ADSORPTION OF BILIRUBIN BY POLYMER SORBENTS

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Ph.D CHEMISTRY

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ABSTRACT

Adsorption of bilirubin from aqueous buffer (pH = 7.8) by PVP, by cholestyramine and by amino acid containing pendants, which have been immobilized onto a polystyrene (Merrifield) resin or a water swellable polyamide resin using the solid phase peptide synthesis methods, have been studied. The adsorption of bilirubin by the pendants on the Merrifield resin was minimal while PVP and cholestyramine adsorbed some bilirubin. However, the best adsorbents were the immobilized amino acids on a water swellable polyamide resin.

A systematic study of the effect of the changes in the amino acid composition of the pendant, both in type and number, on the adsorption by the polyamide resins indicates that the charge density, contributed by the R groups of the amino acids in the pendant, is the major factor in the adsorption process. However, some adsorption also occurs at the α-amino groups. Effects due to the conformation of the peptide chains are also indicated. Of the resins studied, those with peptide pendants containing arginine or lysine form the most efficient adsorbents for bilirubin in aqueous buffer solution.

Studies of the adsorption of bilirubin from bilirubin solutions containing bovine serum albumin as well as studies of desorption of bilirubin from the resins by bovine serum albumin indicates that some resins containing arginine in the pendants can successfully compete with albumin. Stoichiometric binding constants obtained for the polyamide resins by the method of Klotz are of the order of $1 \times 10^3\ M^{-1}$ to $86 \times 10^3\ M^{-1}$. These binding constants are lower than that of the reported values for the first binding site of bilirubin on albumin by a factor of 10^1 to 10^4 and lower by a factor of 10 to 10^3 than the values reported for the second binding site.

Ph.D CHIMIE

D.S. Henning

L'ADSORPTION DE LA BILIRUBINE PAR DES ADSORBANTS POLYMERIQUES

RESUME

Cette étude porte sur l'adsorption de la bilirubine en solution aqueuse tamponnée (pH = 7.8) par le PVP, la cholestyramine et par des acides aminés greffés par la méthode de synthèse de peptides en phase solide sur une résine de polystyrène (Merrifield) ou sur une résine de polyamide gonflée en milieu aqueux. L'adsorption de la bilirubine par les acides aminés greffés sur la résine de Merrifield est minimale alors qu'elle est appréciable pour le PVP et la cholestyramine. Les meilleurs résultats sont obtenus avec les acides aminés greffés sur la résine de polyamide.

En variant la composition des séquences d'acides aminés greffées sur les résines de polyamide il est démontré que le procédé d'adsorption est principalement relié à la densité de charge apportées par les groupements R des acides aminés. Cependant, l'adsorption peut aussi se produire au niveau des groupements α-amino. De plus, des effets dûs à la conformation des chaînes peptidiques sont aussi indiqués. Parmi les résines étudiées, les plus efficaces pour l'adsorption de la bilirubine en milieu aqueux tamponné sont celles possédant des groupes latéraux formés d'arginine ou de lysine.

Des études de l'adsorption de la bilirubine dans des solutions contenant de l'albumine de sérum de boeuf ainsi que des mesures de la désorption de la bilirubine, préalablement adsorbée sur les résines de polyamide, par l'albumine de sérum de boeuf indiquent que certaines résines ayant des arginines dans les groupes latéraux peuvent toujours adsorber la bilirubine malgré la présence d'albumine. Les constantes de fixation des résines de polyamide obtenues de façon stoechiométrique par la méthode de Klotz sont de l'ordre de $1 \times 10^3\ M^{-1}$ à $86 \times 10^3\ M^{-1}$. Ces constantes sont inférieures de 10^1 à 10^4 aux valeurs de la littérature pour le premier site de fixation de la bilirubine par l'albumine et elle le sont de 10 à 10^3 fois pour le second site.

To my family

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

CHAPTER II

ADSORPTION OF BILIRUBIN

BY POLYVINYLPYRROLIDONE AND CHOLESTYRAMINE

TABLE OF CONTENTS (Cont'd) page

TABLE OF CONTENTS (Cont'd)

LIST OF FIGURES

LIST OF FIGURES (Cont'd) page

CHAPTER III

CHAPTER IV

CHAPTER V

LIST OF FIGURES (Cont'd) page

CHAPTER VI

APPENDIX 2

LIST OF TABLES

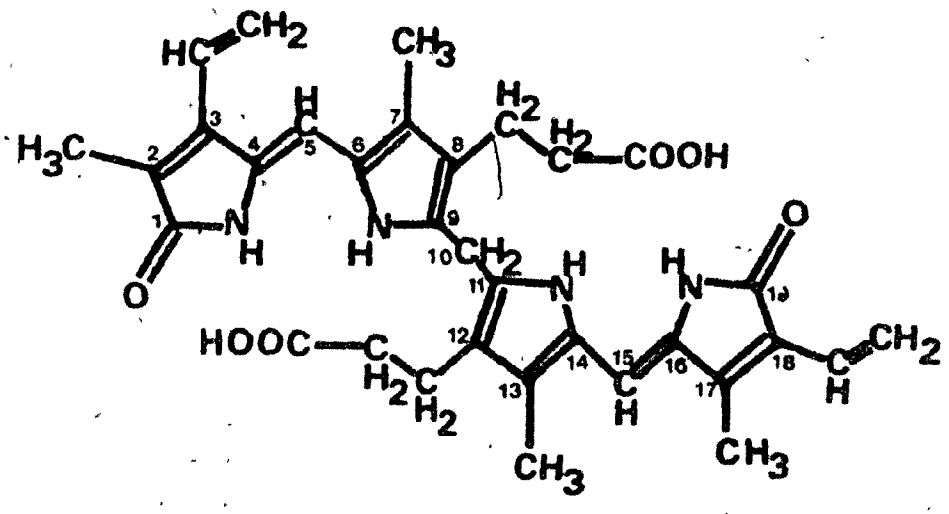
LIST OF TABLES (Cont'd)

CHAPTER I

INTRODUCTION

1) Metabolism of Bilirubin

Bilirubin is a red-orange bile pigment usually represented as a linear tetrapyrrole [1]. It is the



[1]

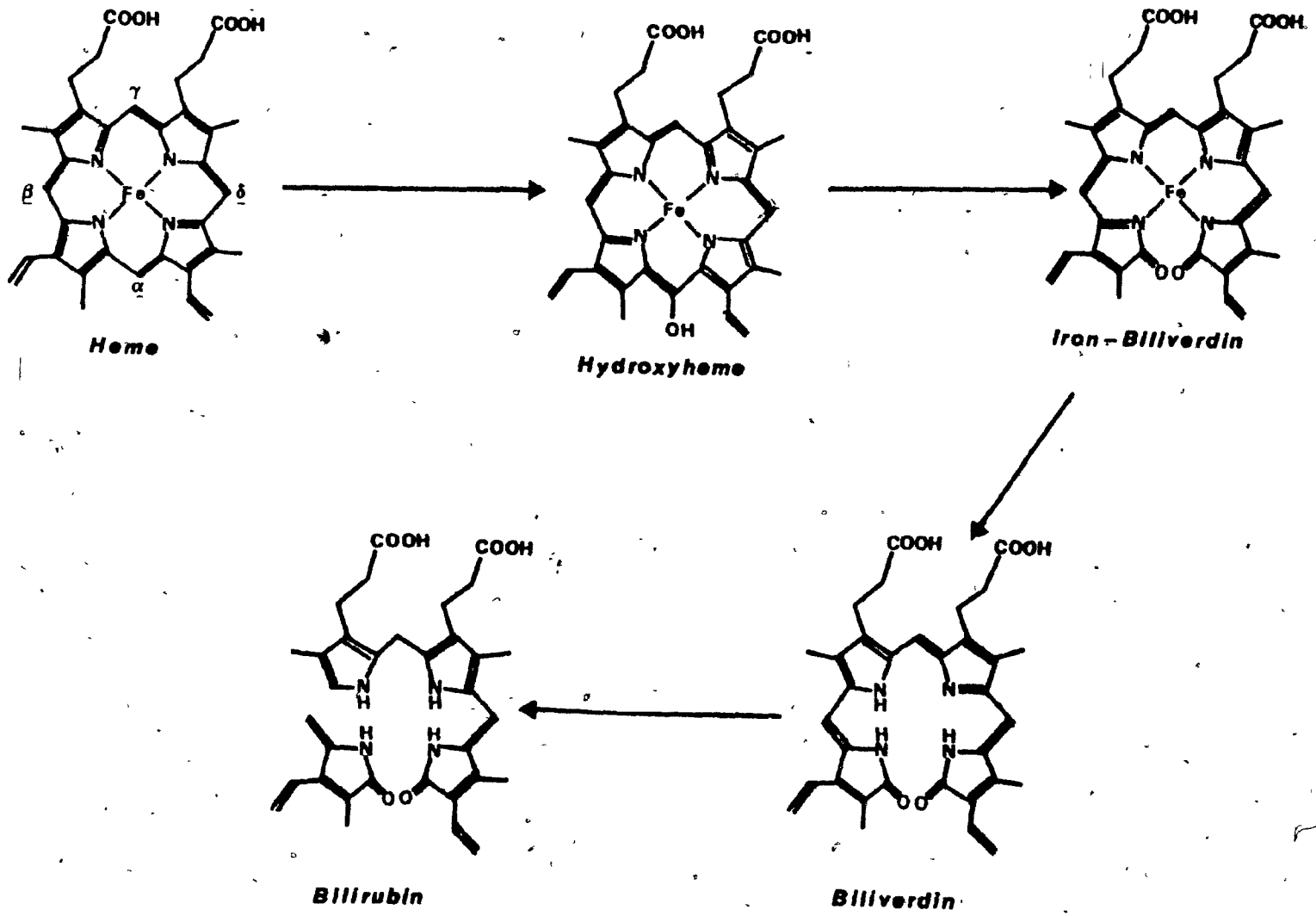
pigment, found in all mammals, that is responsible for the yellow colour seen in the skin of patients with jaundice. Bilirubin is a toxic waste product that serves no known purpose in the body.

In man approximately 4.4 mg of bilirubin is produced daily per kg of body weight (Berk, et al., 1974), of which approximately 70 to 80% comes from the degradation of the heme part of hemoglobin (Fig. 1) in senescent red blood cells. This degradation occurs mainly in the liver, spleen, and bone marrow (Scharschmidt and Gollan, 1979). The remaining 20 to 30% is produced in the liver from free heme (Berk, et al., 1976). Heme degradation is enzyme

FIGURE 1

CHEMICAL STRUCTURE AND SEQUENCE

FOR HEME DEGRADATION


COOH
COOH
γ
β
δ
α
N
Fe
Heme
OH
Hydroxyheme
O
Iron – Biliverdin
Biliverdin
Bilirubin
H

catalysed, by heme oxygenase, and is thought to follow the reaction scheme shown in Fig. 2 (Kikuchi and Yoshida, 1983). Due to the configuration of the enzyme, this reaction is stereoselective, so that the α-methene bridge (Fig. 1) is cleaved to yield bilirubin IXα (Fig. 3) in preference to other isomers (Brown S.B., 1976). In fact, less than 5% of bilirubin isomers other than the IXα are found in appreciable quantity in plasma. Commercial preparations of bilirubin contain another group of the bilirubin isomers, the IIIα and the XIIIα, arising from "isomeric scrambling" (Fig. 3) (McDonagh and Assisi, 1972). These isomers are not formed in the presence of albumin and should not occur in-vivo (McDonagh, 1975).

Once in plasma, bilirubin is transported to the liver as a complex formed with albumin. The purpose of the complexation appears to be two fold: 1. It prevents the toxic bilirubin from crossing the cell barrier and entering the tissues, and 2. It solubilizes the hydrophobic bilirubin. At the liver barrier, the bilirubin free of albumin is taken up by the hepatocytes, probably by a carrier mediated mechanism (Scharschmidt, et al., 1975, Scharschmidt and Gollan, 1979). It is still not clear whether or not the dissociation of bilirubin and albumin occurs near the cell membrane and is followed by transport of bilirubin to the cell membrane or if there are receptor sites for albumin on the membrane where the bilirubin-albumin complex is dissociated and the bilirubin taken up immediately by the liver cell (Weisiger, et al., 1981).

FIGURE 2

PROPOSED MECHANISM OF HEME DEGRADATION

(Kikuchi and Yoshida, 1983)

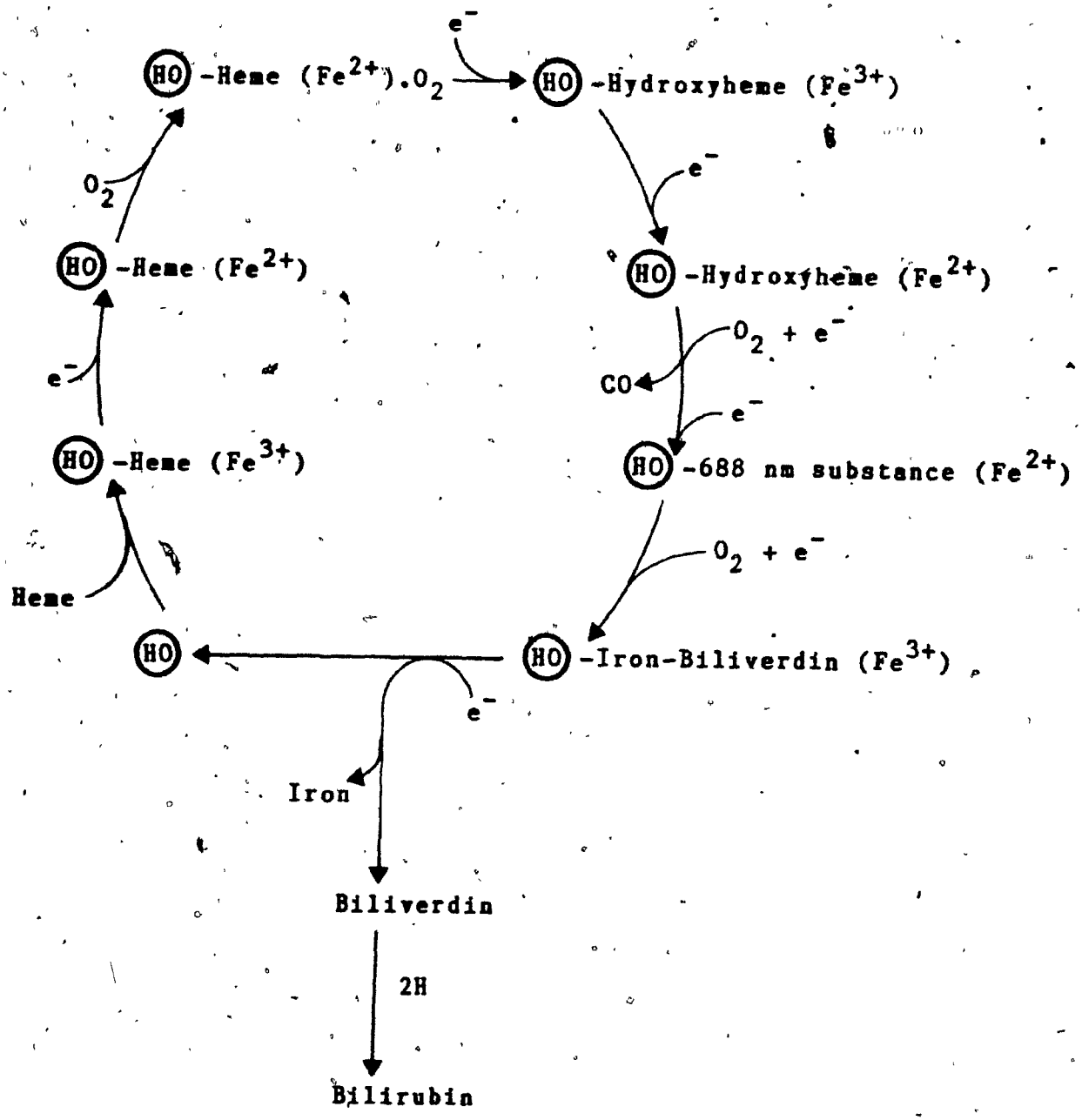
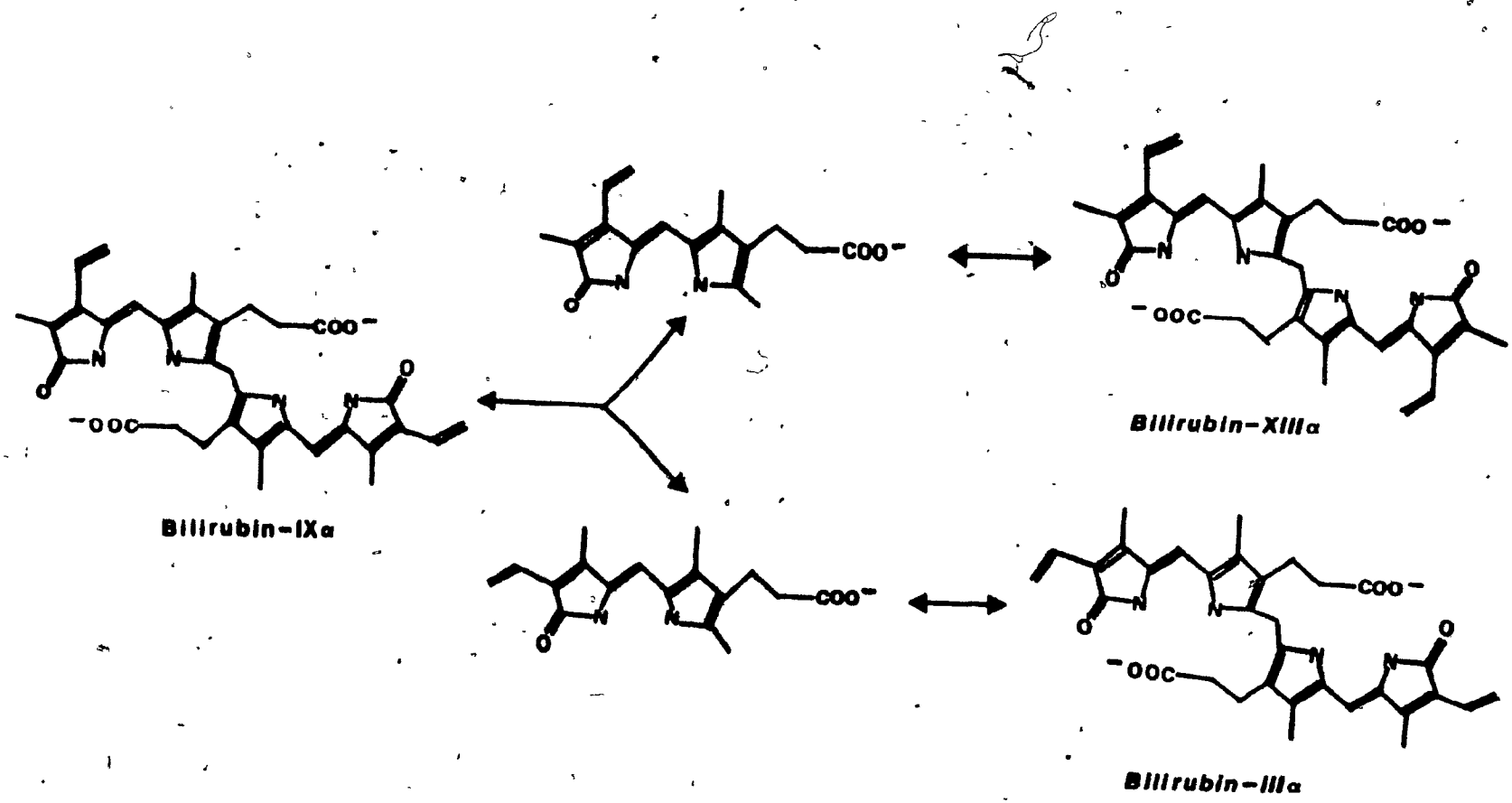

HO -Heme (Fe^{2+}).O_2
e^-
HO -Hydroxyheme (Fe^{3+})
O_2
HO -Heme (Fe^{2+})
e^-
HO -Hydroxyheme (Fe^{2+})
O_2 + e^-
CO
e^-
e^-
HO -Heme (Fe^{3+})
HO -688 nm substance (Fe^{2+})
Heme
O_2 + e^-
HO
HO -Iron-Biliverdin (Fe^{3+})
e^-
Iron
Biliverdin
2H
Bilirubin
HO = Heme Oxygenase

FIGURE 3

ISOMERIC SCRAMBLING OF BILIRUBIN

(McDonagh and Assisi, 1972)


Bilirubin-IXα
Bilirubin-XIIIα
Bilirubin-IIIα

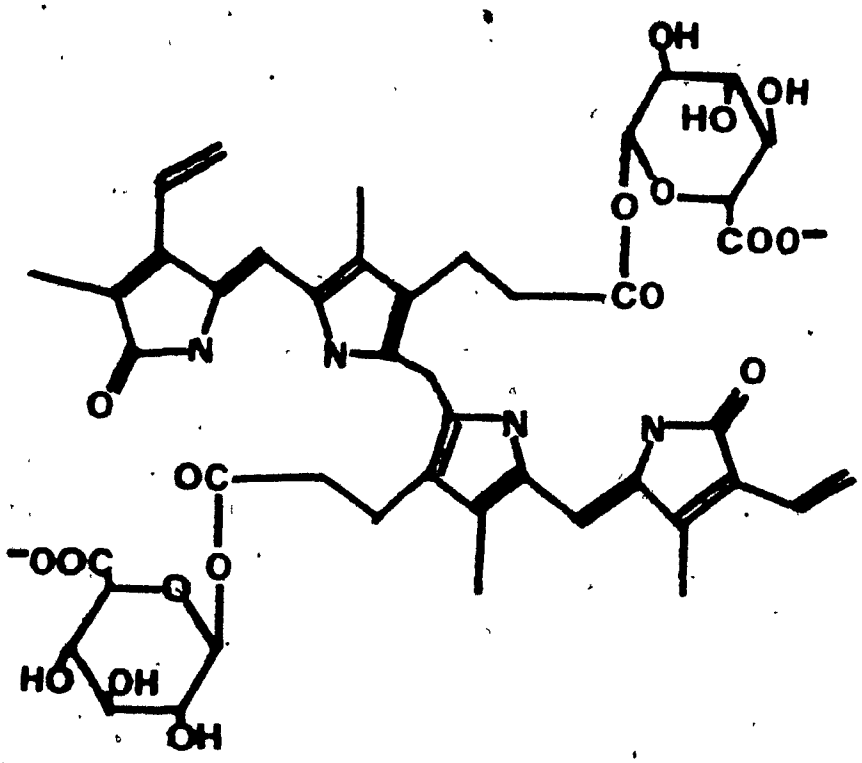
Inside the liver cell bilirubin is bound to cytoplasmic proteins; mainly ligandin and Z protein (Fevery and Heirwegh, 1980). It is then esterified, with glucuronic acid, to form bilirubin diglucuronide or "conjugated" bilirubin (Fig. 4). The first step in this process is enzyme catalyzed by UDP-glucuronyltransferase. Whether or not the second step is catalyzed by the same enzyme (Pathway one) or by a different enzyme, which would tansfer one glucuronic acid moiety from one bilirubin molecule to another to yield one bilirubin diglucuronide and one unconjugated bilirubin (Pathway two), still remains to be determined (Fevery, et al., 1977, Fevery and Heirwegh, 1980).

After conjugation bilirubin diglucuronide is excreted from the liver cells into bile. Apparently conjugation is essential for this process and, under normal circumstances, the amount of unconjugated bilirubin found in bile is very small. The necessity for conjugation may be due to a requirement of the bilirubin to "fit with a specific carrier" (Scharschmidt and Gollan, 1979) or for the disruption of the intramolecular hydrogen bonds to yield more hydrophilic products that are more easily excreted. However, experiments with non α-isomers of bilirubin and with photopigments, both of which lack the hydrogen bonds and are found in bile in their unconjugated form, suggest that the latter postulate is the more probable (Scharschmidt and Gollan, 1979). Finally, bilirubin is transformed to urobilinogens by bacterial flora in the

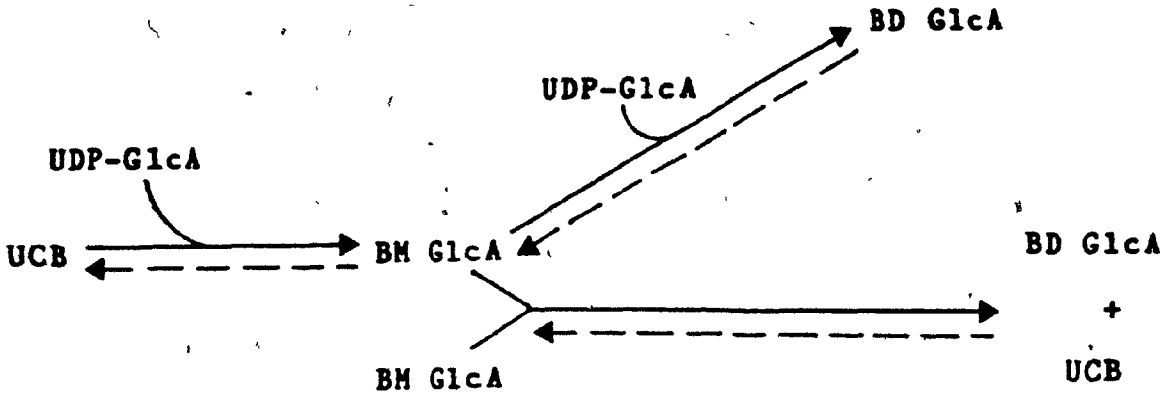
FIGURE 4

A) BILIRUBIN DIGLUCURONIDE

B) PROPOSED PATHWAYS FOR THE CONJUGATION OF BILIRUBIN (Fevery et al., 1977)



[A]



UCB = unconjugated bilirubin
BM = bilirubin monoglucuronide
GlcA = glucuronyl transferase
UDP = uridine 5'-diphosphate

[B]

intestine and excreted in the faeces.

Any defect or illness that effects the above mentioned processes can lead to hyperbilirubinemia (Fevery, et al., 1977, Fevery and Heirwegh, 1980, Petryka and Howe, 1979) (Table I), an abnormally high concentration of bilirubin in the plasma, which under pathological conditions becomes as high as 10^{-3} M (McDonagh, 1979), compared to a concentration of less than 1.7×10^{-5} M in the normal adult (Bloomer, et al., 1971). When hyperbilirubinemia prevails, free bilirubin can enter the tissues where it modifies the activity of many enzymes (Weisiger, et al., 1981). Newborns, especially prematures, are more susceptible than adults to hyperbilirubinemia. In newborns, the bilirubin production is generally higher than it is in adults, due to a faster turnover of the red blood cells (Fevery, et al., 1977, Fevery and Heirwegh, 1980), while their conjugating enzymes are not mature until approximately 14 days after birth (Golan and Schmidt, 1982). Furthermore, premature babies have a lower concentration of albumin and that albumin has a lower binding capacity for bilirubin (Fevery, et al., 1977, Fevery and Heirwegh, 1980). The blood barrier of the newborn is permeable to bilirubin. Thus, the accumulation of bilirubin in the brain, known as kernicterus, is very common in babies with hyperbilirubinemia. Consequences of brain damage caused by kernicterus range from learning disabilities, in mild cases (Johnson and Boggs, 1974), to lasting brain damage or even death, in more severe cases (Hansen, et al., 1979,

TABLE I

HYPERBILIRUBINEMIA

Step in the Metabolism	Abnormality
production	- faster destruction of red cells - increase in destruction of immature red blood cells
transport	- competitive binding - low albumin concentration
hepatic uptake	- competitive binding
conjugation	- enzyme deficiency - inhibition by drugs
secretion in bile	- hepatitis - cirrhosis
elimination	- mechanical obstruction

Scharschmidt and Gollan, 1979).

2) Physical and Chemical Properties of Bilirubin

Before discussing the different methods available for the treatment of hyperbilirubinemia, some of the chemical and physical properties of bilirubin will be considered. Although it has stimulated the curiosity of numerous researchers, many of the chemical and physical properties of bilirubin are still not well known, mainly because of experimental difficulties.

The solubility of bilirubin generally increases with increasing polarity of the solvent. Hence, it is very soluble in formamide, DMSO, CH_2Cl_2, and $CHCl_3$, less soluble in aromatic solvents, such as benzene and pyridine, and virtually insoluble in apolar solvents, such as hexane and cyclohexane. In water bilirubin solubility is low, approximately 0.007 μM (Brodersen, 1979). The solubility increases with an increase in pH. Consequently, bilirubin is very soluble in alkaline solutions in which the two carboxylic protons are lost so that a soluble salt is formed. Thus, aqueous solutions can be prepared by first dissolving the solid in a small amount of base, e.g., NaOH, and diluting with an appropriate buffer. Aqueous solutions of bilirubin near neutral pH are stable for several hours. If they are supersaturated, colloidal suspensions are formed which are not necessarily visible to the naked eye. The tendency of bilirubin to form supersaturated solutions has resulted in large errors in

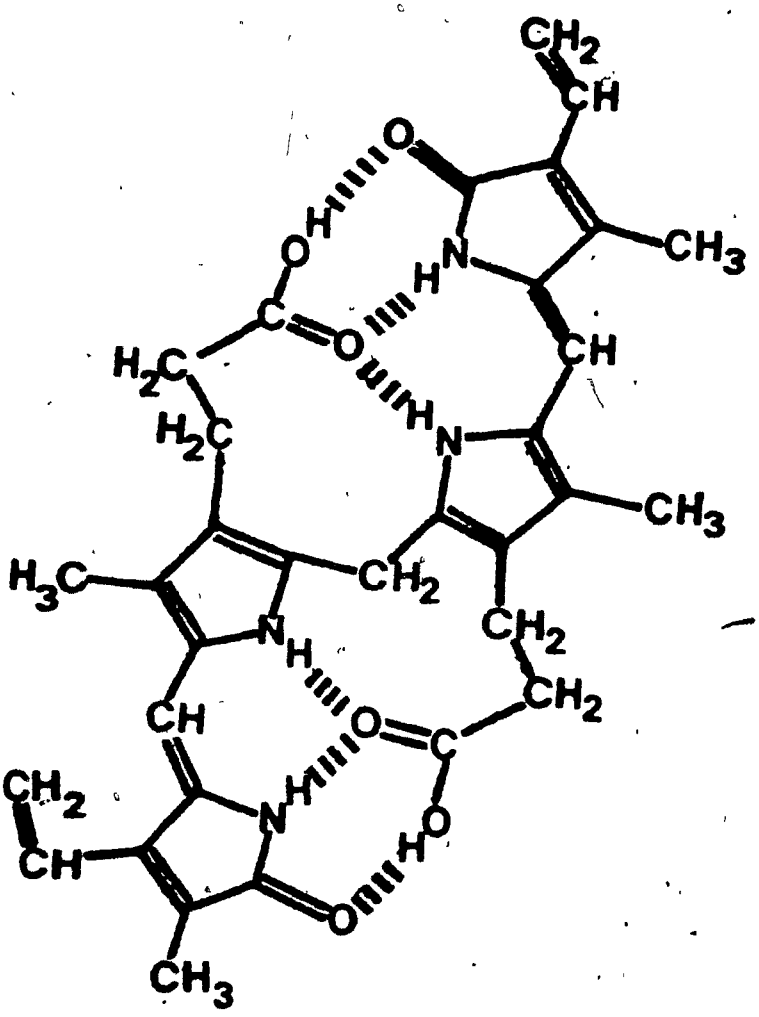
the determination of the solubility of bilirubin in water so that a wide range of values have been reported. For example, a reported solubility as high as 100 μM (Burnstine and Schmidt, 1962) probably refers to a supersaturated solution. Using its solubility at pH 8.5, where bilirubin is more soluble, Brodersen (Brodersen, 1980) calculated the bilirubin solubility at pH 7.4 to be 4 nM, in fair agreement with the value of 7 nM obtained experimentally (Brodersen, 1980).

The hydrophobicity of bilirubin is rather unexpected. Since bilirubin has several groups, such as two carboxyls, two lactam NH's, two pyrrol NH's, and two lactam carbonyls, all with hydrophilic character, it would be thought to be water soluble. The answer to this apparent paradox was first given by Fog and Fellum (Fog and Fellum, 1963) who proposed that the hydrophilic groups may be involved in internal hydrogen bonding. Using X-ray diffraction measurements, Bonnet, et al. (Bonnet, et al., 1978) recently proposed the now accepted structure for crystalline bilirubin (Fig. 5). This "ridge tile" structure has a Z configuration at both the C_5 and C_{15} methene bridges. The presence of six intramolecular hydrogen bonds leaves the hydrophilic groups saturated inside the structure and the hydrophobic groups outside at the surface of the molecule. Whether or not the aqueous bilirubin dianion, obtained by the formation of the soluble bilirubin salt in alkaline medium, retains some of the intramolecular bonds has not been elucidated. However,

FIGURE 5

INTRAMOLECULAR HYDROGEN BONDS IN BILIRUBIN

(Bonnet, et al., 1978)


CH2
CH
O
OH
N
H
CH3
C
O
H2C
CH
H
N
H2C
CH3
H3C
CH2
CH2
N
H
CH2
CH
O
C
CH2
N
H
O
CH2
CH
H
O
CH3

Mugnoli, et al. (Mugnoli, et al., 1983) showed by X-ray diffraction measurements that bilirubin isopropylammonium salt in chloroform retains four of the six intramolecular hydrogen bonds.

Bilirubin absorbs visible light strongly and in aqueous solution an absorption maximum is observed at 438 - 440 nm with an extinction coefficient of the order of 4.7×10^4 l mole^{-1} cm^{-1} (Lee and Gartner, 1976). The absorbance spectum is insensitive to pH, in the region of 8 to 11 (Hansen, et al., 1979), and obeys Beer's law at concentrations below 10 mg/dl. Consequently, UV-visible spectroscopy is a useful analytical tool for the determination of the concentration of bilirubin in aqueous solution. Upon addition of albumin a red "shift" occurs, to λ_{max} = 460 nm, with an increase in the extinction coefficient at the maximum. However, the extinction coefficient at 438 - 440 nm remains essentially unchanged (Shapovalenko and Kolosov, 1978), although some small variation (less than 4%) in the absorbance of the solution can sometimes be observed.

The acid-base properties of bilirubin are still under debate. The lactam groups ionize at a pH in the region of 12 to 14, depending on the solvent (Ostrow and Celic, 1984, Hansen, et al., 1979). However, agreement on the pK_a of the carboxyl groups has not been reached. Numerous authors report pK_a values of approximately 4 - 5 for the carboxyl groups, as expected for propionic acid side chains. For example, Lee, et al. (Lee, et al., 1974)

compared the data for potentiometric titration of bilirubin and of other dicarboxylic acids with the known pK_a in DMF to obtain values of 4.3 and 5.3. From potentiometric titration of bilirubin in DMSO, as well as titration in aqueous solution coupled with ^{13}C NMR studies, Hansen, Thiessen and Brodersen determined that the carboxyls have a pK_a of 4.4 (Hansen, et al., 1979). In contrast, a value of 7 - 8 was also reported for the pK_a of the carboxyl groups based on titration of aqueous bilirubin (Krasner and Yaffe, 1973, Gray, et al., 1961). However, according to McDonagh (McDonagh, 1979) and Brodersen (Brodersen, 1980), the observed inflection point does not necessarly correspond to the pK_a of the acids since bilirubin starts to precipitate around that pH. Thus, the shape of the titration curve is a function not only of pH but also of solubility.

As a result of the intramolecular hydrogen bonds bilirubin-IXα is slightly more stable than other rubins or verdins. In the crystalline form bilirubin can be stable for at least one to two years provided that it is stored in a dark, cool environment (McDonagh, 1979). However, as mentioned previously, aqueous solutions of bilirubin are not stable. In addition to the colloid formation mentioned previously, bilirubin undergoes oxidation in the presence of even trace amounts of oxygen to yield biliverdin and other degradation products. The instability increases with increasing pH, so that alkaline solutions of bilirubin decompose rapidly. Between the precipitation of bilirubin,

at pH's lower than approximately 7.4, and the oxidation at a slightly higher pH, there exists only a narrow margin of pH in which aqueous bilirubin solutions are stable even for a short period of time. Addition of albumin solubilizes bilirubin and slows down the rate of the oxidation process (Lightner, et al., 1976). Thus, albumin/bilirubin solutions are experimentally easier to handle.

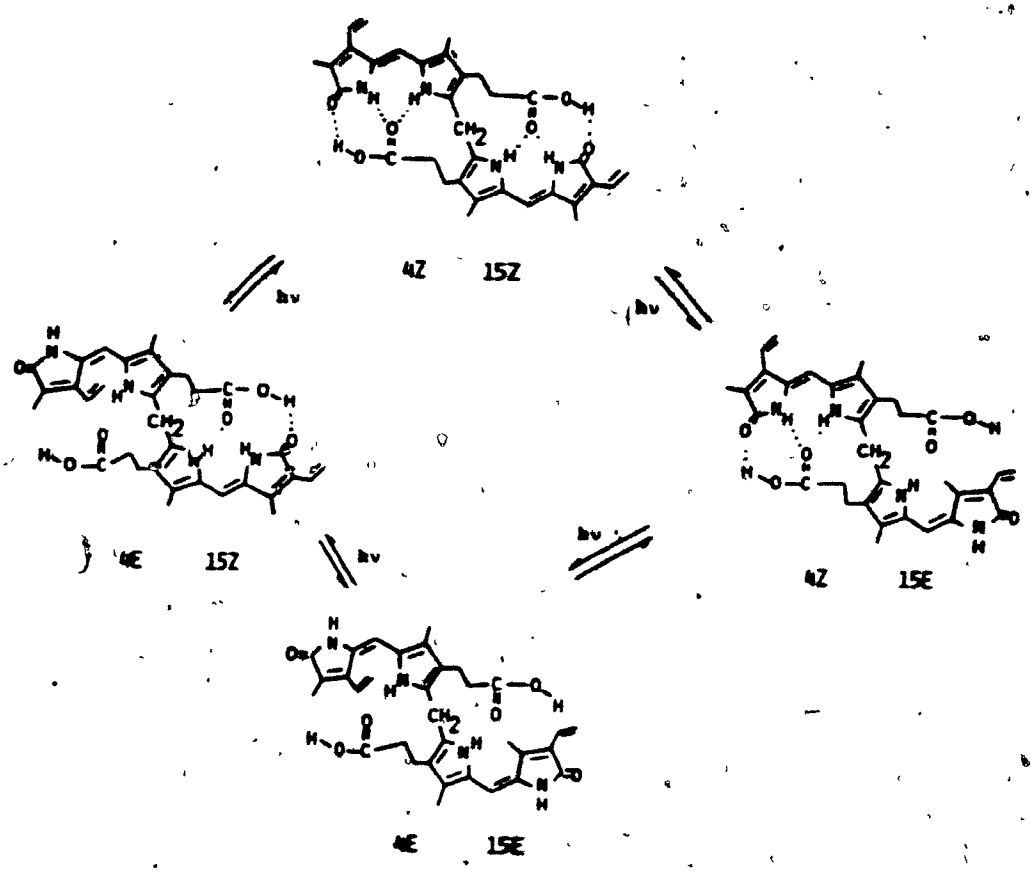
Bilirubin is also light sensitive so that it should be handled under subdued light (McDonagh, 1979). After a few minutes of irradiation, photooxidation occurs to yield several decomposition products. The nature and distribution of these products depends on three factors: the nature of the solvent, the excitation energy, and the presence of additives such as quenchers (Bonnet, 1976). A second process also occurs when a solution of bilirubin is exposed to UV or visible light, it is almost instantaneous and occurs in the absence of oxygen (McDonagh, 1974, 1979). This second process is thought to be the photoisomerization of the ZZ isomer of bilirubin to EZ, ZE, EE isomers and other photoproducts (Fig. 6) (Lightner, et al., 1979, McDonagh, et al., 1979).

3) Treatment of Hyperbilirubinemia

Many methods have been devised for the treatment of hyperbilirubinemia, including phototherapy, hemoperfusion, ingestion of adsorbent, or a combination of phototherapy and ingestion.

FIGURE 6

BILIRUBIN PHOTOISOMERS


4Z 15Z
hν
hν
4E 15Z
hν
hν
4Z 15E
4E 15E

a) Phototherapy and Photobilirubin

The irradiation of the patient with blue light, known as phototherapy, is now the method of choice in the treatment of neonatal hyperbilirubinemia (Kalpoyiannis, et al., 1982, Moseley and Summer, 1983). It was introduced in 1958 as a result of an observation of an "apparent fading away of the yellow pigmentation in the skin of jaundiced babies when they had been a short time in the sunlight" (Cremer, 1958). The mechanism for the elimination of bilirubin during phototherapy seems to be quite complex and is not yet fully understood. It was first thought that photooxidation (Cremer, 1958) or photooxygenation (McDonagh, 1976) was responsible for the reduction of bilirubin during phototherapy. However, new insight into the phototherapy mechanism was obtained when Ostrow (Ostrow, 1971) found that unconjugated bilirubin, along with other water soluble bilirubin derivatives, is excreted in the bile of Gunn rats undergoing phototherapy. Gunn rats lack the ability to conjugate bilirubin and thus cannot normally eliminate it. Since it was known that bilirubin cannot normally be excreted unless it is first conjugated with glucuronic acid, it was postulated that phototherapy must cause bilirubin to be modified in a way such that it can cross the liver barrier. McDonagh (McDonagh, 1976) suggested that phototherapy promotes the formation of a bilirubin isomer which is excreted more easily than the parent pigment but which, once in the gastrointestinal tract, reverts back to the parent

bilirubin isomer. This would account for the presence of the unconjugated bilirubin detected in bile. Direct evidence, by difference UV-visible spectroscopy, for the in-vitro formation of a photolabile bilirubin derivative was first presented by Davies and Keohane (Davies and Keohane, 1970, 1973). The difference spectrum of irradiated and non-irradiated bilirubin solutions showed a "gain" peak at around 490 nm, while a "loss" peak appeared at 465 and 420 nm. This observation was confirmed by Lightner, Wooldridge and McDonagh (Lightner, et al., 1979, McDonagh, et al., 1979). Evidence for the existence of "photobilirubin" was also obtained by Onishi, et al. (Onishi, et al., 1980). On the basis of HPLC data they showed that upon irradiation the bilirubin concentration decreases with simultaneous formation of a new, more polar substance. These products are unstable and thermally revert back to bilirubin; the reversion is enhanced by light, trifluoroacetic acid and iodine.

Due to their instability the products could not originally be isolated or characterized. However, from X-ray crystallography it was known that bilirubin is in the ZZ conformation (Bonnet, 1976). Based on the Z --> E isomerization of other bilirubin-like molecules, Pedersen, et al. (Pedersen, et al., 1977) and Lightner, et al. (Lightner, et al., 1979, McDonagh, et al., 1979) proposed a similar Z --> E isomerization at the C_5 and/or C_{15} bridge of bilirubin to yield the ZE, EZ and EE isomers. These isomers would account for the appearance of bilirubin in

bile. Since they are more polar, hence more water soluble, than bilirubin they could be excreted without conjugation (McDonagh, et al., 1980).

The first isolation of photobilirubin was accomplished by Stoll, Ostrow, Zenone and Zarembo (Stoll, et al., 1979); two pairs of photoisomers IA and IB, IIA and IIB were isolated on silica gel. The more polar and more stable pair, component II, was assigned as being two rotomers of the EE isomer. These rotomers showed spectral and chemical properties similar to those of a major photoproduct detected during phototherapy. The less polar and less stable isomer IA was assigned the ZE conformation while IB was assigned the EZ conformation. These two isomers could well account for the unconjugated bilirubin seen in the bile of rats undergoing phototherapy. Although they had no direct evidence for these stuctures, several observations supported this postulate: 1. Higher polarity arising from the greater freedom of the carboxyl groups to ionize as a result of the disruption of H bonds; 2. Absorption at shorter wavelengths, i.e. lower λ_{max} of the photoproducts due to loss of coplanarity; 3. Appearance of IR bands at 2850 and 2920 cm^{-1}, due to the modification of carboxyl-OH groups, again due to disruption of H-bonds.

Stoll, et al. (Stoll, et al., 1981) showed that ^{14}C-bilirubin, which had been irradiated in $CHCl_3$, evaporated, dissolved in serum, and injected into Gunn rats, was excreted in bile as bilirubin and "photobilirubin". This provided direct evidence that the in-vitro and in-vivo

photoproducts are probably identical. When the different isomers of photobilirubin were isolated and separately injected into the rats, components IA and IB were excreted mainly as the parent bilirubin isomer, although some unreverted isomers could be still be detected. However, photobilirubin II remained unchanged during the passage through the rats.

The first structural evidence for the E --> Z isomerization was provided by Stoll, et al. (Stoll, et al., 1982) from the proton NMR study of very pure photobilirubin II. Analysis of the NMR spectra, together with chemical considerations, suggested that the IIA and IIB isomers are diasterioisomers in which the C_3 vinyl groups have cyclized intramolecularly (Fig. 7). Another photoproduct, photobilirubin III, was also reported and is probably similar to the photobilirubin II but with the EE configuration. A proposed relationship between the different photoproducts of Stoll, et al. and Onishi, et al. (Stoll, et al., 1982, Onishi, et al., 1984) is shown in Figure 7. The structures shown for the cyclobilirubins are those proposed by Onishi, et al. (Onishi, et al., 1984) and are identical to one of the structures proposed originally by Stoll, et al. (Stoll, et al., 1982). The reaction schemes proposed by the two groups are also identical.

