

CHAPTER V

SECTION I: THE INTERACTION OF NUCLEIC ACIDS WITH AMINO ACID-RESIN COMPLEX

With the ion exchange resins saturated with individual amino acids and washed four times with 5.0 ml. distilled deionized water as outlined in Chapter IV, the interaction with nucleic acids was studied in the following manner.

To the saturated amino acid-resin complex in a 50 ml. Erlenmeyer flask was added 0.161 mg. of RNA or 0.150 mg. of DNA dissolved in 5.0 ml. of distilled deionized water, and the mixture shaken by hand for 10 minutes. In each experiment there were two controls, one was the U.V. spectrum of the pure nucleic acid, the other was the U.V. spectrum of the same concentration of nucleic acid after it was shaken with the resin for 10 minutes.

The Interaction of Nucleic Acid with Amino Acid-saturated Strong Cation Exchange Complex

Fig. IV shows the U.V. absorption curves of: (a) pure RNA; (b) RNA after shaking with the cation exchange resin only; (c) RNA after shaking with glutamic acid-saturated cation exchanger complex; and (d) RNA after shaking with arginine-saturated cation exchanger complex. The U.V. absorption curves of RNA after it was shaken with phenylalanine-saturated cation exchange complex and with histidine-saturated cation exchange complex are not shown because they were the same as (c) and (d). Fig. IVI represents the results of the same type of experiment but instead of RNA, DNA was the nucleic acid shaken with the amino acid-saturated cation exchange complex.

FIG. XVIII: U.V. absorption curves (a) pure RNA, (b) RNA after shaking with the strong anion exchanger (OH^-) only, (c) RNA after shaking with histidine-saturated anion exchange complex, (d) RNA after shaking with glutamic acid-saturated anion exchange complex. The same concentration of RNA used here as stated in Fig. XVI.

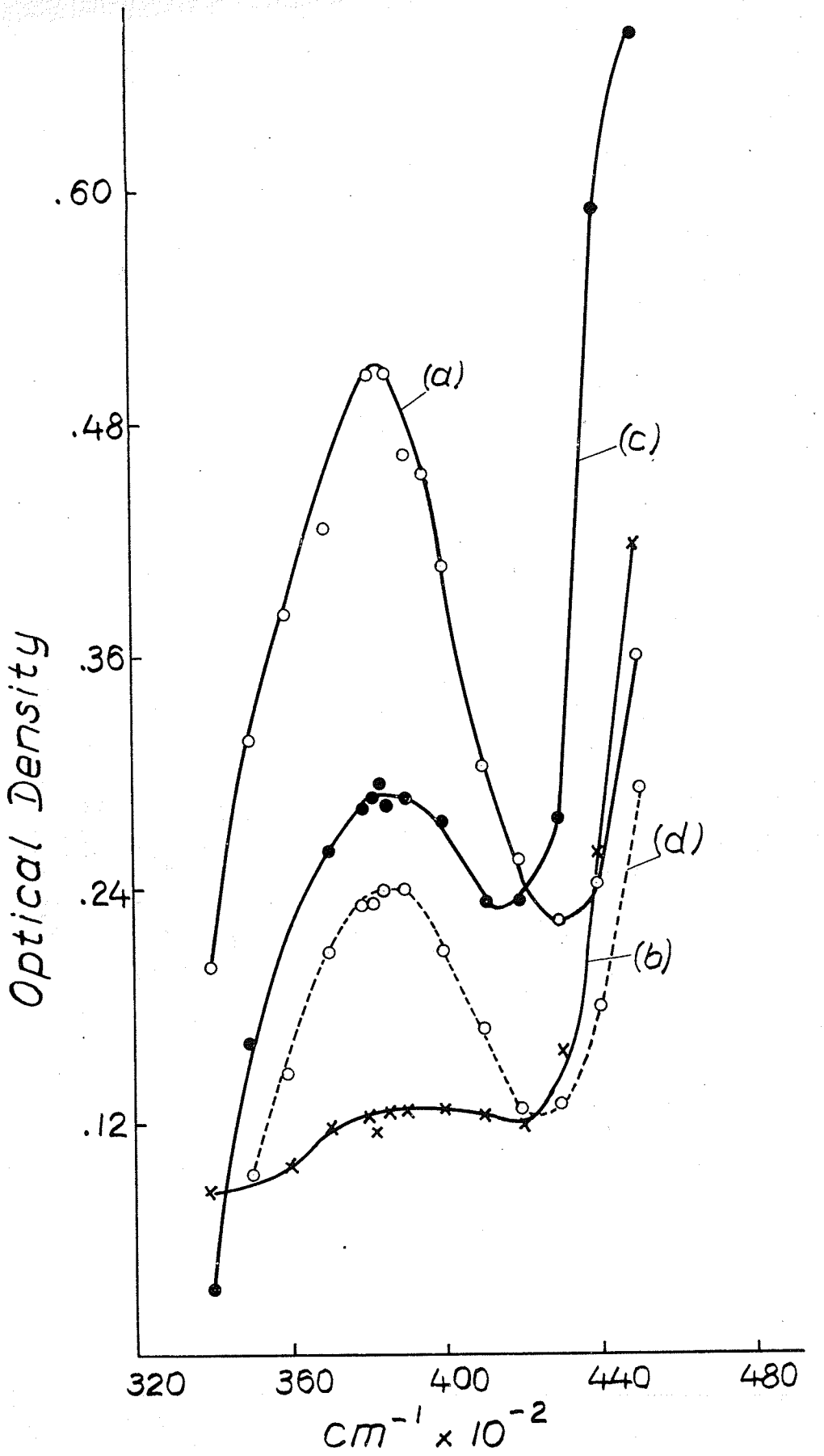

.60
.48
.36
.24
.12
Optical Density
(a)
(c)
(d)
(b)
320
360
400
440
480
cm⁻¹ x 10⁻²

FIG. XVIII

TABLE V

Maximum optical densities at 36500 cm^{-1} of 0.336 mg/ml of DNA and .0322 mg/ml of RNA, and after shaking for 15 minutes with 0.50 gm. Strong Acidic Resin Dowex 50 X-4 (H^+) and with this resin saturated with the amino acids indicated.

Nucleic Acid	Pure Solution	Resin Only	RESIN SATURATED WITH AMINO ACIDS			
			Phenylalanine	Arginine	Glutamic Acid	Histidine
DNA	.54	.72	.64	.65	.65	.65
RNA	.52	.50	.53	.52	.52	.55

TABLE VI

Maximum optical densities at 36500 cm^{-1} of .0336 mg/ml of DNA and .0322 mg/ml of RNA and after shaking for 15 minutes with 0.50 gm. Strong Basic Resin, Amberlite IRA - 400 (OH^-) and with this resin saturated with the amino acids indicated.

Nucleic Acid	Pure Solution	Resin Only	RESIN SATURATED WITH AMINO ACIDS			
			Phenylalanine	Lysine	Glutamic Acid	Histidine
DNA	.54	.43	.43	.44	.43	.43
RNA	.52	.12	.26	.26	.26	.26

The maximal optical densities at 35500 cm^{-1} of the curves in Fig. XV and Fig. XVI are tabulated in Table V. Those obtained with the nucleic acid after it was shaken with phenylalanine and histidine-saturated anion exchange complexes are also given.

The Interaction of Nucleic Acids with Amino Acid-saturated Anion Exchanger Complex

The same concentrations of nucleic acids were used as in the experiments with the anion exchanger. Fig. XVII shows the U.V. absorption curves of: (a) pure DNA; (b) DNA after shaking with the strong anion (OH^-) resin only; (c) DNA after shaking with histidine-saturated anion exchanger complex; and (d) DNA after shaking with glutamic acid-saturated anion exchange complex. The U.V. absorption curves of DNA after shaking with phenylalanine-saturated anion exchange complex and with lysine-saturated complex are not shown because they were the same as (c) and (d).

Fig. XVIII is the result of the same type of experiments, but instead of DNA, RNA was the nucleic acid added to the amino acid-saturated anion exchange complex. The maximal U.V. optical densities of the curves at wave length 35500 cm^{-1} shown in Fig. XVII and Fig. XVIII are tabulated in Table VI. Those obtained with the nucleic acids after they were shaken with phenylalanine and lysine-saturated anion exchanger complexes are also given.

SECTION II. THE INTERACTION OF NUCLEIC ACID WITH HISTONE-IRC-50 (H^+) COMPLEX

In this experiment the nucleic acid was dissolved in 0.01 M potassium phosphate buffer, pH 5.5. The method of measuring the concentration of nucleic acid after it was shaken with the histone-IRC-50 complex was the same as described previously.

Fig. XIX shows the decrease in optical density from 0.57 for the pure RNA to 0.20 for the same concentration of RNA after it was shaken for 12 hours with 1.5 mg. of histone retained on IRC-50 (H^+) resin. Therefore 90% of 0.161 mg. of RNA was withdrawn from solution when RNA was shaken with histone-IRC-50 complex; this, then, must mean that the RNA reacted with histone on the resin.

Fig. XX (a) and (b) shows the percentage of RNA retained by 1.50 mg. of histone on the IRC-50 (H^+) resin, and that 3 hours' shaking was required before equilibrium was achieved. In another experiment 0.30 mg. of RNA and 0.25 mg. of DNA were retained by 3.15 mg. of histone attached to the IRC-50 resin (Figs. XXI A and XXII).

Fig. XXI A and Fig. XXII A show the maximum amounts of RNA and DNA retained by 3.15 mg. of histone attached to IRC-50 (H^+) resin. When it was found that no more nucleic acid was retained by the histone-IRC complex, the supernatant nucleic acid was removed from the solid phase, i.e., the nucleic acid-histone-IRC complex, and the latter washed 4 times with distilled deionized water to remove any trace of the free nucleic acid from the solid phase. The excess of free water was removed from the nucleic acid-histone-IRC complex by pressing it against filter paper. At this point, 5.0 ml. of potassium phosphate buffer, pH 5.5, was added to the Erlenmeyer flask containing the nucleic acid-histone-IRC complex and increasing concentration of solid $MgCl_2$ or solid NaCl was shaken with the complex.

FIG. XIX U.V. absorption curves of (a) 0.0322 mg/ml. of pure HSA in 0.01 M potassium phosphate buffer pH 5.5, (b) and (c) the pure solution of HSA after shaking for 1 and 2 hours respectively with 1.50 mgs of histone retained on 0.50 gm. of Zeo-50 (Na^+).

FIG. XX Equilibrium curves of 0.322 mg/ml of HSA in 0.01 M potassium phosphate buffer pH 5.5 in contact with 1.50 mg. of histone retained on 0.50 mg. of Zeo-50 (Na^+).