Phototherapy has been used successfully in many cases (Kalpoyiannis, et al., 1982). However, some side effects have been noted and the long term morbidity has not yet been assessed (Kalpoyiannis, et al., 1982). The "bronze

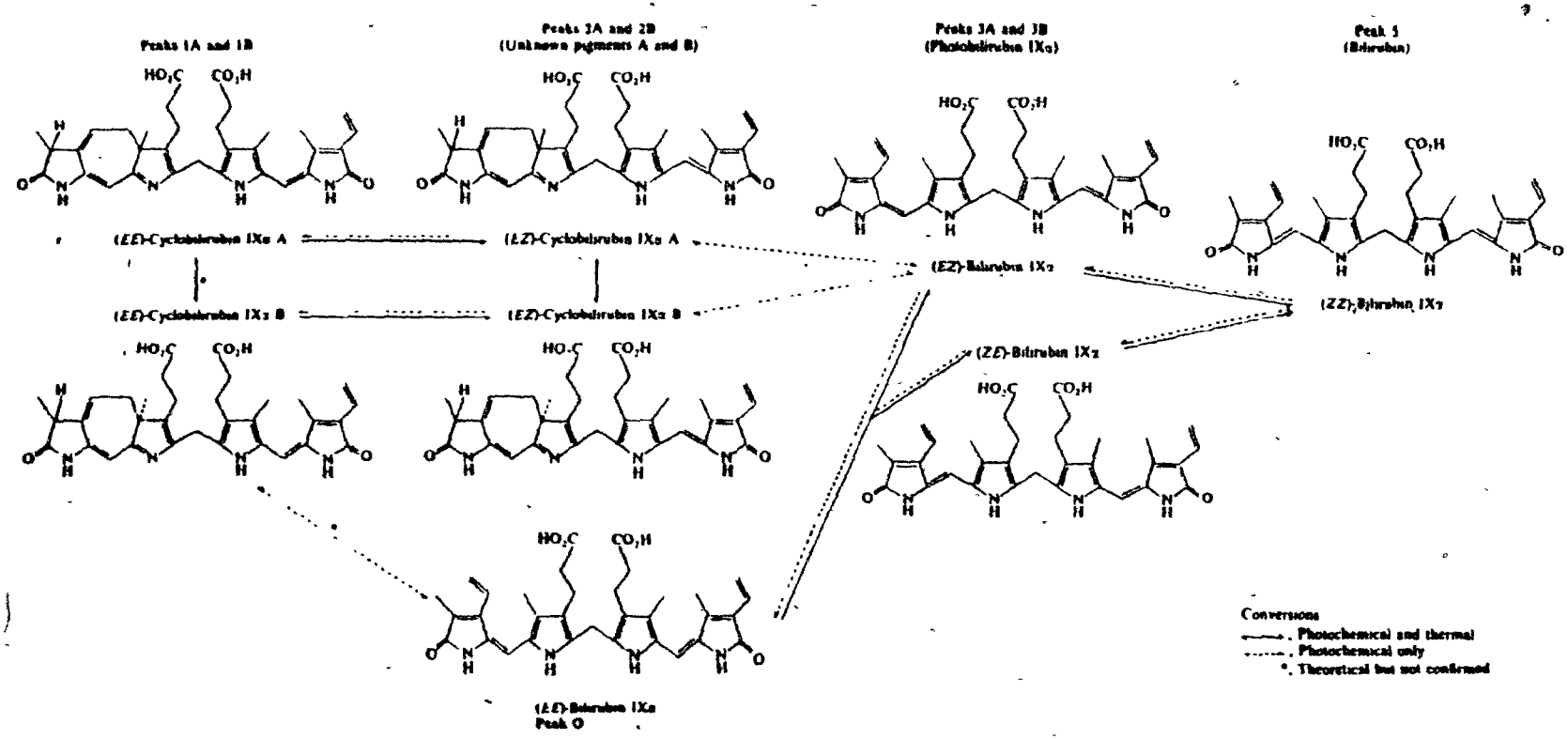
FIGURE 7

STRUCTURE AND RELATIONSHIP

BETWEEN BILIRUBIN AND ITS PHOTOPRODUCTS

(Onishi, et al., 1984)

Photobilirubin I = Peak III
Photobilirubin II = Peak II
Photobilirubin III = Peak I


Peaks 1A and 1B
Peaks 2A and 2B
(Unknown pigments A and B)
Peaks 3A and 3B
(Photobilirubin IXα)
Peak 5
(Bilirubin)
HO2C
CO2H
(EE)-Cyclobilirubin IXα A
(EZ)-Cyclobilirubin IXα A
(EZ)-Bilirubin IXα
(ZZ)-Bilirubin IXα
(EE)-Cyclobilirubin IXα B
(EZ)-Cyclobilirubin IXα B
(ZE)-Bilirubin IXα
(EE)-Bilirubin IXα
Peak 0
Conversions
——→ : Photochemical and thermal
------→ : Photochemical only
*: Theoretical but not confirmed

baby" syndrome, thought to be an accumululation of photoproduct II polymerized to yield an insoluble bile pigment, is one of the side effects of phototherapy (Onishi, et al., 1982, Tan and Jacob, 1982). Carcinogenic potential and damages to the genetic material resulting from exposure to light (Santella, et al., 1978, Ennever, et al., 1983) should also be considered. This is especially true in the case of hyperbilirubinemia since there is evidence that suggests that bilirubin acts as a photosensitizing agent which could enhance the level of DNA damage (Rosenstein and Ducore, 1984).

b) Ingestion of an Adsorbent

The ingestion of an adsorbent to remove bilirubin from the gastrointestinal tract is another method of treating hyperbilirubinemia. It is thought that the addition of a scavenger for bilirubin into the gastrointestinal tract lowers the free bilirubin concentration, thus shifting the equilibrium and promoting bilirubin transfer across the intestinal mucosa (Ennever, et al., 1982) and interrupting the enterohepatic recirculation of bilirubin (Ulstrom, et al., 1964, Ennever, et al., 1982). Lester, et al. (Lester, et al., 1962) first demonstrated that adult Gunn rats have a lower serum bilirubin concentration when they are fed cholestyramine, an anion exchange resin composed of styrene divinylbenzene copolymer with quaternary ammonium ions. Similarly, Arrowsmith, et al. (Arrowsmith, et al., 1975) demonstrated that cholestyramine fed to infants reduces the concentration of serum bilirubin. However, in apparent

contradiction, Schmidt, et al. (Schmidt, et al., 1963) demonstrated that feeding cholestyramine to premature infants is not an effective treatment for the reduction of bilirubin levels in blood. Charcoal, if administered to newborns prior to 4 hours after birth, also facilitates the elimination of bilirubin. However, if administered 12 hours after birth it has no effect (Ulstrom, et al., 1964). Conflicting reports exist concerning the efficiency of agar, a seaweed extract, as an adsorbent for bilirubin removal in the gastrointestinal tract. Although most of the studies indicate that agar does not adsorb bilirubin (Maurer, 1973, Moller, 1974, Blum and Etienne, 1973, Arrowsmith, et al., 1975, Romagnoli, et al., 1975), Poland and Odell (Poland and Odell, 1971) reported that feeding agar to newborns results in a lowering of the bilirubin concentration in the serum. Ingestion of a soluble polymer, polyvinylpyrrolidone (PVP), which can form a complex with bilirubin before it is eliminated, was proposed as a possible adsorbent by Ploussard, et al. (Ploussard, et al., 1972). When applied to Gunn rats, treatment with PVP reportedly gives better results than does cholestyramine. However, when it was applied to newborn infants during the first 8 days of their life, the difference in bilirubin concentration between PVP treated and control groups was significant only during the second and third days (Ploussard, et al., 1972).

It appears that physiological conditions, such as gestational age, birth weight, feeding methods, and time at

which the treatment is started, may greatly influence the bilirubin uptake by adsorbents in the gastrointestinal tract of newborn infants (Ploussard, et al., 1972, Moller, et al., 1974). The presence of only low levels of bilirubin in the gastrointestinal tract of the newborn may also be responsible for the lack of success of the ingestion treatment, which is more successful in older patients (Ploussard, et al., 1972). This method is, apparently, not reliable and may not be applicable in all cases.

Ingestion of a resin in conjunction with phototherapy has also been tried as a method for reducing the level of bilirubin. Phototherapy results in the production of more hydrophilic photoproducts that are more easily excreted than the parent bilirubin. These photoproducts can then revert back to bilirubin and thus increase the concentration of bilirubin in the gastrointestinal tract. This bilirubin could then be adsorbed onto the scavenger. Thus, phototherapy should complement ingestion of an adsorbent for bilirubin very well. It promotes the transfer of more bilirubin into the gastrointestinal tract, thus resulting in a more rapid elimination. Nicopoulos, et al. showed that the duration of phototherapy required to lower the serum bilirubin level was shorter for infants also receiving cholestyramine (Nicopoulos, et al., 1978, 1981). Ingestion of charcoal was also reported to enhance the effect of phototherapy in the Gunn rat (Davies, et al., 1983). Conflicting results of similar (Ebbesen and Moller,

1977) and enhanced effect (Odell, et al., 1983) of phototherapy in conjunction with agar ingestion have been obtained.

c) Hemoperfusion

One other method of treating hyperbilirubinemia is by perfusion of blood through a column packed with an adsorbent. Since plasma contains a higher concentration of bilirubin than does the gastrointestinal tract, this method should be more efficient than the ingestion of adsorbent. Charcoal is one of the adsorbents that has been used in hemoperfusion systems. However, there is disagreement concerning the efficiency of charcoal in removing bilirubin, with some authors reporting good adsorption (Lie, et al., 1976, Lauterburg, et al., 1979, 1980, Otsubo, et al., 1980, Horak, et al., 1980) while others report adsorption to be low or non-existent (Ouchi, et al., 1978, Hew, et al., 1978). Even if charcoal adsorbs bilirubin, it has the disadvantage of non-selectively adsorbing a wide variety of substances. Another major drawback associated with hemoperfusion through charcoal is the loss of platelets by adsorption. Several methods, including microencapsulation in albumin-cellulose nitrate membrane (Chirito, et al., 1977, Chang, 1978), coating with a thin polymer membrane made of polyhydroxyethyl-methacrylate (polyhema) (Hughes, et al., 1980), have been used to improve the biocompatability of charcoal. Polymer coating reduces the amount of adsorption while coating with albumin increases it (Hughes and Williams, 1981).

Nonetheless, it appears that hemoperfusion through charcoal is not an entirely satisfactory method for the treatment of hyperbilirubinemia.

Both charged and uncharged resins have also been used as adsorbents in hemoperfusion columns. Charged resins tested in-vitro include the Dowex 1 family (cholestyramine), a styrene-divinylbenzene copolymer with quaternary ammonium ions (Willson, et al., 1972, Sideman, et al., 1979, 1983), anion exchange resins MCTI-2A (Lopukhin, et al., 1977) and BR 601 (Ouchi, et al., 1978, Asanuma, et al., 1980, Matsubara, et al., 1983), as well as a tertiary amine anion exchange synthetic resin fiber, ionex (Idezuki, et al., 1981), and a macroreticular resin MR (Sideman, et al., 1979, 1982, 1983). Uncharged resins that have been used include Amberlite XAD-7, a styrene-acrylic ester copolymer (Yamazaki, et al., 1979, Ouchi, et al., 1978, Sideman, et al., 1979), Amberlite XAD-2, a styrene-divinylbenzene copolymer (Willson, et al., 1972, Yamazaki, et al., 1979, Sideman, et al., 1979), and AR-1, another styrene-divinylbenzene copolymer with physical characteristics slightly different from Amberlite XAD-2 (Yamazaki et al., 1979). A macroporous gel, composed of 2-hydroxyethylmethacrylate (Skelly and Tighe, 1979) was found to have a better adsorption capacity when it was copolymerized with acrylic acid.

Since no universal methods or standards have been set for the evaluation of adsorption of bilirubin, either in-vitro or in-vivo, it is rather difficult to compare the

results of different studies. Consequently, it is very difficult to relate and compare all the literature data. Furthermore, in some cases incomplete data were also encountered so that accurate evaluation of these resins is not possible. Another problem encountered in the evaluation of the adsorbent is the lack of a standard bilirubin solution for the adsorption. It is expected that the amount of bilirubin adsorbed will vary with the solvent. For example, in in-vitro the presence of albumin will lower the adsorption capacity. Furthermore, the bilirubin to albumin concentration will also affect the adsorption. In in-vivo studies the bilirubin removal rate is expected to vary from patient to patient (Hughes and Williams, 1981).

Due to a lack of complete information, no attempts will be made to compare the various adsorbents directly. Because such a comparison can not be made accurately, misleading conclusions could be inferred. However, a summary of some of the in-vitro studies has been compiled (Table II) from which some results have been omitted because they lack sufficient data. It should be stressed that the data shown are intended only as a representation of the amount of bilirubin adsorbed by the adsorbents under specific conditions and that caution should be exercised in making comparisons between the resins.

Some of the adsorbents mentioned above have also been studied in-vivo, either in animal or man, with varying

TABLE II

ADSORPTION CAPACITY OF CHARGED AND UNCHARGED RESINS FOR BILIRUBIN

Resin	Charge	Solution	Capacity (mg/g)	Reference
ionex	positive	buffer 1% albumin	0.3	Ydezuki, et al., 1981
macroreticular	positive	buffer 4% albumin	16	Sideman, et al., 1981
AR1	none	plasma	1.5	Yamazaki, et al., 1979
XAD-2	none	plasma	0.4	Yamazaki, et al., 1979
XAD-7	none	plasma	0.2	Yamazaki, et al., 1979
XAD-7	none	plasma	0.5	Skelly and Tighe, 1979
BR601	positive	plasma	4.4	Ouchi, et al., 1978
BR601	positive	plasma	80	Mastabura, et al., 1983
Dowex 1X2	positive	Buffer 4% albumin	9	Sideman, et al., 1980
Dowex 1X1	positive	Albumin solution	4	Schmidt, 1965
Dowex 1X4	positive	plasma	0.5	Skelly and Tighe, 1979
HEMA	none	plasma	0.2	Skelly and Tighe, 1979
HEMA(75)-AA(25)	none	plasma	0.4	Skelly and Tighe, 1979
albumin-agarose	both	plasma	0.2	Plotz, et al., 1974
albumin-agarose	both	plasma	0.2	Losgen, et al., 1974

degrees of success. These include the macroreticular resin (Sideman, et al., 1979, 1981, 1983,), the XAD-7, (Ton, et al., 1979), the AR-1 (Yamazaki, et al., 1979), and the BR 601 resins (Ouchi, et al., 1978, Asanuma, et al., 1980, Matsabura, et al., 1983). As with the charcoal hemoperfusion, some compatability problems have been encountered. Once again, coating with albumin proves to be a solution to this problem.

Studies that involve more than one type of resin are more suited for the evaluation of the different factors that lead to a better adsorption since, in these cases, external factors affecting the adsorption are kept identical. Hence, a better comparison of the resins is possible. From such studies it appears that charged resins are more efficient than uncharged. For instance, Willson, et al. (Willson, et al., 1972) showed that in plasma the charged resin Dowex 1X4 adsorbs approximately 3.6 times more bilirubin than does the uncharged resin, Amberlite XAD2. Other charged resins, Amberlyst and Amberlite IRA 400, adsorb 3 and 2.6 times more bilirubin, respectively, than does XAD2. Ouchi et al. (Ouchi, et al., 1978) showed that BR 601, an ion exchange resin, is more efficient than the uncharged XAD-7 resin.

It also appears that the more porous resins are more efficient. Yamazaki, et al. (Yamazaki, et al., 1979) showed that uncharged styrene-divinylbenzene copolymer, AR-1, adsorbs approximately 5 times more bilirubin than the styrene-divinylbenzene copolymer, XAD-2, with smaller pores

and surface area. Sideman et al. (Sideman, et al., 1979, 1983) showed that a strongly basic macroreticular resin has a greater adsorption capacity than do Dowex 1X1 and 1X2 resins, which are also strongly basic but less porous. They also showed that Dowex 1X1 has a higher adsorption capacity and a higher rate of adsorption than Dowex 1X2, resins which differ only in the degree of cross-linking. The former is less cross-linked, hence it is more porous. In agreement with Sideman, Sawchuk and Nairn (Sawchuk and Nairn, 1968) determined that diffusion within the polymer beads seems to be the rate controlling step in the adsorption of bilirubin onto polymer resins. On the basis of the rate constants for the adsorption of bilirubin onto Dowex resins with different degrees of cross-linking and different bead size, it was shown that the resins with a higher percentage of cross-links have a lower rate constant. Furthermore, for a given percentage of cross-links, the resins with the larger bead size have a larger rate constant.

Albumin immobilized onto a polymer matrix has also been used as an adsorbent (Plotz, et al., 1974a, 1974b, Losgen, et al., 1978). Since albumin is known to have only 2 sites that bind bilirubin strongly, this adsorbent should not bind as many different ligands as the other resins on a per unit weight basis. However, it should be remembered that albumin binds a wide variety of substrates. Subsequent testing of this adsorbent proved that bilirubin can be adsorbed successfully (Scharschmidt, et al., 1977,

Logsen, et al., 1978). However, because albumin is a large protein, with a molecular weight of approximately 66,000, that binds only two molecules of bilirubin tightly it is an adsorbent with a low capacity, approximately 0.1 to 0.2 mg/g of adsorbent (Scharschmidt, et al., 1977).

At the present time none of the available adsorbents are entirely satisfactory for use in hemoperfusion or ingestion. The major limitations are lack of specificity, low capacity, and poor biocompatability. While the latter problem has been solved by coating the adsorbent, the other two remain. The use of immobilized albumin for removal of bilirubin through hemoperfusion is an interesting concept. Since only certain parts of the protein, namely the binding sites, are active the adsorption capacity of this adsorbent is low. If a polymer resin could be synthesized to mimic the binding site for bilirubin on albumin, it is expected that an adsorbent with a higher capacity than immobilized albumin would be obtained. However, before attempting to do so a better understanding of albumin and its interactions with bilirubin, particularly the location and identity of the bilirubin binding site, is required.

4) Albumin

In adult man the average concentration of albumin is about 42 $\pm$ 3.5 mg/cm^3, with a range from 35 to 50 mg/cm^3. This represents approximately 60% of the total protein content of plasma (Peters, 1970). The albumin concentration is lower at birth but reaches adult level

after three months (Peters, 1970).

Serum albumins are large, globular proteins with a molecular weight of approximately 66,000. Human serum albumin (HSA) is composed of 584 amino acid residues while bovine serum albumin (BSA) has 581 (Peters, 1975). Due to the preponderence of acidic residues, aspartic and glutamic acids, over basic residues, arginine, lysine, and histidine, serum albumins are the most acidic proteins found in appreciable amount in plasma (Peters, 1975). Both BSA and HSA have a net charge of -18.

Elucidation of the amino acid sequence of both bovine and human serum albumins is largely due to the work of J.R. Brown (Brown J.R., 1977a, 1977b), although the sequence for human serum albumin has also been determined independently by Meloun et al. (Meloun, et al., 1975). Agreement between the two stuctures is within 93% (Peters and Reed, 1977). Brown's sequences for BSA (Brown J.R., 1977a) and for HSA (Brown J.R., 1977b) are shown in Figs. 8 and 9. The missing sequence in BSA, residues 400 to 403, has been reported as being glycine-phenylalanine-glutamine-asparagine by Reed et al. (Reed, et al., 1980).

The structure, as well as the sequence, of albumin has also been determined by Brown (Brown J.R., 1977b). His deduction of the structure (Fig. 10) is based largely on the repeating pattern formed by the amino acid sequence and the presence of a 17 disulphide bridges. The reasoning was as follows: The amino acid sequence and the disulphide bridges suggest the presence of three large repeating units

FIGURE 8

AMINO ACID SEQUENCE OF BOVINE SERUM ALBUMIN

(Brown, 1977a)

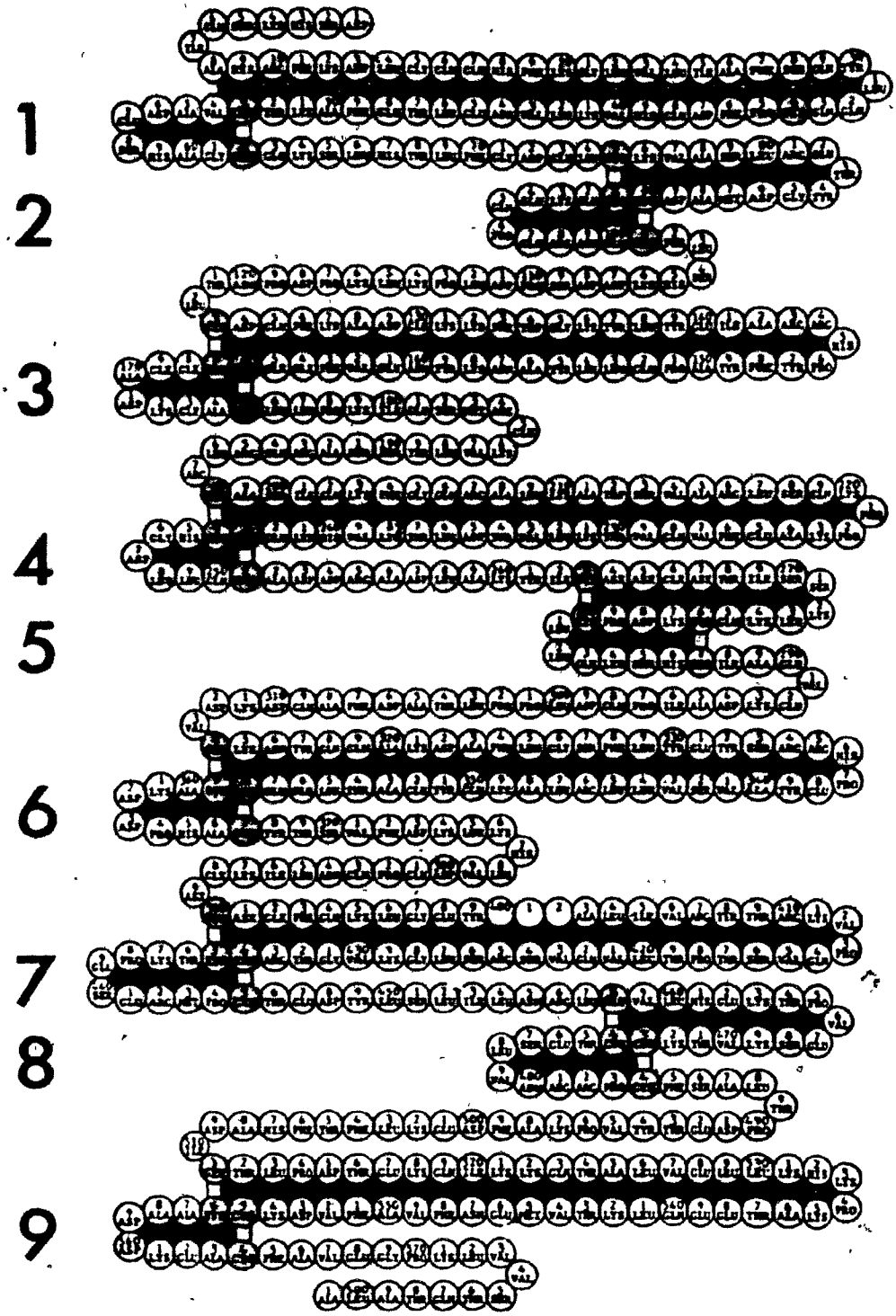

1
2
3
4
5
6
7
8
9

FIGURE 9

AMINO ACID SEQUENCE FOR HUMAN SERUM ALBUMIN

(Brown, 1977b)

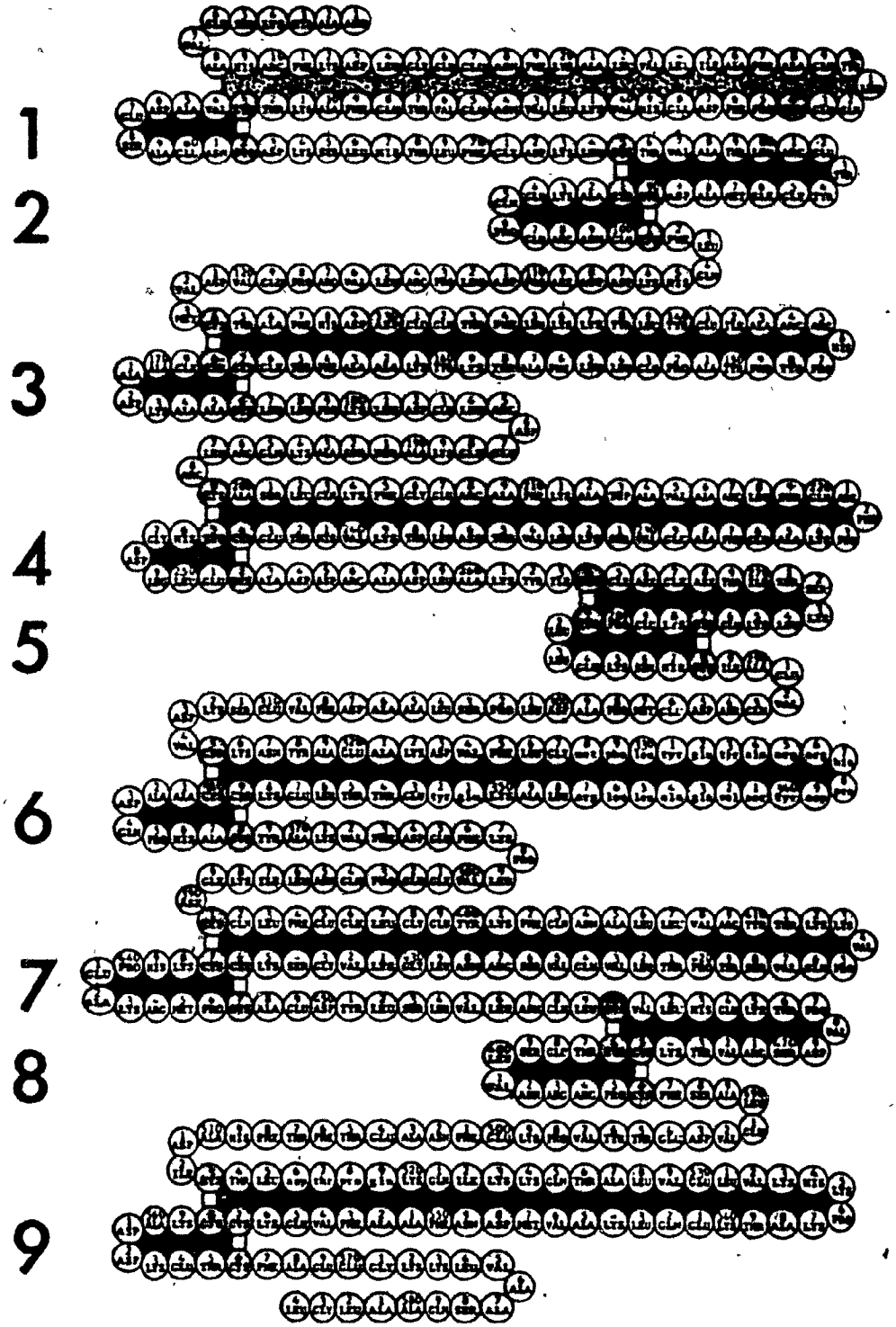
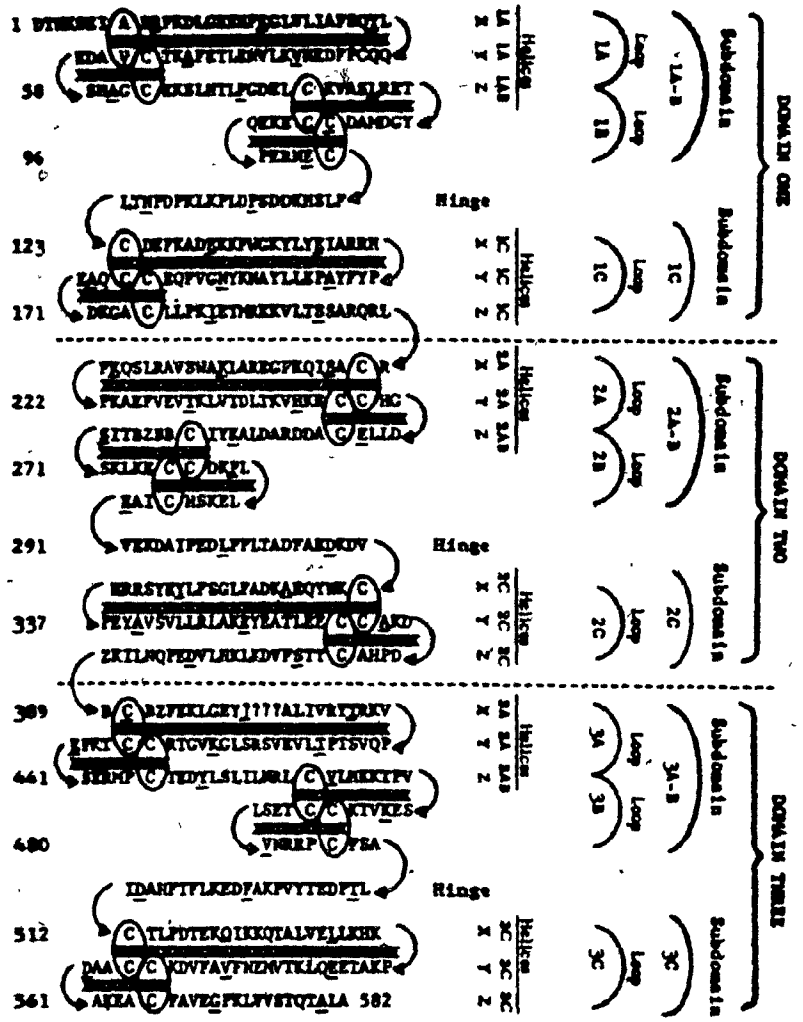

1
2
3
4
5
6
7
8
9

FIGURE 10

STUCTURAL ORGANIZATION OF ALBUMIN

(Brown, 1977b)


DOMAIN ONE
DOMAIN TWO
DOMAIN THREE
Subdomain
Loop
Helices
Hinge

called domains. Each domain is composed of two large loops separated by a smaller loop. Each domain can also be subdivided into two subdomains, the first containing the first large loop plus the small loop, and the second containing the second large loop. Alternatively, each subdomain can be described as being composed of three α-helices, approximately 20 amino acids in length, running parallel to each other. These α-helices are defined by the disulphide bonds at one end, and the proline residues at the other end, both of which impose a folding of the peptide chain. The three helices are arranged in a semi-circle (Fig. 11) with their hydrophobic residues pointing toward the inside. Each domain also contains a flexible segment which is not involved in this helix arrangement. This segment, which is situated between the two subdomains acts as a hinge to permit pairing of the subdomains, so that their hydrophobic sides face each other. This cylindrical structure creates a hydrophobic hole with a cluster of basic residues at the opening. Another possible arrangement of Brown's six half-domains has also been proposed by Brodersen (Brodersen, 1980). In this latter arrangement the subdomains are paired as follows, subdomain 1AB with 3C, 1C with 2AB and 2C with 3AB (Fig. 12). In Brown's model the subdomains are paired as 1AB with 1C, 2AB with 2C and 3AB with 3C.

Albumin forms reversible complexes with a variety of ions, including bilirubin, long chain fatty acids, as well as various drugs and dyes. Many of these ligands can

FIGURE 11

SUBDOMAIN ORGANIZATION OF ALBUMIN

(Brown, 1977b)

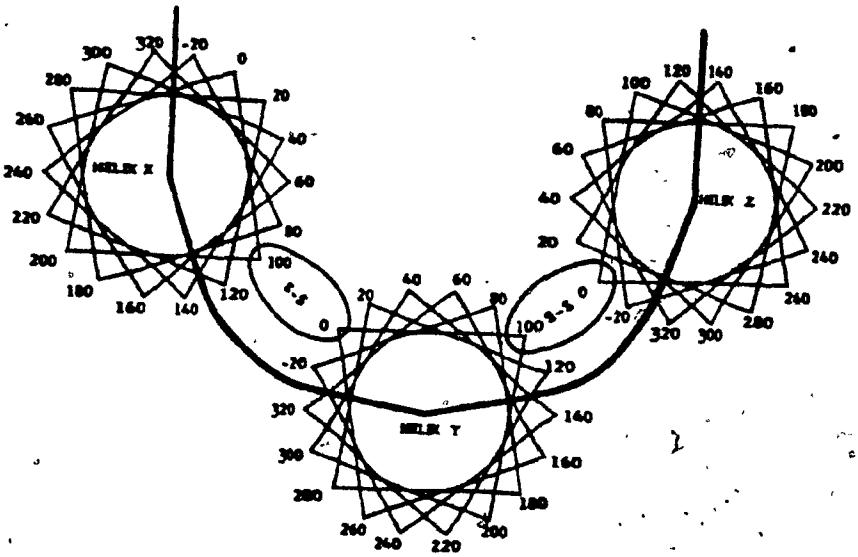
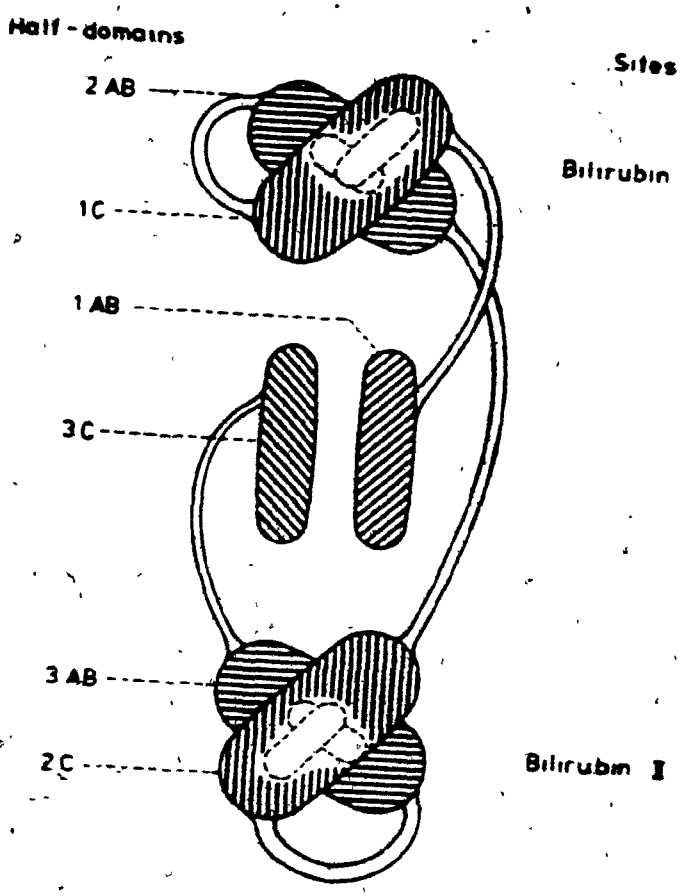

HELIX X
HELIX Y
HELIX Z

FIGURE 12

SUBDOMAIN ORGANIZATION OF ALBUMIN

(Brodersen, 1980)


Half - domains
Sites
2 AB
1C
1 AB
3C
3 AB
2C
Bilirubin I
Bilirubin II

occupy more than one binding site on albumin, the first one being a high affinity site while the others have a lower affinity. Binding of ligands to albumin appears to be non-saturable. Thus, in addition to the strong affinity binding sites already mentioned, albumin also has a multitude of weaker binding sites. Binding to the strong affinity binding sites is sometimes referred to as "specific binding" while binding to the weaker sites is referred to as "unspecific" binding.

A given site may be capable of binding several different ligands, thus giving rise to competitive binding among ligands. Competitive binding and displacing properties of drugs and other metabolites have some clinical consequences since their presence in sufficiently high concentration in the blood could induce a displacement of the bound bilirubin and lead to hyperbilirubinemia. Brodersen (Brodersen, 1978) studied the displacing properties of 150 drugs and found that sulfonamide, some X-ray contrasting media, stabilizers for albumin preparation, acetylsalicylic acid, sodium salicylate, and phenylbutazone can displace bilirubin.

Binding of substrates with different shapes to the same binding site implies that, unlike most proteins, albumin has a considerable adaptability and can assume a number of different configurations. It also invalidates the concept of preformed sites (Karush, 1950). A site should then be thought of as a part of the molecule which can fold in such a way as to accomodate a ligand. Serum albumin has been

compared to a

"human hand which has no specific, preformed sites but serves as a universal carrier by combining fingers and palms in a suitable combination for seizing objects of diverse sizes, shape and texture" (Brodersen, 1980).

5) Binding of Bilirubin to Albumin

Albumin binds two molecules of bilirubin tightly. The first molecule is bound to a high affinity primary binding site while the second one is bound to a lower affinity secondary binding site (Jacobsen J., 1969). The possibility of binding a third molecule to a secondary binding site has been reported (Beaven, et al., 1973, Jacobsen J., 1977). In addition, 10 to 14 weak sites have been reported (Shapovalenko and Kolosov, 1978). This is in agreement with the postulate that albumin is a non-saturable carrier (Brodersen, et al., 1984). Binding constants for the primary binding site for bilirubin on albumin are difficult to determine, due largely to the experimental difficulties involved in working with bilirubin. As a result, a wide range of values have been reported, depending on the method used to obtain the constant (Table III). Obviously, the binding constant for the second binding site is even more difficult to obtain. However, most researchers agree that it is approximately one order of magnitude smaller than that of the first binding constant (Brodersen, 1980) (Table III). Binding constants for HSA are larger (Blauer, et al., 1977) than those for BSA (Table IV).

TABLE III

CONSTANTS FOR BINDING BILIRUBIN TO HUMAN SERUM ALBUMIN

Method	Type	pH	Temp. °C	Ionic Strength M	K_1 X 10^{-6} M^{-1}	K_2 X 10^{-6} M^{-1}	Reference
CD	Site	7.4	26.5	0.1	200	—	Blauer, et al., 1977
peroxidase	Site	7.4	37	0.18	60	—	Jacobsen J., 1976
solubility	Stoi	6.5	37	0.15	2	—	Shapovalenko and Kolosov, 1978
fluorescence	Stoi	7.4	—	0.1	13	6.7	Branca, et al., 1983
peroxidase	Site	7.4	37	0.1	140	0.5	Jacobsen J., 1969
CD	Stoi	8.5	24	0	7	0.1	Beaven, et al., 1973
fluorescence	Stoi	7.5	37	0.07	64	2.6	Berde, et al., 1979
fluorescence	Stoi	7.5	23	0.07	120	4.9	Berde, et al., 1979
kinetics	——	7.4	4	0.1	>4000	—	Gray and Stoupe 1978
peroxidase	——	7.4	37	0.18	55	44	Brodersen, 1978

CD = circular dichroism
Site = site binding constant
Stoi = stoichiometric binding constant

TABLE IV

CONSTANTS FOR BINDING BILIRUBIN TO BOVINE SERUM ALBUMIN

Method	Type	pH	Temp. °C	Ionic Strength	K_1 X 10^{-6} M^{-1}	K_2 X 10^{-6} M^{-1}	Reference
CD	Stoi	5	25	0.2 mM	6	—	Blauer, King 1970
peroxidase	Site	7.4	37	0.1 M	20	—	Reed, et al.,1975
kinetics	——	7.4	37	0.1 M	6	—	Reed, 1977

CD = circular dichroism
Site = site binding constant
Stoi = stoichiometric binding constant

The process of binding bilirubin to albumin involves three to five steps (Faerch and Jacobsen J., 1977, Gray and Stroupe, 1978, Koren, et al., 1982). The first step, the association of bilirubin with albumin, is a fast, bimolecular reaction with a second order rate constant of 2 X 10^7 $M^{-1}s^{-1}$ for HSA (Faerch and Jacobsen J., 1977, Gray and Stroupe, 1978) and 0.9 X 10^6 $M^{-1}s^{-1}$ (Koren, et al., 1982) or 1.5 X10^6 $M^{-1}s^{-1}$ (Reed, 1977) for BSA. A higher value, 8 X 10^7 $M^{-1}s^{-1}$, has also been reported for BSA (Koren, et al., 1982). Association is followed by several unimolecular steps (Faerch and Jacobsen J., 1977, Gray and Stroupe, 1978, Koren, et al., 1982) corresponding to intramolecular rearrangement. Rate constants for these steps have been reported to be in the range of 3.8 to 40 s^{-1} for a two step rearrangement (Gray and Stroupe, 1978) and 0.32 to 48 s^{-1} for a four step rearrangement (Faerch and Jacobsen J., 1977) for HSA. The rearrangement steps in BSA are also rapid. However, changes in light spectra have been observed up to 8 minutes after addition of the albumin (Jacobsen J. and Brodersen, 1983).

6) Bilirubin Binding Site on Albumin

The location and identity of the amino acids forming the primary binding site for bilirubin on albumin are not yet known. However, during the last few years research in this area has been very active and many significant discoveries have been made.

Geisow and Beaven (Geisow and Beaven, 1977) divided BSA into three large fragments by peptic digestion. When complexed with bilirubin, two fragments corresponding to the N terminal of the protein, retained CD spectra similar to that of native BSA-bilirubin complex. The third fragment, corresponding to the carboxyl terminal, did not show this similarity. This strongly suggests that the primary binding site for bilirubin on albumin is situated in the portion of albumin containing the N terminal. Sjodin, et al. (Sjodin, et al., 1977) cleaved albumin with trypsin and found that a large fragment containing residues 182-585 retains the capacity to bind bilirubin. Reed et al. (Reed, et al., 1975) subjected BSA to tryptic and peptic hydrolysis and obtained twelve different fragments of the native protein; three of these, containing fragments with residues 1-385, 1-306 and 186-306 retained a binding capacity for bilirubin similar to that of native BSA. These fragments have residues 186 to 306 in common. However, another fragment containing part of the overlapping region, residues 239-306, did not retain a binding capacity high enough to suggest the presence of the primary binding site. Thus, most of the primary binding site appears to be located in the region roughly comprising residues 186-240, or loop 4, situated in the N terminal of the protein. Of course this does not mean that some residues lying outside, but near by, are not involved in the binding site, but rather that they may be of secondary importance.

Using a carbodiimide derivative, C. Jacobsen (Jacobsen C., 1976) covalently linked ^{14}C-labelled bilirubin to an amino group of HSA (Fig. 13A). Subsequently this labelled albumin was cleaved by trypsin. When the peptide fragments were isolated, the bilirubin probe was located on the fragment consisting of residues 240 to 258, which contains only one residue with an amino group, namely lysine 239. Thus, it was concluded that lysine 239 must be at, or near, the primary binding site for bilirubin on HSA.

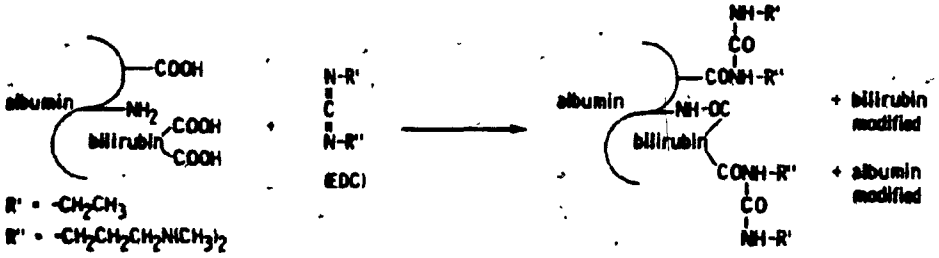
Kuenzle, et al. (Kuenzle, et al., 1976) modified ^{14}C-labelled bilirubin with Woodward reagent to yield an activated bilirubin enol ester, BW. The modified bilirubin was equilibrated for two hours with albumin at pH = 7.4. After equilibration nucleophilic substitution was initiated by addition of imidazole (Fig. 13B). Subsequently the labelled albumin was cleaved into seven peptide fragments, using CNBr. This showed that bilirubin is attached to two peptide fragments, that consisting of residues 124-293 and that with residues 446-547 (Gitzelmann-Cumarasamy, 1976).

Jori, et al. (Jori, et al., 1980) showed that bilirubin and/or its photoproducts undergo a photo-induced covalent binding with BSA. After fragmentation of the resulting photoproduct, using CNBr, the covalently linked bilirubin was found in a peptide with an amino acid content closely related to peptide fragment 187-397 of BSA. In contrast, Hutchinson and Mutopo (Hutchinson and Mutopo, 1979) used CNBr to fragment HSA previously labelled by

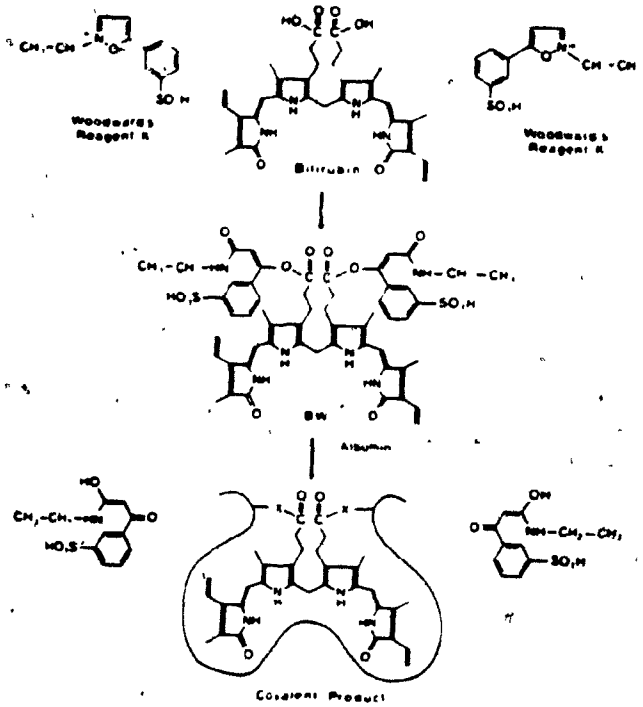
FIGURE 13

COVALENT BINDING OF BILIRUBIN TO ALBUMIN

A) Jacobsen, 1976
B) Kuenzle, 1976


albumin
COOH
NH2
bilirubin
COOH
COOH
R' = -CH2CH3
R' = -CH2CH2CH2N(CH3)2
N-R'
C
N-R'
(EDC)
NH-R'
CO
CONH-R'
albumin
NH-OC
bilirubin
CONH-R'
CO
NH-R'
+ bilirubin modified
+ albumin modified

[A]


Woodward's Reagent K
Bilirubin
Woodward's Reagent K
BW
Albumin
Covalent Product

[B]

photocovalent binding to ^{3}H bilirubin. They found that 62% of the label is in peptides 1-124 while 32% is in peptides 125-297.

C. Jacobsen (Jacobsen C., 1972) showed that chemical modification of 10 arginines of HSA with glyoxal or of 2 histidines with diethylpyrocarbonate or of 8 tyrosines with tetranitromethane, results in a decrease of the binding affinity of HSA for bilirubin. Complete modification of the tryptophan and cysteine residues, as well as of 10% of the carboxyl groups and of 22 of the amino groups does not change the binding affinity. In all cases, the tertiary structure of albumin is not significantly altered as a result of the modification. It was concluded that that arginine, histidine and tyrosine are involved in the high affinity sites of bilirubin while tryptophan and cysteine are not. No firm conclusion could be drawn concerning the role of amino and carboxyl groups. In subsequent papers Jacobsen (Jacobsen C., 1974, Jacobsen C. and Jacobsen J., 1979) showed by reaction of lysine residues with dansyl chloride, and by trinitrophenylation with picryl chloride or 2,4,6-trinitrobenzenesulphonic acid, that at least two different lysine residues are involved in the primary binding site of HSA. It is significant that the binding affinity of HSA modified by these reagents, in the presence of bilirubin, remains unchanged. This implies that, when bilirubin is present, these amino acids are protected from reaction with these reagents. In the absence of bilirubin, the reagents used

actually modify the residues involved in the bilirubin binding site.