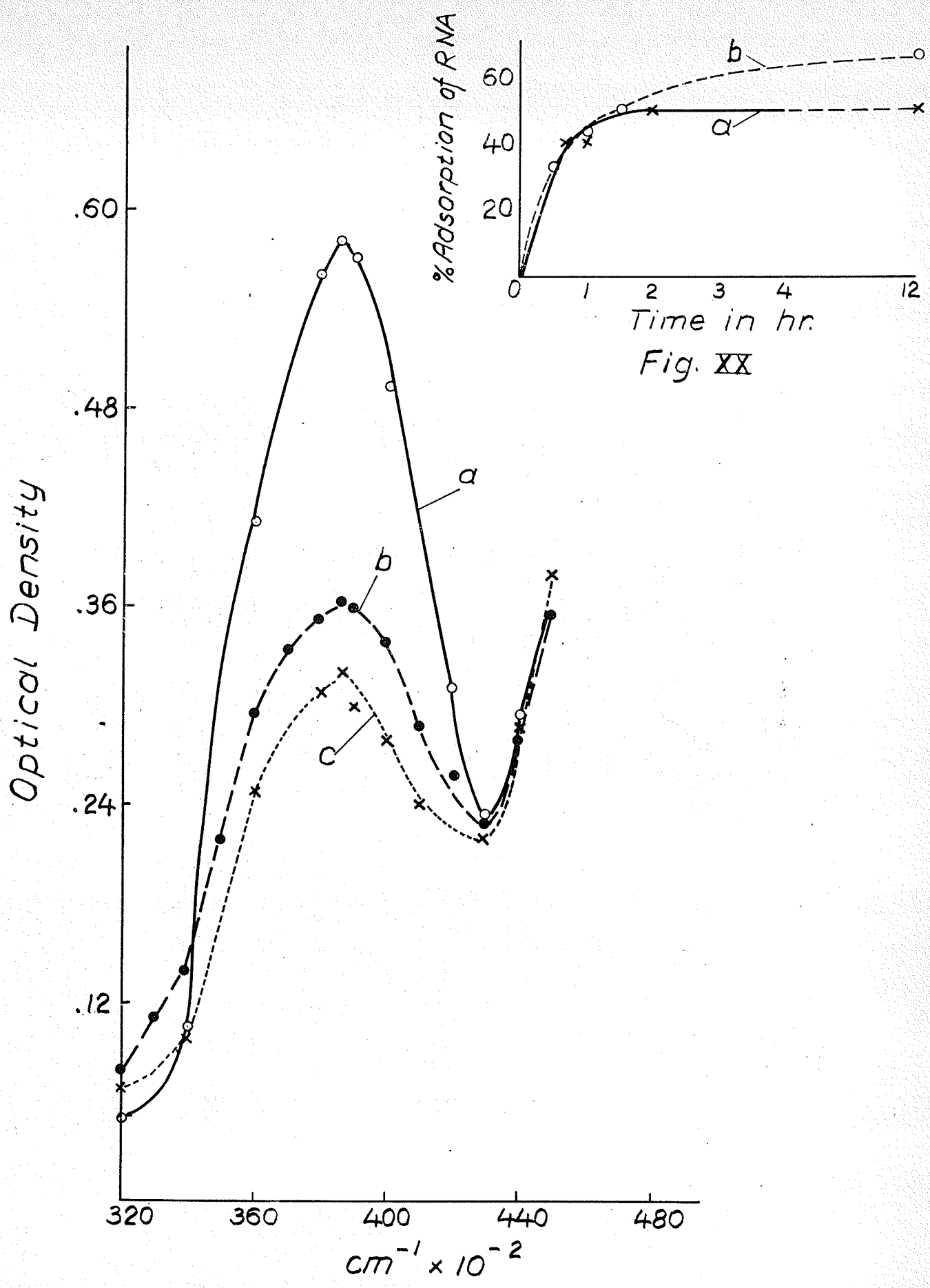


Fig. XX

FIG. XIX

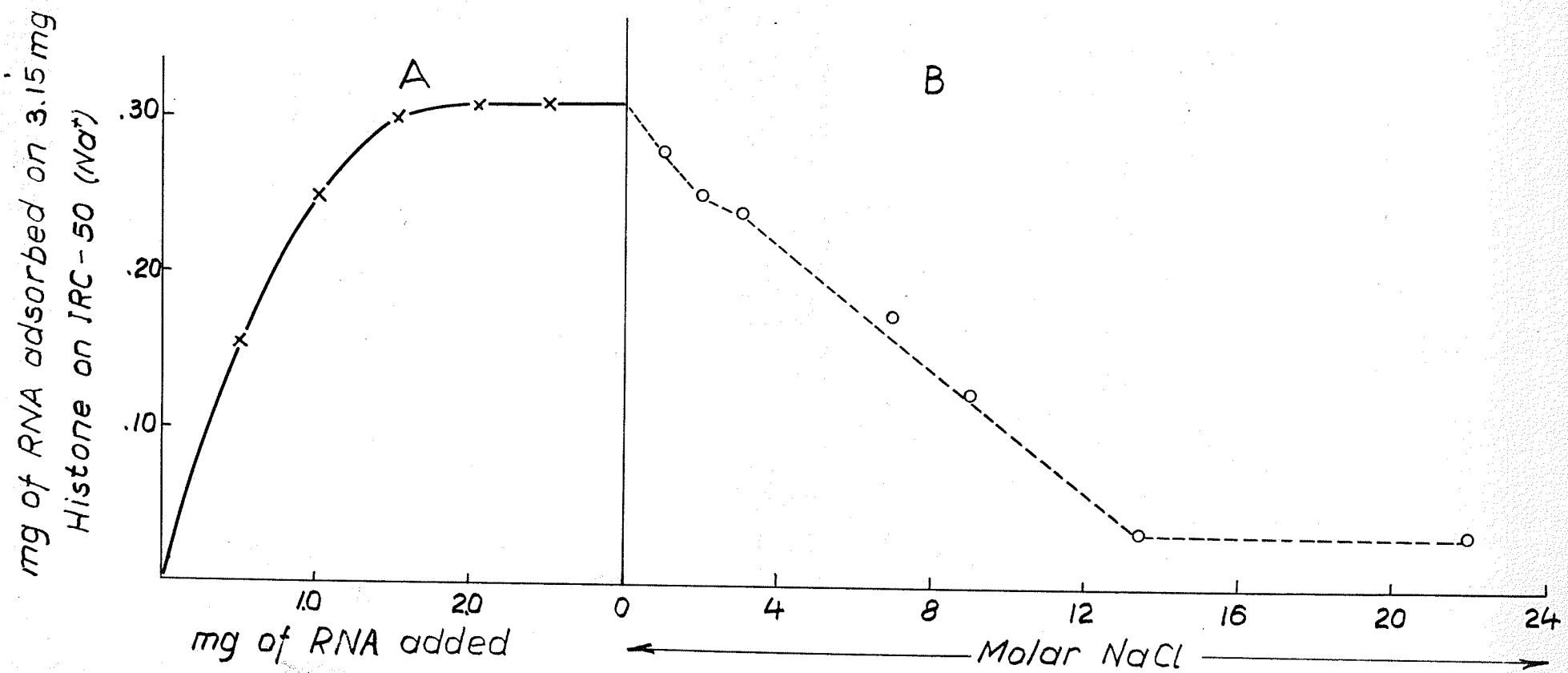
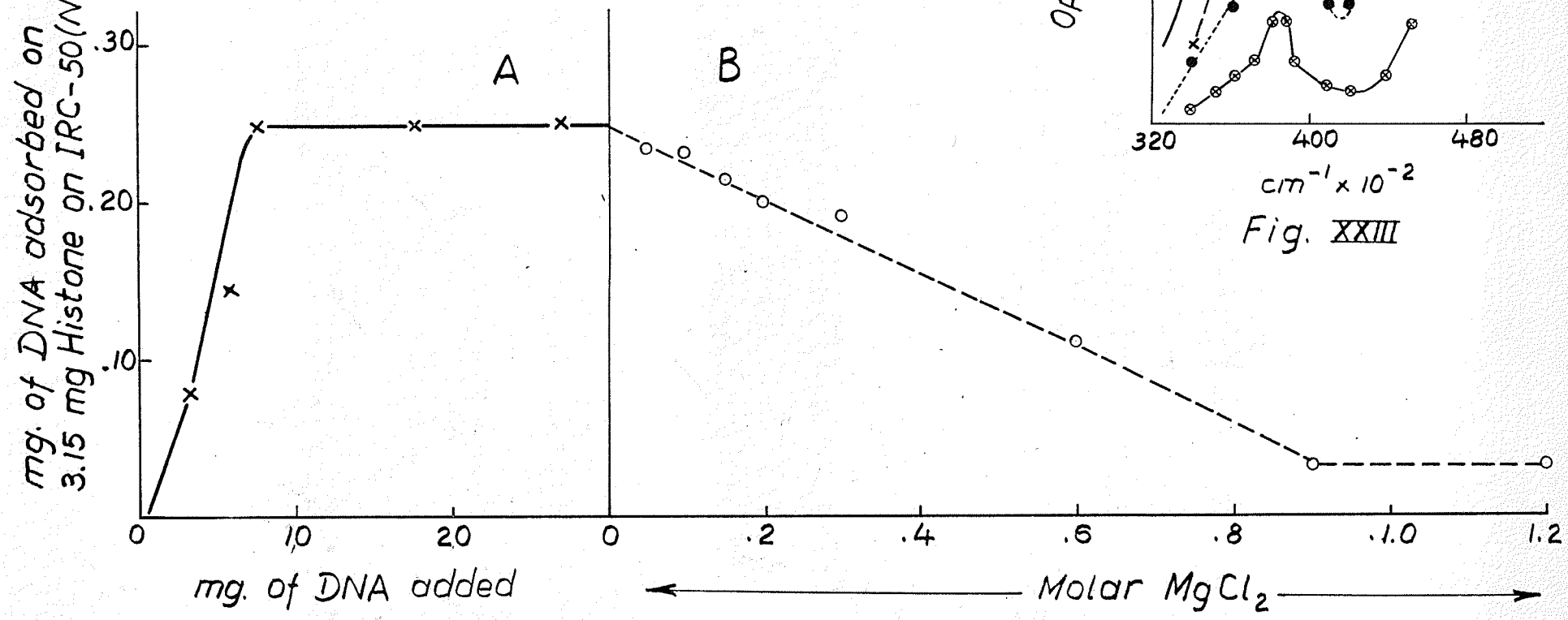


FIG. XXI

A. Maximum amount of RNA in 0.01 M potassium phosphate buffer pH 5.5 retained by 3.15 mg. histone attached to 0.50 gm. of IRC-50 (Na^+).

B. Milligrams of RNA remaining attached to the 3.15 mg. of histone after shaking for 30 minute intervals with increasing concentrations of NaCl. in 5.0 ml. 0.01 M potassium phosphate buffer pH 5.50.

FIG. XXII



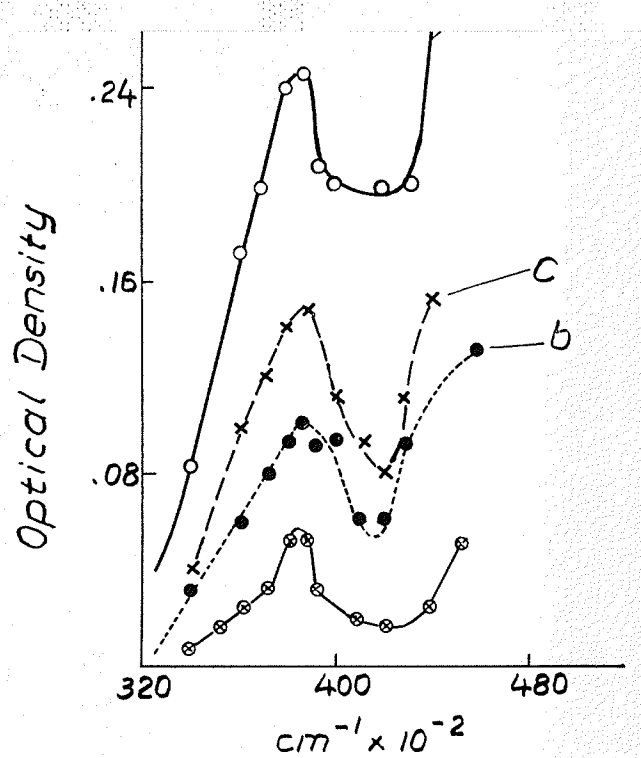


Fig. XXIII

FIG. XXII: A. Maximum amount of DNA in 0.01 M potassium phosphate buffer pH 5.5 retained by 3.15 mg. histone attached to 0.50 gm. of IRC-50 (Na^+).
B. Milligrams of DNA remaining attached to the 3.15 mg. of histone after shaking for 30 minute intervals with increasing concentration of $MgCl_2$ in 5.0 ml. of 0.01 M potassium phosphate buffer pH 5.50.

FIG. XXIII: U.V.absorption curves of the DNA released from the DNA histone-IRC complex by increasing concentration of $MgCl_2$.

FIG.XXIV U.V. ABSORPTION CURVES OF THE RNA released from the RNA histone-HRC complex by increasing concentration of NaCl.