Based on evidence obtained by CD and ORD spectroscopy, Blauer and coworkers suggested that bilirubin bound to BSA or HSA assumes a dissymmetric configuration which is altered by a change in pH and ionic strength (Blauer and King, 1970, Blauer, et al., 1970). The ORD and CD spectra obtained when bilirubin is complexed with BSA differ from those with HSA which suggests that, in spite of the similar amino acid sequences and conformations of the proteins, the binding of bilirubin to HSA is slightly different from the binding to BSA (Blauer, et al., 1970). This was confirmed by Lee and Gillespie (Lee and Gillispie, 1981) who suggested, on the basis of evidence obtained by fluorescence spectroscopy, that bilirubin is buried more deeply within the HSA molecule while that bound to BSA results in a more open conformation of the bilirubin molecule which is also situated closer to the surface.

THE PRESENT STUDY

This thesis is divided into six chapters and is concerned mainly with the study of new adsorbents for use in either hemoperfusion devices or by direct ingestion.

Chapter I is a general introduction. The possibility of using a commercially available insoluble, crosslinked polyvinylpyrrolidone (PVP) as an adsorbent for bilirubin has been investigated and is compared with the adsorption

behaviour of cholestyramine in Chapter II. This study was based on the results of Ploussard, et al. (Ploussard, et al., 1972) which suggest that soluble PVP interacts with bilirubin.

One of the best adsorbents tested previously is immobilized albumin. The major limitation of this adsorbent is a low capacity which arises from the fact that albumin is a large protein, molecular weight > 66,000, and can bind bilirubin tightly only at two high affinity binding sites. Thus, the possibility of preparing improved adsorbents by mimicking the binding sites for bilirubin with small peptide pendants immobilized onto a suitable solid polymeric matrix has also been investigated and is presented in Chapters III and IV. Chapter III considers the adsorption of bilirubin by polystyrene divinylbenzene (Merrifield) resins onto which single amino acids have been attached as pendants. A systematic study of the adsorption of bilirubin by small peptide pendants consisting of several different combinations of amino acids synthesized onto a water swellable polyamide backbone is presented in Chapter IV. Sequences consisting of the amino acids lysine, arginine, tyrosine, and histidine have been immobilized onto this polymer matrix. The choice of amino acids is based on the work of Jacobsen (Jacobsen C., 1972, 1974, Jacobsen C. and Jacobsen J., 1979), which suggests that these amino acids are involved in the primary binding site for bilirubin on albumin.

Comparison of the binding affinities of these polyamide resins and of serum albumin was tested in studies of desorption of bilirubin from the polyamide resins in the presence of albumin and by studies of the adsorption of bilirubin by these resins from bilirubin-albumin solutions. These results are presented in Chapter V. Chapter VI deals with the binding constants for the adsorption of bilirubin by the polyamide resins. These chapters are followed by a summary of the contributions to original knowledge and some suggestions for future work.

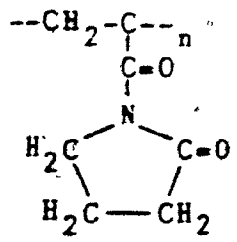
Appendix I describes the equipment and the reaction schemes used for the synthesis of the peptide pendants on the polyamide resins. Appendix II deals with the experimental data used to draw the figures shown in the preceding chapters. Part of this work has already been published and the three reprints are given in Appendix III.

CHAPTER II

ADSORPTION OF BILIRUBIN BY POLYVINYLPYRROLIDONE AND CHOLESTYRAMINE

INTRODUCTION

The first adsorbent for bilirubin investigated in this study was polyvinylpyrrolidone (PVP) [II]. The choice of



[II]

this adsorbent was based on a previous report by Ploussard et al (Ploussard, et al., 1972) of a "red shift" in the visible spectrum of bilirubin as well as the protection against photodegradation obtained upon addition of water-soluble, i.e., non-cross-linked, PVP to an aqueous bilirubin solution.

In this first series of experiments, insoluble, cross-linked PVP was used instead of the soluble, non-cross-linked polymer used by Ploussard (Ploussard, et al., 1972). The main advantage of using the insoluble PVP is that it could be used in a hemoperfusion system where the physiological factors that affect the adsorption of bilirubin in the gastrointestinal tract seem to be of lesser importance. However, insoluble PVP could still be used in the gastrointestinal tract if it were required.

The purpose of this first series of experiments is to determine if the interaction between soluble PVP and bilirubin is still present in the insoluble polymer and if

this interaction is strong enough to permit it to be used as a scavenger. For the latter reason, the adsorption capacity of PVP will be compared to that of the ion-exchange resin cholestyramine (Dowex-1X2). Cholestyramine has already been tested for its adsorption capacity for bilirubin and proved to be one of the better resins (Table II, Chap. I).

EXPERIMENTAL

1) Preparation of the Resins

Cross-linked PVP (Aldrich) and cholestyramine (Dowex 1X2, Aldrich) were pretreated in the following manner: The PVR was washed overnight with buffer (50°C) to remove any trace of soluble polymer. Subsequently, both cholestyramine and PVP were washed overnight with methanol, twice with 1.0 M HCl (20 minutes), twice with 0.010 M HCl (20 minutes), and twice with 0.10 M $NaOH/KH_2PO_4$ (pH = 7.8 ± 0.05) buffer. Finally, they were filtered, using a glass sintered filter, dried at 100°C under vacuum and the fraction with mesh size 100-140 was stored in a dessicator until use.

2) Bilirubin Solutions

Solutions of bilirubin (10 mg/dl) were prepared daily by dissolving bilirubin powder (from gallstones, Sigma) in 0.10 M NaOH and adjusting to volume with $KH_2PO_4/NaOH$ (0.10 M) buffer, made weekly, to achieve a final pH of 7.8 ± 0.05 and a concentration of 10 mg/dl. This stock

solution was diluted with KH_2PO_4/NaOH (0.10 M, pH = 7.8) buffer as required. Solutions were kept in the dark at 0°C and all experiments were made in a dark room using a red light.

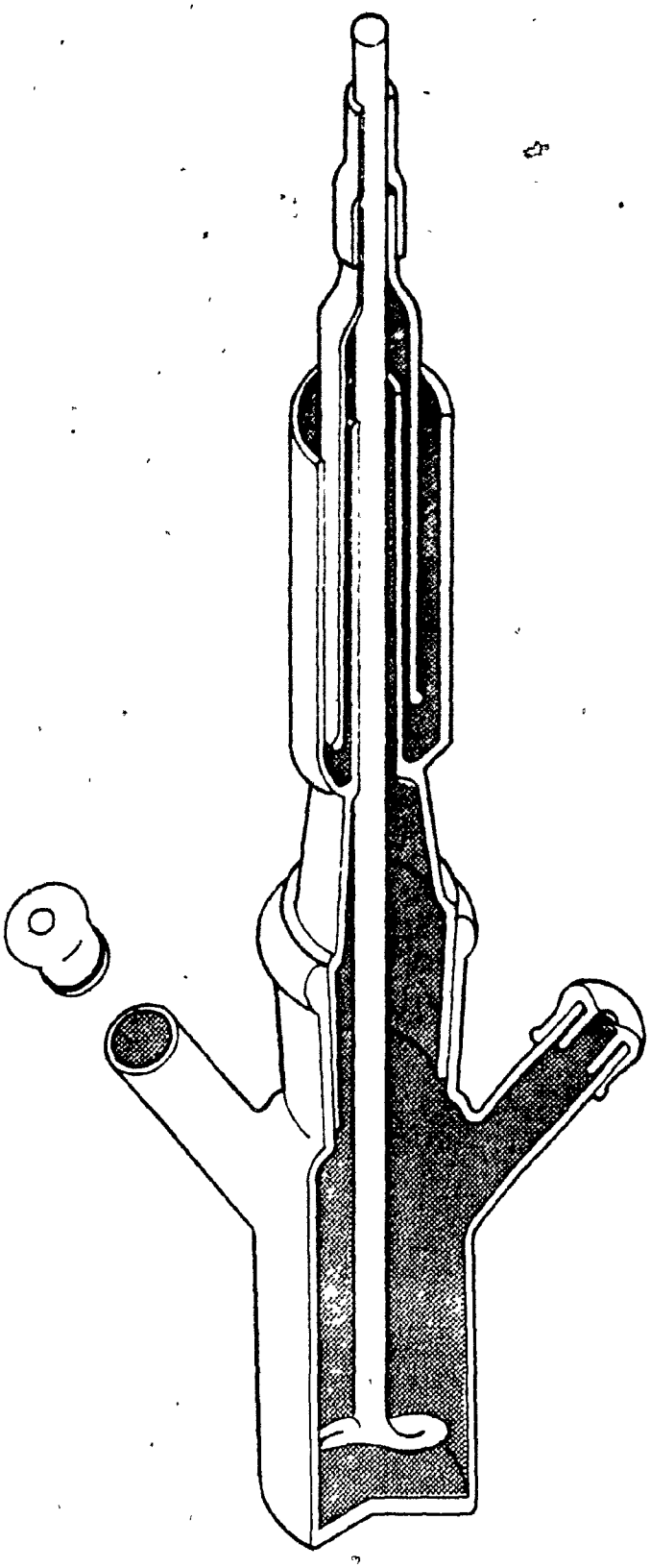
3) Kinetics of Adsorption and Adsorption Isotherms

The adsorption studies were done using a specially designed adsorption flask, shown schematically in Fig. 14. It was built so as to prevent formation of scratches which encourage the precipitation of bilirubin. Consequently, the glass stirrer was placed so that it did not touch the surface of the flask. In addition to the inlet neck for the stirrer, the flask had two other necks, each of which was stoppered with a rubber septum to facilitate the purging of the flask with an inert gas and the withdrawal of solution during the experiment. Before the first adsorption the flask was coated, with dimethyldichlorosilane overnight, washed with ethanol and distilled water. It was then washed with a bilirubin solution (25 ml, 10 mg/dl) for two hours. The solution was discarded, the flask rinsed with distilled water, and dried. Subsequently, the flask was simply rinsed with distilled water and dried between each adsorption experiment.

The flask containing the bilirubin solution (25 ml, 10 mg/dl) was placed in a thermostatted water bath or ice water bath and purged continuously with nitrogen. Adsorption was initiated by adding the appropriate weight

FIGURE 14

ADSORPTION FLASK



of resin to the flask. Aliquots (1 ml) were withdrawn at 15 minute intervals for PVP and 30 minute intervals for cholestyramine. The concentration of bilirubin in the supernatant solution was determined from its absorbance at 438 nm measured with a Beckmann model 25 double beam spectrophotometer using the buffer solution as the reference. Calibration experiments with bilirubin solutions of known concentrations yielded a linear Beer's law plot, in the concentration range of these studies, with a molar extinction coefficient of 4.44×10^4 l $mole^{-1}$ cm^{-1}. The amount of bilirubin adsorbed was obtained from the difference in concentration between the initial and final bilirubin concentrations.

For the studies of the kinetics of adsorption a continuous flow system was used. The solution was withdrawn from the adsorption flask, entered into the bottom of a 1 mm spectrophotometer flow cell, withdrawn from the top of the cell by a piston pump and returned to the flask. Glass tubing was used throughout except at the connections to the cell, pump, and flask. Corrections were made for the rate of loss of bilirubin in the absence of adsorbent.

RESULTS

1) Effect of Soluble PVP on the Bilirubin Spectrum

Preliminary experiments were first made to verify the report of interactions between bilirubin and PVP made by

Ploussard et al. (Ploussard, et al., 1972). When PVP was added to aqueous bilirubin solution, pH = 7.8, at a weight ratio of bilirubin:PVP of 0.5 the absorbance maximum, λ_{max}, shifted from 435 to 420 nm, i.e, a "blue shift" rather than the reported "red shift" was observed (Fig. 15A). However, when the weight ratio of bilirubin to PVP was decreased to 0.05, a shoulder appeared at 460 nm on the peak with λ_{max} at 420 nm (Fig. 15A). A further decrease in the bilirubin:PVP ratio down to 0.005 resulted in a relative decrease in the absorbance at 420 nm and an increase at 460 to give two peaks (Fig. 15B). Finally at a bilirubin:PVP ratio of 0.0012 the peak at 420 nm was seen only as a shoulder on the main peak that appears at λ_{max}= 460nm (Fig. 15B), corresponding to a "red shift". This is in agreement with the report of Ploussard et al (Ploussard, et al., 1972) since their studies were made at a bilirubin:PVP ratio of $5x10^{-4}$, i.e., under conditions where the peaks at 420 nm are no longer observed.

2) Kinetics of Adsorption

The substantial effect of PVP on the bilirubin spectrum suggested that adsorption studies were warranted. The study of the adsorption of PVP and cholestyramine was begun with an investigation of the relative rate of adsorption by the resins. The kinetic studies at 10°C showed that bilirubin adsorption by PVP is complete by 20 minutes while at least 60 minutes are required by cholestyramine to reach its ultimate level (Fig. 16). When

FIGURE 15

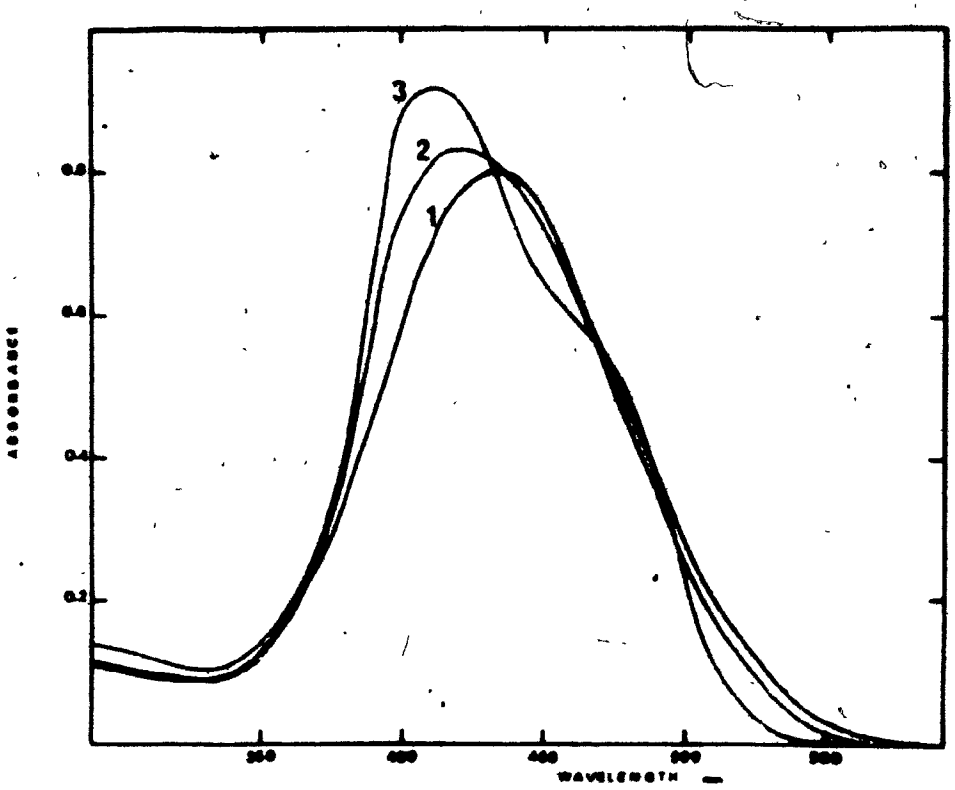
EFFECT OF SOLUBLE PVP ON THE BILRUBIN SPECTRUM

A)

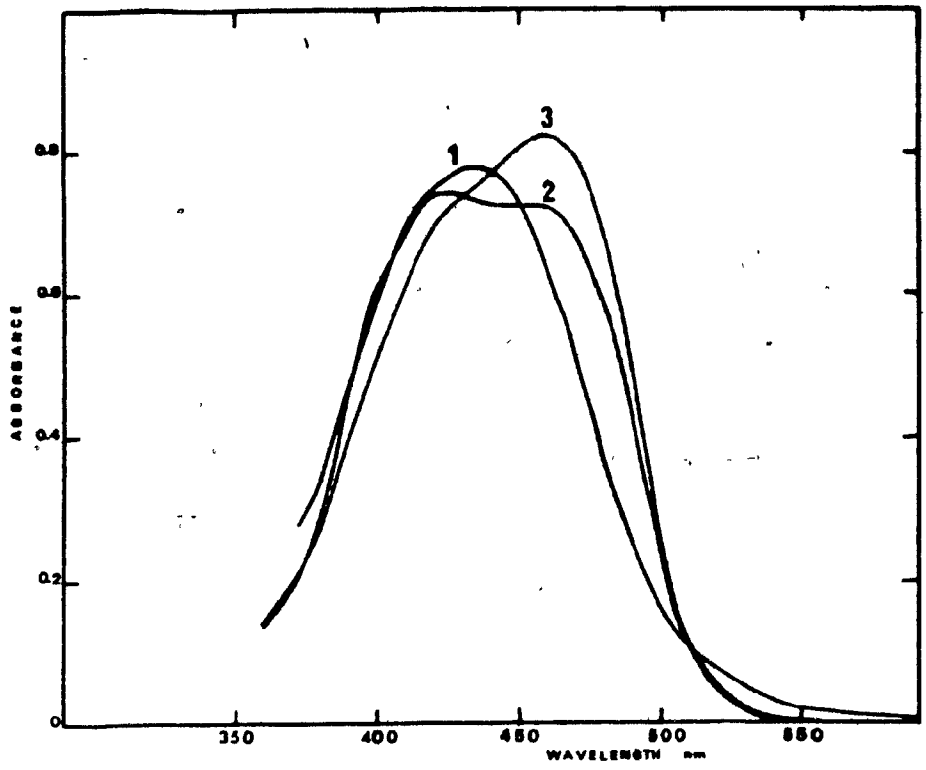
[bilirubin] = 10 mg/dl
pathlength = 0.1 cm
1 reference
2 bilirubin/ PVP = 0.49
3 bilirubin/ PVP = 0.05

B)

[bilirubin] = 10 mg/dl
pathlength = 0.1 cm
1 reference
2 bilirubin/PVP = 0.0012
3 bilirubin/PVP = 0.0046


3
2
1
ABSORBANCE
WAVELENGTH

[A]


3
1
2
ABSORBANCE
0.2
0.4
0.6
0.8
0
350
400
450
500
550
WAVELENGTH nm

[B]

FIGURE 16

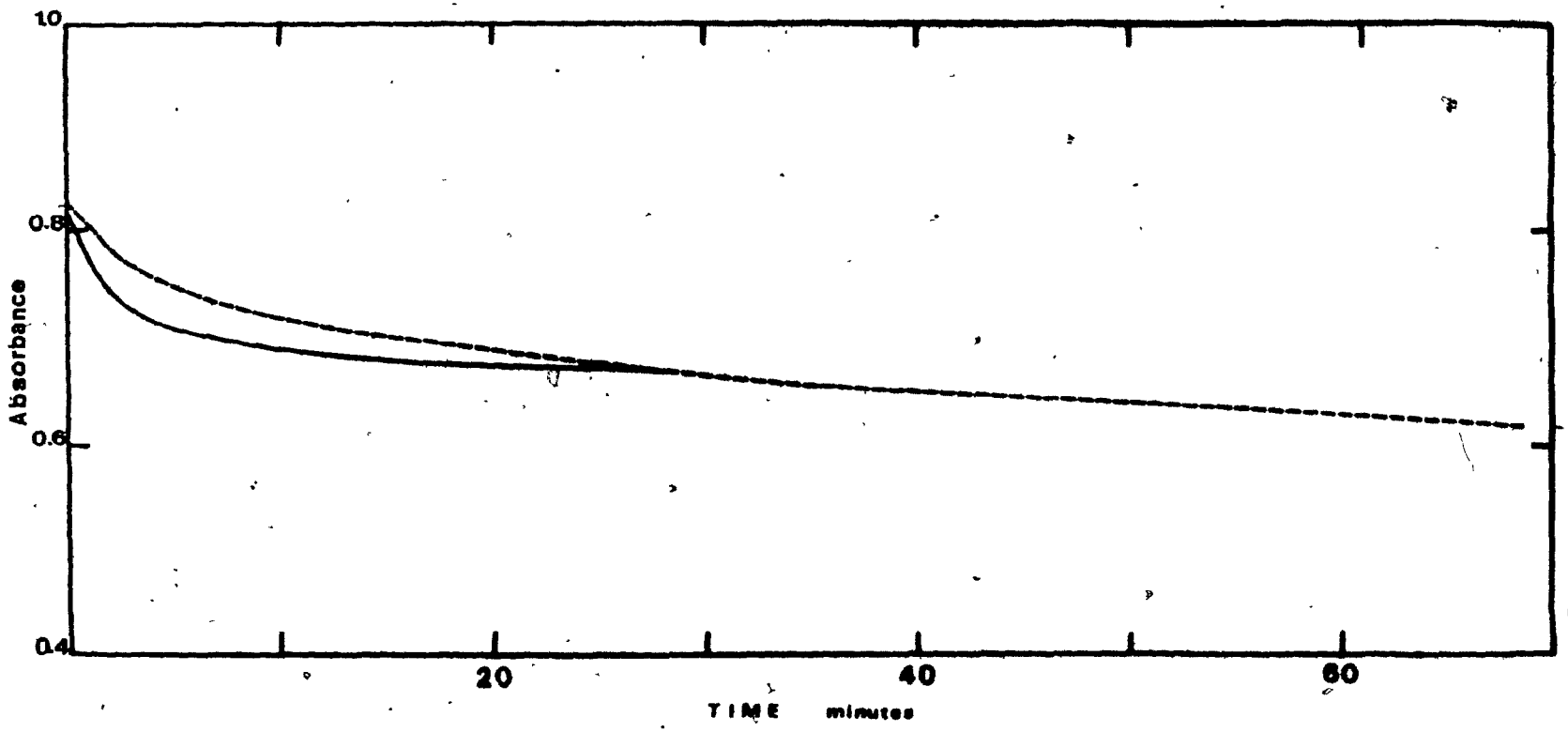
KINETICS OF ADSORPTION OF BILIRUBIN

BY CHOLESTYRAMINE AND PVP

$[\text{bilirubin}]_{\text{initial}}$ = 10 mg/dl

--- cholestyramine (15 mg)

—— PVP (15 mg)


1.0
0.8
0.6
0.4
Absorbance
20
40
60
TIME minutes

PVP is added to the bilirubin solution, a sharp decrease in the absorbance occurs and is followed by a levelling off after about 20 minutes. The completion of adsorption is clearly defined. In contrast, the initial rate of decrease in absorbance for adsorption by cholestyramine is less pronounced and adsorption extends over a longer period of time while the point at which adsorption is complete is difficult to assess.

3) Adsorption Isotherms

On the basis of the rate studies, adsorption isotherms at several temperatures were obtained as the amount of bilirubin adsorbed per gram of adsorbent (Fig. 17, 18). The amount of bilirubin adsorbed, calculated from the decrease in bilirubin concentration, was taken after 30 minutes of adsorption for PVP and after 90 minutes for cholestyramine. It appears from these isotherms that no improvement in the adsorption capacity compared to that of cholestyramine is obtained with PVP. Furthermore, while improved binding capacity with increasing temperature is obtained for cholestyramine, no temperature effect was observed for PVP.

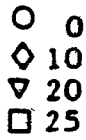
DISCUSSION

1) Effect of Soluble PVP on the Bilirubin Spectrum

The red and blue shifts observed in the absorption spectrum of bilirubin as a result of the addition of soluble, non-crosslinked PVP indicates a definite

FIGURE 17

ADSORPTION OF BILIRUBIN BY PVP



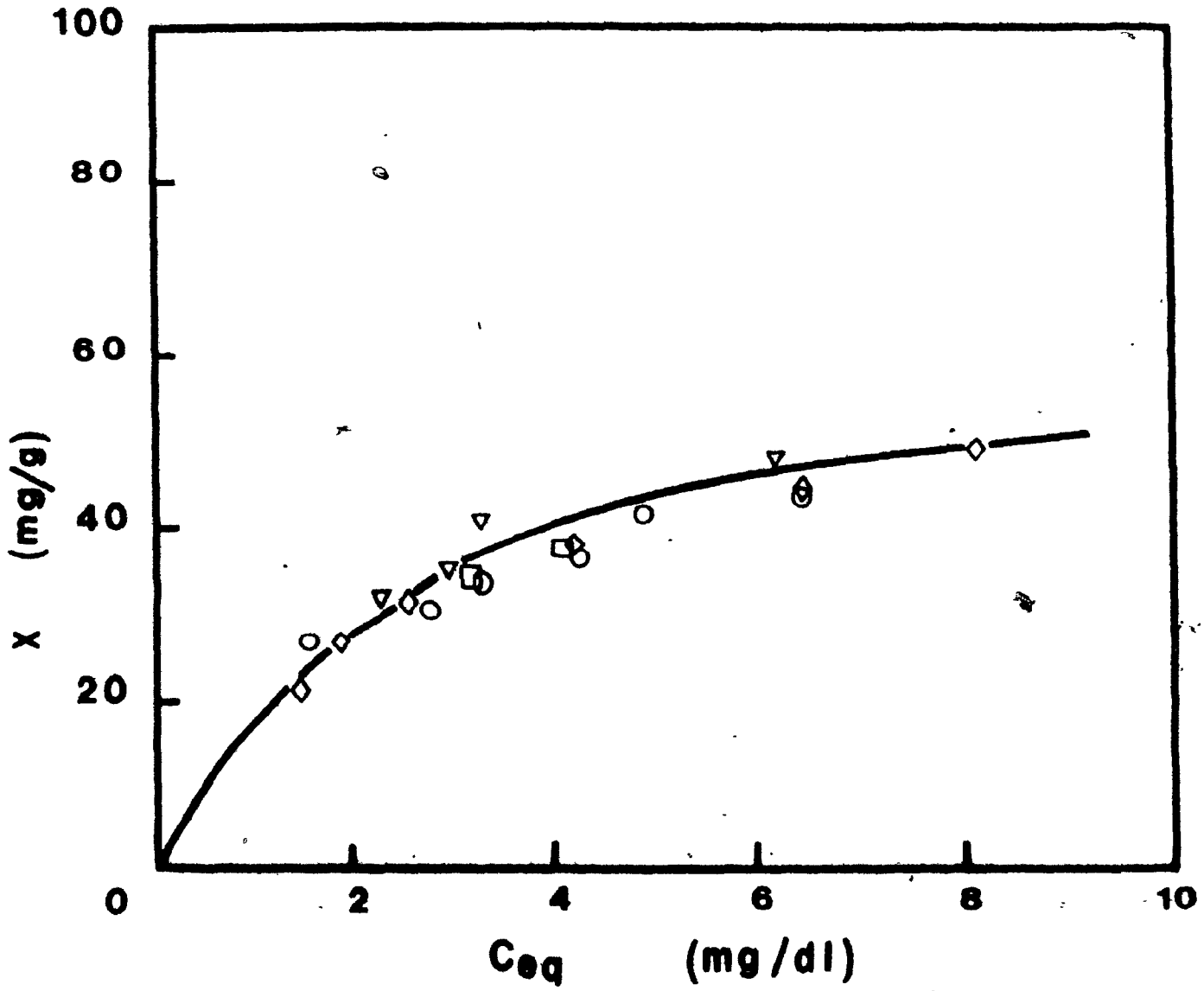
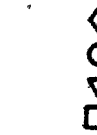
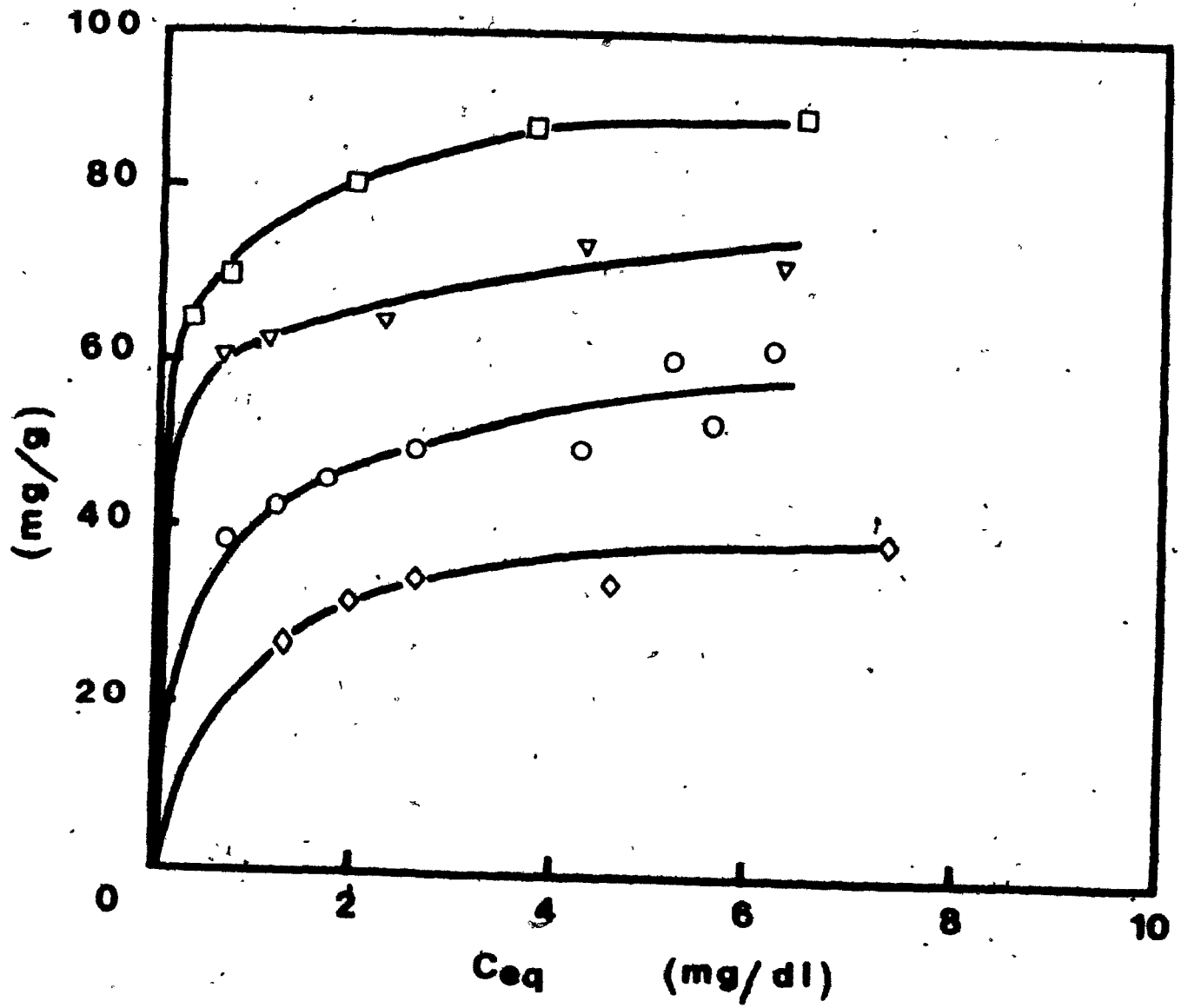

100
80
60
40
20
0
X (mg/g)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 18

ADSORPTION OF BILIRUBIN BY CHOLESTYRAMINE




100
80
60
40
20
0
(mg/g)
2
4
6
8
10
Ceq (mg/dl)

interaction. The existence of two different shifts, depending on the bilirubin to PVP ratio, also suggests that more than one complex can be formed. This fact was not noticed in the Ploussard (Ploussard, et al., 1972) report since that study was conducted at a fixed bilirubin to PVP ratio of 5×10^{-4}, i.e., under conditions where the blue shift is no longer detected. It is interesting to note that the two new λ_{max} obtained are essentially identical to the gain peaks obtained upon irradiation of bilirubin with visible light. Previous reports on the use of PVP as an adsorbent in chromatography of phenolic compounds and aromatic acids (Clifford, 1974, Olsson and Samuelson, 1974, Plaizier-Vercammen and de Neve, 1982) or for various drugs (Horn and Ditter, 1982) indicate that binding to PVP is generally the result of a H-bond formation, presumably to the C=O function on the PVP. Thus, it is possible that PVP has the same effect on bilirubin as does light, i.e., disruption of some of the bilirubin intramolecular hydrogen bonds. The shift in maximum to longer wavelength upon addition of soluble PVP is also similar to the shift in the absorption maximum of bilirubin to 460 nm obtained upon addition of albumin. Upon binding to albumin bilirubin assumes a dissymmetric conformation (Blauer and King, 1970, Blauer, et al., 1970) which once again would involve breaking the hydrogen bonds and a rotation of the chromphores relative to each other.

2) Kinetics of Adsorption and Adsorption Isotherms

The differences in kinetics and temperature dependence of the adsorption of bilirubin onto cholestyramine and onto crosslinked PVP indicate different adsorption processes. PVP is more water swellable than cholestyramine which in spite of having charged functional groups, does not swell extensively in water as a result of its hydrophobic polystyrene backbone. Thus, access and diffusion to the binding sites should be enhanced in PVP as compared to cholestyramine. However, the adsorption capacity for bilirubin is higher for cholestyramine than for PVP. Unlike PVP, cholestyramine has a charged side chain, namely a quaternary ammonium group. It is likely that PVP forms hydrogen bonds with the weakly acidic protons on the bilirubin lactam rings while cholestyramine form stronger salt linkages with the bilirubin carboxylic acid groups.

Although insoluble, crosslinked PVP adsorbed some bilirubin, its adsorption capacity is only approximately one half that of the reference resin, cholestyramine. However, PVP is favoured kinetically since adsorption of bilirubin reaches saturation in a few minutes. At short times, PVP adsorbs more bilirubin than does cholestyramine. This could be an important factor if the resins were used in a column for perfusion, since during a perfusion the contact time between the bilirubin and the adsorbent would be relatively brief. A resin adsorbing more rapidely could then well be more efficient than one with higher capacity but slower kinetics. Nonetheless, in order to maximize the

efficiency of the adsorbent, improvement in the adsorption capacity would still be more desirable.

CHAPTER III

ADSORPTION OF BILIRUBIN BY MERRIFIELD RESINS

INTRODUCTION

As discussed in Chapter I, transport of bilirubin in the body becomes possible as a result of binding to albumin. Each molecule of HSA is known to bind tightly two molecules of bilirubin. This information was used in seeking to design and synthesize a special sorbent for bilirubin. Instead of immobilizing the entire HSA molecule, with a molecular weight greater than 66,000, onto a suitable support it was decided that attempts would be made to synthesize, onto a suitable polymer backbone, pendants consisting of sequences of amino acids. Since bilirubin interacts with the amino acids in albumin, there is a strong possibility that it can also do so with immobilized amino acids.

For the purpose of this study it was assumed that the primary structure, and maybe the secondary stucture, of albumin play the major role in the binding of bilirubin. Any contribution from the tertiary structure, which cannot be represented with small peptides, was ignored even though it is recognised that tertiary structure of a protein plays a role in the binding of small ligands to that protein. Due to the unusually high conformational adaptability of serum albumins and the possibility that the binding sites are not preformed (Karush, 1950) it was expected that small peptides, mimicking the binding sites reasonably well, could still be synthesized.

As a starting point single amino acids were attached to Merrifield resin (a chloromethylated or hydroxymethylated polystyrene divinylbenzene resin) (Merrifield, 1963). The amino acids to be tested included the four amino acids, arginine, lysine, histidine, and tyrosine, proposed by Jacobsen (Jacobsen C., 1972, 1975, Jacobsen C. and Jacobsen J., 1979), to be active in the binding of bilirubin to albumin, as well as some other amino acids with uncharged R groups (Fig. 19).

EXPERIMENTAL

1) Synthesis of the Protected Amino Acid

A series of sorbents was prepared by chemically attaching single amino acids to the Merrifield resin. For this synthesis the amino acids, lysine, histidine and tyrosine were protected at the α-amino group and at the reactive side chain group with the ter-butyloxycarbonyl (t-BOC). Arginine was protected only at the α-amino group and purchased as such from Sigma.

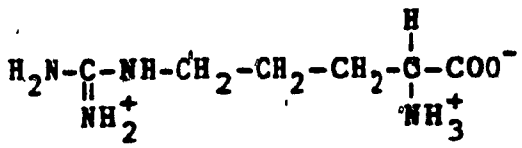
Protected amino acids were synthesized from the mono-BOC amino acids (N-α-t-BOC-L-histidine, N-α-t-BOC-L-tyrosine, Sigma) or from the unprotected amino acids (L-histidine HCl, L-lysine HCl, Sigma) by adding di-tert-butyl-dicarbonate (1.1 equivalent for the mono-BOC and 2.2 equivalent for the unprotected amino acids) to a solution of the amino acid (1 equivalent) and triethylamine (1-2 equivalents) (MC&B) and dimethylaminopyridine (2.4 mg/mmole

FIGURE 19

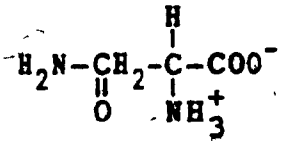
STUCTURE OF THE AMINO ACIDS

IMMOBILIZED ONTO THE MERRIFIELD RESIN

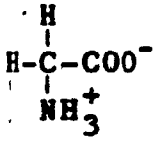
Arginine



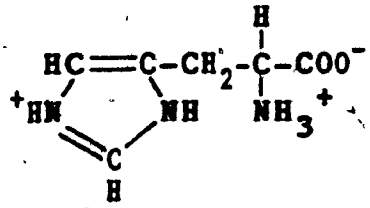
Asparagine



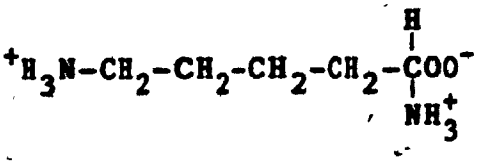
Glycine



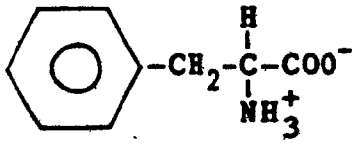
Histidine



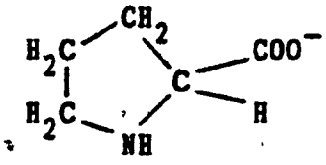
Lysine



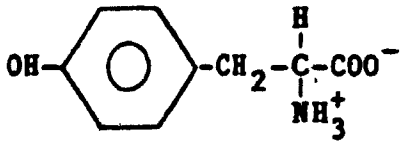
Phenylalanine



Proline



Tyrosine



of amino acid) (Aldrich) in dichloromethane for the mono-BOC or 50:50 water peroxide-free dioxane for the unprotected amino acids. The reaction occurred under N_2 at room temperature and was allowed to proceed for 18 hours.

The bis-BOC amino acids were isolated and purified using the following procedures:

From unprotected amino acid

- The water/dioxane solution containing the bis-BOC amino acids was washed with water (15 ml) and ethyl acetate (20ml).
- The aqueous layer was washed with ethyl acetate (20 ml).
- The aqueous phase was acidified with citric acid (1 g/20 ml).
- The bis-BOC amino acids were extracted with ethyl acetate (3 X 20 ml).
- The combined extracts were washed with water (3 X 10 ml), dried over $MgSO_4$, and filtered.
- The solvent was evaporated using a rotary evaporator and the bis-BOC amino acids were dried overnight under vacuum.

From mono-BOC

- The organic phase was washed with a citric acid solution (5%, 3 X 10 ml) and water (1 X 10ml).
- The organic phase was dried over $MgSO_4$, filtered, the solvent was evaporated, and the bis-BOC amino acids were dried under vacuum.

Samples were identified by H^1 NMR and their purity was checked by TLC.

2) Grafting of the Amino Acids onto the Merrifield Resins

The protected amino acids were attached to either the chloromethylated resin, in the case of tyrosine, arginine

and lysine, or to the hydroxymethylated resin in the case of histidine. To attach the amino acids to the chloromethylated resin, a solution of the amino acid (1 equivalent per equivalent of Cl present) in absolute ethanol (2 - 5 ml/g resin) was added to the resin. Triethylamine (0.9 equivalent) was added and the mixture was refluxed at 90°C for 24 hours (65 hours in the case of arginine). The resin was then washed (3 X 10 minutes) with absolute ethanol, water, methanol and dicholoromethane.

To attach histidine to the hydroxymethylated resin (Bachem), bis-BOC-histidine (1 equivalent) was added to an Erlenmeyer flask containing a solution of 1-hydroxybenzotriazole hydrate (HOBT)(1.2 equivalent) in dry tetrahydrofuran (2 ml) and dichloromethane (15 ml) at 0°C. Dicyclohexylcarbodiimide (DCC) was added and the mixture was stirred for 5 minutes before the resin (0.3 equivalent of OH, 0.5 g) was added. The reaction mixture was stirred, using a magnetic bar, under N_2 at room temperature, for 60 hours. The resin was washed (3 X 10 minutes) with dichloromethane, absolute ethanol, water and dichloromethane. Merrifield resins with proline, glycine, phenylalanine and asparagine pendants were purchased from Sigma as the N-t-BOC-amino acid ester.

The attached amino acids were deprotected by stirring the resin in trifluoroacetic acid (TFA) (40% in dichloromethane, 40 minutes) and neutralized with triethylamine. The resins were then thoroughly washed with dichloromethane and ethanol. The presence of the amino

acids was tested using the picric acid test (Gisin, 1972).

3) Adsorption Studies

The Merrifield resin (~60 mg) was added to the bilirubin stock solution (25 ml) in a stoppered flask. The suspended resin was mixed by inversion and left to stand for 30 minutes. The resin was filtered, washed first with buffer and then with dichloromethane. Presence or absence of bilirubin was judged from the colour of the resin.

RESULTS

1) Adsorption Studies

For none of the amino acids immobilized onto the Merrifield resin was sufficient adsorption obtained to cause a measurable decrease in the concentration of bilirubin in the adsorbate solution. However, since bilirubin is highly coloured, qualitative observations were possible based on the colour of the resin after washing with CH_2Cl_2 (Fig. 20) (Table V). A yellow colour was taken as an indication that sorption of bilirubin had occurred while a white resin indicated a lack of or a weaker interaction. Two resins, one with lysine and the other with arginine pendants, showed colour with the latter being more intense. It is of interest to note that lysine and arginine are two of the four amino acids proposed by Jacobsen to be located at or near the binding sites of HSA for bilirubin (Jacobsen C., 1972, 1975, Jacobsen C. and Jacobsen J., 1979).

FIGURE 20

COLOUR OF SOME SUBSTITUTED MERRIFIELD RESINS

AFTER THE ADSORPTION OF BILIRUBIN

A) Arginine Containing Resin

B) Unsubstituted Resin

C) Glycine Containing Resin

D) Lysine Containing Resin

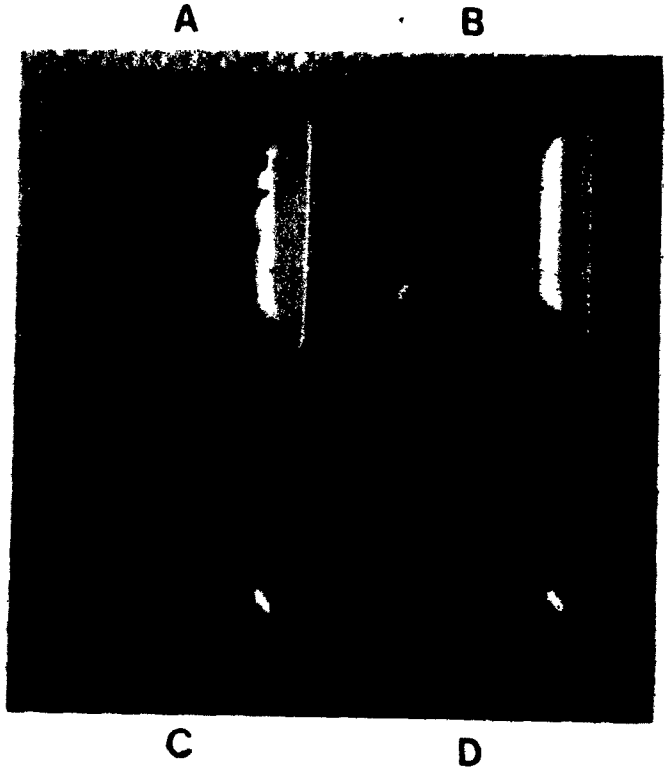

A
B
C
D

TABLE V

AMINO ACID CONTAINING MERRIFIELD RESINS FOR SORPTION OF BILIRUBIN

Amino Acid	R Group Status at pH of 7.8	Colour
no acid (resin only)	no charge	white
Lysine	positive charge	yellow
Arginine	positive charge	yellow
Glycine	no charge	white
Histidine	no charge	white
Tyrosine	no charge	white
Asparagine	no charge	white
Phenylalanine	no charge	white
Proline	no charge	white

These qualitative observations indicate that the presence of suitable amino acid pendants on the resin can result in strong interaction with bilirubin. Both, the lysine- and the arginine-containing resin, retained a significant amount of yellow colouration even after washing with CH_2Cl_2. The failure to adsorb larger quantities of bilirubin appeared to result from poor contact with the adsorbate solution. It would seem that resins with immobilized amino acids could give good adsorbents provided that water wettability could be obtained. Attempts were made to achieve better wettability by increasing the length of the amino acid pendants. Pendants consisting of Arg_1Ala_6, Arg_2Ala_6, Arg_1Ala_9, and Arg_2Ala_9 on a benzyhydrilamine resin were synthesized, however, wettability was not improved and no visible improvement in the adsorption was obtained.

DISCUSSION

1) Adsorption Studies

Adsorption of bilirubin by the Merrifield resins with lysine or arginine seems to confirm Jacobsen's postulate (Jacobsen C., 1972, 1975, Jacobsen C. and Jacobsen J., 1979) that lysine and arginine must be at or near the binding site for bilirubin on serum albumin. Of all the amino acids tested in this part of the study (which included all the amino acids proposed by Jacobsen as well as some other uncharged amino acids), arginine and lysine

were the only two showing a detectable interaction with bilirubin. Arginine and lysine are also the only two amino acids with a charged side group at a pH of 7.8. Furthermore, bilirubin is known to exist as a dianion in aqueous solution at this pH. Consequently, it is possible that a salt linkage is formed between the carboxylic acid groups of bilirubin and the positively charged R group of the amino acids and is responsible for the observed interactions. The R groups of the other amino acids are not ionized at a pH of 7.8. Thus, they are unable to form salt linkages. However, the lack of a detectable color in the resins containing these amino acids cannot be taken as indicating no interactions with bilirubin, since the possibility of weaker interactions than salt linkages still remains. These interactions could take place at the α-amino group which is also charged for all the resins. Hydrogen bonding, hydrophobic interactions, and/or specific non-ionic interaction with the R groups, can also be possible.