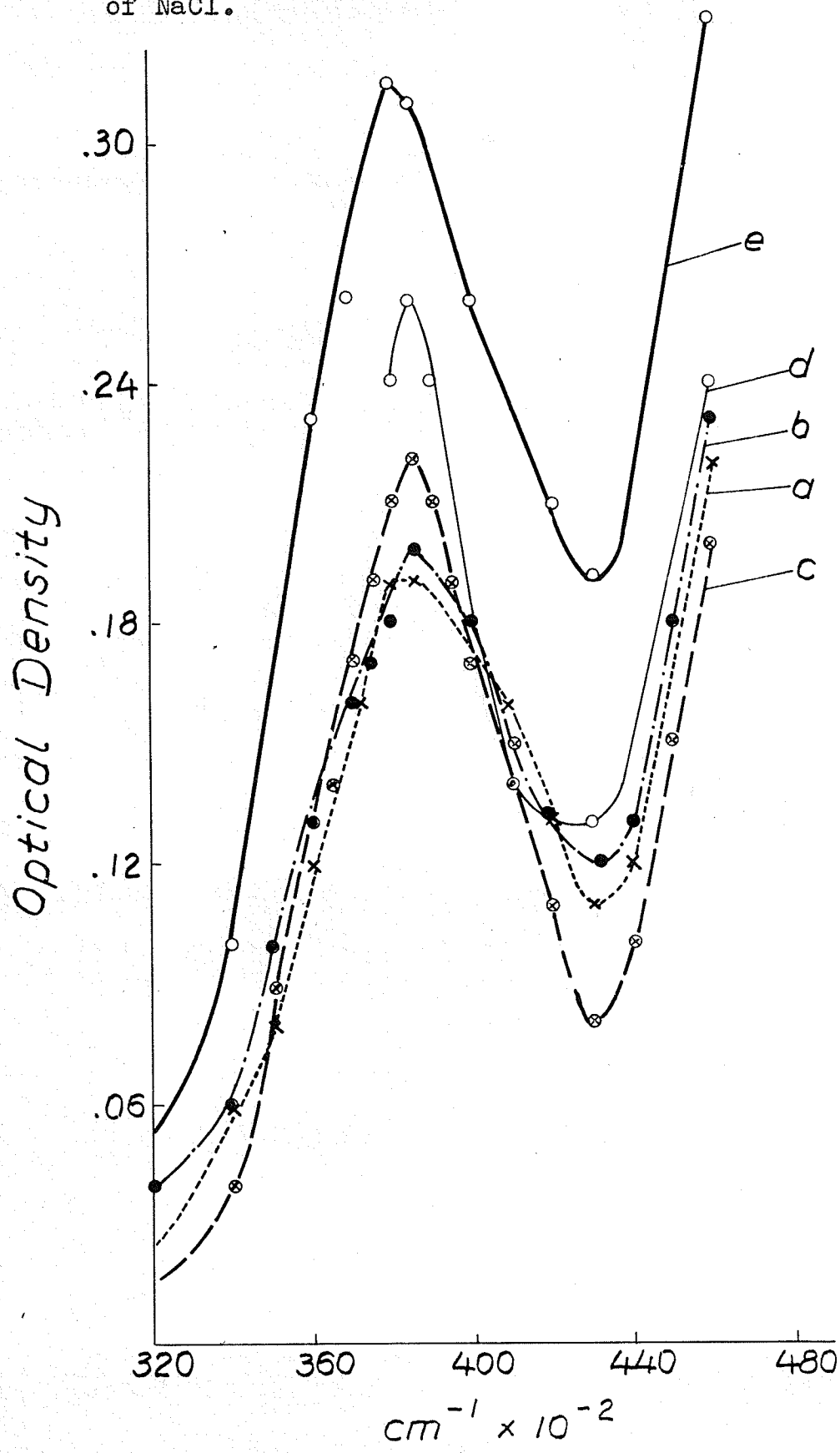


FIG.XXIV

If a chemical bond similar to that existing in native nucleoprotein was formed when nucleic acids interacted with histone, then the addition of either $HgCl_2$ or NaCl to the nucleic acid-histone-ZnO complex should result in a sudden rupture of this bond, giving rise to a sudden increase in the amount of nucleic acid in the solution. The amount of DNA removed from this complex by 0.05 N $HgCl_2$ was followed for 1 hour at 10 minute intervals, and the same procedure was followed for 0.10 N NaCl and RNA. This showed that after 30 minutes no more of the nucleic acids were removed from the nucleic acid-histone-ZnO complex by the above concentration of salts.

Fig. XXI B and Fig. XXII B show the concentration of DNA and RNA left attached to the histone-ZnO complex after shaking for 30 minutes with increasing concentrations of $HgCl_2$ and NaCl respectively. These results are tabulated in Table VII, and indicate that there was no sudden increase in the amount of nucleic acids released from the nucleic acid-histone-ZnO complex by either the $HgCl_2$ or NaCl. On the contrary, the amount of nucleic acids released was proportional to the concentration of salts added.

Fig. XXIII and Fig. XIV are the U.V. absorption spectra of DNA and RNA respectively, which were obtained after they were removed from DNA-histone-ZnO complex and RNA-histone-ZnO complex by $HgCl_2$ and NaCl. These curves also show that only the nucleic acids and none of the histone were removed by $HgCl_2$ and NaCl.

TABLE VII

Retention of Mn and Zn by 1.15 mg. of Nafion retained by 100-50 (Na) resin in equilibrium with various concentrations, $MgCl_2$ and NaCl respectively. Both salts dissolved in 5.0 ml. of potassium phosphate buffer pH 5.5

Molar $MgCl_2$ added	mg Mn left attached to Nafion-100-50	Molar NaCl added	mg Zn left attached to Nafion-100-50
0.0	.250	0.0	.330
.05	.243	"	"
.10	.230	.10	.260
.15	.220	.14	.250
.20	.200	"	"
.30	.180	.30	.210
.60	.120	.70	.105
.90	.030	.98	.130
1.20	.010	1.35	.045
		2.76	.045

Discussion

The increased optical density which occurred when the nucleic acids were shaken with the amino acids retained on the cation exchanger made it impossible to tell whether an interaction had occurred with the nucleic acids and the amino acids. This increased optical density effect was markedly less than that obtained when the nucleic acids were shaken with the free ion exchange resins, and gave rise to the question of whether the nucleic acids interacted with a part of the resin surface not affected by the amino acids.

The positively charged end of the amino acids was free when the amino acids were retained on the anion exchanger: R -NH_3^+, COO-R-NH_3^+, and, therefore, it was quite possible that a salt linkage might have occurred with the positively charged amino acid and the negatively charged nucleic acid. However, although a decrease of the optical density of the nucleic acids occurred when they were shaken with the amino acid-anion exchange complex, it was not definite that a reaction had taken place with the nucleic acid and the amino acid, because the nucleic acids were also retained by the anionic resin which had no amino acids retained upon it.

Both RNA and DNA interacted with the histone that was retained on IRC-50^H. It was not possible to ascertain whether a salt linkage was formed between the histone retained on the ion exchange resin and the nucleic acids. Increasing concentrations of either $MgCl_2$ or NaCl removed increasing quantities of the nucleic acids from the nucleic acid-histone-IRC complex.

Conclusion

The strong anion exchange resin was not particularly suitable for the study of the interaction between nucleic acids and amino acids and histone, because even when it was saturated with amino acids, the strong anion exchange resin still caused an increase of the optical density of the nucleic acids, and thus made it difficult to interpret the observations. This difficulty was not encountered with the weak anion exchanger.

The results obtained when the nucleic acids were shaken with the amino acid-saturated anion exchange complexes were not conclusive because the nucleic acids were also retained by the amino acid free anion exchanger. This result could be verified if the amino acid was retained on a weak ion exchange resin that did not interact with nucleic acids.

The reactions which occurred with nucleic acids and histone show that nucleic acids are in some way related to native nucleo-proteins.

Some of the positively charged amino groups on the histone must have caused its retention on the weak anion exchanger. However, since histone has numerous basic amino groups, it was quite possible that some of these basic groups were not adhering to the anionic resin surface but were free to form a salt linkage with the nucleic acids. If histone was retained on an anionic resin by means of its carboxylic groups, then it would be certain that all its positively charged amino groups would be free to interact with the negatively charged nucleic acid. This experiment was attempted, but the results were masked by the fact that the anion exchange resin caused the histone solution to become very cloudy. On the other hand, if an anion exchange resin could be found, that does not cause this effect upon histone solution, but retains the histone in such a

center and leaves all the other groups free, then any interaction that occurs with the Histone and the nucleic acid would be definitely due to the formation of a salt linkage.

ACKNOWLEDGMENT

The author wishes to thank The Manitoba Cancer Treatment and Research Foundation for the use of their laboratory equipment.

BIBLIOGRAPHY

1. Morgan, T.H., J. Exptl. Zool., 11, 365, (1911).

2. Caspersson, T.O., Nature 143, 294, (1939).

3. Brachet, J., Arch. of Biol. Liege, 53, 207, (1942).

4. Caldwell, P.C., Hanbor, E.L. and Hinshelwood, C., J. Chem. Soc., 3151, (1950).

5. Nalngren, B., and Heden, C.G., Acta Path. Microbiol. Scand., 24, 427, (1947).

6. (a) Gale, E.F., and Folkes, J.P., Biochem. J., 53, 483, (1953).

   (b) Ben-Ishai, R., Biochimica et Biophysica Acta., 26, 477, (1957).

7. Brachet, J., The Nucleic Acids, Vol. 1, 487, Academic Press N.Y. (1955).

8. Jensen, E.E., and Jensen, E., Exptl. Cellular Research, 3, 679, (1952).

9. Davidson, J.N., Cold Spring Harbour Symposia Quant. Biol., 12, 50, (1947).

10. Mandel, P., Jacob, H., and Mandel, L., Bull. soc. chim. biol., 32, 80, (1950).

11. Campbell, E.H., and Kenterlitz, F.W., Endocrinology, 6, 308, (1950).

12. Levy, H.B., Smith, E.E., and Schaeler, A.L., Arch. Biochem., 24, 198, (1949).

13. Mizuno, T., and Nakamura, T., Bull soc. chim. biol., 33, 1169, (1951).

14. Daly, M.M., Allfrey, V.G., and Mirsky, A.E., J. Gen. Physiol., 36 173, (1952).

15. Smellie, R.M.S., Melrose, W.M., and Davidson, J.N., Biochim. and Biophys. Acta., 11, 559, (1953).

16. Brachet, J., and Chantrenne, H., The Nucleic Acids, Vol. 2, 295, Academic Press, N.Y. (1955).

17. Lindenstrøm-Lang, K., Bull. soc. chim. biol., 22, 339, (1940).

18. Hancock, H., Physiol. Rev., 32, 806, (1952).

19. Lipmann, F., Federation Proc., 8, 597, (1949).

20. Spiegelman, S., Cold Spring Harbour Symposia Quant. Biol., 12, 256, (1946).

21. Dreeler, S.S., and Mikayan, E.I., Doklady Akad. Nauk., S.S.S.R., 72, 79, (1950.

22. Lounsbo, A.L., Enzymologia, 15, 251, (1951).

23. Neurath, F., Quart. Rev. Biol. 24, 93, (1949).

24. Neurath, F., Chemistry and Biology of Proteins, Academic Press, 334, (1950.

25. Ganer, G., Nature, 173, 318, (1954).

26. Borot, J.L., Arch. Sc. Phys. Nat., 10, 139, (1953).

27. Doole, R.N., Harris, R.J.C., and Rao, R.M.F., J. Chem. Soc., 1031, (1952).

28. Cassilli, P., Comptes Rendus Soc. de Biol., 146, 285, (1952).

29. Winters, F., and Rubin, R., Ind. Eng. Chem., 41, 460, (1949).

30. Partridge, S.M., And Brimley, R., Biochem. J., 51, 620, (1952).

31. Moore, S. and Stein, W., J. Biol. Chem., 178, 53, (1949).

32. Mirsky, A.E., and Pollister, A.W., J. Gen. Physiol., 30, 117, (1946).

33. Stedman, E. and Stedman, E., Nature, 152, 556, (1943).

34. Behov, S.S., Biokhimya, 16th, 334, (1951).

35. Lucy, J.A. and Butler, J.A.V., Biochem. et Biophys. Acta, 16, 431, (1955).

36. Kressman, T.R.E., Kitchener, J.A., J. Chem. Soc., 1190, (1949).

37. Kitchener, J.A., Ion Exchange Resin, Methuen's Chemical Monograph (1956).

38. Adams, B.A., Holmes, E.L., J. Soc. Chem. Ind., 54, 1 T, (1935).

39. The Dow Chemical Company, Midland, Michigan, Publication No. 2.

40. Davies, C.W., Biochem. J., 44, 30, (1949).

41. Hale, D.K., and Reichenberg, D., Disc. Faraday Soc., No. 7, 79, (1949).

42. Boyd, G.E., Adamson, A.W., and Myers, L.S., J. Amer. Chem. Soc., 69, 2836, (1947).

43. Kressman, T.R.E. and Kitchener, J.A., Disc. Faraday Soc., No. 7, 90, (1949).

44. Partridge, S.M., Nature, 158, 270, (1946).

45. Kunin, R., Anal. Chem., 22, 87, (1949).

46. Vickery, H.B., and Schmidt, C.L.A., Chem. Rev., 9, 169, (1931).

47. Cohn, E.J., Edsall, J.T., Proteins, Amino Acids and Peptides, Monography Series No. 1, 75, (1943).

48. Cohn, E.J., Physiol. Rev. 5, 349, (1925).

49. Cannan, R.K., Ann. N.Y. Acad. Sc., 47, 154, (1946).

50. Kunin, R., and Myers, R.J., Colloid Symp. Amer Chem. Centen June (1947)

51. Moore, S., and Stein, W.H., Ann. New York Acad. Sci., 49, 265, (1948).

52. Boissy, B.S., Kirschner, L.B., and Brandon, C., Arch. Biochem. and Biophys., 58, 145, (1955).

53. Crampton, C.F., Stein, W.H., and Moore, S., J. Biol. Chem., 225, 363, (1957).

54. Stocken, L., and Stocken, L., Nature, 166, 780, (1950).

55. Lusk, J.M., Cook, M.A., Eldredge, M.C., Haley, M.I., Rupke, D.W., and Ingraham, F.A., Arch. Biochem. and Biophys., 65, 449, (1956).