The preference of bilirubin to form an interaction with arginine and lysine is in agreement with the observation of Jacobsen (Jacobsen C., 1972, 1975, Jacobsen C. and Jacobsen J., 1979) and the recent rediscovery of δ-bilirubin (Wu, 1984). The presence of this bilirubin covalently linked to albumin in the sera of jaundiced infants was demonstrated by Wu. It was suggested that δ-bilirubin is bound through an amide bond between the bilirubin COOH and one of the amino acid NH_2's on the

albumin (Wu, 1984). It is, thus, quite likely that an in-vivo interaction occurs between the R group NH_2 of certain amino acids and bilirubin.

The Merrifield resin provided a first indication that immobilized amino acids have some potential as adsorbents for bilirubin. Clearly, even if bilirubin can be adsorbed by these materials, the amount of adsorption is insufficient to justify the replacement of cholestyramine by the Merrifield-amino acid resins. Nonetheless, the observed differences in interaction that result when different amino acids form the side chains provide strong evidence that it should be possible to prepare a sorbent that has a high capacity and perhaps even specificity for bilirubin by appropriate selection of the polymer backbone and of the amino acids on the peptide pendants.

CHAPTER IV

ADSORPTION OF BILIRUBIN BY POLYAMIDE RESINS

INTRODUCTION

The adsorption of bilirubin, albeit in very small amounts, by the arginine-Merrifield and lysine-Merrifield resins is an indication that these two amino acids may be useful in forming a good adsorbent. Clearly, the hydrophobic nature of the Merrifield resins offers a strong impediment to good adsorption from aqueous solution. Furthermore, it seems quite possible that the pendant amino acids, which were attached in the presence of organic solvents that cause the resin to swell, lie buried within the polymer beads and inaccessible to the aqueous bilirubin solution.

In this part of the study of the adsorption of bilirubin the use of a water-swellable "polyamide" resin will be presented. The amino acids used predominently as the active portion of the pendants are arginine and lysine. However, since interactions with other amino acids cannot be ruled out conclusively on the basis of the studies of the Merrifield resin, adsorption by resins containing histidine and tyrosine, the other two amino acids proposed by Jacobsen as being involved in the binding of bilirubin by albumin, will also be studied. The pendants grafted onto the polyamide resins will be composed of various series of amino acids to provide a systematic study of the effect of various factors such as charge density, length of the pendants, distance between the active amino acids and the polymer backbone, and distance separating the different

active amino acids within a pendant.

EXPERIMENTAL

1) Synthesis of the Pendants on the Polyamide Resins

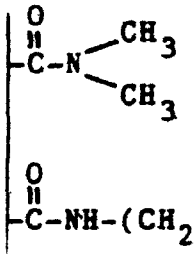
A polyamide resin (Chemalog), which is a water swellable copolymer of dimethylacrylamide and N-acryl-1,6-diaminohexane reticulated with bisacrylyldiaminoethane (14%) (Fig. 21), was used as the polymer backbone onto which the amino acid pendants were synthesized. The first amino acid of the pendant was attached directly to the resin NH_2 groups via the amino acid COOH group thus forming an amide bond. A trialanine "spacer" portion was always included next to the polymer backbone to extend the active unit and so make it more accessible for sorption.

The reaction scheme for the synthesis of the pendants onto the polyamide resin is given in Appendix I. The synthesis of the peptide pendants was achieved using a Vega model 250 automatic synthesizer (Appendix I) according to one of the sequences given in Appendix I. The different schemes correspond to improvements in the wash cycles as more syntheses were completed.

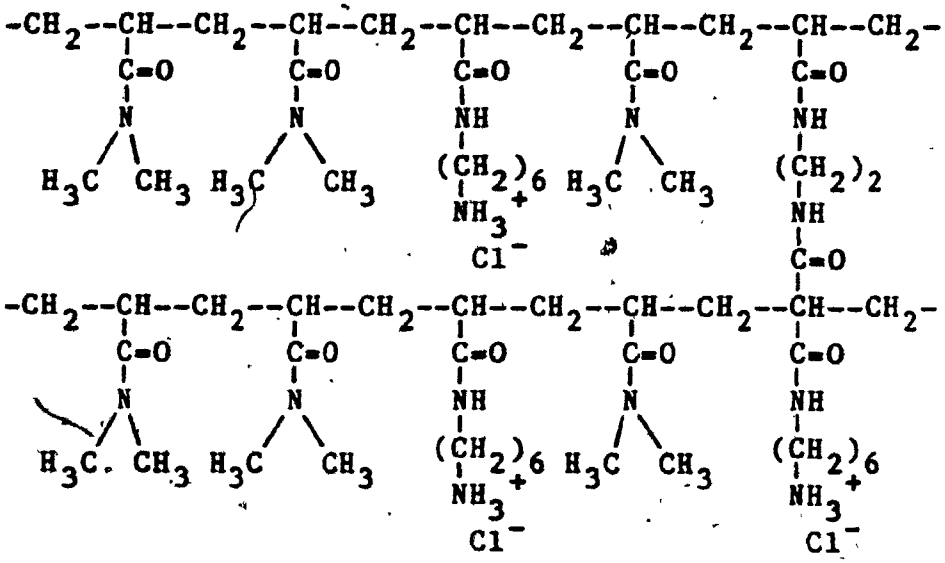
The resin was first swollen in dichloromethane and the hydrochloride salt was displaced with 40% diisopropylethylamine (DEA) in dichloromethane (30 minutes). Fully protected amino acids, N-α-t-BOC-L-alanine, N-α-t-BOC-2-bromo-CBZ-L-tyrosine, N-α-t-BOC-N-ε-2,4-dichloro-CBZ-L-lysine, N-α-t-BOC-N-im-tosyl-L-

FIGURE 21

POLYAMIDE RESIN



(Functional Groups)



histidine, and N-α-t-BOC-N-ω-nitro-L-arginine were obtained from Chemalog. The symmetrical anhydrides of the amino acids used as the active intermediates in the coupling step (Wiland, et al., 1973) were obtained by mixing a solution of two equivalents of the amino acid in dichloromethane or tetrahydrofuran (for arginine) with one equivalent of N-N'-dicyclohexylcarbodiimide (DCC)(10% in CH_2Cl_2). The reaction occurred at 0°C for 20 minutes. Upon completion of the reaction the dicyclohexyl urea obtained as a by-product was filtered off and the filtrate containing the reactive intermediate was added to the resin.

Upon completion of the coupling step the α-amino groups were deprotected with 40% trifluoroacetic acid (TFA), neutralized with 5% DEA and washed. The next amino acid was added and the synthesis sequence was repeated. Completion of the coupling step and deprotection were checked with the ninhydrin test.

The sorbents were synthesized in different batches. To obtain the various pendant lengths in a given batch, part of the resin was withdrawn when a given sequence length was complete. The synthesis was resumed with the remaining sample. After the last amino acid in the desired sequence had been introduced, deprotected, neutralized and washed, the resin was washed a last time with anhydrous ethyl ether and dried overnight under vacuum.

The R groups were then deprotected by treatment with anhydrous HF (Sakakibara and Shimonishi, 1965). The resin was introduced into the reaction flask of the HF line

(Appendix I). Next, one ml of anisol was added and the flask was securely screwed onto the line. The reaction mixture was cooled with liquid N_2 and HF (15 - 20 ml per gram of resin) was distilled in the reaction flask. The mixture was warmed to 0°C while the resin was stirred with a magnetic stirrer to ensure the complete penetration of the resin by HF. Once the HF had melted the stirring was kept to a minimum to avoid grinding the resin. After one hour the HF was evaporated, the resin was washed with anhydrous ethyl ether, dried under vacuum and kept in a vacuum dessicator until use.

2) Amino Acid Analysis

Completion of each coupling step and deprotection of the α-amino groups were checked with the ninhydrin test which can detect less than 1% of free NH_2 (Kaiser, 1970) so that a minimum of 99% completion of each coupling step is indicated. Hence, for the longest pendant, consisting of 8 amino acids, at least 93% of the pendants should have the complete, correct sequence (Meinhoffer, 1973). A recent study on the efficiency of the solid phase peptide synthesis (Sarin, et al., 1984) reveals that even under less than optimal conditions, the concentration of deletion peptides, i.e., peptides missing one residue, is less than 1%, and, by forcing the conditions, it can be reduced to less than 0.05%. Furthermore, efficiency is not affected as the peptide chain is lengthened. Nonetheless, an amino acid analysis was obtained, from the laboratory of Dr. S.A.

St-Pierre, Department of Pharmacology, Centre Hospitalier Universitaire de Sherbrooke, for the resin containing the Arg_2Ala_3 pendants. The sample was submitted to hydrolysis in 6 N HCl for 20 hours at 110°C under vacuum. Upon completion of the hydrolysis the HCl was evaporated and the amino acids were lyophilized. The residue obtained was dissolved in a citrate buffer (pH = 2) and analysed with a Beckman 119 CL amino acid analyser.

3) Adsorption Studies

For the study of the adsorption of bilirubin by the polyamide resins, bilirubin solution at an appropriate concentration, prepared as for the studies of adsorption by PVP (Chap. II), was added to an accurately weighed amount of the resin (~10 mg) contained in the adsorption flask (Fig. 14). The flask was placed in an ice water bath and purged continuously with nitrogen. For the study of the kinetics of adsorption, aliquots (0.5 ml) were withdrawn at desired times. To obtain the adsorption isotherms, only one aliquot was withdrawn at 60 minutes. The bilirubin concentration was determined as for PVP and cholestyramine.

4) Adsorption in the Presence of Competitors

Adsorption of bilirubin by polyamide resins with pendants of Arg_5Ala_3 and Arg_1Ala_3 was also studied in the presence of arginine (bilirubin to arginine ratio = 1/4 to 1/7). The arginine HCl (Sigma) was added, either to the resin (10 mg) before the bilirubin solution (5 - 10 mg/dl,

25 ml) was added or directly to the bilirubin solution before it was added to the resin. In the latter case, the bilirubin-arginine solution was stirred for 15 minutes before it was added to the resin. In both cases adsorption was carried out in the usual manner once the three reagents were present.

Acetyl salicylic acid (10 - 13 mg/dl) was added to the polyamide resin with Arg_5Ala_3 pendants (10 mg) while urea (20 mg/dl) and creatinine (10 mg/dl) were added to polyamide resin with Arg_1Ala_3 pendants (10 mg) before addition of the bilirubin solution (10 mg/dl, 25 ml). Uric acid (mg/dl) was dissolved in a small amount of 0.10 M NaOH and added to a bilirubin solution (10 mg/dl). This solution (25 ml) was added to polyamide resin with Arg_1Ala_3 pendants (10 mg). Adsorption was carried out as usual.

RESULTS

1) Amino Acid Analysis

The recorder trace from the amino acid analysis confirmed the presence of arginine and alanine. The area under the peak for each these acids were compared to the area under the corresponding peak of reference sample containing a known and equal amount of these two acids. A ratio of 2.8 alanines to 2 arginines was obtained as compared to the expected ratio of 3 to 2.

2) Kinetics of Adsorption

Preliminary experiments showed that the use of a polymer with water swellable backbone greatly increases the adsorption capacity as compared to that of the hydrophobic Merrifield resins. To determine the time the polyamide resins should stay in contact with the bilirubin solution before adsorption could be considered complete the adsorption of bilirubin was followed as a function of time for the polyamide resins with pendants consisting of Arg_1Ala_3, Arg_2Ala_3, and Arg_5Ala_3 (Fig. 22). Adsorption is rapid during the first 15 minutes but then slows down. A small continuous decrease in absorbance between 60 and 90 minutes suggests that adsorption is maintained as a slow process after the initial burst. However, the change in absorbance reading as a function of time is very small, not significantly different from experimental error, and may well reflect loss of bilirubin due to factors other than adsorption, e.g., oxidation or deposition on the stirrer and flask. Thus, for further studies, adsorption was considered complete at the end of 60 minutes.

3) Adsorption by the Polyamide Resins

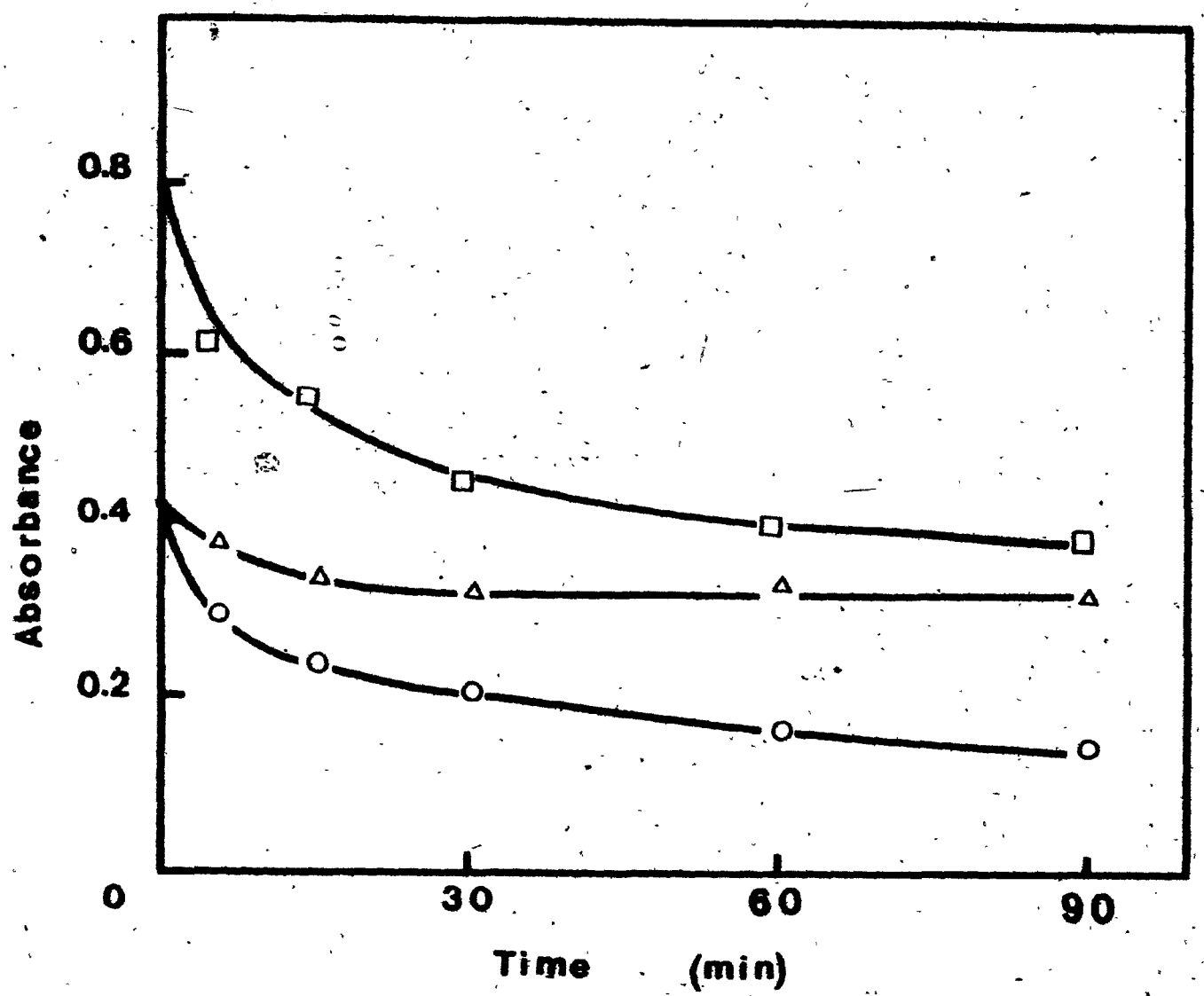
3a) Effect of pendant composition

All of the polyamide resins incorporated an Ala_3-polyamide structure as the starting point. To establish a reference point for comparison of adsorption characteristics, isotherms were determined for the adsorption of bilirubin by the unsubstituted polyamide

FIGURE 22

KINETICS FOR THE ADSORPTION OF BILIRUBIN

BY THE POLYAMIDE RESINS


0.8
0.6
0.4
0.2
0
Absorbance
30
60
90
Time (min)

resin and by the Ala_3-polyamide resin at 0°C. Figure 23 shows the amount of bilirubin per equivalent of pendants, adsorbed after 60 minutes, plotted as a function of the final equilibrium concentration. The adsorption capacity of the polyamide resin is not significantly increased by the addition of the trialanine spacer. This might be expected since the unsubstituted resin already has NH_2 as functional groups which could be considered similar to the terminal α-amino groups of the trialanine spacer.

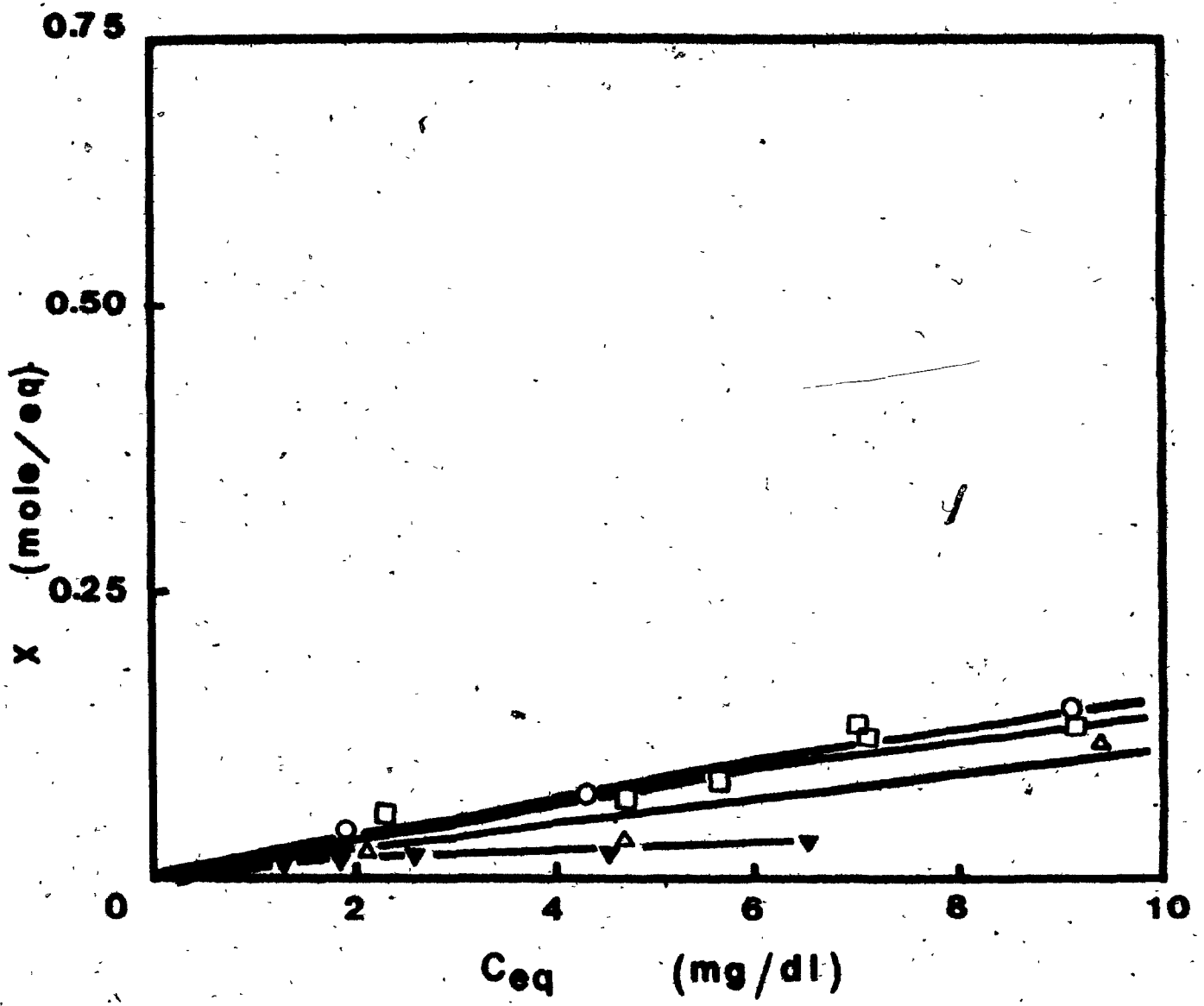
Acetylation of the terminal α-amino groups of the trialanine spacer reduces the adsorption capacity of the Ala_3-polyamide to less than one half (Fig. 23). This is a good indication that the α-amino groups contribute substantially to the adsorption of bilirubin by the polyamide resins. However, there is some residual adsorption which may be attributed to adsorption by the polymer backbone or, perhaps, to a loss of bilirubin by some process other than adsorption by the resin.

Also included in Fig. 23 for the purpose of comparison is the isotherm, determined under similar conditions, for the adsorption of bilirubin by cholestyramine (Chap. II). On the basis of equivalents of active sites, both the polyamide and the Ala_3-polyamide resins have the ability to adsorb bilirubin in amounts that significantly exceed that adsorbed by cholestyramine. However, because of the higher substitution of cholestyramine it still has a greater capacity for bilirubin when it is expressed on a per gram of sorbent basis.

FIGURE 23

ADSORPTION OF BILIRUBIN BY THE REFERENCE RESINS

□ Unsubstituted Polyamide Resin
○ Ala_3
△ Ala_3 (Acetylated)
▼ Cholestyramine

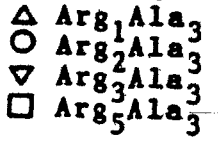

0.75
0.50
0.25
0
x (mole/mole)
2
4
6
8
10
Ceq (mg/dl)

The adsorption isotherms of various polyamide resins, to which were attached pendants synthesized with the four amino acids, arginine, lysine, histidine and tyrosine were determined at 0°C. Duplicate experiments indicate that the precision of individual points is about ± 10% in conditions where adsorption is small, e.g., for the reference resins and the resins containing histidine and tyrosine, or at low equilibrium concentration (≤ 2 mg/dl). For experiments in which the amounts of bilirubin adsorbed are larger the precision is better. Figures 24 to 27 show isotherms for resins with the structure X_nAla_3-polyamide, where X is one of the four amino acids mentioned by Jacobsen and n varies from 1 to 5.

The adsorption isotherms for the polyamide resins with pendants containing arginine, one of the two amino acids which showed some sign of interaction with bilirubin when immobilized onto the Merrifield resin, are shown in Fig. 24. The resin with the Arg_1Ala_3 shows enhanced adsorption as compared to the unsubstituted resin and the resin containing the trialanine spacer (NOTE: In comparing Fig. 23 with Fig. 24 the change in the scale of the y axis should be noted). As the number of arginines in the pendant is increased from one to two a dramatic increase in the adsorption capacity is obtained. However, no further enhancement is obtained when three arginines, instead of two, are present in the pendants. A further increase is obtained in the adsorption capacity of the resins as the pendant length is increased from three to five arginines.

FIGURE 24

ADSORPTION OF BILIRUBIN BY THE ARGININE-CONTAINING RESINS



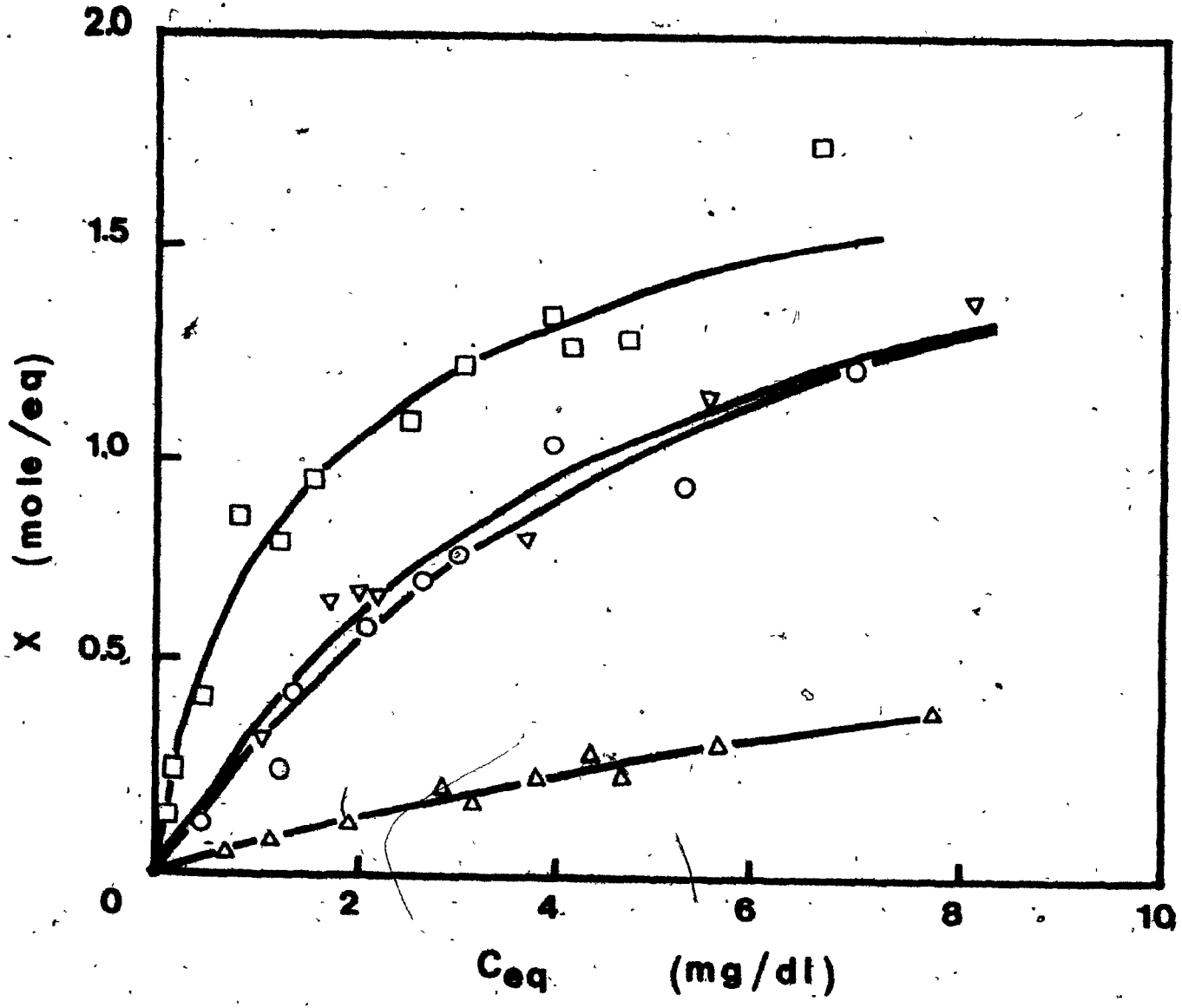
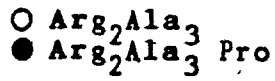

2.0
1.5
1.0
0.5
0
X (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 25

EFFECT OF PROTECTING THE R GROUP OF ARGININE PENDANTS ON POLYAMIDE RESINS



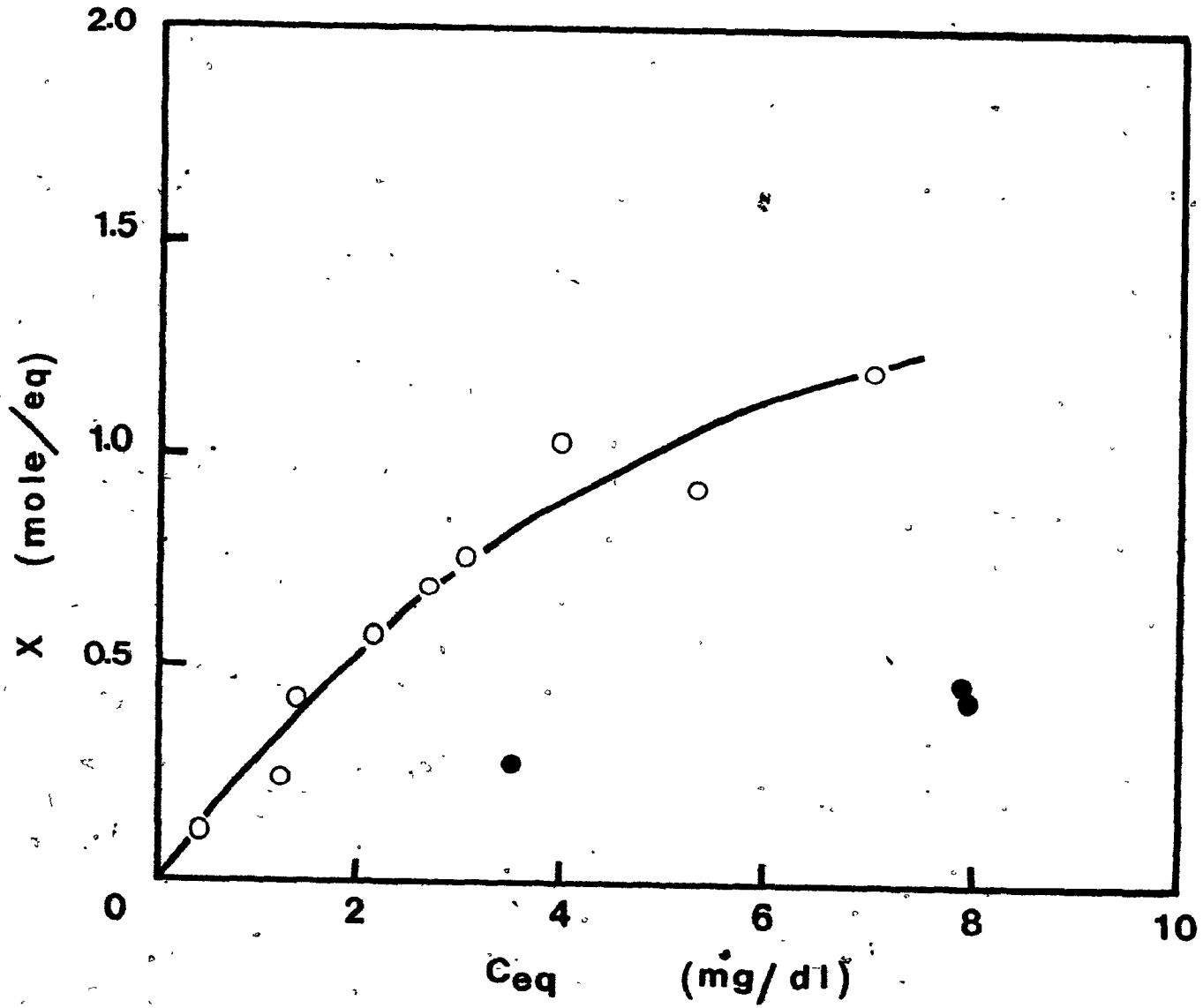

2.0
1.5
1.0
0.5
0
X (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 26

ADSORPTION OF BILIRUBIN BY THE LYSINE-CONTAINING RESINS

△ Lys_1Ala_3
○ Lys_2Ala_3
□ Lys_5Ala_3

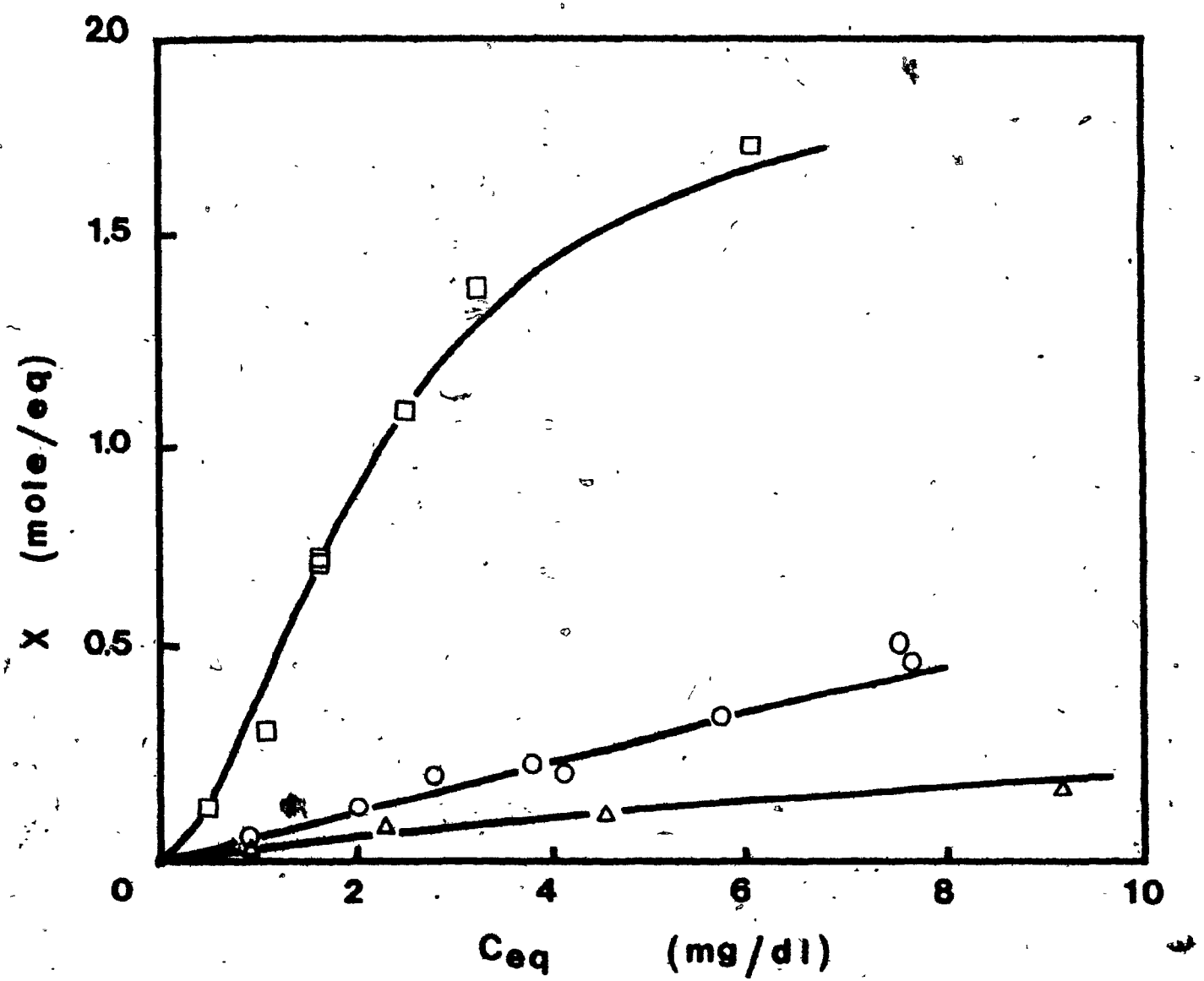
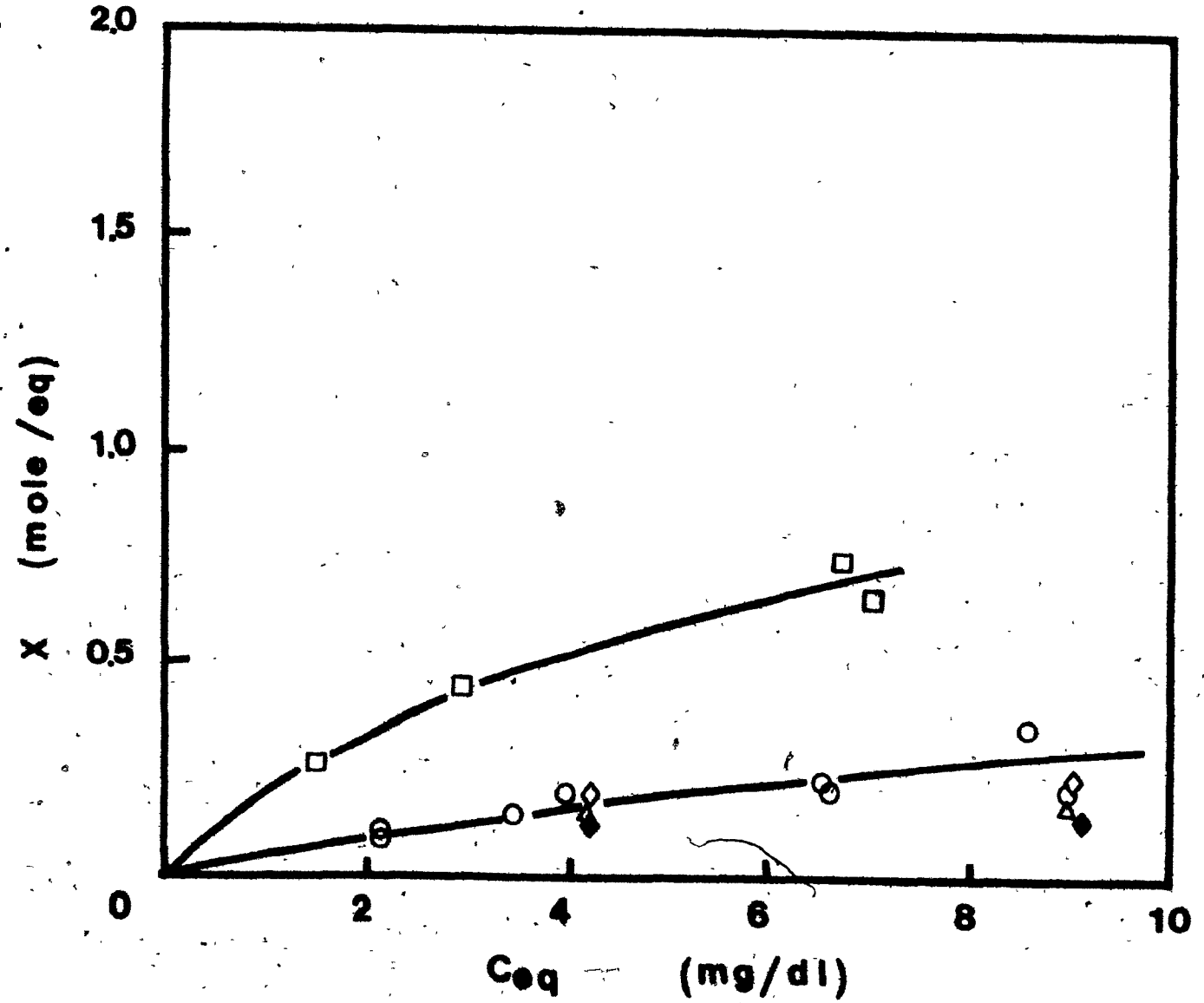

2.0
1.5
1.0
0.5
0
X (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 27

ADSORPTION OF BILIRUBIN

BY THE HISTIDINE- OR TYROSINE-CONTAINING RESINS

△ His_1Ala_3
○ His_2Ala_3
□ His_5Ala_3
◆ Tyr_1Ala_3
◇ Tyr_2Ala_3


2.0
1.5
1.0
0.5
0
x (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

However, this increase is less than the increase obtained between the resins containing one and two arginines.

The Arg_2Ala_3 resin was also tested with the two guanido groups still protected by nitro groups but with the α-amino groups free. The adsorption capacity of this resin is reduced to one third that of the Arg_2Ala_3 resin but it still remains slightly more active than the Arg_1Ala_3 resin (Fig. 25). This behaviour, like that of Ala_3 pendants with an acetylated α-amino group which retained a capacity less than one half that of Ala_3 (Fig. 23), gives strong evidence that the adsorption of bilirubin is not due solely to the interaction with the R group on the amino acid pendants. As shown by the adsorption behaviour of these "protected" resins, the α-amino group is also active. This interaction was not detected with the glycine-Merrifield resin, probably because of its lack of water swellability. The fact that the arginine-Merrifield and lysine-Merrifield resins adsorbed bilirubin shows that, as might be expected, the interactions with the charged R groups are more powerful.

The adsorption isotherms of the polyamide resins containing pendants with lysine (Fig. 26), the second amino acid showing signs of interaction with bilirubin when immobilized onto the Merrifield resin, also show an increased adsorption capacity as compared to the unsubstituted and the trialanine containing resins (Fig. 23). However, the adsorption capacity of the resins containing the Lys_1Ala_3 pendants is smaller than that of

the resin containing the Arg_1Ala_3 pendants. The increase in adsorption capacity obtained when one extra lysine is added to the pendants to yield a polyamide resin with a Lys_2Ala_3 pendant is also smaller than the increase which was obtained between one and two arginines. Thus, the adsorption capacity of the polyamide containing the Lys_2Ala_3 pendants is smaller than the corresponding resin, Arg_2Ala_3, in the arginine series. In fact, it is similar to the adsorption capacity of the resin containing the pendant Arg_1Ala_3. However, the resin containing the Lys_5Ala_3 pendants breaks the trend of the lysine-containing resins showing a lower adsorption capacity than their arginine-containing counterparts. The increase in adsorption capacity between the resin containing the Lys_2Ala_3 and the resin containing the Lys_5Ala_3 is larger than any increase in adsorption capacity obtained previously. Thus, although the resin containing the Lys_5Ala_3 has an adsorption capacity which is lower than that of Arg_5Ala_3 at low equilibrium concentrations, it becomes greater at higher equilibrium concentrations.

The adsorption isotherms for the resins containing histidine in the pendants, an amino acid mentioned by Jacobsen but which did not show any visible sign of interaction with bilirubin when immobilized onto the Merrifield resin, are shown in Fig. 27. Although the resin containing only one histidine does not show any increase in adsorption capacity as compared to that of the resin containing the trialanine spacer, the other resins

containing pendants with more than one histidine show some improvement in adsorption capacity (Fig. 27). However, the increase in the adsorption capacity is smaller than that obtained for either the arginine- or lysine-containing resins. The resin containing the His_2Ala_3 pendants has an adsorption capacity which is situated somewhere between the adsorption capacity of the resins containing Lys_1Ala_3 and Lys_2Ala_3 pendants while it is approximately similar to that of the resins containing the Arg_1Ala_3 pendants. The resin containing the His_5Ala_3 pendants has a higher capacity than the resin containing the Lys_2Ala_3, but it is still smaller than that of the resin containing the Arg_2Ala_3 pendants.

The adsorption isotherms for the tyrosine-containing resins, the fourth amino acid mentioned by Jacobsen but which like histidine did not show any visible signs of interaction with bilirubin when it was immobilized onto the Merrifield resin, are also shown on Fig. 27. These resins behave like their histidine-containing counterpart, with the resin containing the Tyr_1Ala_3 pendants showing almost no improvement in the binding capacity as compared to that of the trialanine containing resin while a small increase is obtained for the resin with the Tyr_2Ala_3 pendants.

In general, the adsorption capacity for the substituted resins with a given number (less than 5) of active amino acids follows the order arginine > lysine > histidine = tyrosine, i.e., decreasing with decreasing basicity. At pH = 7.8 it is expected that both arginine and lysine pendants would be positively charged while histidine and tyrosine

would be uncharged. This is based on the assumption that the pK_a of the amino acids in the pendants is similar to those of free amino acids, as is known to be the case for small peptides in solution (Lehninger, 1975)

Doubling the number of active amino acids in the pendant, i.e., increasing n from 1 to 2, generally results in an increased capacity for bilirubin when X is a charged amino acid with a basic pK_a, such as arginine or lysine (Figs. 24 and 26), but a smaller increase is obtained when X consists of the pendants with uncharged histidine and tyrosine (Fig. 27). This suggests that charge density is an important factor in the adsorption of bilirubin by these resins. A confirmation of the importance of charge density is demonstrated by experiments involving resins with pendants containing an even higher number of arginine, lysine or histidine units in the pendants (Figs. 24, 26, 27). In the case of arginine, increasing the number of charged amino acids from two to three, has no apparent effect on the adsorption isotherm, but a further increase to five enhances the adsorption capacity by a factor of 4.5 as compared to that of the resin whose pendants contain only one arginine. For lysine the adsorption is enhanced by a factor of 11 when the pendant length is increased from one to five while for histidine the enhancement is about 6. A summary of the isotherms, based on the amount of bilirubin adsorbed at high equilibrium concentration (C_{eq} = 6 mg/dl), where the change in adsorption with concentration is small, is given in Table VI.

TABLE VI

RELATIVE ADSORPTION CAPACITIES FOR BILIRUBIN BY SUBSTITUTED POLYAMIDE RESINS

	Number of Amino Acids Attached to the Ala_3			
Amino Acid	1	2	3	4
Arginine	3	10	10	14
Lysine	1,3	4	---	16
Histidine	1	2.5	---	6
Tyrosine	1	2.5	---	---

It is of interest to note that although the resin with the Arg_2Ala_3 pendants has a greater capacity for bilirubin per equivalent than does the Lys_2Ala_3-containing resin, the capacity of the Arg_5Ala_3 resin is somewhat less than that of the Lys_5Ala_3 resin. Rather surprisingly, the resins with Arg_2Ala_3 and Arg_3Ala_3 pendants have an approximately equal capacity for bilirubin. However, adding a histidine in place of an arginine to the Arg_2Ala_3 pendants to yield $His_1Arg_2Ala_3$ pendants, produces a resin with an adsorption capacity slightly larger than that of the resins containing two or three arginines (Fig. 28). Clearly some synergistic role is played by this added histidine. The fact that histidine is uncharged and has a small adsorption capacity by itself suggests that factors other than charge density, perhaps conformation of the peptide chain, also play a significant role in the adsorption process.