56. Craft, H.J., Mauritzen, C.M., Stocken, L., Nature, 174, 580, (1954).

57. (a) Khouvine, Y. and Baron, P., Bull. Soc. Chem. Biol., 33, 229, (1951).

(b) Khouvine, Y. and Gregoire, J., Comp. rend., 231, 1100, (1950).

58. Davison, P.F., Jones, R.N.F., Shooter, K.V., Butler, J.A.V., Biochem. et Biophys. Acta., 15, 435, (1954).

59. Davison, P.F., Butler, J.A.V., Biochem. et Biophys. Acta, 15, 439, (1954).

60. Conrad, H.F., Ann. Rev. Biochem., 25, 291, (1956).

61. Hirsky, A.E. and Pollister, A.W., J. Gen. Physiol., 29, 117, (1946).

62. Boissy, B.S., Kirschner, L.B., and Brandon, C., Arch. Biochem. and Biophys., 58, 145, (1955).

63. Daly, M.M., and Hirsky, A.E., J. Gen. Phys., 28, 405, (1955).

64. Kossel, A., Z. physiol. chem., 8, 511, (1884).

65. Kossel, A., The Proteamines and Histones: Longmans Green, New York and London, (1928).

66. Crampton, C.F., Lipshitz, R., and Gurgacz, J. Biol. Chem., 210, 499, (1954).

67. Crampton, C.F., Moore, S., and Stein, W.H., J. Biol. Chem., 215, 787, (1955).

68. Crampton, C.F., Moore, S., and Stein, W.H., J. Biol. Chem., 225, 363, (1957).

69. Crampton, C.F., Moore, S., and Stein, W.H., J. Biol. Chem., 221, 185, (1957).

70. Brown, G.L., and Watson, J.D., Nature, 172, 339, (1953).

71. Bowman, H.F. and Partridge, S.M., Biochem. J., 59, 543, (1955).

72. Trautman, R. and Crampton, C.F., Abstracts American Chemical Society 130th Meeting Atlantic City 9 C Sept. (1956).

73. Altman, R., Arch. Anat. u. physiol, Physiol. Abt. 528 (1889).

74. Kossel, A., Z. Physiol. Chem., 11, 284, (1887).

75. Hammarsten, O., Z. Physiol. Chem., 19, 19, (1894).

76. Lovenz, P.A. and London, E.J., J. Biol. Chem., 85, 721, (1929).

77. Holliday, E.R. and Johnson, E.A., Nature, 161, 216, (1948).

78. Watson, J.D. and Crick, F.H., Nature, 171, 737, (1953).

79. Cohn, W.E., J. Am. Chem. Soc., 72, 2471, (1950).

80. Cohn, W.E., Science, 109, 377, (1949).

81. Moore, S., Stein, W.H., J. Biol. Chem., 176, 337, (1948).

82. Leland, E.G. and Cynerod, W.G., Acta Chem. Scand., 8, 192, (1954).

83. Wahlin, E., Ehym, J.E., and Cohn, W.E., J. Am. Chem. Soc., 75, 1503, (1953).

84. Mirsky, A.E. and Pollister, A.W., J. Gen. Physiol., 30, 117, (1946).

85. Littlefield, E., Z. Physiol. Chem., 10, 473, (1894).

86. Kutscher, G., Z. Physiol. Chem., 28, 215, (1903).

87. Pollister, A.W. and Mirsky, A.E., J. Gen. Physiol., 30, 101, (1946).

88. Kirsky, A.E. and Pollister, A.W., Proc. Natl. Acad. Sc. U.S., 28, 234, (1942).

89. Chargaff, E., The Nucleic Acids Vol. I, 313, Academic Press, N.Y., (1955).

90. Kerr, S.E.H. and Bernhardsen, E., J. Biol. Chem., 180, 1203, (1949).

91. Sanchis, M., Florio, A., Carracedo, L.R., J. Biol. Chem., 181, 713, (1949).

92. Fels, M.H., Butler, J.A.V., Biochem. J., 46, 399, (1950).

93. Butler, J.A.V., Conway, B.E., Jones, D.W., J. Am. Chem. Soc., 70, 612, (1954).

94. Schmidt, C.L.A., (Editor) The Chemistry of Amino Acids and Proteins, Thomas, Springfield, Illinois, 217, (1938).

95. Moore, S. and Stein, W.H., J. Biol. Chem., 176, 367, (1948).

96. Moore, S. and Stein, W.H., J. Biol. Chem., 211, 907, (1954).

97. Kroll, H. and Gerard, R.W., J. Biol. Chem., 210, 803, (1953).

98. Brick, G., Biochimica et Biophysica Acta, 11, 11, (1953).

99. Hunt, R.E. and Academia, A., Biochimica et Biophysica Acta, 12, 161, (1953).

100. Tolbin, B., J. Am. Chem. Soc., 73, 1533, (1951).

101. Zamenhof, S. and Chargaff, E.J., Biol. Chem., 195, 49, (1952).