3b) Effect of Pendant Length

The effect of the length of the pendants as opposed to charge density was tested using arginine-containing resins with extra alanine spacers either at the beginning of the chain, i.e., before the first arginine, or between arginines (Fig. 29). Each of these resins can be related to another resin with either the same number of amino acids or with the same number of arginines in the pendant. As might be expected, the resin with Arg_1Ala_4 pendants behaves like the resin with Arg_1Ala_3 pendants, which has the same number of arginines, rather than like the resin containing the Arg_2Ala_3 pendants, which has the same number of amino

FIGURE 28

EFFECT OF THE ADDITION OF AN HISTIDINE TO ARG_2ALA_3

○ Arg_2Ala_3
▽ Arg_3Ala_3
▼ $His_1Arg_2Ala_3$

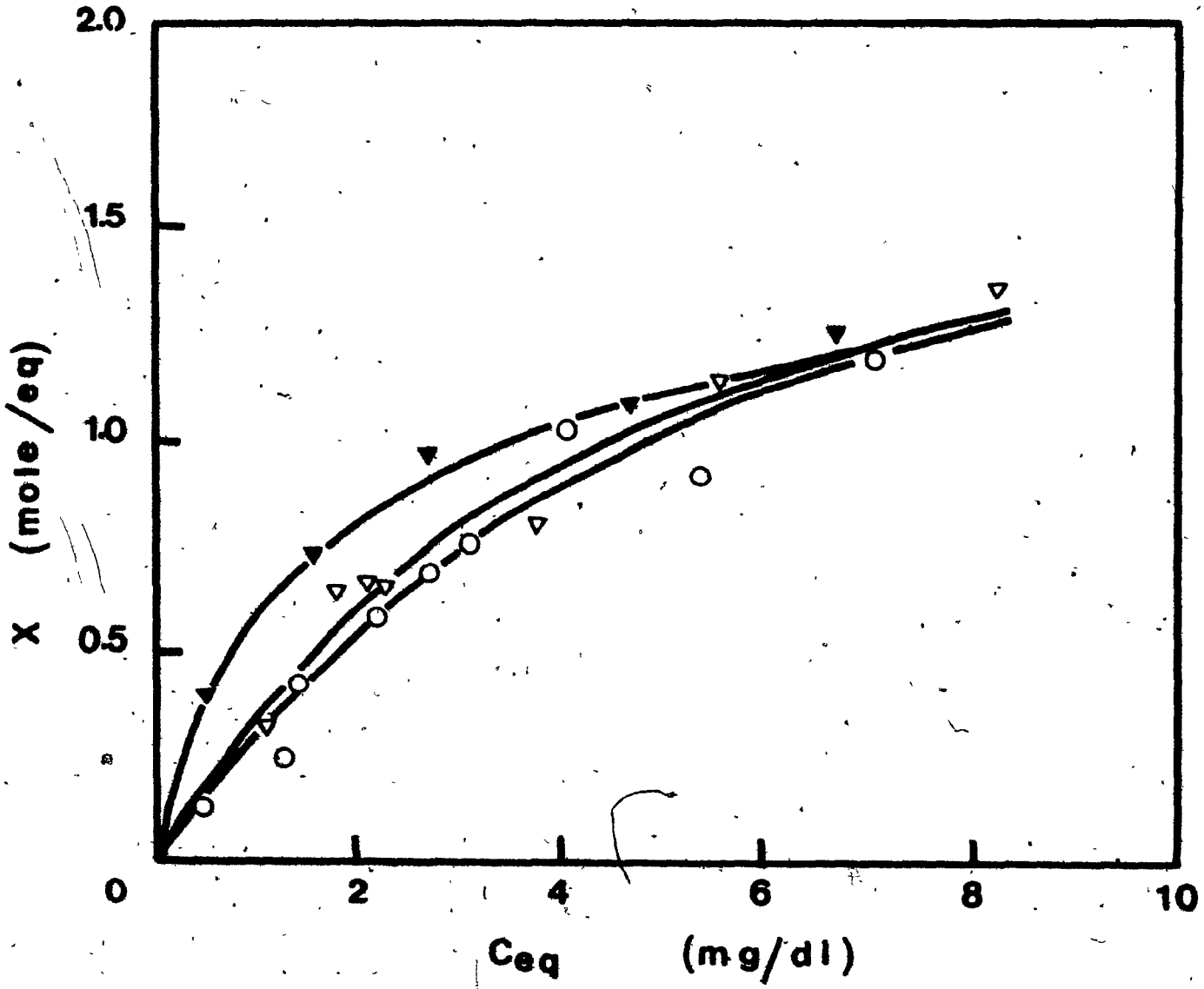
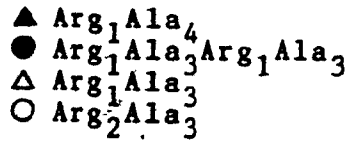
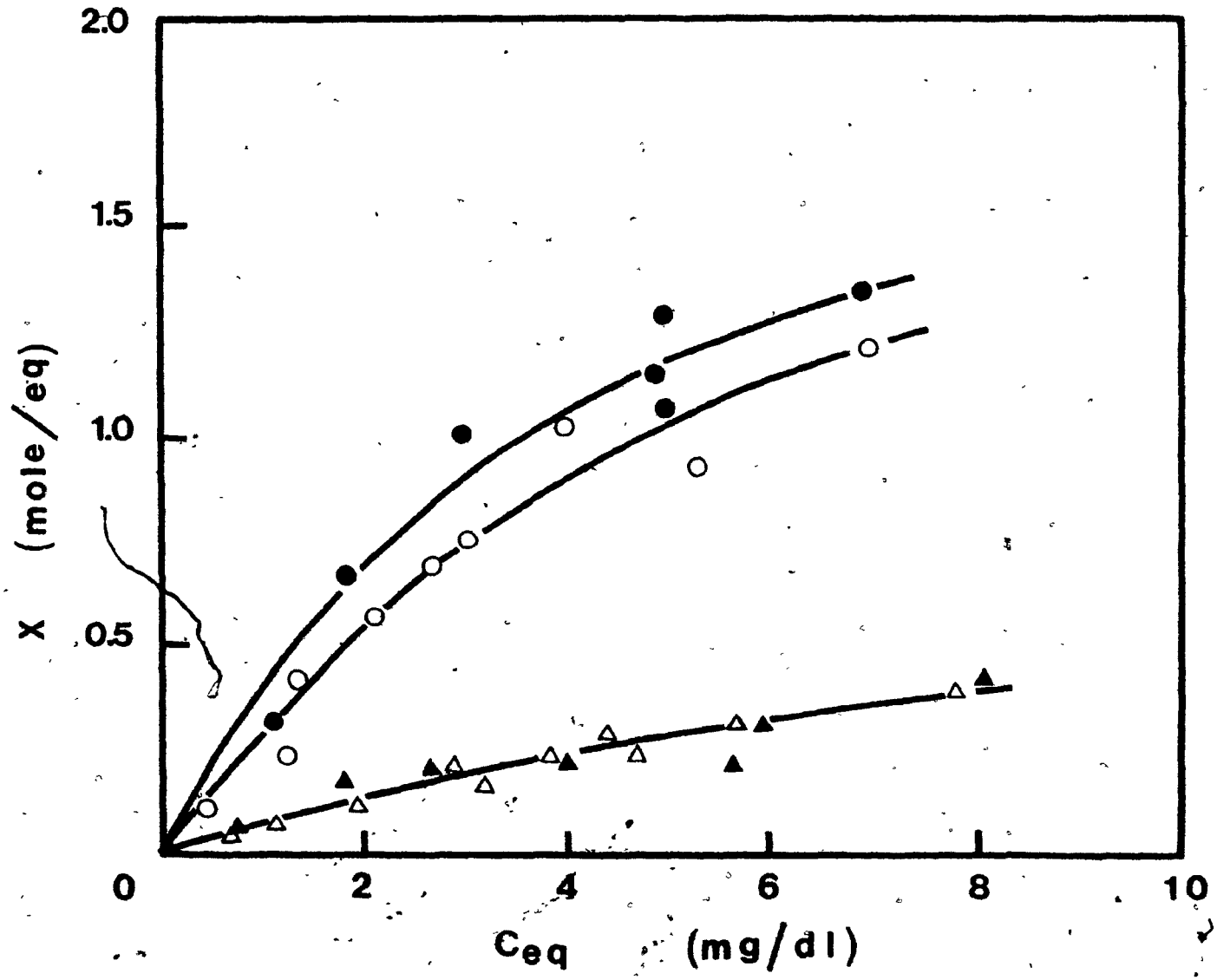

2.0
1.5
1.0
0.5
0
2
4
6
8
10
X (mole/eq)
Ceq (mg/dl)

FIGURE 29

EFFECT OF THE ADDITION OF A SPACER




2.0
1.5
1.0
0.5
0
2
4
6
8
10
X (mole/eq)
Ceq (mg/dl)

acids in the pendants. However, it is somewhat surprising that the resin with the $Arg_1Ala_3Arg_1Ala_3$ pendants has an adsorption capacity which is higher than that of the Arg_2Ala_3 resin, containing the same number of arginines in the pendants. However, it is still lower than that of the resin with the Arg_5Ala_3 pendants, the resin with the same number of amino acids in the pendants. Thus, the Ala_3 spacer separating the arginines apparently has a beneficial, albeit minimal, effect on the adsorption capacity that seems to be more dependent on the number of arginines in the pendants than on the length of the pendants. It appears that increasing the distance separating the "active" amino acids, in this case arginine, has a positive effect on the adsorption capacity.

4) Effect of Ionic Strength

An adsorption isotherm for the Arg_2Ala_3 resin was also obtained at lower ionic strength of 0.010 M as compared to previous studies at 0.10 M. Although, adsorption at low equilibrium concentration seems to be enhanced somewhat, no changes are apparent for adsorption at higher equilibrium concentrations (Fig. 30).

5) Adsorption with Competitors

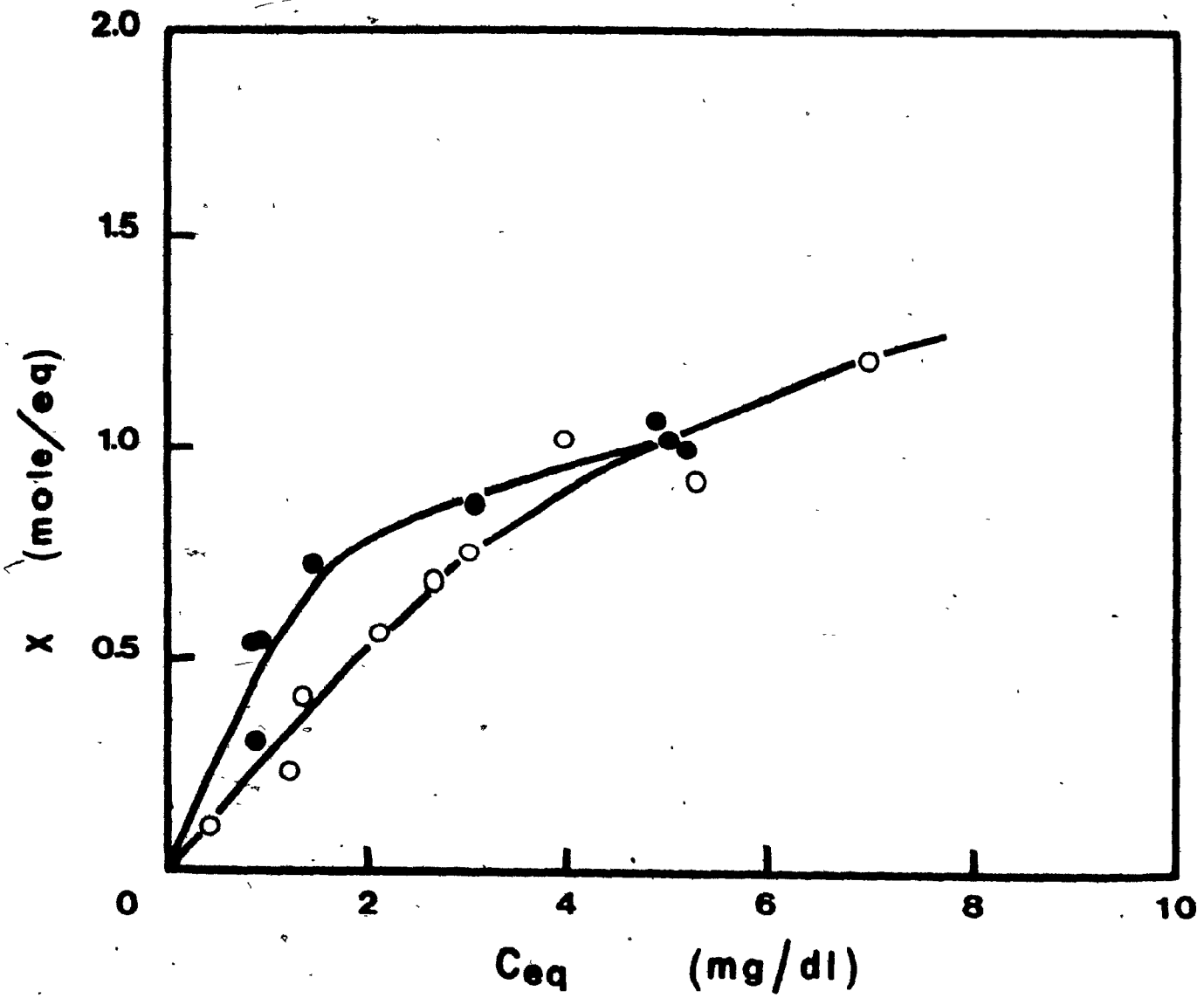
Of all the competitors tested, arginine, uric acid, creatinine and urea, none had any detectable effects, either positive or negative, on the adsorption of bilirubin by the resins containing Arg_5Ala_3 and Arg_1Ala_3 pendants.

FIGURE 30

EFFECT OF IONIC STRENGTH ON

ADSORPTION OF BILIRUBIN BY ARG_2ALA_3

○ Arg_2Ala_3 0.10 M
● Arg_2Ala_3 0.010 M


2.0
1.5
1.0
0.5
0
2
4
6
8
10
x (mole/eq)
Ceq (mg/dl)

DISCUSSION

1) Comparison between Polyamide and Cholestyramine

Comparison of the adsorption isotherm of the polyamide resins to that of cholestyramine clearly shows that former are more effective on a per equivalent of pendants basis. Even the unsubstituted resin and the Ala_3 resin themselves have capacity for bilirubin 4 to 5 times greater than that of cholestyramine when compared on an active unit basis. However, cholestyramine has a much higher substitution, more than 16 times greater than the unsubstituted polyamide resin. Hence, since steric hindrance from the already adsorbed bilirubin can influence further binding, this comparison may be somewhat unjustified. For this reason a comparison on a weight basis may be more appropriate. On a weight basis, the resin containing Arg_1Ala_3 pendants is the resin with an adsorption capacity closest to that of cholestyramine. Resins with more than one arginine residue as well as those with the Lys_2Ala_3, Lys_5Ala_3, and His_5Ala_3 pendants all have a much higher adsorption capacity. These, even on a weight basis are still more effective adsorbents than cholestyramine. Moreover, on a active unit basis, the Arg_5Ala_3 resin has a capacity which is 70 times greater than that of cholestyramine.

2) Comparison between Polyamide and Other Bilirubin Sorbents

Adsorption capacities for other sorbents used previously for the removal of bilirubin have been presented in Chap. I (Table II). For adsorption of bilirubin from aqueous solutions, the polyamide resins are of the order of 100 times more efficient than most of those sorbents. It is of particular interest to note that the immobilized albumin has a capacity of approximately 0.2 mg/g (Plotz, et al., 1974a, 1974b). By comparison, the resin with Arg_5Ala_3 pendants resin has a capacity for bilirubin that is approximately 500 times as large. However, most of the resins listed in Chap. I have been tested for adsorption of bilirubin in plasma where the presence of albumin may cause some interference with the adsorption process.

3) Factors Affecting the Adsorption of Bilirubin by the Polyamide Resins

The adsorption of bilirubin by the polyamide resin can be affected directly by several features of the adsorbent. These include basicity or charge density on the pendants, the distance separating two charges, distance between the active part of the pendant and the polymer matrix, and total chain length. In addition, other factors such as the conformation of the pendants may have a less direct effect.

Comparison of the polyamide resins among themselves reveals that the charge on the pendants is undoubtedly the main factor in the adsorption of bilirubin. Charges on the pendants can come from two sources, the R group of the

constituent amino acids and the terminal α-amino group. The carboxylic acid does not contribute to the charge since it is involved in the peptide bond linking the amino acids in the pendant.

The charge on the R group of the amino acid residues is of primary importance and has a profound effect on the adsorption capacity which increases with increasing basicity of the R groups. In the resins with one residue beyond the Ala_3 spacer, those with a charged amino acid, such as arginine and lysine, adsorb much more bilirubin than the resins containing the uncharged histidine and tyrosine. Moreover, arginine, which is more basic than lysine (pK_a of 12.48 as compared to 10.55), is more active. The importance of charge density of the R group is also confirmed by the trend seen in the adsorption capacity of the resins with more than one residue after the Ala_3 spacer. Adding more charged amino acid residues enhances the adsorption to a greater extent than adding uncharged residues, except in the case of $His_1Arg_2Ala_3$ pendants.

Electrostatic interactions are also suggested by the increase in adsorption capacity of the resin with the Arg_2Ala_3 pendants when the ionic strength of the adsorbate solution is reduced from 0.10 M to 0.010 M. Electrostatic interactions, resulting in an increase in binding capacity as the ionic strength is reduced, have also been reported for the binding of bilirubin to albumin (Jacobsen J., 1977).

At the pH of the adsorption studies, the terminal α-amino group, $pK_a \simeq 9$, is charged for all the amino acids, and its contribution to the total adsorption capacity is not negligible, as shown by the appreciable adsorption capacity of the Ala_3 resin. This is confirmed by the fact that although the resins with protected pendants have a lower capacity than the resins with unprotected pendants, they still adsorb a significant quantity of bilirubin. Moreover, the unsubstituted resin itself adsorbs. This resin, when not substituted, possesses an NH_3^+ group which can be considered similar to the α-amino group. In the substituted resins, only the terminal α-amino group is charged, all the others are involved in the peptide bonds and do not contribute to the overall charge. Thus, for longer peptide chains and for albumin, contribution from the α-amino group should be much less important than contributions from the R groups.

Further interesting insight into the specifics of the binding of bilirubin to the polyamide resins can be obtained by varying the organization of the residues within a pendant. Figure 29 shows that a resin containing a pendant with the structure $Arg_1Ala_3Arg_1Ala_3$ is a better adsorbent than the resins containing the Arg_2Ala_3 pendants. The enhancement cannot be due to the increase in the alanine content since the resin containing the Arg_1Ala_4 pendants has the same activity as that with the Arg_1Ala_3. Thus, the observed enhancement is probably due to an increase in the distance between the charges. In this case

it is expected that binding of a second bilirubin to the second arginine in the pendants would be facilitated as compared to the case of the resin with the Arg_2Ala_3 pendants.

Several observations cannot be explained on the basis of charge density alone. For instance, a large increase in binding capacity was obtained between the resin containing Arg_2Ala_3 pendants and the resin containing Arg_1Ala_3 pendants, while a smaller increment was observed between the resin with Arg_5Ala_3 pendants and the resin with Arg_2Ala_3 pendants. For the lysine series, the reverse was obtained with a smaller increase between the resin with Lys_2Ala_3 pendants, and the resin with Lys_1Ala_3 pendants than between the resin with the Lys_5Ala_3 pendants and that with Lys_2Ala_3 pendants. The equal capacities of the resin with the Arg_2Ala_3 pendants and with Arg_3Ala_3 and the increase in capacity obtained when one histidine instead of an arginine is added to Arg_2Ala_3 also indicates that factors other than the charge on the pendants are involved in the binding process. These differences may indicate a difference in the chain conformation, giving different binding sites.

Confirmation that factors other than electrostatic may be involved in the adsorption process, can also be inferred from the behaviour of the resins with protected pendants (Fig. 25), which still adsorb more than the unsubstituted resin or the Ala_3 resin. If the charge on either the R or α-amino groups were the sole factors responsible for the

adsorption, these resins should adsorb bilrubin to approximately the same extent as the reference resins.

The presence of a simultaneous interaction of the first bilirubin bound with more than one residue is indicated by the complexe trends in the adsorption capacities of various resins. The second interaction need not to be electrostatic, especially in the case of the resin with the $His_1Arg_2Ala_3$ pendants. Interactions of bound ions with uncharged residues in addition to the primary electrostatic interactions have been suggested previously by Karush (Karush, 1950) for the binding of alkyl sulphate to albumin.

Formation of specific interactions between biliprotein, a protein with a bilirubin-like moiety, and histidine immobilized onto sepharose gel, has been reported (Rabier, et al., 1983). These interactions are at their maximum at a pH of 5 which is above the isoelectric point of the protein and below that of histidine. These biliproteins also exhibit an interaction with lysine and arginine, the other positively charged amino acids. Although a proton exchange or charge transfer mechanism was proposed in that study, salt linkages seem equally probable. Non-electrostatic interaction between bilirubin and polypeptides have been reported by Van der Eyk (Van der Eyk, et al., 1980). Apparently bilirubin does not interact with protonated polyhistidine (pK = 9.4) but does with unprotonated polyhistamine (pK = 5.2). Hydrogen bonding between the weakly acidic pyrrolic nitrogen and the weakly

basic protons of the bilirubin lactam rings, rather than electrostatic interactions, was proposed. The present study does no negate such interactions but these are obviously weaker than the electrostatic interactions which predominate in such resins as that with the Arg_5Ala_3 pendants.

4) Possible Binding Sites on Albumin

Although the elucidation of the primary binding site of albumin for bilirubin is not the main goal of this study, the adsorption studies offer some insight into this problem. It is interesting to note that the sequence His-Arg-Arg appears twice in native albumin. This sequence is located at residues 143-145 and and 334-336 of the Brown's sequence for BSA (Brown J.R., 1977a) and residues 144-146 and 336-338 of the Brown's sequence for HSA (Brown J.R, 1977b). These sequences are situated in loop 3 and 6. and are located at the tips of two loops. Thus, they should be near the surface of the globular protein. From the literature survey given in Section 6 of Chap. I, it appears that the primary bilirubin binding site should be situated in the region containing residues 180 to 250 or roughly loop 4. In the case of BSA, this loop contains one arginine (197) at the beginning of the loop and one histidine (245) on the opposite helix. Since residues 244 and 198 are two cysteines, bound through a disulphide bridge, the arginine 197 and histidine 245 are close together and may be in a favorable conformation for

binding. Located near this sequence is lysine 238 corresponding to lysine 239 (240) of HSA which was proposed by Jacobsen (Jacobsen C., 1978) to be involved in the primary binding site for bilirubin. This region also contains 2 other arginines (193, 195), one other histidine (240) and two other lysine (203, 241). In the case of HSA the corresponding amino acids would be arginine 198 at the tip of one loop with histidine 246 at the other end of the disulphide bridge. Lysine 239 (240) is near by and some other arginine (196), histidine (241), and lysines (194, 204) are present. Based on the present results that a combination of arginine and histidine and/or a high arginine content increases the bilirubin affinity of the peptide containing resins, loop 4 seems a likely location for the primary binding site for bilirubin on albumin.

Although the polyamide resins with peptide pendants synthesized and tested in this study do not exactly mimick the location on loop 4 mentioned previously or any other possible binding site for bilirubin on albumin, their adsorption capacities in aqueous solutions indicate that these resins are good adsorbents for bilirubin in aqueous buffer. However, a measure of their adsorption capacity in the presence of albumin is required to obtain a more thorough evaluation of their binding strength.

CHAPTER V

ADSORPTION AND DESORPTION FROM BILIRUBIN-ALBUMIN SOLUTIONS

INTRODUCTION

In the previous chapter efficient adsorption of bilirubin from aqueous buffer solution by polyamide resins containing various peptide pendants was demonstrated. To further characterize the adsorption by these resins, their behavioun in the presence of BSA, a molecule that is known to bind bilirubin tightly, is studied. In this chapter the desorption of bilirubin from the polyamide resins containing peptide pendants by a buffer solution and by albumin in buffer solution as well as the adsorption of bilirubin by these resins from a bilirubin-albumin solution are considered.

EXPERIMENTAL

1) Interaction between Albumin and Polyamide Resins

An albumin-buffer solution (1.0 g/l, 25 ml) was added to either Ala_3Arg_5 or Ala_3Lys_5 resin (~10 mg). The mixture was stirred for an hour under the same conditions as for the adsorption of bilirubin, i.e., 0°C and purged with N_2. At the end of the adsorption time, an aliquot was withdrawn and diluted 1 in 4 with the buffer. The absorbances of the solution and of the diluted solution were measured at 278 nm. The absorbance readings were matched against a calibration curve obtained from diluted samples of the same albumin-buffer stock solution.

2) Desorption of Bilirubin with Buffer

Adsorption of bilirubin was carried out as described previously (Chap. IV). Following the adsorption, an absorbance reading was taken at 438 nm to determine the amount of bilirubin adsorbed. The supernatant was decanted, using a Pasteur pipette, and 25 ml of buffer was added to the resin. A 0.5 ml aliquot was withdrawn immediately and an absorbance reading was taken to determine the amount of bilirubin not adsorbed but not removed by decantation. The remaining mixture of buffer and resin with adsorbed bilirubin was then stirred for one hour under conditions identical to those used for the adsorption. At the end of the desorption period, an absorbance reading was taken at 438 nm. The amount of bilirubin desorbed was calculated from the absorbance readings.

3) Desorption of Bilirubin with Albumin/Buffer Solutions

Bilirubin was adsorbed onto polyamide resins with pendants of Arg_2Ala_3, Arg_5Ala_3, Lys_5Ala_3, and $His_1Arg_2Ala_3$ as described previously. Upon completion of the adsorption, an absorbance reading was obtained to determine the amount of bilirubin adsorbed and a known amount of bovine serum albumin (5-200 mg, Sigma Chemical fraction V) was added to the remaining supernatant. The resulting mixture was then stirred under the same conditions used for adsorption. To determine the kinetics of desorption, aliquots were withdrawn at several intervals over a period

of 3 hours.

To compare the relative amounts of bilirubin desorbed from each resin, the initial adsorption was made using approximately equal amounts of each resin. The bilirubin stock solution was used as the adsorption medium. Upon completion of the adsorption step, the same amount of albumin (80-85 mg) was added to the supernatant for each resin. Desorption was permitted to continue for one hour under the usual conditions.

For each resin, additional data were obtained by varying the initial concentration of bilirubin in the adsorption step. Thus, different amounts of bilirubin were adsorbed onto the resins. Additional data were also obtained by varying the amount of albumin added at the beginning of the desorption step. For these additional points, desorption was allowed to continue for two hours.

4) Desorption of Bilirubin at 0.010 M Ionic Strength

For polyamide resins with a Arg_2Ala_3 pendant, adsorption was carried out in 0.010 M buffer solution instead of the usual 0.10 M. The adsorption was followed by decanding, addition of a 0.010 M buffer (25 ml), and desorption for one hour. After this albumin was added and desorption was followed as mentioned previously. Absorbance readings were obtained after adsorption, after the addition of the buffer, after desorption with buffer for one hour, and after the desorption with albumin.

5) Adsorption of Bilirubin from Albumin-Bilirubin Solutions (Competition)

Adsorption of bilirubin from albumin-bilirubin solutions was carried out at one initial concentration of bilirubin, 10 mg/dl. The stock bilirubin solutions were prepared and handled in the usual manner. Immediately before the adsorption process was initiated, the stock bilirubin solution (40 ml) was added to a known amount of solid bovine serum albumin (5-200 mg, Sigma Chemicals fraction V). The resulting solution was stirred gently with a glass rod or by gentle shaking until the albumin was completely dissolved. It was then allowed to stand for approximately 10 minutes. The resulting albumin-bilirubin solution (25 ml) was added to one of the polyamide resins (~10 mg) contained in the adsorption flask. The adsorption was allowed to progress as for the adsorption from bilirubin solutions. This procedure was repeated several times with different amounts of albumin to yield different bilirubin to albumin ratios.

RESULTS

1) Desorption of Bilirubin with 0.10 M Buffer

Desorption studies were made using the Arg_2Ala_3 polyamide resin which, after adsorption, contained 1.02 moles of bilirubin adsorbed per equivalent of pendants at an equilibrium concentration of 5.0 mg/dl. After desorption into buffer for one hour the bilirubin

concentration attained a value of 0.8 mg/dl which corresponds to 0.86 moles of bilirubin per equivalent of pendants still adsorbed. Thus, less than 20% of the adsorbed bilirubin was desorbed. Reference to the isotherm (Fig. 24) shows that the resin retains much more bilirubin than expected for reversible adsorption; 0.86 moles instead of 0.25 moles at an equilibrium concentration of 0.8 mg/dl. Due to the limited desorption, the difficulty in removing excess bilirubin, and the number of steps, each of which introduces an error of ~ 5-10%, these results must be considered to be semi-qualitative. However, it is clear that very little is desorbed.

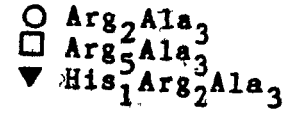
2) Desorption of Bilirubin with Bovine Serum Albumin

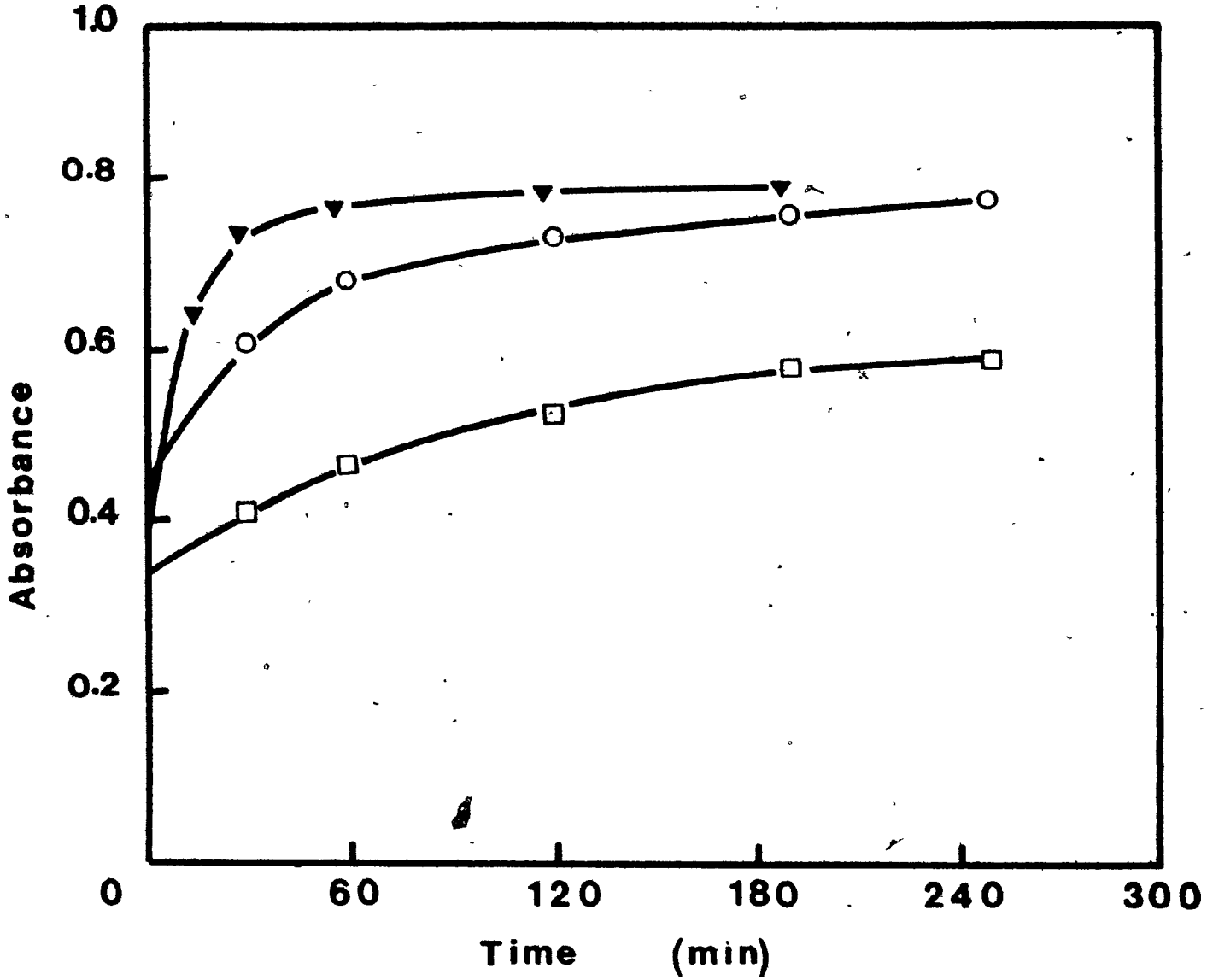
Better success in desorption was realized when a solution of bovine serum albumin (BSA) in buffer was added to a resin with adsorbed bilirubin. To begin this study the possibility of an interaction between the resins and BSA was considered. However, experiments showed that albumin is not adsorbed onto the resin with the Ala_3Arg_2 pendants when this resin is added to an albumin buffer solution since the absorbance of this solution after one hour of stirring remained the same as the absorbance of the calibration curve at the same albumin concentration.

Studies of the kinetics of the desorption of bilirubin from the resins with Arg_2Ala_3 and Arg_5Ala_3 pendants by BSA/buffer solution give strong indication that it is a slow process (Fig. 31). Although, desorption is relatively

FIGURE 31

KINETICS OF DESORPTION OF BILIRUBIN FROM THE POLYAMIDE RESINS




1.0
0.8
0.6
0.4
0.2
0
Absorbance
60
120
180
240
300
Time (min)

fast during the first hour, bilirubin is still desorbing even after three hours. It is of interest to note that desorption from Arg_2Ala_3 resin is faster than from the Arg_5Ala_3 resin. Furthermore, the former loses considerably more bilirubin. Desorption from the resin with the $His_1Arg_2Ala_3$ pendants is faster than desorption from the resin with Arg_2Ala_3 pendants and appears to be complete before two hours (Fig. 31).

The desorption from the resins with Arg_2Ala_3, Arg_5Ala_3, Lys_5Ala_3 pendants and from cholestyramine by BSA/buffer solution were tested after one hour of desorption. The relative amounts desorbed from these resins after the addition of a fixed amount of albumin can be obtained by comparing the amount of bilirubin desorbed from the resins which have been loaded to the same extent (Table VII). Loading to the same extent corresponds to the amount of bilirubin adsorbed by 10 mg of resin from 25 ml of the stock solutions (10 mg/dl). This corresponds to a point on the adsorption isotherm where the resins are almost saturated, i.e., near plateau values. Upon the addition of 1.25 $\pm$ 0.05 μmole of albumin, 17% of the bilirubin adsorbed onto the resin with Lys_5Ala_3 pendants was still retained, 16% remained on the resins with $His_1Arg_2Ala_3$ pendants, 57% was still adsorbed on the resin with Arg_2Ala_3 pendants, while 82% remained on the resin with Arg_5Ala_3 pendants and 100% remained on cholestyramine (Table VII). The addition of this amount of BSA is sufficient to tightly bind all of the bilirubin in the system assuming that each

Table VII

DESORPTION OF BILIRUBIN IN THE PRESENCE OF BSA/BUFFER

Pendants	Total Bilirubin * (μmole)	Albumin Added (μmole)	Bilirubin Retained (μmole)	Bilirubin Retained %
Arg_2Ala_3	1.78	1.2	1.01	57
Arg_5Ala_3	2.44	1.3	2.00	82
Lys_5Ala_3	2.77	1.3	0.33	12
$His_1Arg_2Ala_3$	2.04	1.2	0.31	16
Cholestyramine	2.01	1.2	2.01	100

* total bilirubin = total amount of bilirubin adsorbed

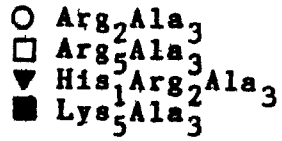
BSA molecule binds two bilirubins. Capacity for retaining bilirubin, once it is adsorbed, follows the order cholestyramine > Arg_5Ala_3 > Arg_2Ala_3 > Lys_5Ala_3. To remove 20% of the adsorbed bilirubin by the resin with Lys_5Ala_3 pendants only 15 mg (0.22 μmole) of BSA is required. This represents less than 1/5 of the BSA required to desorb the same amount from the resin with Arg_5Ala_3 pendants. Of the three resins, the resin containing the Lys_5Ala_3 pendants is the only one from which can be desorbed more than one bilirubin per albumin (Table VII).

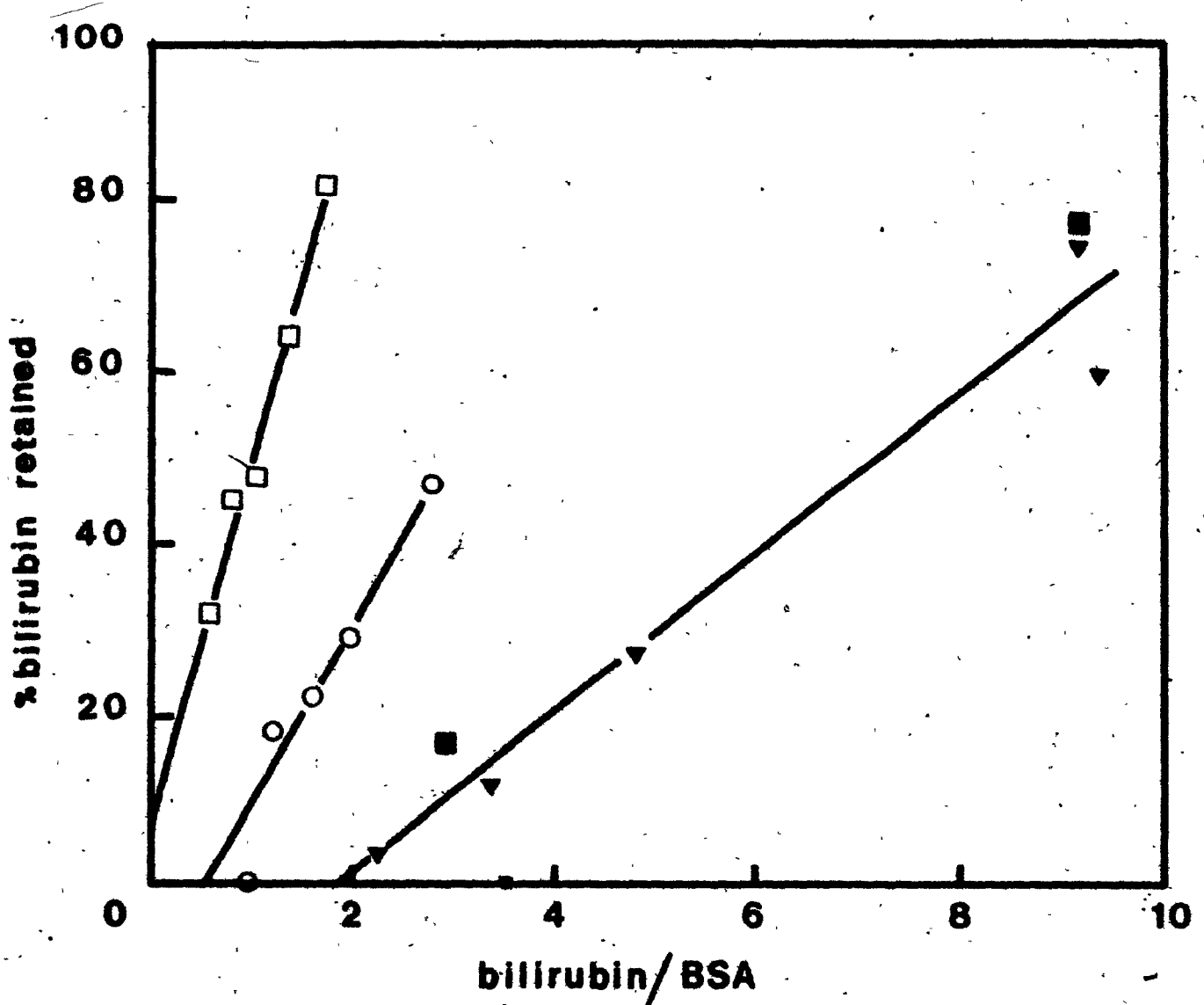
Further desorption studies were made on these resins to test the effect of varying the BSA level or the bilirubin adsorbed on the resins to obtain different equilibrium bilirubin to BSA ratios. To ensure equilibrium desorption, results were taken two hours after the addition of albumin.

For the resin containing the Arg_5Ala_3 pendants the percentage of bilirubin retained increases rapidly as the ratio of bilirubin to albumin increases, i.e., as the binding sites for bilirubin on albumin are being occupied (Fig. 32). It should be noted that even at bilirubin to albumin ratios of less than one, i.e., below the saturation of the first binding site of the added albumin, not all of the bilirubin is desorbed from the resin while less than 20% desorption occurs at a ratio of two bilirubin per albumin, i.e., at saturation of the second binding site on albumin.

FIGURE 32

DESORPTION OF BILIRUBIN WITH BOVINE SERUM ALBUMIN




100
80
60
40
20
0
% bilirubin retained
2
4
6
8
10
bilirubin/BSA

As the ratio of bilirubin to albumin increases, fraction of adsorbed bilirubin retained by the resin containing the Arg_2Ala_3 pendants increases less rapidly than it does for the resin containing the Arg_5Ala_3 pendants (Fig. 32). At saturation of the first binding site of the added albumin, desorption from the former is complete while at saturation of the second binding site desorption is of the order of 70%, i.e., only 30% of adsorbed bilirubin is retained.

Desorption from the resins containing the Lys_5Ala_3 and the $His_1Arg_2Ala_3$ pendants decreases even less rapidly than it does for the resin containing the Arg_2Ala_3 pendants (Fig. 32). For these two resins less than 20% of the adsorbed bilirubin is retained after the saturation of the second binding site.

In the case of cholestyramine no desorption was obtained upon addition of albumin.

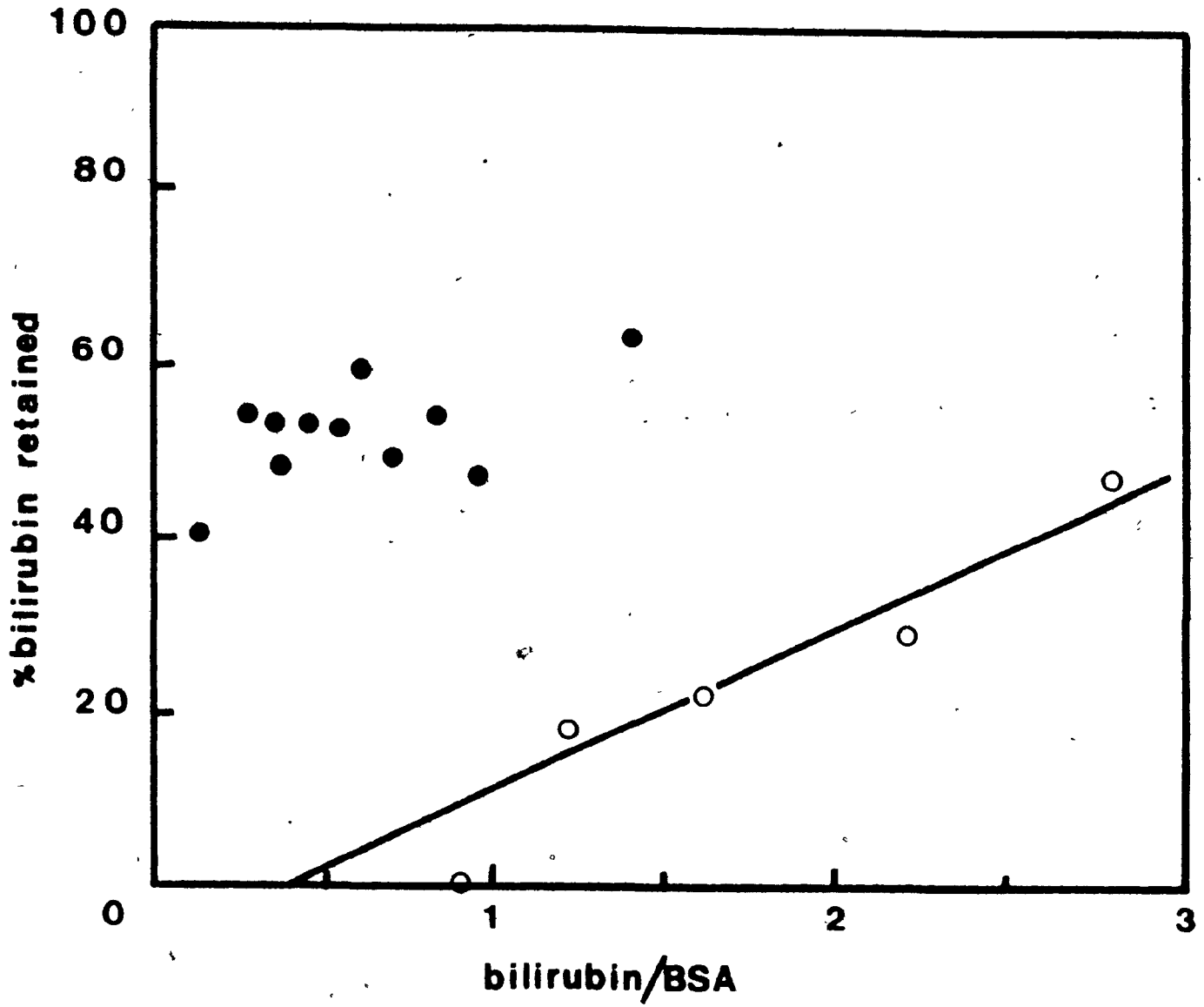
3) Desorption of Bilirubin with 0.010 M Buffer

Attempts were also made to desorbed bilirubin from the resin containing Arg_2Ala_3 pendants using a buffered solution at 0.010 M (Fig. 33). It should be recalled that at this ionic strength the adsorption was higher at low equilibrium concentration of bilirubin than for adsorption from 0.10 M buffer. However, the extent of desorption was less than that with 0.10 M and appears to be independent of the equilibrium bilirubin to BSA ratio with the possible exception of the two points at either end. In this case

FIGURE 33

DESORPTION OF BILIRUBIN FROM ARG_2ALA_3 WITH BOVINE SERUM ALBUMIN AT 0.010 M

○ 0.10 M
● 0.010 M


100
80
60
40
20
0
% bilirubin retained
1
2
3
bilirubin/BSA

higher desorption would start only below saturation of the first binding site.

4) Adsorption of Bilirubin from Albumin-Bilirubin Solutions

The capacity of some of the resins to adsorb bilirubin which has previously been allowed to form a complex with bovine serum albumin was also evaluated. This is essentially the complement to the desorption experiments in which the resins were first allowed to adsorb bilirubin that was then desorbed by addition of BSA. The amount of bilirubin adsorbed, X, on each resin as a function of the equilibrium bilirubin/BSA ratio is shown in Figures 34 to 37. Also plotted in the same figures is the initial free bilirubin concentration assuming that albumin binds either one or two molecules immediately, i.e., before adsorption by the resin begins to occur.

For the resin containing the Arg_5Ala_3 pendants (Fig. 34) there is one point at a bilirubin to BSA ratio of 1.7 at which the initial free bilirubin concentration is negative, i.e., all of the bilirubin should be bound to albumin assuming that albumin binds two molecules of bilirubin. Thus, although there is no free bilirubin, adsorption by the resin still occurs. This indicates that the resin containing the Arg_5Ala_3 pendants can compete with the second binding site for bilirubin on BSA. Above this ratio of two bilirubins per albumin the adsorption increases significantly indicating that the resin can definitely compete successfully with the weaker binding

FIGURE 34

ADSORPTION OF BILIRUBIN BY ARG_5ALA_3

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

▲ Bilirubin Adsorbed
◇ Free Bilirubin Assuming One Binding Site
△ Free Bilirubin Assuming Two Binding Sites

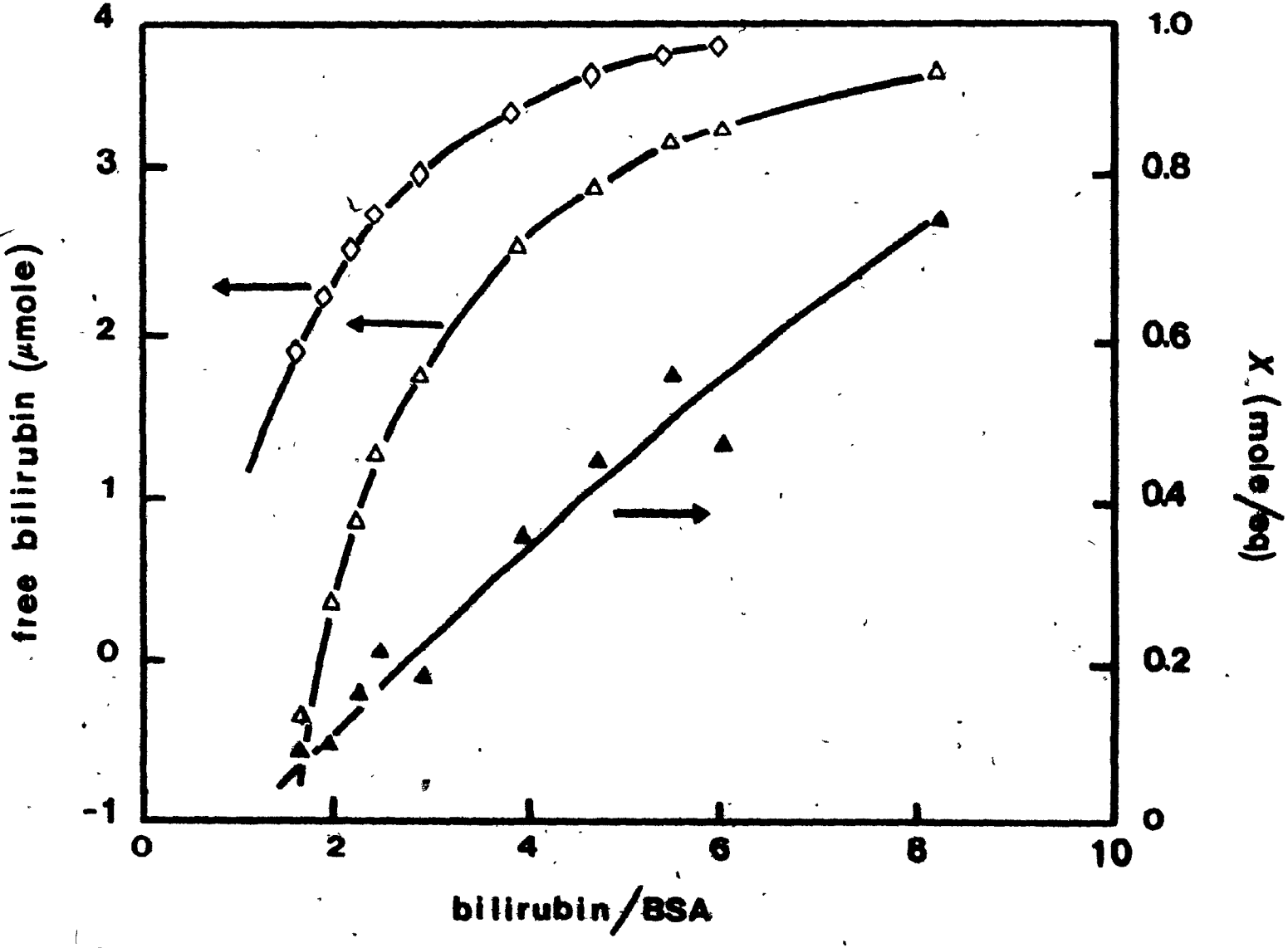

4
3
2
1
0
-1
0
2
4
6
8
10
1.0
0.8
0.6
0.4
0.2
0
free bilirubin (μmole)
X (mole/eq)
bilirubin/BSA

FIGURE 35

ADSORPTION OF BILIRUBIN BY ARG_2ALA_3

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

▲ Bilirubin Adsorbed
◇ Free Bilirubin Assuming One Binding Site
△ Free Bilirubin Assuming Two Binding Sites

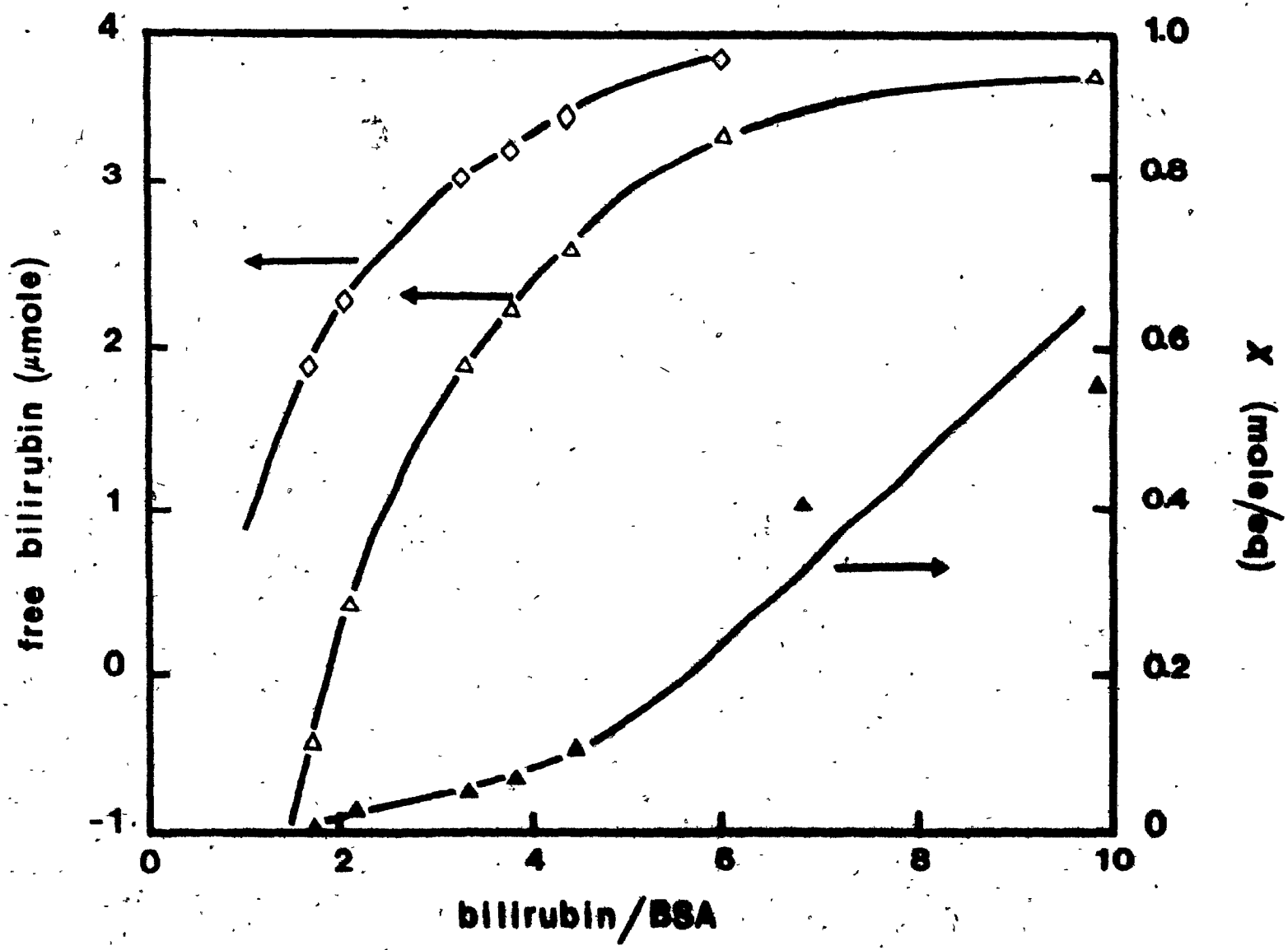

4
3
2
1
0
-1
free bilirubin (μmole)
0
2
4
6
8
10
bilirubin/BSA
1.0
0.8
0.6
0.4
0.2
0
X (mole/eq)

FIGURE 36

ADSORPTION OF BILIRUBIN BY LYS_5ALA_3

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

▲ Bilirubin Adsorbed
◇ Free Bilirubin Assuming One Binding Site
△ Free Bilirubin Assuming Two Binding Sites

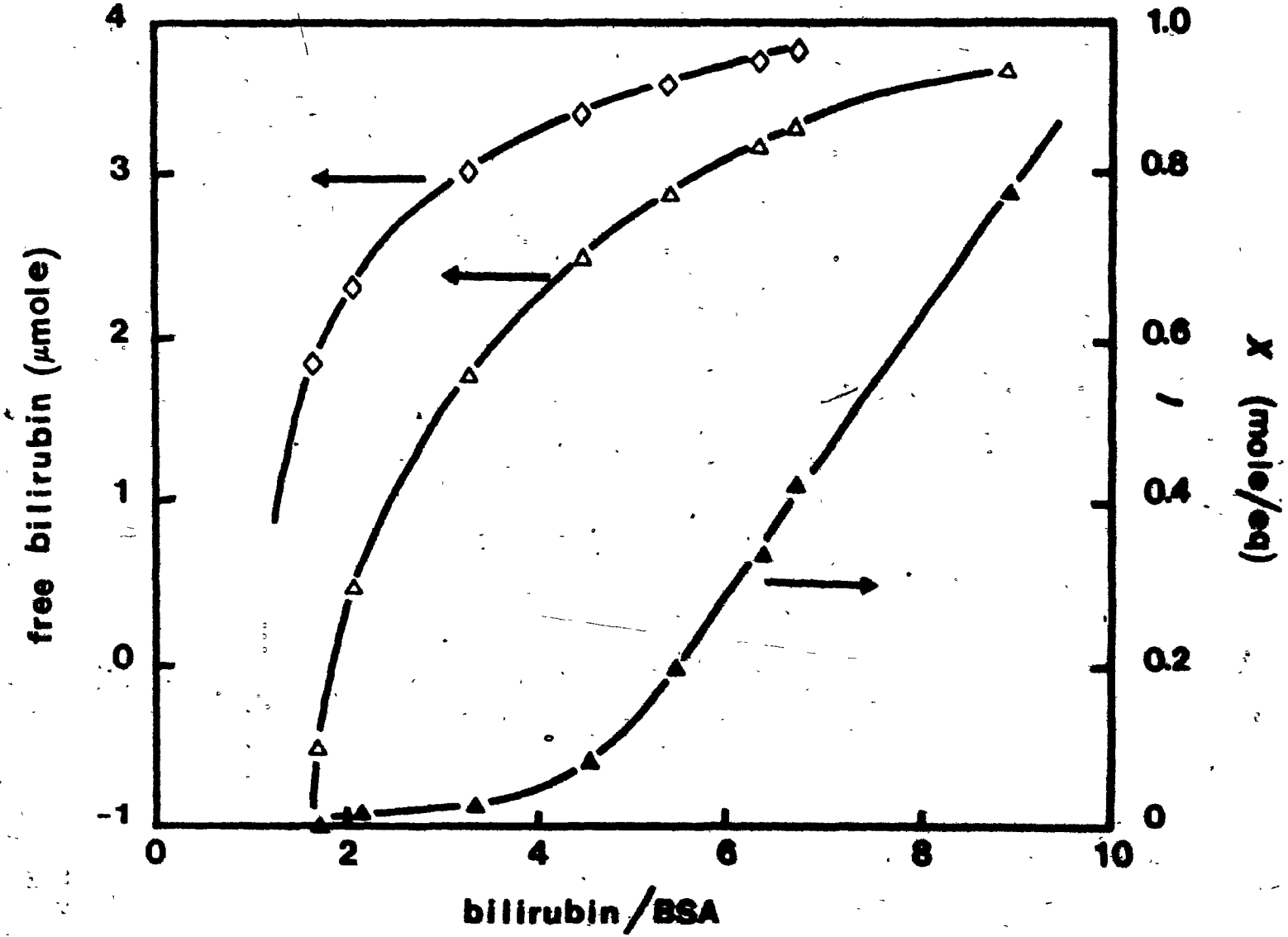
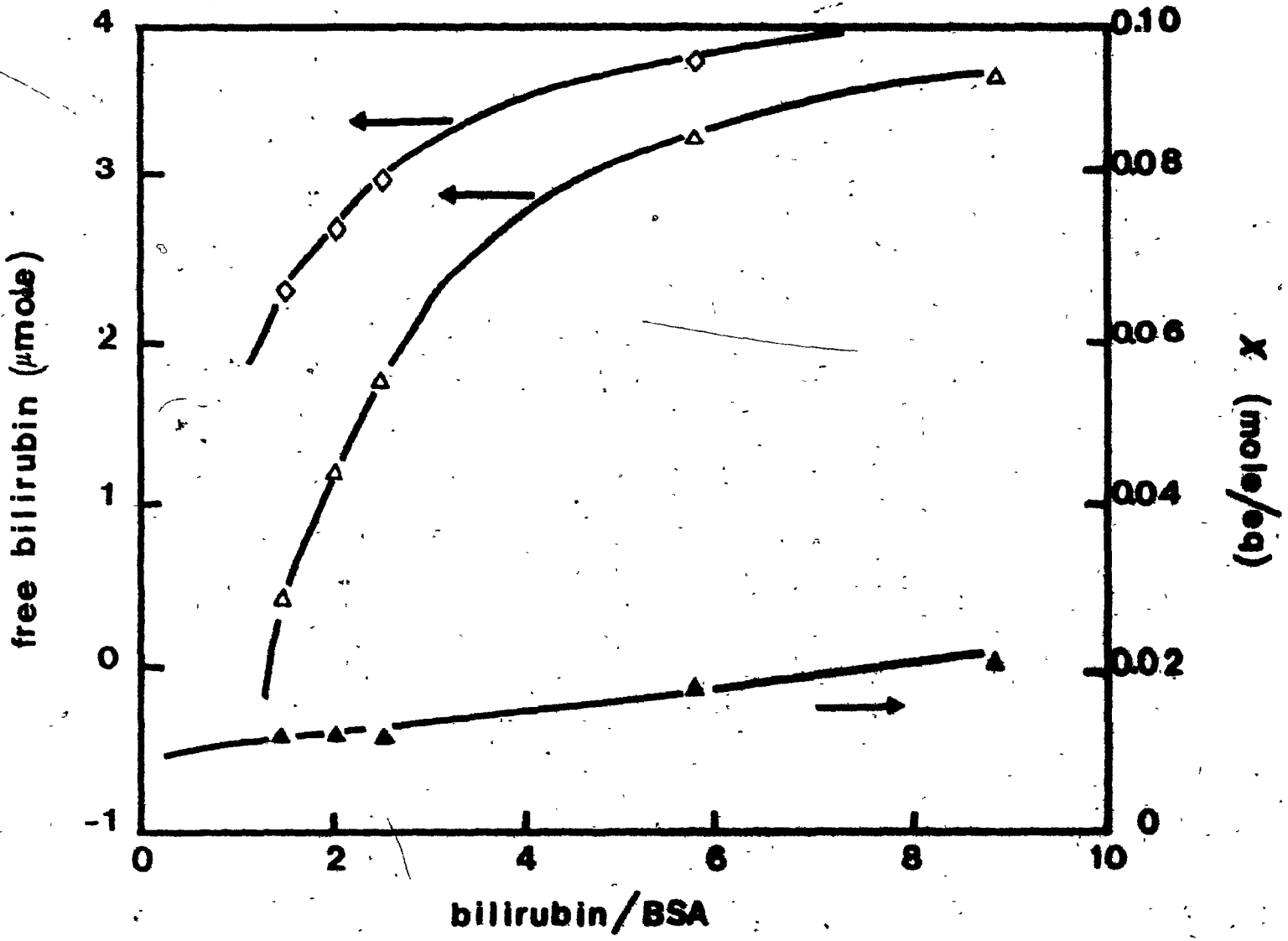

4
3
2
1
0
-1
0
2
4
6
8
10
1.0
0.8
0.6
0.4
0.2
0
free bilirubin (μmole)
bilirubin/BSA
X (bo/eo/ow)

FIGURE 37

ADSORPTION OF BILIRUBIN BY CHOLESTYRAMINE

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

▲ Bilirubin Adsorbed
◇ Free Bilirubin Assuming One Binding Site
△ Free Bilirubin Assuming Two Binding Sites


free bilirubin (μmole)
bilirubin/BSA
(bo/olow) X
4
3
2
1
0
-1
0
2
4
6
8
10
0.10
0.08
0.06
0.04
0.02
0

sites for bilirubin on albumin. This is in complete accord with the desorption data shown in Fig. 32 that indicate that all of the bilirubin adsorbed by the resin is retained at bilirubin to BSA ratios greater than two.

When there is no free bilirubin present in solution, again based on the assumption that albumin can bind two bilirubin molecules, the resin containing the Arg_2Ala_3 pendants does not adsorb bilirubin (Fig. 35). Thus, it appears that this resin cannot even compete with the second binding site on BSA. As the initial free bilirubin concentration is increased the adsorption also increases but not as much as for the resin containing the Arg_5Ala_3 pendants. Actually, adsorption does not increase significantly until a ratio of four bilirubins per albumin is reached. Again this is reflected to some degree in the desorption data (Fig. 32) which indicate that only 30% of the adsorbed bilirubin on the resin with the Arg_2Ala_3 pendants is retained at a bilirubin to albumin ratio of two. This indicates that while the Arg_2Ala_3 can compete with the weaker binding sites it is not as efficient as the Arg_5Ala_3. The resin containing the Lys_5Ala_3 shows the same behaviour as the resin containing the Arg_2Ala_3 pendants (Fig. 36), i.e., it cannot compete with the second binding site but it will compete with the weaker binding sites with the adsorption increasing significantly after a ratio of four bilirubins per albumin.

In the case of cholestyramine, the amount of bilirubin adsorbed is not significantly affected by the presence of

albumin (Fig. 37). This is in good agreement with the fact that albumin could not desorb bilirubin from cholestyramine. However, Figure 37 once again emphasizes the very limited adsorption capacity of cholestyramine.

Thus, the adsorption of bilirubin from bilirubin-albumin solution shows that competition between albumin and the adsorbents follows the order Arg_5Ala_3 > Arg_2Ala_3 ≥ Lys_5Ala_3. These results are in agreement with the desorption results. Both suggest that as competitors, the Arg_5Ala_3 resin binds bilirubin more strongly than does the resin with the Arg_2Ala_3 pendants which in turn binds more strongly than does the resin with Lys_5Ala_3 pendants.

DISCUSSION

1) Adsorption of bilirubin in the Presence of Albumin

Relative adsorptivities of the resins as competitors with BSA does not follow the same order as their adsorption capacity. When complexation with albumin preceded the addition of the resins to the resulting buffer solution of albumin and bilirubin, the resins containing the Arg_2Ala_3 and Lys_5Ala_3 pendants proved to be poor competitors with the albumin for available bilirubin and adsorbed very little below levels corresponding to approximately 5 bilirubin molecules per albumin molecules. They are apparently unable to compete with even the second binding site for bilirubin bound to albumin.

The resin with the Arg_5Ala_3 pendants proved to be a better adsorbent and shows some signs of competition with the second binding site on BSA. However, the data suggest that it is not able to compete with the primary binding site for bilirubin on albumin.

On the other hand, in terms of capacity for bilirubin, the resin with the Lys_5Ala_3 pendants is comparable to the resin with Arg_5Ala_3 pendants. Hence, it might have been expected that it would have been comparable to that resin in a competitive situation and that it would definitely be better than the resin with Arg_2Ala_3 pendants. However, such is not the case and at least a partial answer may be found in a careful inspection of the adsorption isotherm of the resin with the Lys_5Ala_3 pendants (Fig. 26, Chap. IV) which reveals that at equilibrium concentrations below 1 mg/dl, Lys_5Ala_3 follows approximately the same isotherm as the resin with the Arg_2Ala_3 pendants (Fig. 24, Chap. IV) and does not reach the adsorption capacity of the resin containing the Arg_5Ala_3 pendants until the equilibrium concentration of bilirubin reaches approximately 2.5 mg/dl. At low equilibrium concentrations of bilirubin the resin with the Lys_5Ala_3 pendants has a low adsorption capacity which increases rapidly as the concentration of bilirubin increases. Thus, it appears that adsorption of bilirubin is not efficient at low concentrations and that the adsorption improves in some manner as concentration increases, perhaps becoming more favourable after small amounts have been adsorbed. In the presence of added

albumin, which binds available bilirubin strongly and quickly, the bilirubin concentration is low resulting in a low adsorption capacity for Lys_5Ala_3 containing resin. Consequently, this resin is a poor competitor. The synergism obtained with increasing bilirubin concentration may be an indication that the conformation of the peptide chain may play an important role in the adsorption of bilirubin. For example, in the absence of adsorbed bilirubin, the Lys_5Ala_3 pendants may have a unfavourable conformation for the binding of bilirubin. However, the adsorption of small amounts of bilirubin to the pendant may change this conformation to a more favourable one.

2) Desorption of Bilirubin with Bovine Serum Albumin

Desorption from the resins due to the addition of albumin follows the same trend as does the adsorption when albumin is present in the solution. However, the partition between the bilirubin adsorbed by the resin and that bound to albumin depends on which of the two competitors comes into contact with bilirubin first.

In good agreement with the desorption results indicating a high desorption from the resin containing the Lys_5Ala_3 pendants, the results for the adsorption from bilirubin-albumin solution show that this resin is also a poor adsorbent in the presence of BSA. The BSA creates a low equilibrium concentration of free bilirubin corresponding to a region of the adsorption isotherm where

the resin with the Lys_5Ala_3 pendants is a relatively poor adsorbent.

The large degree of desorption from the resin containing the $His_1Arg_2Ala_3$ pendants in the presence of BSA is rather surprising. From its higher adsorption capacity as compared to the resin with Arg_2Ala_3 it would have been expected that this resin would be a better competitor than the resin with the Arg_2Ala_3 pendants. However, it is actually as poor a competitor as the resin with the Lys_5Ala_3 pendants. One way to explain this phenomenon is to consider that the abnormality lies with the resin containing the Arg_2Ala_3 pendants and not with the resin containing the $His_1Arg_2Ala_3$ pendants. The resin with the Arg_2Ala_3 pendants exhibits much slower kinetics of desorption than does that with the $His_1Arg_2Ala_3$ (Fig. 31). Thus, given enough time, the resin containing the Arg_2Ala_3 pendants may eventually desorb enough bilirubin to reach the same level as the resin with the Lys_5Ala_3. Prediction of larger desorption from the resin with the Arg_2Ala_3 pendants also correlates with the small amount of bilirubin adsorbed in the presence of albumin, which in fact is similar to adsorption by the resin containing the Lys_5Ala_3 pendants. Under those terms, the resin with Arg_2Ala_3 pendants would be expected to be a poor competitor. On the other hand, if the same logic were applied to the resin with the Arg_5Ala_3 pendants, which also shows slow kinetics for desorption, it would be expected that the resin would also be a poor competitor. Yet, the adsorption in the

presence of albumin clearly demonstrates that the resin with the Arg_5Ala_3 pendants can successfully compete with albumin.

True competition where the two competitors would be added to and be in contact with the adsorbate solution simultaneously, would provide a better comparison of the relative binding strength of each resin as opposed to that of albumin. However, this is not possible experimentally since adsorption by the resins involves a diffusion within the polymer beads and is thus fairly slow while complexation with albumin is rapid with a rate constant of the order of 10^6 $M^{-1}s^{-1}$ (Reed, 1977).

In the presence of albumin the efficiency of the adsorbents tested for the adsorption of bilirubin follows the order $Arg_5Ala_3 > Arg_2Ala_3 > Lys_5Ala_3 \sim His_1Arg_2Ala_3$. This order is not similar to the adsorption capacity in the absence of albumin. This difference was unexpected and may be taken as a further indication that although charge density is a primary factor in the adsorption of bilirubin by the polyamide resins, other factors such as the conformation of the peptide pendants may also play an important role.

CHAPTER VI

BINDING CONSTANTS FOR BILIRUBIN/POLYAMIDE RESIN SYSTEMS

INTRODUCTION

In Chap. IV it has been demonstrated that certain oligopeptides bound to the water swellable polyamide resin are capable of efficiently adsorbing bilirubin from aqueous buffer solution. Adsorption of bilirubin in the presence of albumin indicated that the strength of adsorption of bilirubin to certain of these materials is sufficient to compete with albumin for bilirubin (Chap. V). This chapter describes the derivation of binding constants for the adsorption of bilirubin to the various sorbents from the adsorption isotherms.

Since the pendants on the resins are constituted of amino acids, binding constants can be obtained by considering each pendant as a small protein, P, which can bind a maximum number, n, of ligands, A. Two different types of binding constants, site and stoichiometric, can be defined.

1) Site Binding Constants

The most commonly used constant is the site binding constant which considers the binding of a ligand to a specific site on the protein regardless of how many other ligands are already bound to that protein. The equilibrium between the protein and the ligand can be represented as:

$$
\begin{aligned}
{}_1P + A &\longrightarrow {}_1PA \\
{}_2P + A &\longrightarrow {}_2PA \\
{}_nP + A &\longrightarrow {}_nPA
\end{aligned}
\qquad (1)
$$

where $_iP$ represents a specific binding site i on the protein, A is the ligand, and $_iPA$ is the protein with one ligand attached to binding site, i.

The binding constants associated with these equations are given by:

$$k_1 = \frac{[_1PA]}{[_1P][A]} \tag{2}$$

$$k_2 = \frac{[_2PA]}{[_2P][A]}$$

$$k_n = \frac{[_nPA]}{[_nP][A]}$$

The experimental parameter X, defined previously as the molar ratio of bound ligand to total protein (or number of pendants), can be expressed as:

$$X = \frac{[_1PA]}{[_1P]+[_1PA]} + \frac{[_2PA]}{[_2P]+[_2PA]} + \cdots + \frac{[_nPA]}{[_nP]+[_nPA]} \tag{3}$$

$$X = \sum_{i=1}^{n} \frac{[_iPA]}{[_iP]+[_iPA]} \tag{4}$$

Substitution of the expression for the binding constants into these equations yields:

$$X = \sum_{i=1}^{n} \frac{k_i[_iP][A]}{[_iP]+k_i[_iP][A]} \tag{5}$$

$$X = \sum_{i=1}^{n} \frac{k_i[A]}{1+k_i[A]} \qquad (6)$$

Equation (6) is in the form of the well-known Scatchard equation (Scatchard, 1949):

$$X = \sum_{i=1}^{n} \frac{k_i[A]}{1+k_i[A]} + \sum_{j=1}^{m} \frac{k_j[A]}{1+k_j[A]} + \ldots \qquad (7)$$

Each summation represents one class of equivalent binding sites and n and m are the number of binding sites per protein in each class. Thus, as is evident from comparison of equation (6) and (7), the Scatchard method gives <u>site</u> binding constants although this is not always taken into account when it is used. As a result, misleading numbers will be obtained if the limitations of a site binding constant are not recognized.

The major limitation of the site binding constant model is that it assumes that the sites are independent, i.e., the presence of a ligand at one site will not affect binding at another site. Thus, the values of the site binding constants are not expected to change throughout the whole binding process. Obviously, for most binding processes this assumption will not hold. If interactions occur, $n2^{n-1}$ site binding constants, where n is equal to the maximum number of ligands which can be bound per protein, will be required to describe the system (Klotz and Hunston, 1975), or, alternatively, the constants k_i can be replaced by some empirical constants k_α, k_β, etc.

However, these constants need not necessarily bear any relationship to the site binding constants they represent (Klotz and Hunston, 1975, 1979).

2) Stoichiometric Binding Constants

An alternative way of describing the same system (Klotz, 1946) is to consider the sequential addition of ligands to the protein regardless of the site to which those ligands are bound. In this case, the binding process can be described as:

$$P + A \longrightarrow PA_1 \qquad (8)$$
$$PA_1 + A \longrightarrow PA_2$$
$$PA_{n-1} + A \longrightarrow PA_n$$

Where PA_i is a protein to which i ligands are bound.

The corresponding stoichiometric binding constants are given by:

$$K_1 = \frac{[PA_1]}{[P][A]} \qquad (9) \qquad [PA_1] = K_1[P][A] \qquad (10)$$

$$K_2 = \frac{[PA_2]}{[PA_1][A]} \qquad [PA_2] = K_1K_2[P][A]^2$$

$$K_n = \frac{[PA_n]}{[PA_{n-1}][A]} \qquad [PA_n] = K_1K_2\ldots K_n[P][A]^n$$

Under these conditions, X is given by:

$$X = \frac{[PA_1] + 2[PA_2] + \ldots + n[PA_n]}{[P] + [PA_1] + [PA_2] + \ldots + [PA_n]} \qquad (11)$$

Substitution of the stoichiometric binding constants yields:

$$X = \frac{K_1[A] + 2K_1K_2[A]^2 + \ldots + nK_1K_2\ldots K_n[A]^n}{1 + K_1[A] + K_1K_2[A]^2 + \ldots + K_1K_2\ldots K_n[A]^n} \quad (12)$$

which is sometimes called the Adair equation (Van Holde, 1971).

This analysis carries no underlying assumptions and is applicable even in the case of non-independent binding sites.

3) Interaction between Sites

The stoichiometric approach does not provide information about the individual binding sites although some insight can be obtained as to how the different sites interact. When the sites are equivalent and non-interacting, the stoichiometric binding constants can be related to an overall binding constant, K_o (Klotz, et al., 1946), as follows: Each protein can be treated as consisting of a series of independent sites, y, each of which can form a binary complex yA with a ligand. The overall binding constant K_o can then be defined by:

$$K_o = \frac{[Ay]}{[A]\,[y]} \quad (13)$$

This equation can be rewritten in terms of the measurable experimental parameters P and A since:

[Ay] = concentration of bound ligand = bound
= sites occupied

[A] = concentration of free ligand

[y] = concentration of free sites
= total protein (P) times maximum number of binding sites per protein (n) minus the sites occupied
= nP - bound

Thus,

$$K_o = \frac{[\text{bound}]}{[A]\,[nP - \text{bound}]} \qquad (14)$$

or

$$[\text{bound}]\,(1 + K_o\,[A]) = n\,K_o\,[P]\,[A] \qquad (15)$$

and

$$X = \frac{[\text{bound}]}{[P]} = \frac{n\,[A]}{1/K_o + [A]} \qquad (16)$$

Equation (12) gives the expression for X in terms of the individual constants, K_i. When it is recognised that the numerator in equation (12) is simply [A] times the derivative of the denominator, then

$$X = \frac{f'\,[A]}{f} \qquad (17)$$

where

$$f = 1 + K_1[A] + K_1K_2[A]^2 + \ldots + K_1K_2\ldots K_n[A]^n \qquad (18)$$

Substitution of equation (16), which is the expression for X in terms of the overall binding constant, K_o, into equation (17) yields:

$$X = \frac{f'[A]}{f} = \frac{n\,[A]}{1/K_o + [A]} \qquad (19)$$

Integration of equation (19) yields the function f in terms of K_o, i.e.,

$$f = \left(1 + \frac{[A]}{1/K_o}\right)^n \qquad (20)$$

Equating both functions, i.e., f's from equations (18) and (20), yields:

$$1 + K_1[A] + K_1K_2[A]^2 + \ldots + K_1K_2\ldots K_n[A]^n = \left(1 + \frac{[A]}{1/K_o}\right)^n \qquad (21)$$

By differentiating n times and solving for the constants a series of equations is obtained,

$$K_1K_2\ldots K_n = K_o^n \qquad (22)$$

$$K_1K_2\ldots K_{n-1} = n\,K_o^{n-1} \qquad (23)$$

$$K_1K_2\ldots K_i = \frac{(n!/i!)\,K_o^i}{(n-i)!} \qquad (24)$$

Individual binding constants can now be obtained by taking suitable ratios. Hence,

$$\frac{K_1K_2\ldots K_i}{K_1K_2\ldots K_{i-1}} = \frac{(n - (i - 1))\,K_o}{i} = K_1 \qquad (25)$$

$$i(K_i)_{ideal} = K_o(n + 1) - K_o\,i \qquad (26)$$

Thus, in the ideal situation of equivalent, non-interacting binding sites a plot of iK_i as a function of i, referred to as an affinity plot, should be linear with an x-intercept at $i = n + 1$ at which point $K_i = 0$ (since i cannot be zero). In the case of a positive interaction, K_i will be larger than $(K_i)_{ideal}$ for equivalent sites. Thus, the slope of the line between K_{i-1} and K_i will be less negative than the ideal line. For a negative interaction K_i will be smaller than $(K_i)_{ideal}$ and the slope will also be more negative (Klotz, 1974, Klotz and Hunston, 1975).

When the sites are not equivalent, the affinity profile is more complex and depends on the number of classes of equivalent sites, the number of sites in each class, and their interactions with each other. Affinity profiles for several different cases have been computed by Klotz and Hunston (Klotz and Hunston, 1979).

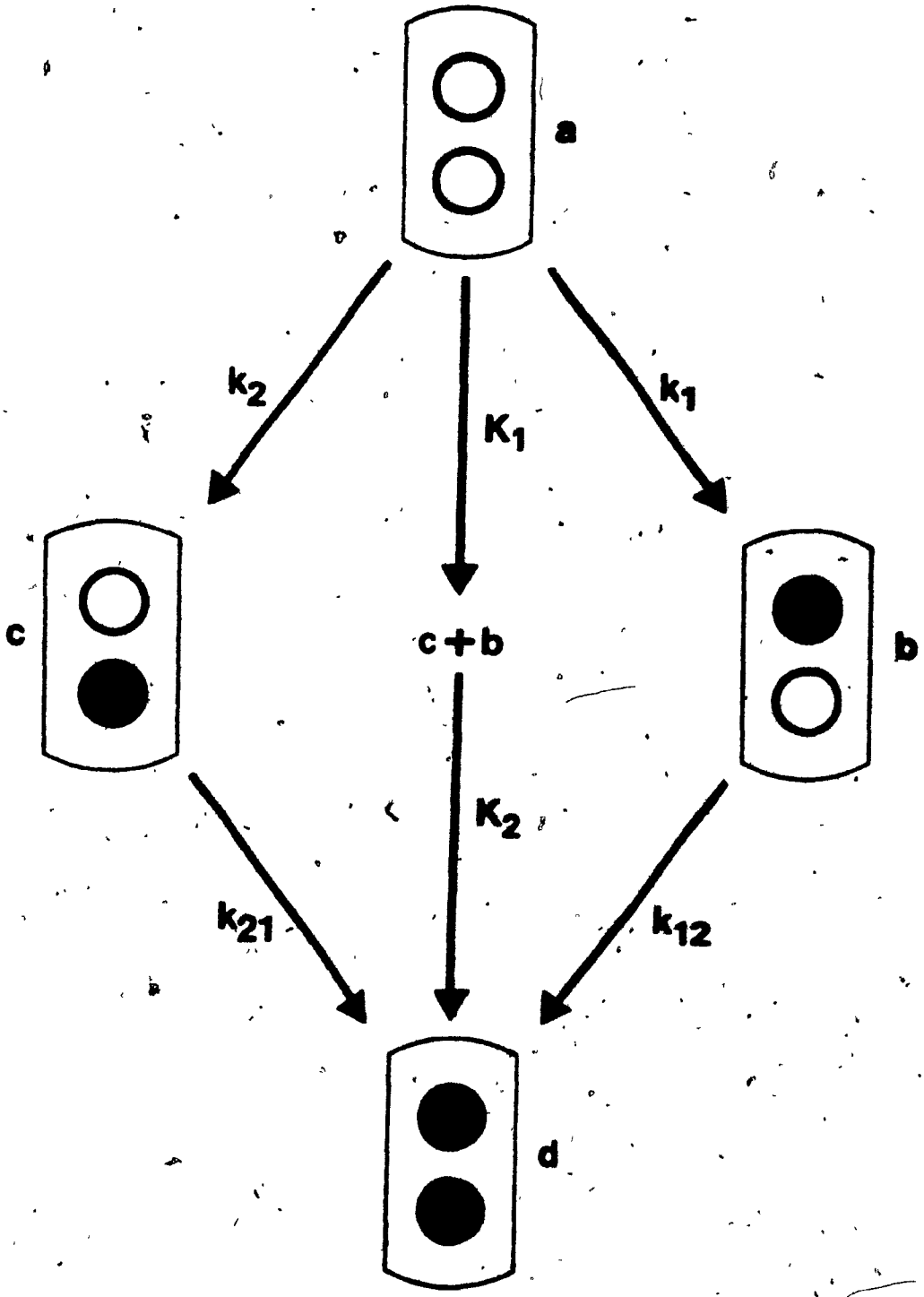
As an example, the case of a protein with 2 binding sites will be considered in the following discussion: To obtain the relationship between the sites in the case of a protein with two <u>non-equivalent</u> and <u>interacting</u> sites, it is convenient to relate the stoichiometric binding constants to the site binding constants. For a two site system, four site binding constants are needed as shown schematically in Fig. 38. These constants can be represented by:

$$k_1 = \frac{[b]}{[a]\,[A]} \quad (27) \qquad k_2 = \frac{[c]}{[a]\,[A]} \quad (28)$$

FIGURE 38

RELATIONSHIP BETWEEN SITE (k_1)

AND STOICHIOMETRIC (K_1) BINDING CONSTANTS


a
k_2
K_1
k_1
c
c+b
b
K_2
k_{21}
k_{12}
d

$$k_{12} = \frac{[d]}{[b]\ [A]} \quad (29) \qquad k_{21} = \frac{[d]}{[c]\ [A]} \quad (30)$$

Where [a], [b], [c], and [d] represent the concentrations of the four different protein-ligand complexes present (Fig. 38). The corresponding stoichiometric binding constants are represented by:

$$K_1 = \frac{[b] + [c]}{[a]\ [A]} \quad (31)$$

$$K_2 = \frac{[d]}{[b]\ [A] + [c]\ [A]} \quad (32)$$

The relationships between the two types of binding constants can be obtained by substituting equations (27) to (29) into equations (31) and (32) to yield

$$K_1 = k_1 + k_2 \quad (33)$$

$$K_2 = \frac{k_1 k_{12}}{k_1 + k_2} \quad (34)$$

In an ideal case of <u>equivalent</u> and <u>non-interacting</u> sites, the site binding constants are equal, i.e.,

$$k_1 = k_2 = k_3 = k_4 = k \quad (35)$$

and

$$K_1 = 2k \quad (36)$$

$$K_2 = k/2 \quad (37)$$

$$K_2 = K_1/4, \quad (38)$$

Equation (38) is in agreement with equation (25) which also predicts that in an ideal case the ratio of K_1 to K_2 is four when n = 2. In a plot of iK_1 as a function of i, the ideal line, which is the line connecting K_1 at i=1 and 0 at i= n+1, will give $K_2 = K_1/2$. Thus, $2K_2$ will indeed fall on the ideal line.

If the sites are <u>equivalent</u> but <u>non-independent</u>,

$$k_1 = k_2 = k \tag{39}$$

$$K_1 = 2k \tag{40}$$

$$K_2 = \frac{k\, k_{12}}{2k} = \frac{k_{12}}{2} \tag{41}$$

$$K_2 = \frac{K_1}{4}\ \frac{k_{12}}{k} \tag{42}$$

In a case of a positive interaction, $k_{12} > k$. Thus, $2K_2$ lies above the ideal line. In the case of negative interaction, $k_{12} < k$, and $2K_2$ lies below the ideal line.

In the case of <u>non-equivalent</u> sites with <u>interaction</u> it is convenient to define an interaction parameter, I, as:

$$I + 1 = \frac{k_{12}}{k_2} = \frac{k_{21}}{k_1} \tag{43}$$

If k_2 is assigned such that it is larger than k_1, a difference parameter, δ, can also be defined:

$$\delta + 1 = \frac{k_2}{k_1} \tag{44}$$

According to equation (38), to lie on the ideal line, $K_1 = 4K_2$ so that according to equations (33) and (34)

$$k_1 k_{12} = \frac{(k_1 + k_2)^2}{4} \tag{45}$$

Thus, from equation (44)

$$\delta k_1 + 2k_1 = k_1 + k_2 \tag{46}$$

and

$$k_1 = \frac{k_2}{(\delta + 1)} \tag{47}$$

Substitution of equation (46) into equation (45) and division by k_1^2 yields:

$$\frac{k_{12}}{k_1} = \frac{(\delta + 2)^2}{4} \tag{48}$$

Similarly, substitution of equation (47) into equation (48) yields:

$$\frac{k_{12}\ (\delta + 1)}{k_2} = \frac{(\delta + 2)^2}{4} \tag{49}$$

When equation (49) is substituted in equation (43) the resulting parameter for the interaction parameter is:

$$I = \frac{\delta^2}{4(\delta + 1)} \qquad (50)$$

Thus, when $I > \delta^2 / 4(\delta+1)$, $2K_2$ will fall above the ideal line; if it is smaller, then $2K_2$ will be below. For non-interacting sites $I = 0$, so that the $2K_2$ point will always be below the ideal line, since by definition the δ containing term is always positive. For negatively interacting sites, I is negative. Thus, the $2K_2$ point will also always be below the ideal line. However, if positive interactions are present, the $2K_2$ point can be anywhere depending on the magnitude of I and δ. For a divalent system, it is then impossible to differentiate between equivalent, negatively interacting sites and non-equivalent sites as well as between positively interacting equivalent sites or non-equivalent sites (Table VIII).

In this chapter, the constants for the binding of bilirubin to the polyamide resins will be obtained using stoichiometric approach. These constants will then be be compared to the binding constants for bilirubin to HSA. Finally, for the resins containing more than one binding site, the interaction between those sites will also be considered.

RESULTS

1) Stoichiometric Binding Constants in an Ideal Case

Stoichiometric binding constants for the binding of bilirubin to the various polyamide resins were derived

TABLE VIII

EQUIVALENCE AND INTERACTION IN AFFINITY PROFILES

$2K_2$ Situated	Equivalence	Type of Interaction
above ideal line	equivalent	positive
	non-equivalent	positive
on ideal line	equivalent	none
	non-equivalent	positive
below ideal line	equivalent	negative
	non-equivalent	none
		positive
		negative

using the binding model described by equation (12). Since some of the adsorption isotherms show signs of saturation below one molecule of bilirubin per pendant while others level off somewhere between one and two molecules of bilirubin per pendant it would appear that either a one or two binding site model should describe these systems. In the case of one binding site equation (12) reduces to:

$$X = \frac{K_1[A]}{1 + K_1[A]} \tag{51}$$

while a model with two binding site results in:

$$X = \frac{K_1[A] + 2K_1K_2[A]^2}{1 + K_1[A] + K_1K_2[A]^2} \tag{52}$$

As a first approximation, the binding constants were determined from a plot of 1/X as a function of 1/[A], since according to equation (16):

$$\frac{1}{X} = \frac{1}{K_o n} \frac{1}{[A]} + \frac{1}{n} \tag{16a}$$

Assuming the sites to be equivalent and independent, this plot should be linear with a slope of $1/nK_o$. The values of K_1 and K_2 can then be calculated by substituting K_o into equation (25).

Using the data obtained for the adsorption isotherms in Chap. IV for these plots yields the values for K_1 and K_2 given in Table IX as determined by linear regression analysis. Since equivalence and independence are assumed,

TABLE IX

BINDING CONSTANTS FOR THE ADSORPTION OF BILIRUBIN TO VARIOUS POLYAMIDE RESINS ASSUMING IDEAL BEHAVIOUR

Pendants	One Site	Two Sites	
	K_1 10^{-3} M^{-1}	K_1 10^{-3} M^{-1}	K_2 10^{-3} M^{-1}
resin	1.4		
Ala_3	1.6		
Ala_3 (acetylated)	0.4		
Arg_1Ala_3	5.0		
Arg_2Ala_3		13	3.3
Arg_3Ala_3		22	5.4
Arg_5Ala_3		74	18
Lys_1Ala_3	2.8		
Lys_2Ala_3	3.9		
Lys_5Ala_3		14	3.4
His_2Ala_3	3.5		
His_5Ala_3	12		
Arg_1Ala_4	7.4		
$Arg_1Ala_3Arg_1Ala_3$		19	4.5
$His_1Arg_2Ala_3$		23	5.8

a plot of 1/X as a function of 1/[A] does not necessarily yield accurate values of K_o. Moreover, such a plot of 1/[A] compresses the data at high free ligand concentrations, [A], i.e., at high equilibrium values of bilirubin, and tends to hide deviations from ideality to give apparent straight lines (Rodbard, 1973, Klotz, 1974). This limited accuracy is reflected in the experimental data by the following observations:

a) A negative value for the y-intercept is obtained in certain cases (Fig. 39). This is unreasonable since, according to equation (16a), the reciprocal of the y-intercept should give the maximum number of binding sites, n.

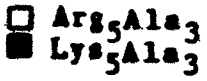
b) The calculated isotherms obtained by resubstituting the derived values for K_1 and K_2 into equation (51) or equation (52) are generally not a good representation of the experimental data, particularly for the resins reaching saturation above one molecule of bilirubin per pendant. An example of the resulting fit for the adsorption by the Arg_5Ala_3 resin is shown in Fig. 40.

2) Stoichiometric Binding Constants in an Non-ideal Case

Using a non-linear iterative computer program, NLIN from SAS (SAS Institute, 1982), binding constants were derived for each resin assuming a model with one binding site but making no assumption about equivalence and independence, i.e., by solving equation (51). The criterion for the best fit was a minimum value for the sum

FIGURE 39

DOUBLE RECIPROCAL PLOT TO DETERMINE THE BINDING CONSTANTS AT 0°C FOR VARIOUS POLYAMIDE RESINS



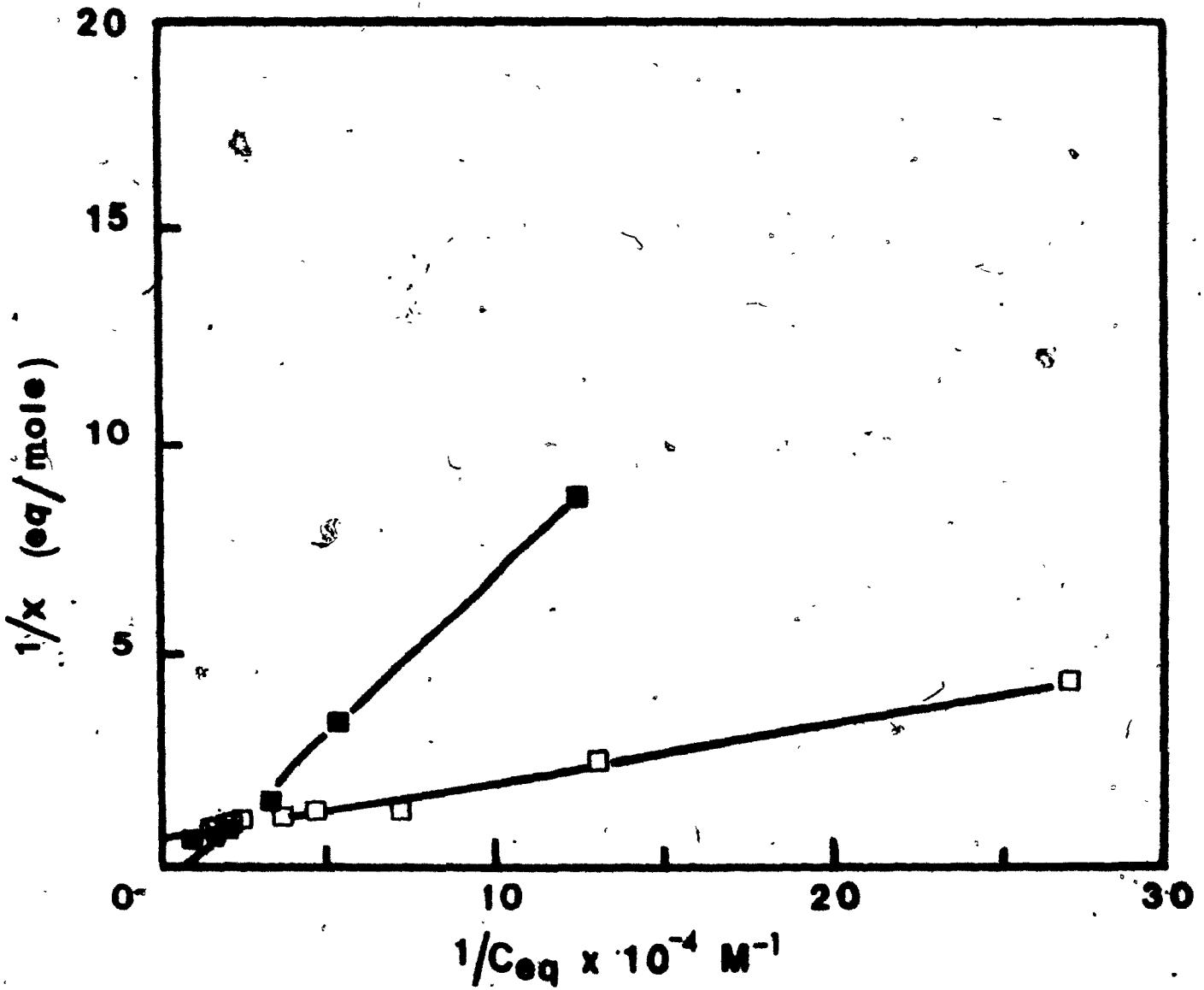
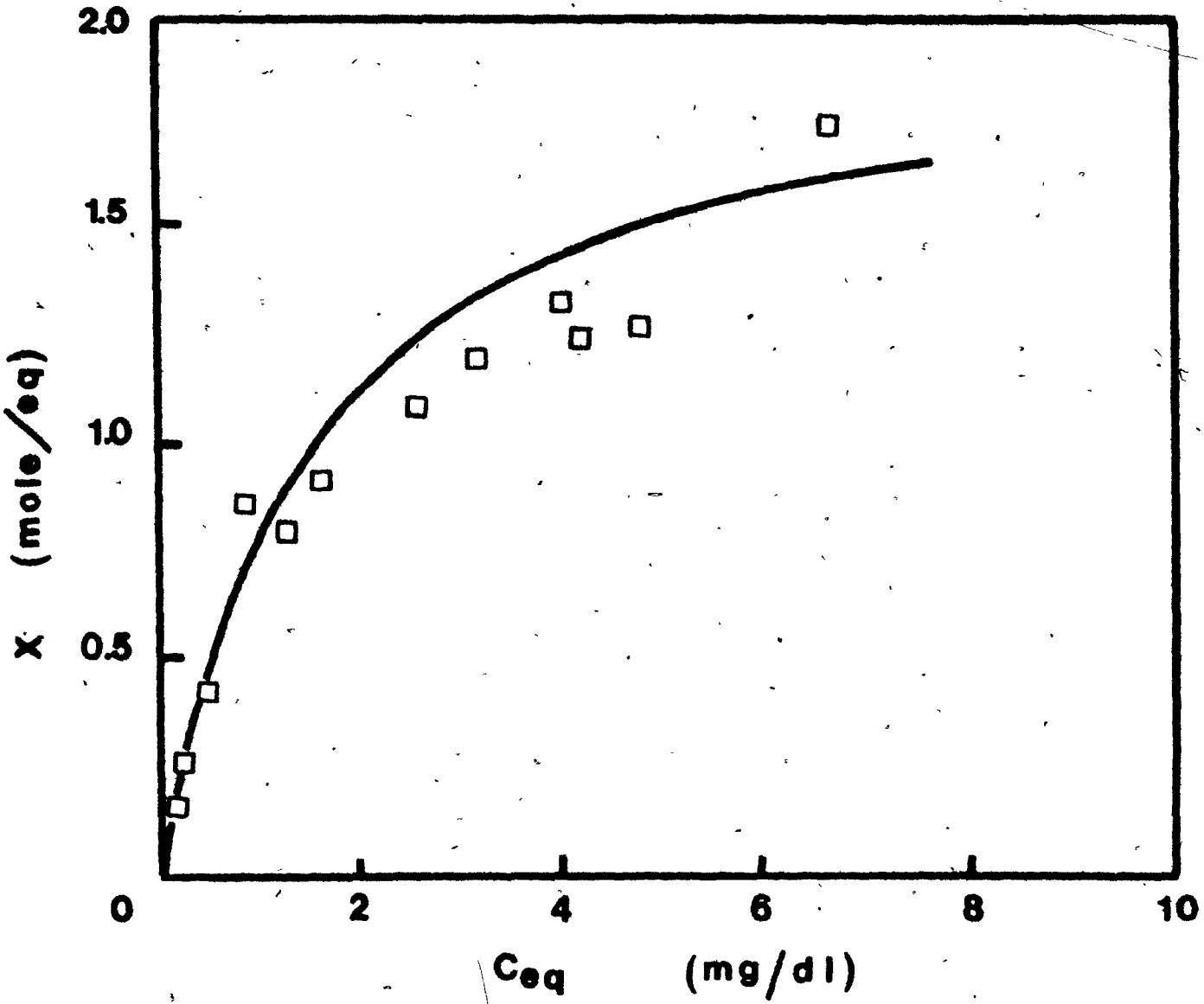

20
15
10
5
0
10
20
30
1/x (eq/mole)
1/Ceq x 10⁻⁴ M⁻¹

FIGURE 40

CALCULATED ISOTHERM FOR ARG_5ALA_3

BASED ON A IDEAL CASE, ASSUMING A TWO SITE MODEL

— Calculated isotherm
□ Experimental data


2.0
1.5
1.0
0.5
0
2
4
6
8
10
X (mole/eq)
Ceq (mg/dl)

of the squares of the differences between the experimental and calculated values obtained from the model equation. To begin the iteration, the constants, K_i, obtained from the previous method were entered. The values obtained for K_1 by this method are given in Table X.

These values of K_1 were substituted into equation (51) to obtain a second set of calculated isotherms assuming a one binding site model. For the unsubstituted resin, and for the resins with pendants of Ala_3, Arg_1Ala_3, Lys_1Ala_3, Lys_2Ala_3, His_2Ala_3, His_5Ala_3 and Arg_1Ala_4, this one binding site model appeared to describe the system adequately. However, as might be expected, for resins with pendants of Arg_2Ala_3, Arg_3Ala_3, Arg_5Ala_3, Lys_5Ala_3, $His_1Arg_2Ala_3$ and $Arg_1Ala_3Arg_1Ala_3$, the one binding site model predicts X values which are too high at low free bilirubin concentrations and too low at high concentrations. Fig. 41 shows, as an example, the case of Arg_5Ala_3.

A two binding site model was then assumed. By solving equation (52), again using the non-linear regression methods, binding constants and a third set of calculated isotherms was then obtained as for the one binding site model. For all of the resins the non-linear regression method, assuming either a one or a two binding site model depending on the resin, yields satisfactory curves with the experimental values scattered uniformly below and above the predicted curve (Figs. 42-46).

TABLE I

BINDING CONSTANTS FROM NON-LINEAR REGRESSION ANALYSIS OF THE ADSORPTION OF BILIRUBIN BY VARIOUS SUBSTITUTED POLYAMIDE

Pendants	One Site	Two Sites	
	K_1 10^{-3} M^{-1}	K_1 10^{-3} M^{-1}	K_2 $10^{-3} \cdot M^{-1}$
resin	1.0	1.0	0
Ala_3	1.1	1.1	0.07
Ala_3 (acetylated)	0.7	---	---
Arg_1Ala_3	4.5	4.2	0.03
Arg_2Ala_3	42	19	7.8
Arg_3Ala_3	57	24	7.2
Arg_5Ala_3	187	86	12
Lys_1Ala_3	1.6	1.7	0
Lys_2Ala_3	5.3	3.3	2.9
Lys_5Ala_3	130	1.0	700
His_2Ala_3	2.8	2.8	0
His_5Ala_3	17	13	1.1
Arg_1Ala_4	4.2	4.2	0
$Arg_1Ala_3Arg_1Ala_3$	112	25	10
$His_1Arg_2Ala_3$	114	61	4.2

Binding consants are considered to be accurate within ± 10%

FIGURE 41

CALCULATED ISOTHERMS FOR ARG_5ALA_3

BASED ON A NON-IDEAL CASE

One site model
Two site model
Experimental data

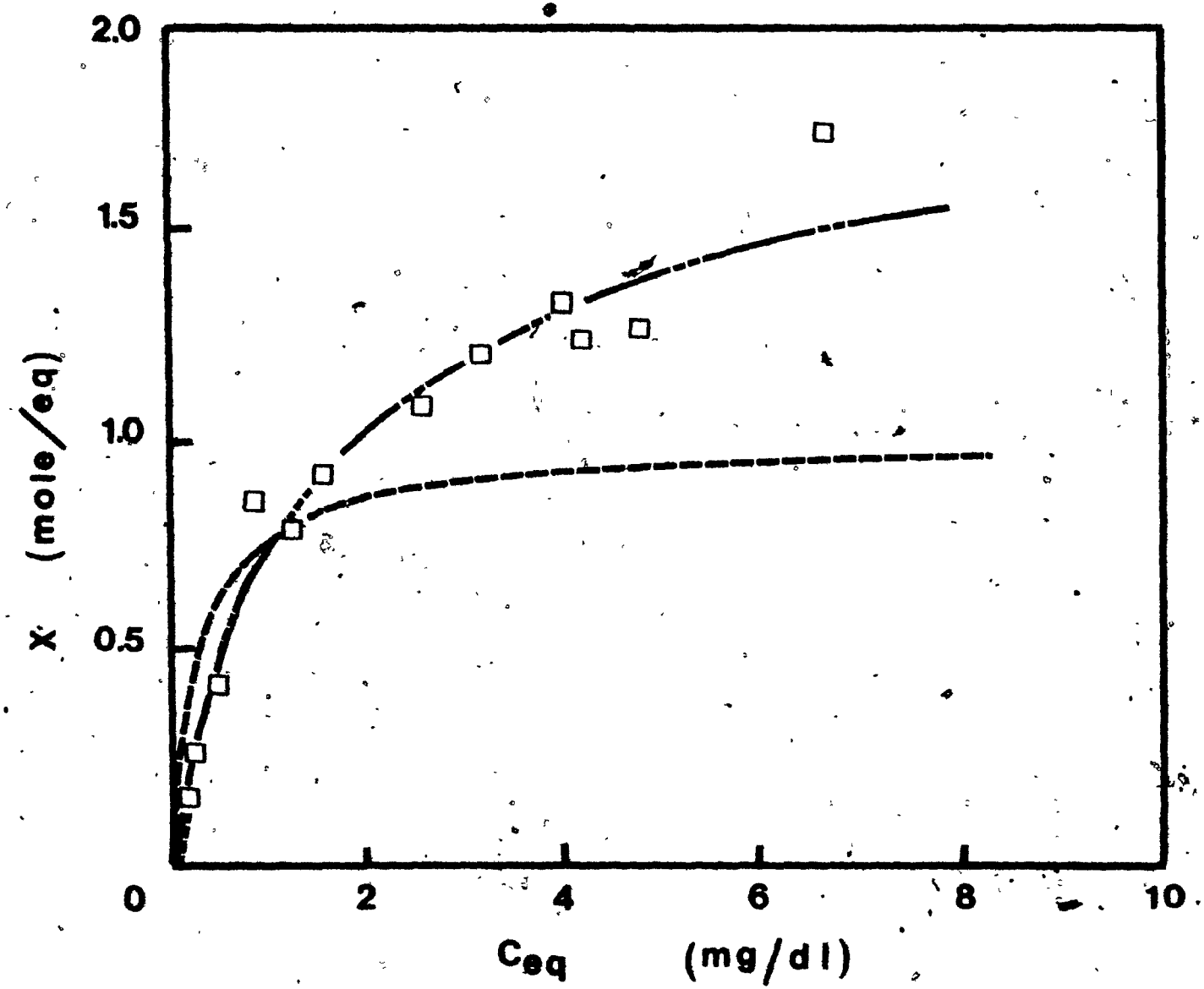
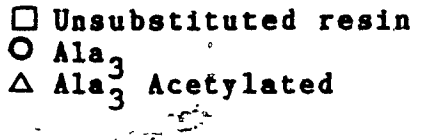

2.0
1.5
1.0
0.5
0
X (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 42

CALCULATED ISOTHERMS FOR THE REFERENCE RESINS

NON-IDEAL CASE, ASSUMING A ONE SITE MODEL



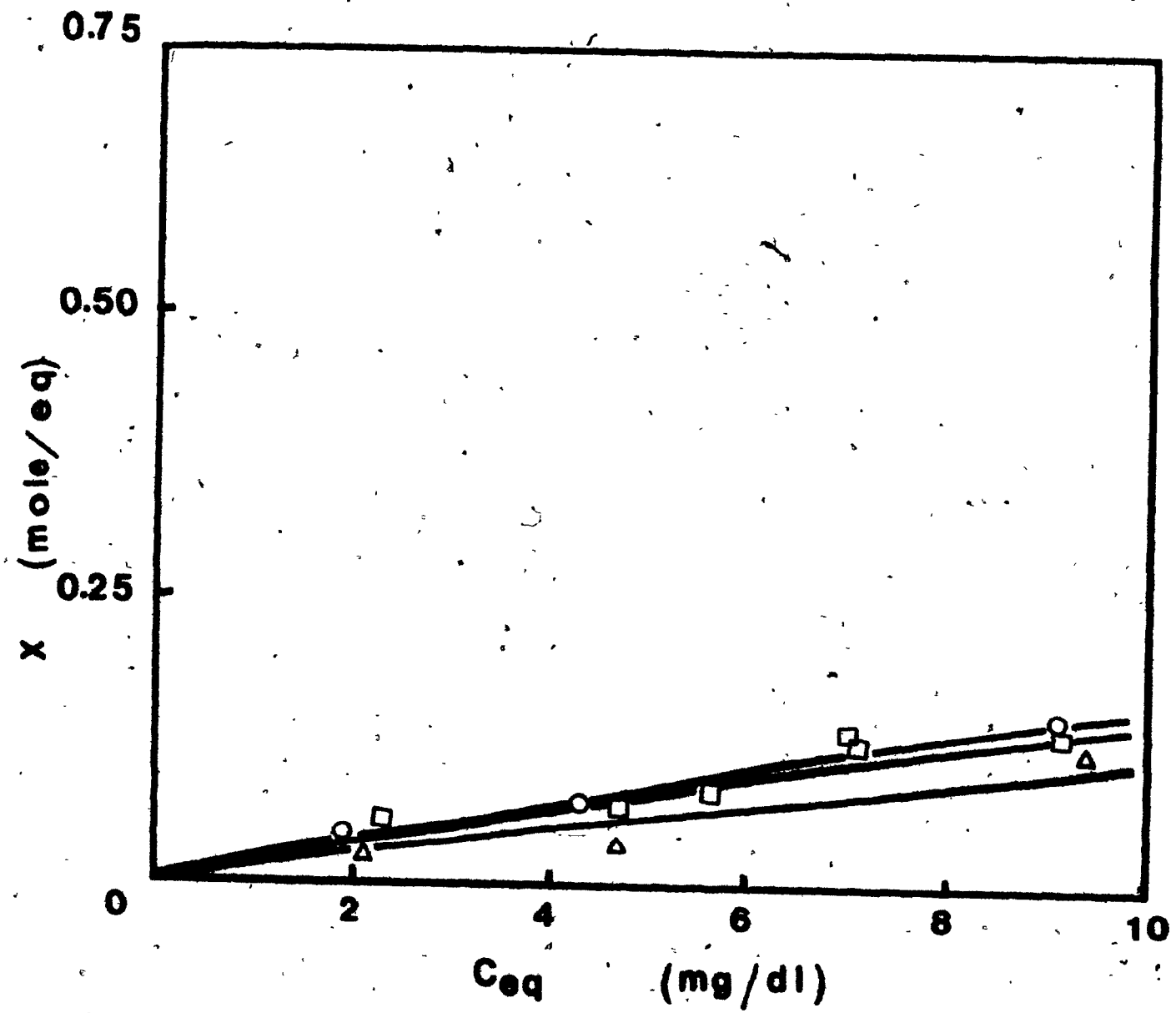
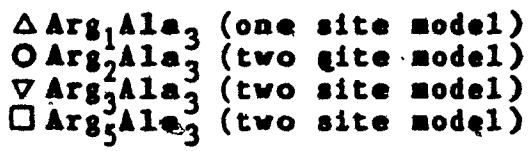

0.75
0.50
0.25
0
X (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 43

CALCULATED ISOTHERMS FOR THE ARGININE-CONTAINING RESINS

NON-IDEAL CASE



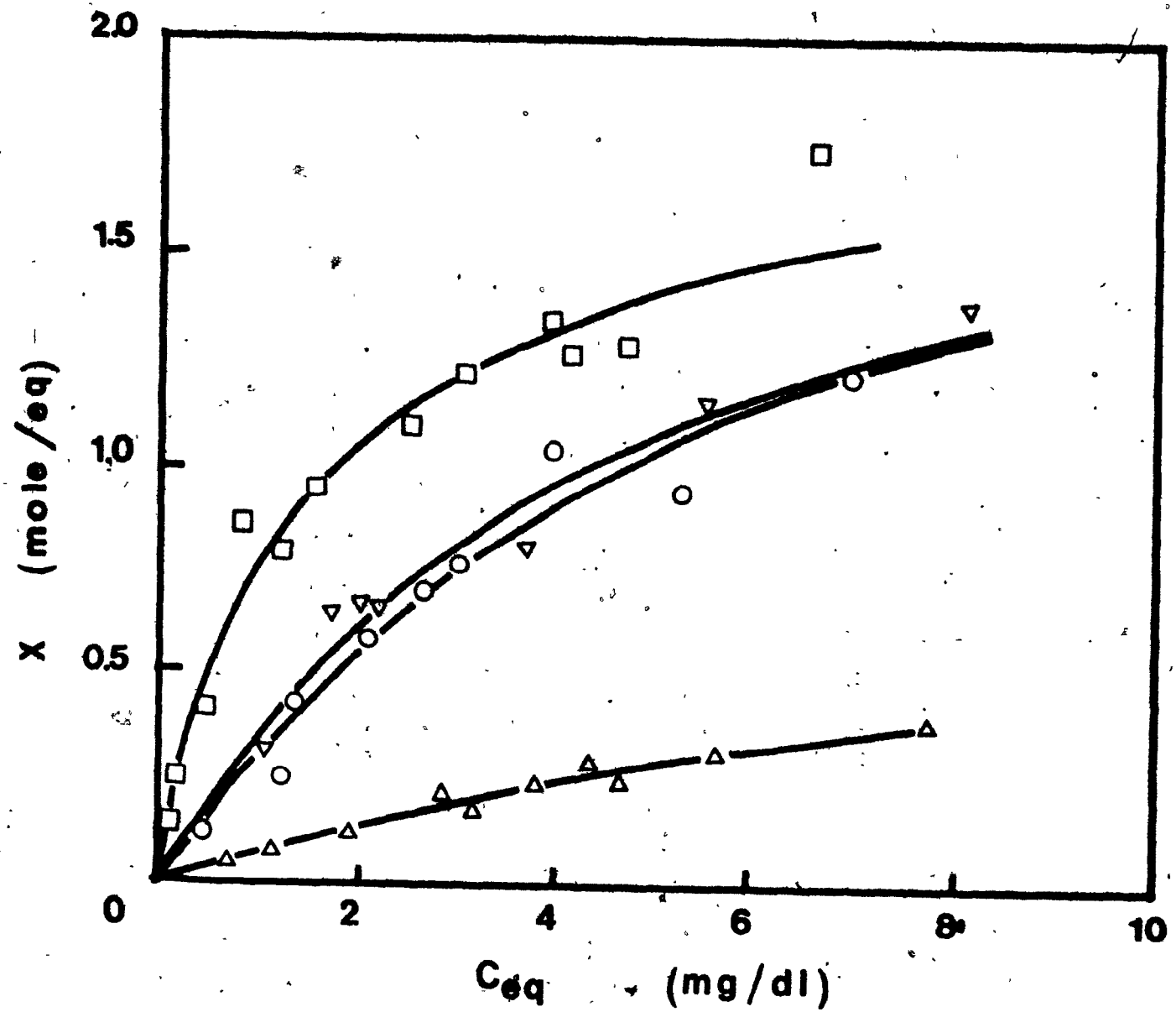

2.0
1.5
1,0
0.5
0
2
4
6
8
10
X (mole/eq)
$C_{éq}$ (mg/dl)

FIGURE 44

CALCULATED ISOTHERMS FOR THE LYSINE-CONTAINING RESINS

NON-IDEAL CASE

△ Lys_1Ala_3 (one site model)
○ Lys_2Ala_3 (one site model)
□ Lys_5Ala_3 (two site model)

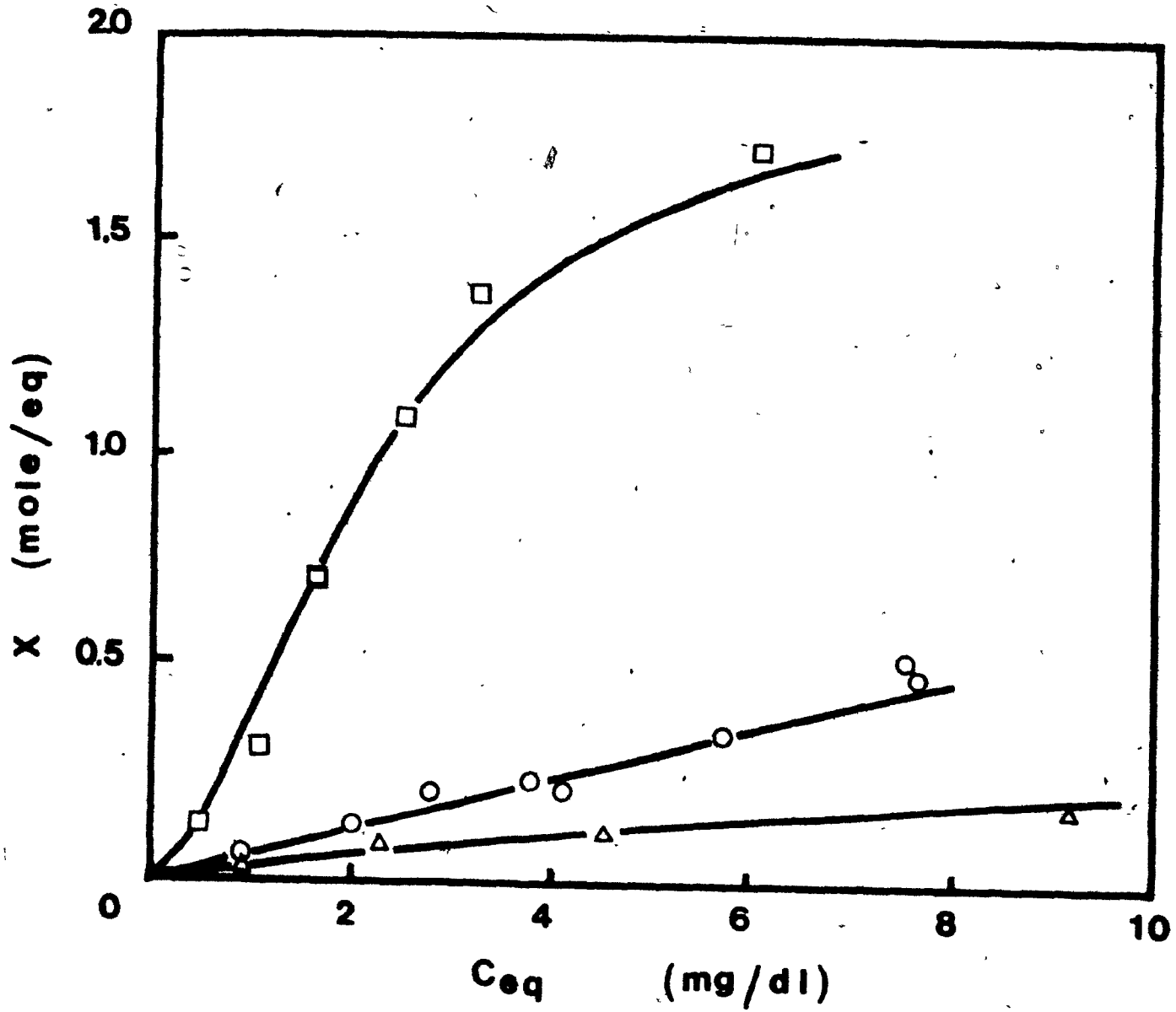

2.0
1.5
1.0
0.5
0
X (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 45

CALCULATED ISOTHERMS FOR THE HISTIDINE-CONTAINING RESINS

NON-IDEAL CASE

○ His_2Ala_3 (one site model)
□ His_5Ala_3 (one site model)

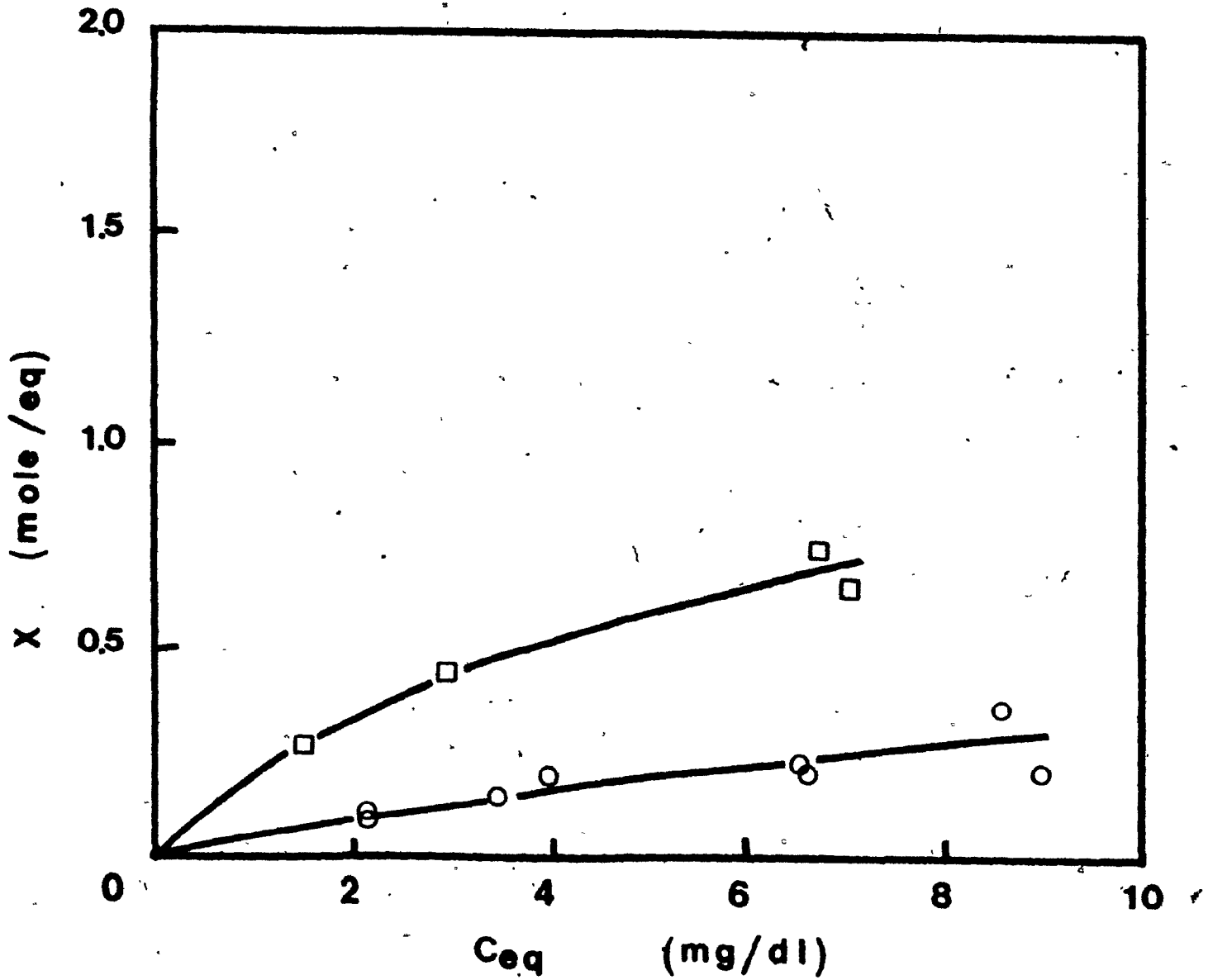
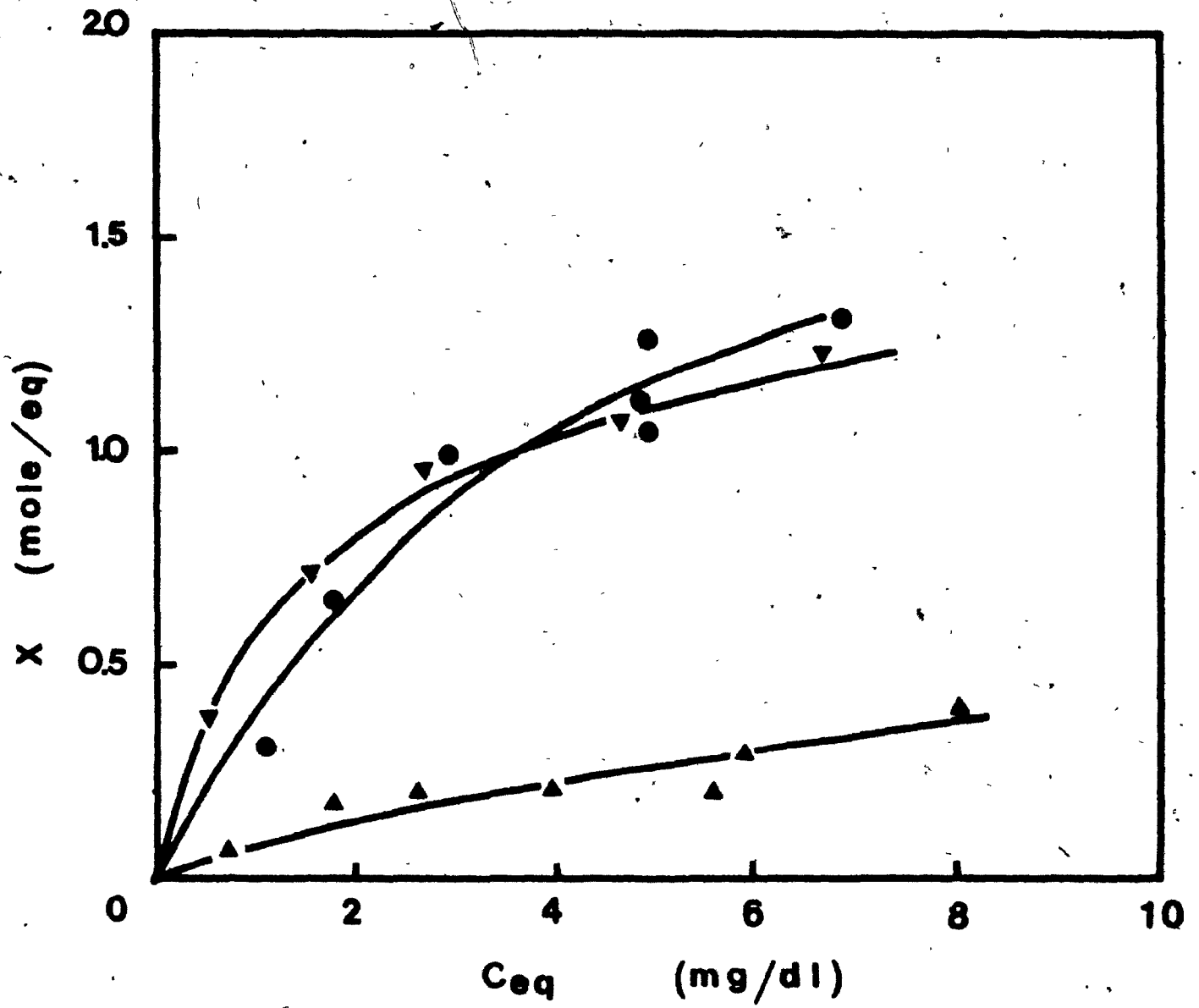

2.0
1.5
1.0
0.5
0
x (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

FIGURE 46

CALCULATED ISOTHERMS FOR OTHER POLYAMIDE RESINS

NON-IDEAL CASE

▲ Arg_1Ala_4 (one site model)
▼ $His_1Arg_2Ala_3$ (two site model)
● $Arg_1Ala_3Arg_1Ala_3$ (two site model)


2.0
1.5
1.0
0.5
0
x (mole/eq)
2
4
6
8
10
Ceq (mg/dl)

3) Thermodynamic Parameters

The free energy change accompanying the binding process was calculated according to:

$$\Delta G^{\circ} = -RT \ln K_i \qquad (53)$$

where K_i is the stoichiometric binding constant obtained from the non-linear regression. The values for the various systems are reported in Table XI.

4) Interaction between Sites

To determine the interactions between sites, a plot of iK_i as a function of i was made for the resins that appear to have two binding sites (Fig. 47). The affinity profiles for Arg_2Ala_3, Arg_3Ala_3, Lys_5Ala_3 and $Arg_1Ala_3Arg_1Ala_3$ show that the slope between K_1 and K_2 is greater than the ideal slope thus indicating positively interacting sites, either equivalent or non-equivalent (Table VIII). For the resins with Arg_5Ala_3 and $His_1Arg_2Ala_3$ pendants this slope is smaller than the slope of the ideal line. Hence, it is not possible to determine the type of interaction since, as mentioned in the Introduction, these results are consistent with either non-equivalent sites with no or positive or negative interactions as well as with equivalent sites with negative interactions (Table VIII).

DISCUSSION

1) Stoichiometric Binding Constants in an Ideal Case

Since stoichiometric binding constants can be obtained without making any assumptions about equivalence or

TABLE XI

FREE ENERGY CHANGE FOR BINDING OF BILIRUBIN TO VARIOUS SUBSTITUTED POLYAMIDE RESINS

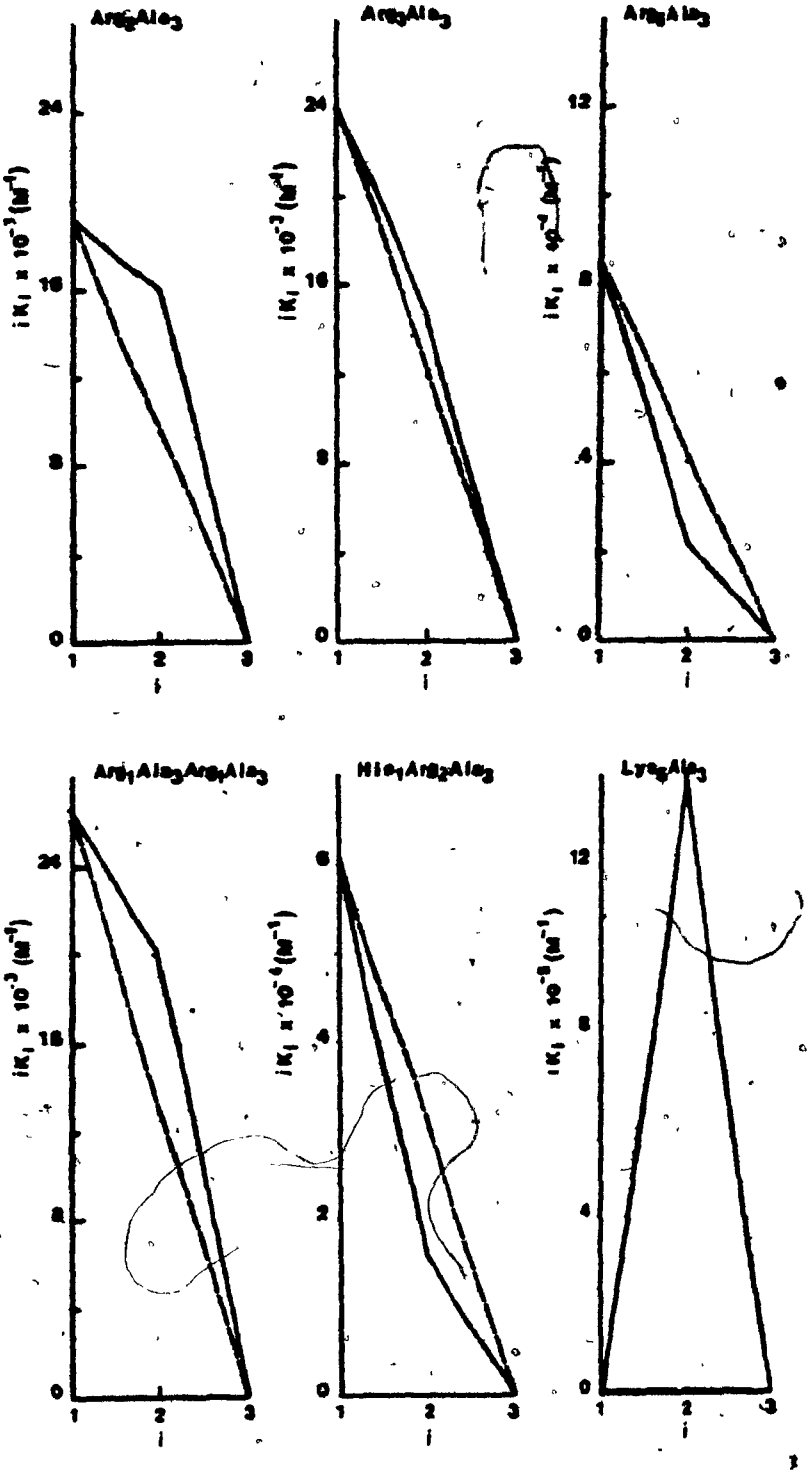
Pendants	$-\Delta G^{o}$ (K_1) Kjoule/mole	$-\Delta G^{o}$ (K_2) Kjoule/mole
resin	16	-----
Ala_3	16	-----
Ala_3 (acetylated)	15	-----
Arg_1Ala_3	19	-----
Arg_2Ala_3	22	20
Arg_3Ala_3	23	20
Arg_5Ala_3	26	21
Lys_1Ala_3	17	-----
Lys_2Ala_3	19	-----
Lys_5Ala_3	21	31
His_2Ala_3	18	-----
His_5Ala_3	22	-----
Arg_1Ala_4	19	-----
$Arg_1Ala_3Arg_1$	23	21
$His_1Arg_2Ala_3$	25	19

FIGURE 47

AFFINITY PROFILES FOR BINDING OF BILIRUBIN

TO VARIOUS SUBSTITUTED POLYAMIDE RESINS

—— Ideal line
----- Experimental line


Arg2Ala3
Arg3Ala3
Arg4Ala3
Arg1Ala3Arg2Ala3
His1Arg2Ala3
Lys2Ala3
iKi x 10-3 (M-1)
i

interactions between binding sites, they are preferred to the site binding constants in the data treatment. As a first approximation, the simple graphical method of plotting 1/X as a function of 1/[A] was used to obtain the binding constants. However, this method did not yield satisfactory results for some of the resins as indicated by a poor correlation between the isotherms calculated according to the Klotz model, using the derived binding constants, and the experimental data (e.g., data for Arg_5, Fig. 48). This is probably due to two factors:

a) The intrinsic property of such a plot to compress the data at high free ligand, i.e., bilirubin, concentration and thus hide deviation from ideality to give an apparent straight line (Rodbard, 1973, Klotz, 1974).

b) Such a plot is valid only in the case of non-interacting, equivalent binding sites, which is not the case for a number of polyamide resins as shown by the affinity profiles.

Since deviation from the experimental values occurs mainly for the resins with two binding sites per pendant, the assumption of equivalence and non-interaction of the binding sites is probably the major drawback of this method for use in certain cases being studied here.

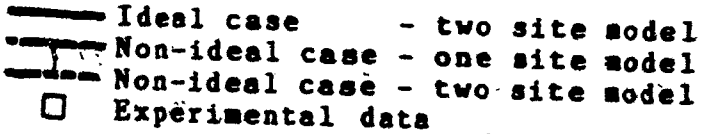
2) Stoichiometric Binding Constants in a Non-ideal Case

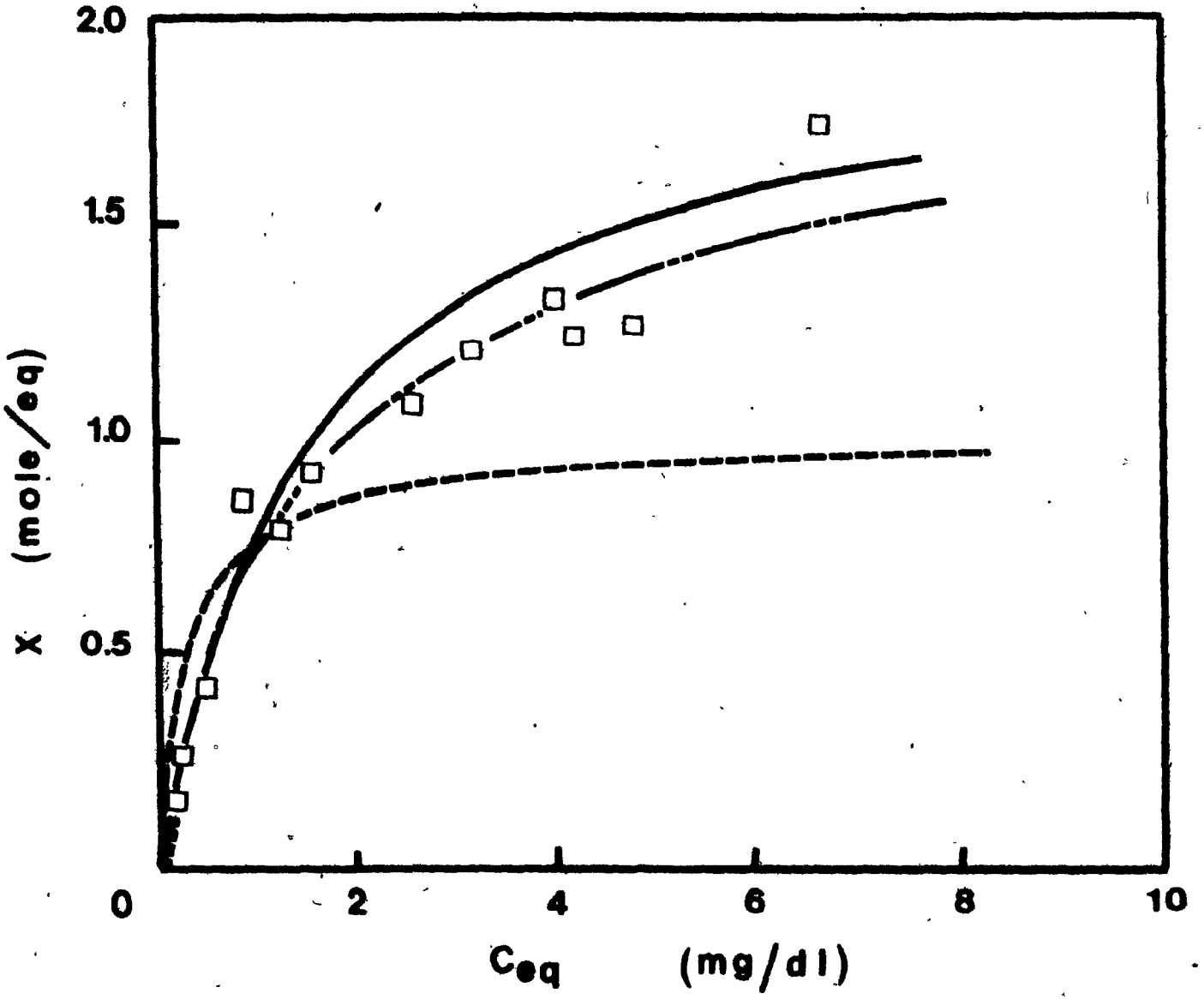
Better correlation between the experimental data and data predicted from the Klotz model was obtained for all the resins when the binding constants were obtained by a

FIGURE 48

CALCULATED ISOTHERMS FOR ARG_5ALA_3

BASED ON IDEAL AND NON-IDEAL CASES




2.0
1.5
1.0
0.5
0
2
4
6
8
10
X (mole/eq)
Ceq (mg/dl)

non-linear regression method. In this case no assumptions are made regarding the equivalence and interactions between the sites. Initially, a one binding site model was assumed for all the resins. In cases when the system could not be described adequately with these calculations, extra sites were added until the calculated isotherm gave a satisfactory representation of the experimental data (e.g. Arg_5Ala_3 resin, Fig. 48).

To verify whether or not the number of binding sites assumed was correct, the analysis was carried out for a model assuming one site more than the minimum number of sites deemed necessary to describe the system adequately. For the resins with one binding site model, assuming a two binding site model resulted in a K_1 which was essentially identical to K_1 obtained using a one binding site model while the resulting K_2 was equal or close to zero (Table X). However, assuming a three binding site model for the resins adequately described by a two site model did not always yield a K_3 equal to zero. In the case of 3 or more variables many different combinations of variables will describe these systems. Derived binding constants will depend on the estimated values of K_i used to start the iterations. Hence, very good first estimates of K_i are needed. Even then the mathematical limitations of the method should be recognized and the values of K_i obtained should be treated accordingly. In the case of less than 3 variables this problem is not as serious and for all the resins submitted to the non-linear curve fitting, the same

values of K_1 were obtained, regardless of the values used to start the iteration.

In the case of the resins containing the Lys_2Ala_3 and His_5Ala_3 pendants a one site model seemed to describe the system adequately but the value of K_1 obtained using a two site model was not equal to K_1 obtained using a one site model. For further consideration, the lowest possible number of binding sites was assumed. It should be kept in mind that this number does not necessarily represent the "true" number of binding sites but rather the minimum number of sites required to describe the system adequately.

Since it is based on the law of mass action, the Klotz model requires that the system should be reversible and at equilibrium. Desorption with albumin, although slow for some resins, shows that adsorption of bilirubin by the polyamide resins is reversible. Furthermore, the small changes in the bilirubin concentration in the adsorbate solution after one hour adsorption indicate that the system must be at or very near equilibrium.

Adsorbents following a one binding site model are mainly those with only one amino acid beyond the Ala_3 spacer as well as the unsubstituted resin and the resin with the Ala_3 spacer. Binding constants for these resins once again show that the adsorption follows the order arginine > lysine > histidine > alanine or increasing binding strength with increasing basicity of the pendants.

Some other observations originally made on the basis of the adsorption isotherms are also confirmed by the derived

binding constants. For example, the binding constants for the unsubstituted resin and the Ala_3 resins correspond to within 10%. This is well within experimental error and confirms the previous postulate that the α-amino group on the pendants and the resin NH_3^+ are equivalent as far as the adsorption process is concerned. The derived binding constants for the resins with Arg_1Ala_3 and with Arg_1Ala_4 also correspond within the limit of experimental error. This strengthens the observation that chain spacer length is not an important factor.

All of the resins following a two binding site model have at least two amino acids beyond the Ala_3 spacer indicating that a given amino acid will bind only one molecule of bilirubin. Thus, a terminal amino acid cannot bind a molecule of bilirubin at the α-amino site and another at the R group. The binding constant for the primary site on these resins is at least one order of magnitude larger than the binding constant for any of the resins fitting the one binding site model. The second binding constant is always at least as large as the constant for the resin with the corresponding single amino acid that fits the one site model. This indicates that the addition of active amino acids not only increases the number of binding sites on the pendants of the resin but also increases the affinity of these sites. If the affinity of the sites would remain unchanged while only the number of sites was increased, the first binding constant

should remain unchanged while, since $K_2 = K_1/4$, the second constants should be smaller.

Unlike the resins fitting the one binding site model, where the binding constants do not offer much added information over that available from the original adsorption isotherms, binding constants for the resins with two binding sites give more detailed information. For instance, a small increase in the value of K_1 and a decrease in the value of K_2 can be observed for the resin containing the Arg_3Ala_3 pendants as compared to the values obtained for the resin containing the Arg_2Ala_3 pendants. The decrease in the second binding constant is very small, less than 10%, and may well reflect experimental error. However, it is also possible that the addition of the third arginine changes the conformation of the binding sites somehow hindering the binding of a second bilirubin molecule. The more substantial increase observed in the first binding constant is probably real and is probably due to an increase in charge density. The binding constant for the first site on $His_1Arg_2Ala_3$ is larger than that of Arg_2Ala_3 or of Arg_3Ala_3, as expected from the higher adsorption isotherm. Once again, the second binding constant is lower. Thus, the addition of a histidine instead of an arginine enhances the binding of the first molecule of bilirubin by the pendant at the expense of the binding of the second bilirubin, implying that the addition of a histidine may affect the conformation of the binding sites.

$Arg_1Ala_3Arg_1Ala_3$ has the same first binding constant, within experimental error, as Arg_3Ala_3 but its second binding constant is larger. Evidently, in this case, the longer chain length has a slightly positive effect on the binding. The larger distance between the arginines and the charges seems to favour the binding of a second molecule of bilirubin slightly.

The lysine-containing resins show a different pattern from the other resins, with their second binding constant as large as the first one in the case of Lys_2Ala_3 (if two binding sites are assumed) and even larger in the case of Lys_5Ala_3. As shown by its isotherm (Fig. 26, Chap. IV), the larger second binding constant of the Lys_5Ala_3 containing resin has the effect of increasing its adsorption capacity to a higher level than the capacity of Arg_5Ala_3 at high equilibrium concentrations (Fig. 24). Second binding constants that are larger than the first have also been reported previously for some proteins (Klotz and Hunston, 1979) and appear to be a results of a large positive interaction between the sites. Thus, it appears that binding of a first bilirubin molecule by the Lys_5Ala_3 pendant affects the conformation of this pendant in such a way as to facilitate the binding of a second bilirubin molecule.

3) Interaction between Sites

Positive interactions between the sites for the Lys_5Ala_3 resin are clearly demonstrated by the affinity

profile. Certain other resins also show deviations from ideality but the interactions are not nearly as pronounced. Although it was not possible to distinguish between the different combinations of equivalence and interactions for a two site model for many of these resins, it is apparent that a second binding constant higher than that predicted by the ideal case, which will be referred to as positive behaviour, is more favorable to the binding capacity than a lower than ideal K_2 (negative behaviour). An understanding of the factors controlling this behaviour would be an important asset in the design of future adsorbents.

The positive interaction between the sites of some resins rule out charge density as the only determining factor. Since the binding of the first bilirubin molecule should decrease the overall charge density on the pendant, this should always have a negative effect on the binding of the second molecule if the electrostatic charge effects were the sole determining factor. It appears that chain length can also be excluded as the sole factor since the various resins with 8 amino acids show different types of behaviour, e.g., Arg_5Ala_3 shows a negative behaviour while Lys_5Ala_3 and $Arg_1Ala_3Arg_1Ala_3$ have positive interaction. Similar behaviour can be seen for the resins with 6 amino acids; Arg_3Ala_3 shows a positive behaviour while $His_1Arg_2Ala_3$ has negative behaviour. The identity of the amino acids does not seem to have any correlation with the behaviour of the sites since, for example, the various arginine-containing resins show either positive or negative

interaction. It seems very possible that the interaction between the sites is not governed by a single factor but rather by a complex combination of the above mentioned factors, as well as possibly certain others.

4) Thermodynamic Parameters

The derived values for the change in free energy associated with the binding of a bilirubin molecule to the various resins at 0°C are in the range of -15 to -31 Kjoule/mole. Excluding the values for the resin, Ala_3 resin and acetylated Ala_3 resin, the average value ΔG° for the first binding site is -21 ± 3 kJ/mole while the corresponding average value for the second binding site is -22 ± 5, where the uncertainty represents the standard deviation. The overall average value, excluding the resin, the Ala_3 resin, and acetylated Ala_3 resin, is - 21 ± 3 kJ/mole. Since the adsorption isotherms showed no indication of temperature dependence, ΔH° for the entire process must be zero. Hence, ΔS° would be in the range 55 to 113 joule $mole^{-1} degree^{-1}$. Using the average value for ΔG° together with $\Delta H^\circ = 0$ yields $\Delta S^\circ = 79 \pm 11$.

Contradictory thermodynamic parameters for the binding of bilirubin to HSA (Table XII) have been reported by Shapovalenko and Kolosov (Shapovalenko and Kolosov, 1978) and by Jacobsen (Jacobsen J., 1976). While ΔG° is negative in both studies, ΔH° and ΔS° were reported to be positive by Shapovalenko and Kolosov (Shapovalenko and Kolosov, 1978) while negative values are reported in Jacobsen's

TABLE XII

THERMODYNAMIC PARAMETERS FOR THE BINDING OF BILIRUBIN TO HSA

Reference	ΔH^o K joule mole	T °C	ΔG^o K joule mole	ΔS^o joule mole deg
Jacobsen J. 1977	- 57	20	- 46.0	- 35.6
		25	- 46.0	- 35.1
		30	- 45.6	- 36.0
		37	- 45.6	- 35.1
Shapovalenko and Kolosov 1978	38	3	- 28	240
		6	- 30	
		12	- 31	
		20	- 34	
		37	- 37	

study (Jacobsen J., 1976). Thus, the thermodynamic parameters obtained from the binding of bilirubin to the polyamide resin are in better agreement with the values of Shapovalenko and Kolosov (Shapovalenko and Kolosov, 1978). It should be remembered that the reported constants for the binding of bilirubin to albumin span a wide range of values. Furthermore, the range of temperatures at which they can be determined is very limited. Hence, a reliable evaluation of the thermodynamic parameters is experimentally very difficult. It should also be noted that the binding of bilirubin to albumin involves several steps, in particular some rearrangement of the albumin conformation, which is expected to be quite different from that which can occur with the small peptide fragments involved in this study. Thus, similarity between the thermodynamic parameters is not really expected.

5) Comparison between Polyamide-Bilirubin and Albumin-Bilirubin Binding Constants

Constants for binding bilirubin to albumin have been reported by several authors (Table III and IV) and for most, K_1 varies from 10^6 to 10^8 while K_2 varies from 10^5 to 10^6. Comparison of these values with the derived binding constants for the polyamide resins indicate that the resins bind bilirubin somewhat more weakly than does albumin. These lower binding constants are in good agreement with the fact that, although some of the resins are very good adsorbents in aqueous solutions, most cannot compete with albumin.

However, the resin containing the Arg_5Ala_3 pendant shows some competition, even with the first binding site of albumin and can definitely compete with the second binding site. This resin has a first binding constant of roughly 10^5 or depending on the source, approximately 10 to 100 fold lower than the reported values for the first binding site for bilirubin on albumin and 1 to 10 fold lower than that of the second binding site. On this basis the ability of the Arg_5Ala_3 resin to compete for bilirubin with the first binding site on BSA is somewhat unexpected and suggests that the values reported in the literature for the binding constants of HSA are somewhat high. Certainly better agreement is obtained if the lower values for these binding constants are accepted. It is also possible that the binding of bilirubin to BSA is not as strong as the binding to HSA.

The general conclusion that can be derived from the various aspects of this study is that the best polyamide resin made for the adsorption of bilirubin is one with pendants of high arginine content. Adsorption of bilirubin seems to be favored by pendants with a strongly basic character.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Throughout this project, a number of original findings have been made. These were discussed in detail in the preceding chapters and are summarized below.

1) It was shown that although crosslinked PVP adsorbs bilirubin more quickly than does cholestyramine, which is one of the most widely used adsorbents for bilirubin, the former has a much lower capacity and thus, its use as an adsorbent is not feasible.

2) Using eight different single amino acids residues, immobilized by solid phase peptide synthesis onto a polystyrene divinylbenzene resin (Merrifield resin), it was demonstrated that an interaction exists between bilirubin and two of these. The amino acids which showed the best interactions were arginine and lysine which are the only two with a positive charge at a pH of 7.8. These are also two of the four amino acids that have been proposed previously to be situated at the bilirubin binding site on albumin.

3) Using longer peptide pendants immobilized onto a water swellable polyamide resin increased the adsorption capacity for bilirubin of the polyamide resins. An interaction between bilirubin and tyrosine and histidine, the other two amino acids thought to be situated at the

binding site for bilirubin on albumin was also demonstrated.

4) A systematic study of the adsorption capacity for bilirubin by polyamide resins containing different combinations of arginine, lysine, tyrosine, histidine, and alanine, the last acting as a spacer, revealed some of the factors controlling the adsorption of bilirubin by immobilized amino acids to be:

a) The α-amino group: This group contributes moderately to the adsorption of bilirubin as shown by the fact that acetylating the α-amino group of a Ala_3 pendants results in a decrease in the adsorption capacity.

b) The R groups: The nature of the R groups of the pendants is the main contributor to the adsorption of bilirubin.

c) Charge density: Resins containing the charged amino acids, arginine and lysine, adsorb more bilirubin than do resins containing the uncharged amino acids tyrosine and histidine. Furthermore, as the number of amino acids in the pendants increases, the corresponding enhancement in the adsorption capacity is higher when the amino acids are charged. Arginine and lysine were found to be the strongest adsorbents with histidine and tyrosine showing weaker binding.

d) Spacing: The distance from the base resin to the active part of the pendants does not appear to

play an important role.

Adding a spacer between two active amino acids residues has a positive effect on the adsorption capacity. The resin containing the pendants $Arg_1Ala_3Arg_1Ala_3$ demonstrating a higher adsorption capacity than a resin containing the pendants Arg_2Ala_3.

e) Mixed residues: Combinations of active residues in a pendant can demonstrate synergistic behaviour.

f) Conformation and/or other factors: Discontinuities in adsorption capacities and binding constants suggest that steric and conformational effects are present.

5) Desorption by albumin showed that resins containing pendants with arginine bind bilirubin more strongly than those composed of lysine.

6) Adsorption from solutions containing both bilirubin and albumin showed that some resins, in particular the resin containing Arg_5Ala_3 pendants, can compete favourably with albumin for bilirubin.

7) Binding constants for bilirubin, obtained for each of the polyamide resins containing peptides pendants using the stoichiometric binding constant approach, were in the range of 10^3 to 10^5. The binding constant for the best resin, that containing the Arg_5Ala_3 pendants, is approximately two to three order of magnitude lower than

the binding of the first site for bilirubin on human serum albumin. For pendants with more than one site an interaction between the sites was generally detected.

8) Binding sites for bilirubin on albumin: The present data give some insight to the deeply researched question concerning the site at which the two strongly bound bilirubin molecules are attached to albumin. Sites where access to two arginine residues is possible are considered to be prime places for consideration. By virtue of its high arginine, lysine and histidine content, the tip of loop 4 (Fig. 8-9, Chap I) should also be considered.

SUGGESTION FOR FUTURE WORK

1) In-vivo studies of the adsorption characteristics for bilirubin by these resins should be obtained.

2) The synthesis and study of the adsorption capacity for bilirubin of resins containing peptide pendants similar to those found in native human serum albumin and/or bovine serum albumin should be considered. This could lead to better adsorbents and a better characterization of the binding sites for bilirubin on albumins.

3) Since it is suspected that conformation of the pendants may play an important role in the adsorption process, a study of the conformation of the immobilized peptide pendants is suggested.

4) Synthesis of active peptide pendants onto a more highly substituted resin should be considered. This should lead to a resin with a increased capacity.

5) Synthesis of pendants made of amino acids derivatives which would be non-hydrolizable should also be undertaken. This type of resin could then be used for removal of bilirubin from the gastrointestinal tract.

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APPENDIX I

SOLID PHASE PEPTIDE SYNTHESIS

FIGURE A1

REACTION SCHEME FOR SOLID PHASE PEPTIDE SYNTHESIS

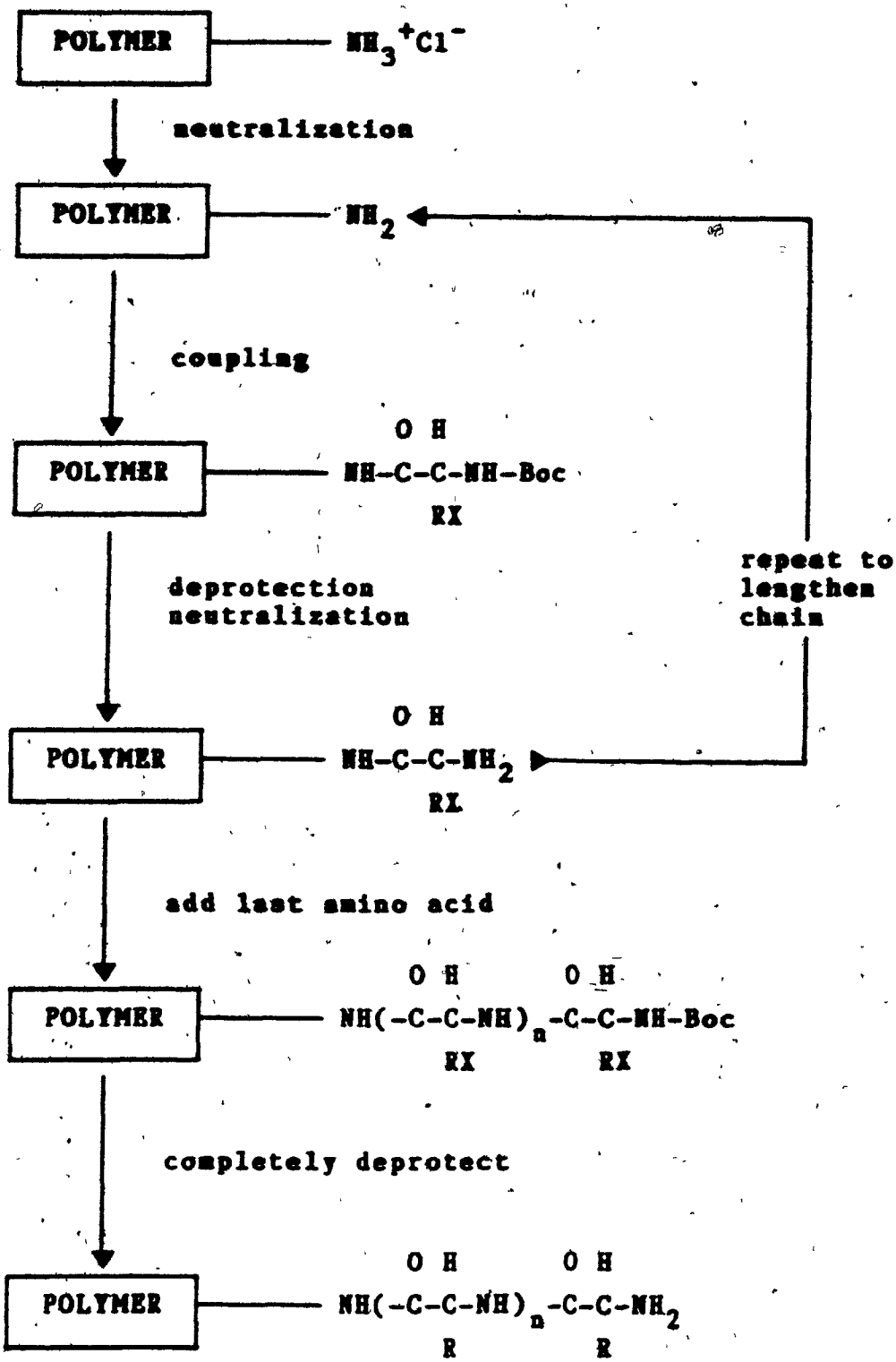
POLYMER
$NH_3^+Cl^-$
neutralization
POLYMER
NH_2
coupling
POLYMER
O H
NH-C-C-NH-Boc
RX
deprotection
neutralization
repeat to
lengthen
chain
POLYMER
O H
NH-C-C-NH_2
RX
add last amino acid
POLYMER
O H O H
NH(-C-C-NH)$_n$-C-C-NH-Boc
RX RX
completely deprotect
POLYMER
O H O H
NH(-C-C-NH)$_n$-C-C-NH_2
R R

FIGURE A2

AUTOMATIC PEPTIDE SYNTHESIZER

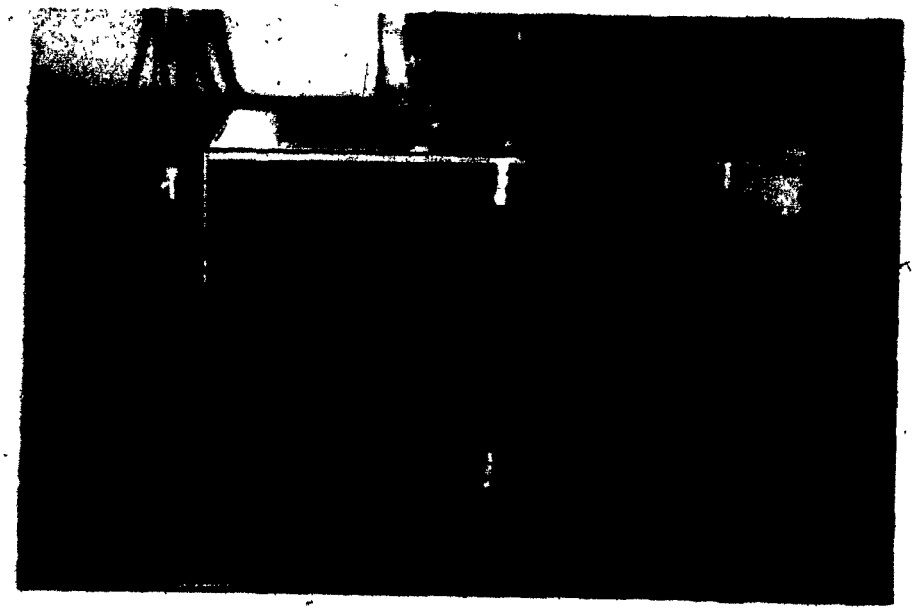

FIGURE A3

HF LINE

TABLE A-I

PROGRAM FOR AUTOMATED SOLID PHASE PEPTIDE SYNTHESIS I

Step	Operation	Reagent	Number of Repetitions	Time (minutes)
1	coupling	symmetrical anhydride	1	until completion
2	washing	CH_2Cl_2	4	2
3	deprotection	40% TFA in CH_2Cl_2	1	20
4	washing	CH_2Cl_2	2	0.75
5	washing	5% DEA in CH_2Cl_2	2	0.75
6	neutralization	5% DEA in CH_2Cl_2	1	5
7	washing	CH_2Cl_2	2	2
8	washing	DMF	2	2
9	washing	CH_2Cl_2	2	2
10	next coupling back to step 1			

TABLE A-II

PROGRAM FOR AUTOMATED SOLID PHASE PEPTIDE SYNTHESIS II

Step	Operation	Reagent	Number of Repetitions	Time (minutes)
1	coupling	symmetrical anhydride	1	until completion
2	washing	CH_2Cl_2	2	0.5
3	washing	hexane	2	0.5
4	washing	CH_2Cl_2	2	0.5
5	deprotection	40% TFA in CH_2Cl_2	1	20
6	washing	CH_2Cl_2	2	1
7	washing	5% DEA in CH_2Cl_2	2	0.75
8	neutralization	5% DEA in CH_2Cl_2	1	5
9	washing	CH_2Cl_2	3	0.5
10	next coupling back to step 1			

TABLE A-III

PROGRAM FOR AUTOMATED SOLID PHASE PEPTIDE SYNTHESIS III

Step	Operation	Reagent	Number of Repetitions	Time (minutes)
1	coupling	symmetrical anhydride	1	until completion
2	washing	CH_2Cl_2	2	1
3	washing	DMF	1	1
4	washing	CH_2Cl_2	3	1
5	deprotection	40% TFA in CH_2Cl_2	1	20
6	washing	CH_2Cl_2	2	1
7	washing	dioxane/CH_2Cl_2	2	1
8	washing	CH_2Cl_2	2	1
9	washing	DMF	2	1
10	washing	5% DEA in CH_2Cl_2	2	0.75
11	neutralization	5% DEA in CH_2Cl_2	1	5
12	washing	CH_2Cl_2	4	2
13	next coupling back to step 1			

APPENDIX II

EXPERIMENTAL DATA

This appendix contains, in tabular form, the experimental data contained in Figures 17 and 18 in Chapter II, Figures 22 to 30 in Chapter IV, Figures 31 to 37 in Chapter V, and Figures 39 to 48 in Chapter VI.

FIGURE 17

ADSORPTION OF BILIRUBIN BY PVP

Temperature = 0°C

C_{eq} (mg/dl)	X (mg/g)
1.5	26.3
2.7	30.2
3.2	33.1
4.2	35.9
4.8	41.6
6.4	43.5

Temperature = 10°C

C_{eq} (mg/dl)	X (mg/g)
1.4	20.3
1.8	26.0
2.5	31.6
4.1	37.6
6.4	44.4
8.1	48.8

Temperature = 20°C

C_{eq} (mg/dl)	X (mg/g)
2.2	31.9
2.9	34.8
3.2	41.0
6.1	48.1

Temperature = 25°C

C_{eq} (mg/dl)	X (mg/g)
4.0	37.0
3.1	33.9

FIGURE 18

ADSORPTION OF BILIRUBIN BY CHOLESTYRAMINE

Temperature = 0°C

C_{eq} (mg/dl)	X (mg/g)
1.3	27.0
1.9	32.0
2.6	35.1
4.5	34.4
6.5	60.4

Temperature = 10°C

C_{eq} (mg/dl)	X (mg/g)
0.7	38.8
1.2	42.9
1.7	46.0
2.6	49.8
4.2	49.2
5.1	60.0
5.5	52.7
6.1	63.0

Temperature = 20°C

C_{eq} (mg/dl)	X (mg/g)
0.1	45.1
0.6	60.9
1.1	62.4
2.2	64.8
4.2	74.3
6.2	71.8

Temperature = 25°C

C_{eq} (mg/dl)	X (mg/g)
0.3	64.9
0.7	69.9
1.9	81.1
3.7	88.1
6.4	89.8

FIGURE 22

KINETICS FOR THE ADSORPTION OF BILIRUBIN

BY THE POLYAMIDE RESINS

TIME (min)	Arg_1Ala_3 Absorbance	Arg_2Ala_3 Absorbance	Arg_5Ala_3 Absorbance
0	0.448	0.448	0.850
5	0.375	0.300	0.626
15	0.340	0.240	0.560
30	0.325	0.205	0.462
60	0.330	0.160	0.400
90	0.320	0.140	0.380

FIGURE 23

ADSORPTION OF BILIRUBIN BY THE REFERENCE RESINS

Unsubstituted Resin

C_{eq} (mg/dl)	X (mole/eq)
2.5	0.060
4.7	0.075
4.8	0.064
7.1	0.140
7.2	0.130
9.2	0.146

Cholestyramine

C_{eq} (mg/dl)	X (mole/eq)
1.3	0.013
1.9	0.016
2.6	0.017
4.5	0.017
6.5	0.025

Ala_3

C_{eq} (mg/dl)	X (mole/eq)
1.9	0.044
4.4	0.072
9.3	0.160

Ala_3 Acetylated

C_{eq} (mg/dl)	X (mole/eq)
2.4	0.014
4.7	0.030
9.5	0.120

FIGURE 24

ADSORPTION OF BILIRUBIN BY THE ARGININE-CONTAINING RESINS

Arg_1Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.7	0.06
1.2	0.08
1.9	0.11
3.1	0.19
3.2	0.17
3.8	0.24
4.3	0.28
4.7	0.23
5.6	0.31
7.7	0.38

Arg_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.11
1.3	0.25
1.4	0.42
2.1	0.58
2.7	0.71
3.0	0.77
4.0	1.02
5.3	0.93
7.0	1.20

FIGURE 24 cont'd

Arg_3Ala_3

C_{eq} (mg/dl)	X (mole/eq)
1.1	0.32
1.7	0.64
2.1	0.67
2.2	0.66
3.7	0.80
5.4	1.09
8.2	1.37

Arg_5Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.1	0.16
0.2	0.24
0.5	0.42
0.8	0.85
1.3	0.80
1.6	0.91
2.6	1.07
3.1	1.20
4.0	1.33
4.1	1.25
4.7	1.27
6.6	1.74

FIGURE 25

EFFECT OF PROTECTING THE R GROUP OF ARGININE PENDANTS ON POLYAMIDE RESINS

Arg_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.11
1.3	0.25
1.4	0.42
2.1	0.58
2.7	0.71
3.0	0.77
4.0	1.02
5.3	0.93
7.0	1.20

Arg_2Ala_3 protected

C_{eq} (mg/dl)	X (mole/eq)
3.5	0.27
7.9	0.48
8.0	0.42

FIGURE 26

ADSORPTION OF BILIRUBIN BY THE LYSINE-CONTAINING RESINS

Lys_1Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.9	0.04
2.2	0.08
4.6	0.11
9.2	0.19

Lys_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.8	0.05
2.0	0.13
2.8	0.21
3.8	0.25
4.2	0.21
5.8	0.35
7.6	0.56
7.7	0.50

Lys_5Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.12
1.1	0.30
1.7	0.67
1.7	0.70
2.6	1.10
3.3	1.40
6.1	1.74

FIGURE 27

ADSORPTION OF BILIRUBIN

BY THE HISTIDINE- OR TYROSINE-CONTAINING RESINS

His_1Ala_3

C_{eq} (mg/dl)	X (mole/eq)
4.3	0.13
9.2	0.15

His_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
2.1	0.11
2.2	0.20
3.5	0.16
4.0	0.19
6.5	0.23
6.6	0.20
8.5	0.37
9.0	0.21

His_5Ala_3

C_{eq} (mg/dl)	X (mole/eq)
1.5	0.25
3.0	0.44
6.8	0.76
7.0	0.66

Tyr_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
4.3	0.20
9.1	0.23

Tyr_1Ala_3

C_{eq} (mg/dl)	X (mole/eq)
4.3	0.14
9.2	0.16

FIGURE 28

EFFECT OF THE ADDITION OF AN HISTIDINE TO ARG_2ALA_3

Arg_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.11
1.3	0.25
1.4	0.42
2.1	0.58
2.7	0.71
3.0	0.77
4.0	1.02
5.3	0.93
7.0	1.20

Arg_3Ala_3

C_{eq} (mg/dl)	X (mole/eq)
1.1	0.32
1.7	0.64
2.1	0.67
2.2	0.66
3.7	0.80
5.4	1.09
8.2	1.37

$His_1Arg_2Ala_3$

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.40
1.6	0.76
2.8	0.96
4.7	1.06
6.8	1.23

FIGURE 29

EFFECT OF THE ADDITION OF A SPACER

Arg_1Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.7	0.06
1.2	0.08
1.9	0.11
3.1	0.19
3.2	0.17
3.8	0.24
4.3	0.28
4.7	0.23
5.6	0.31
7.7	0.38

Arg_2Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.11
1.3	0.25
1.4	0.42
2.1	0.58
2.7	0.71
3.0	0.77
4.0	1.02
5.3	0.93
7.0	1.20

Arg_4Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.7	0.07
1.8	0.18
2.6	0.21
4.0	0.20
5.6	0.20
5.9	0.29
8.0	0.41

$Arg_1Ala_3Arg_1Ala_3$

C_{eq} (mg/dl)	X (mole/eq)
1.1	0.31
1.8	0.65
3.0	1.00
4.9	1.13
5.0	1.04
5.0	1.26
7.01	1.32

FIGURE 30

EFFECT OF IONIC STRENGTH ON

ADSORPTION OF BILIRUBIN BY ARG_2ALA_3

Arg_2Ala_3

Ionic Strength = 0.1 M

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.11
1.3	0.25
1.4	0.42
2.1	0.58
2.7	0.71
3.0	0.77
4.0	1.02
5.3	0.93
7.0	1.20

Arg_2Ala_3

Ionic Strength = 0.010 M

C_{eq} (mg/dl)	X (mole/eq)
0.9	0.33
0.9	0.56
0.9	0.55
1.4	0.73
3.0	0.89
4.8	1.09
4.9	1.05
5.0	1.02

FIGURE 31

KINETICS OF DESORPTION OF BILIRUBIN FROM POLYAMIDE RESINS

Time (min)	Arg_2Ala_3 Absorbance	Arg_2Ala_3 Absorbance	Lys_5Ala_3 Absorbance
0	0.452	0.345	0.390
15	---	---	0.662
30	0.610	0.409	0.754
60	0.678	0.467	0.781
120	0.734	0.520	0.823
190	0.758	0.576	0.821
250	0.774	0.593	---

FIGURE 32

DESORPTION OF BILIRUBIN WITH BOVINE SERUM ALBUMIN

Arg_2Ala_3

bilirubin / BSA	bilirubin retained %
0.87	0
1.19	18
1.61	22
2.02	29
2.81	47

Arg_5Ala_3

bilirubin / BSA	bilirubin retained %
0.54	32
0.80	45
1.07	48
1.43	65
1.74	82

$His_1Arg_2Ala_3$

bilirubin / BSA	bilirubin retained %
2.32	5
3.65	11
4.81	27
9.24	75
9.43	60

Lys_5Ala_3

bilirubin / BSA	bilirubin retained %
2.93	17
9.22	78

FIGURE 33

DESORPTION OF BILIRUBIN FROM ARG_2ALA_3

WITH BOVINE SERUM ALBUMIN AT 0.010 M

Arg_2Ala_3

Ionic Strength = 0.10 M

bilirubin / BSA	bilirubin retained %
0.13	40
0.27	54
0.37	53
0.39	48
0.47	53
0.54	52
0.60	59
0.70	49
0.84	54
0.95	46
1.40	63

Arg_2Ala_3

Ionic Strength = 0.010 M

bilirubin / BSA	bilirubin retained %
0.87	0
1.19	18
1.61	22
2.02	29
2.81	47

FIGURE 34

ADSORPTION OF BILIRUBIN BY ARG_5ALA_3

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

bilirubin / BSA	X (mole/eq)	free bilirubin 1 binding site	free bilirubin 2 binding sites
1.72	0.09	1.97	- 0.31
2.02	0.10	2.32	0.40
2.23	0.16	2.61	0.89
2.50	0.22	2.82	1.33
2.95	0.18	3.06	1.83
3.94	0.36	3.45	2.59
4.78	0.45	3.65	2.97
5.51	0.57	3.76	3.22
6.07	0.47	3.78	3.28
8.45	0.75	3.98	3.67

FIGURE 35

ADSORPTION OF BILIRUBIN BY ARG_2ALA_3

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

bilirubin / BSA	X (mole/eq)	free bilirubin 1 binding site	free bilirubin 2 binding sites
1.70	0.004	1.90	- 0.46
2.08	0.027	2.33	0.40
3.26	0.058	3.09	1.85
3.76	0.062	3.28	2.23
4.45	0.11	3.47	2.62
6.80	0.41	3.84	3.36
9.89	0.56	4.01	3.71

FIGURE 36

ADSORPTION OF BILIRUBIN BY LYS_5ALA_3

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

$\frac{\text{bilirubin}}{\text{BSA}}$	X (mole/eq)	free bilirubin 1 binding site	free bilirubin 2 binding sites
1.74	0	1.88	- 0.48
2.13	0.019	2.36	0.46
3.29	0.031	3.07	1.81
4.37	0.083	3.42	2.53
5.39	0.20	3.63	2.93
6.36	0.32	3.76	3.23
6.68	0.43	3.83	3.34
8.96	0.79	4.01	3.71

FIGURE 37

ADSORPTION OF BILIRUBIN BY CHOLESTYRAMINE

IN THE PRESENCE OF BOVINE SERUM ALBUMIN

bilirubin / BSA	X (mole/eq)	free bilirubin 1 binding site	free bilirubin 2 binding sites
1.68	0.0118	2.33	0.41
2.11	0.0115	2.72	1.2
2.54	0.0129	3.00	1.76
5.63	0.0181	3.77	3.30
8.90	0.2018	3.95	3.66

FIGURE 39

DOUBLE RECIPROCAL PLOT TO DETERMINE

THE BINDING CONSTANTS AT 0°C FOR VARIOUS POLYAMIDE RESINS

Arg_5Ala_3

$1/C_{eq}$ x 10^{-4} (M^{-1})	1/X (eq/mole)
0.88	0.57
1.24	0.79
1.41	0.80
1.47	0.75
1.87	3.83
2.28	0.93
3.64	1.00
4.67	1.25
7.02	1.18
12.8	2.38
26.9	4.12

Lys_5Ala_3

$1/C_{eq}$ x 10^{-4} (M^{-1})	1/X (eq/mole)
0.95	0.57
1.76	0.71
2.26	0.91
3.43	1.49
3.46	1.44
5.18	3.31
12.54	8.58

FIGURE 40

CALCULATED ISOTHERM FOR ARG_5ALA_3

BASED ON A IDEAL, ASSUMING A TWO SITE MODEL

$K_1 = 74 \times 10^3\ M^{-1}$
$K_2 = 18 \times 10^3\ M^{-1}$

Arg_5Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.1	0.16
0.2	0.24
0.5	0.42
0.8	0.85
1.3	0.80
1.6	0.91
2.6	1.07
3.1	1.20
4.0	1.33
4.1	1.25
4.7	1.27
6.6	1.74

FIGURE 41

CALCULATED ISOTHERM FOR ARG_5ALA_3

BASED ON A NON-IDEAL CASE

One site model

$K_1 = 187 \times 10^3\ M^{-1}$

Two sites model

$K_1 = 86 \times 10^3\ M^{-1}$
$K_2 = 12 \times 10^3\ M^{-1}$

Arg_5Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.1	0.16
0.2	0.24
0.5	0.42
0.8	0.85
1.3	0.80
1.6	0.91
2.6	1.07
3.1	1.20
4.0	1.33
4.1	1.25
4.7	1.27
6.6	1.74

FIGURE 42

CALCULATED ISOTHERMS FOR THE REFERENCE RESINS

NON-IDEAL CASE, ASSUMING A ONE SITE MODEL

Unsubstituted Resin

$K_1 = 1.0 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
2.5	0.060
4.7	0.075
4.8	0.064
7.1	0.140
7.2	0.130
9.2	0.146

Ala_3 Acetylated

$K_1 = 1.1 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
2.4	0.014
4.7	0.030
9.5	0.120

Ala_3

$K_1 = 0.7 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
1.9	0.044
4.4	0.072
9.3	0.160

FIGURE 43

CALCULATED ISOTHERMS FOR THE ARGININE-CONTAINING RESINS

NON-IDEAL CASE

Arg_1Ala_3

$K_1 = 4.5 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.7	0.06
1.2	0.08
1.9	0.11
3.1	0.19
3.2	0.17
3.8	0.24
4.3	0.28
4.7	0.23
5.6	0.31
7.7	0.38

Arg_2Ala_3

$K_1 = 19 \times 10^3 M^{-1}$
$K_2 = 7.8 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.11
1.3	0.25
1.4	0.42
2.1	0.58
2.7	0.71
3.0	0.77
4.0	1.02
5.3	0.93
7.0	1.20

FIGURE 42 Cont'd

Arg_3Ala_3

$K_1 = 24 \times 10^3 M^{-1}$
$K_2 = 7.2 \times 10^3 M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
1.1	0.32
1.7	0.64
2.1	0.67
2.2	0.66
3.7	0.80
5.4	1.09
8.2	1.37

Arg_5Ala_3

$K_1 = 86 \times 10^3 M^{-1}$
$K_2 = 12 \times 10^3 M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.1	0.16
0.2	0.24
0.5	0.42
0.8	0.85
1.3	0.80
1.6	0.91
2.6	1.07
3.1	1.20
4.0	1.33
4.1	1.25
4.7	1.27
6.6	1.74

FIGURE 44

CALCULATED ISOTHERMS FOR THE LYSINE-CONTAINING RESINS

NON-IDEAL CASE

Lys_1Ala_3

$K_1 = 1.6 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.9	0.04
2.2	0.08
4.6	0.11
9.2	0.19

Lys_2Ala_3

$K_1 = 5.3 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.8	0.05
2.0	0.13
2.8	0.21
3.8	0.25
4.2	0.21
5.8	0.35
7.6	0.56
7.7	0.50

Lys_5Ala_3

$K_1 = 1.0 \times 10^3\ M^{-1}$
$K_2 = 700 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.12
1.1	0.30
1.7	0.67
1.7	0.70
2.6	1.10
3.3	1.40
6.1	1.74

FIGURE 45

CALCULATED ISOTHERMS FOR THE HISTIDINE-CONTAINING RESINS

NON-IDEAL CASE

His_2Ala_3

$K_1 = 1.0 \times 10^3\ M^{-1}$
$K_2^1 = 700 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
2.1	0.11
2.2	0.20
3.5	0.16
4.0	0.19
6.5	0.23
6.6	0.20
8.5	0.37
9.0	0.21

His_5Ala_3

$K_1 = 1.0 \times 10^3\ M^{-1}$
$K_2^1 = 700 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
1.5	0.25
3.0	0.44
6.8	0.76
7.0	0.66

FIGURE 46

CALCULATED ISOTHERMS FOR OTHER POLYAMIDE RESINS

NON-IDEAL CASE

Arg_1Ala_4

$K_1 = 4.2 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.7	0.07
1.8	0.18
2.6	0.21
4.0	0.20
5.6	0.20
5.9	0.29
8.0	0.41

$Arg_1Ala_3Arg_1Ala_3$

$K_1 = 25 \times 10^3 M^{-1}$
$K_2 = 700 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
1.1	0.31
1.8	0.65
3.0	1.00
4.9	1.13
5.0	1.04
5.0	1.26
7.01	1.32

$His_1Arg_2Ala_3$

$K_1 = 61 \times 10^3 M^{-1}$
$K_2 = 4.2 \times 10^3\ M^{-1}$

C_{eq} (mg/dl)	X (mole/eq)
0.5	0.40
1.6	0.76
2.8	0.96
4.7	1.06
6.8	1.23

FIGURE 47

AFFINITY PROFILES FOR THE BINDING OF BILIRUBIN

TO VARIOUS SUBSTITUTED POLYAMIDE RESINS

Arg_2Ala_3

i	$iK_i \times 10^3$
1	19
2	16
3	0

Arg_3Ala_3

i	$iK_i \times 10^3$
1	24
2	14
3	0

Arg_5Ala_3

i	$iK_i \times 10^3$
1	86
2	23
3	0

$Arg_1Ala_3Arg_1Ala_3$

i	$iK_i \times 10^3$
1	25
2	20
3	0

$His_1Arg_2Ala_3$

i	$iK_i \times 10^3$
1	61
2	8
3	0

$Lys_5Arg_1Ala_3$

i	$iK_i \times 10^3$
1	1:0
2	1400
3	0

FIGURE 48

CALCULATED ISOTHERM FOR ARG_5ALA_3

BASED ON A IDEAL AND NON-IDEAL CASE

Ideal case
$K_1 = 74 \times 10^3\ M^{-1}$
$K_2 = 18 \times 10^3\ M^{-1}$

Non-ideal case

One site model

$K_1 = 187 \times 10^3\ M^{-1}$

Two sites model

$K_1 = 86 \times 10^3\ M^{-1}$
$K_2 = 12 \times 10^3\ M^{-1}$

Arg_5Ala_3

C_{eq} (mg/dl)	X (mole/eq)
0.1	0.16
0.2	0.24
0.5	0.42
0.8	0.85
1.3	0.80
1.6	0.91
2.6	1.07
3.1	1.20
4.0	1.33
4.1	1.25
4.7	1.27
6.6	1.74

APPENDIX III

PUBLICATIONS

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The adsorption of bilirubin from aqueous solution onto solid cholestyramine and polyvinylpyrrolidone

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KEY WORDS

Bilirubin
Adsorption
Cholestyramine
Polyvinylpyrrolidone
Isotherms
Kinetics

ABSTRACT

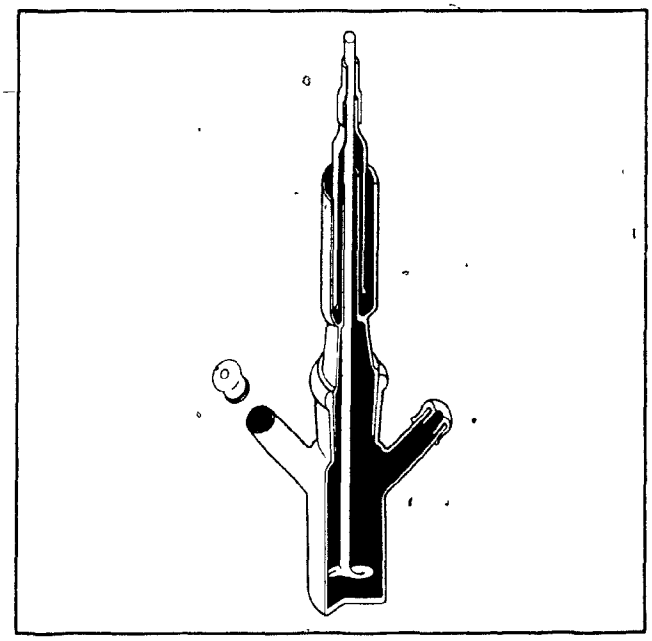
Shifts in the visible spectrum of aqueous bilirubin (BR) resulting from the addition of soluble polyvinylpyrrolidone (PVP) suggest specific interactions. Hence, isotherms were determined for the adsorption of BR from aqueous solution onto solid, cross-linked PVP and onto cholestyramine (CA) (used as a reference adsorbent) at 0, 10, 20 and 25°C. Although adsorption onto PVP reaches equilibrium values more rapidly than for CA, the latter adsorbent has a larger capacity. Furthermore the isotherms for PVP are independent of temperature while those for CA show an increase in BR adsorbed with an increase in temperature.

INTRODUCTION

Bilirubin (BR), a bile pigment produced in the blood by degradation of hemoglobin, is normally conjugated with glucuronic acid in the liver, then metabolized to urobilinogen by bacterial flora in the intestine and excreted. Transport to the liver requires the formation of a complex with albumin. When complexation, or conjugation, is incomplete, either because of an albumin deficiency or a defect in the conjugation mechanism, the level of free BR in the blood will rise, causing hyperbilirubinemia. The free BR diffuses into the tissues causing severe illness, e.g., jaundice and kernecticus, which may result in brain damage or death. This aliment is particularly common in newborn children and in eastern countries may affect as many as 30% of all newborns.

One possible method for treatment of hyperbilirubinemia is adsorption onto an ingested absorbent so that removal occurs in the gastrointestinal tract thus preventing the re-absorption of BR into the cardio-vascular system. Various degrees of success have been reported for the use of charcoal (Ulstrom et al. 1964), agar (Romagnoli et al. 1975), and cholestyramine (a copolymer of styrene and 2% divinylbenzene containing quaternary ammonium ions) (Lester et al. 1962; Arrowsmith et al. 1975) as adsorbents. In some cases, resin feeding has been attempted to assist simultaneous phototherapy (Ebbesen and Møller 1977, Nicolopoulos et al. 1978; Maurer et al. 1973). However, many factors such as gestational age and feeding methods as well as physiological factors seem to be of importance. For example, although ingestion of agar alone generally has little or no effect (Romagnoli et al. 1975; Møller 1974; Maurer 1974; Maurer et al. 1973, Blum and Etienne 1973; Schellong 1974), there has been a report of its effective use by Poland and Odell 1971. The ingestion method of treatment

Fig. 1 Adsorption flask



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is of limited application and is generally impractical for use with newborn children.

Physiological factors seem to be of lesser importance when adsorption of BR occurs during perfusion of plasma through the adsorbent Columns of activated charcoal (Barakat and MacPhee 1971; Lauterburg et al. 1979) and of various polymer resins have been used successfully In general, charged resins such as Dowex, 1X (Cholestyramine) tend to be more efficient than uncharged resins such as Amberlite XAD-2 in removing BR Sideman has reported the use of a «macroreticular MR ion exchange resin» with improved performance for removal of BR by hemoperfusion (Sideman et al 1982) It has also been found the immobilized albumin is useful as a biocompatible substance of adsorption of BR (Scharschmidt et al. 1974; Ton et al. 1979).

Although the various adsorbents mentioned have been used with varying degrees of success for the removal of BR each suffers from a lack of specificity and often have undesirable side effects, especially those which are ingested. A report that the addition of water-soluble polyvinylpyrrolidone (PVP) to BR solution causes a «red-shift» in the visible spectrum of BR (Ploussard et al. 1972) suggested that a specific interaction may be involved. The purpose of this study was to determine whether this interaction could be the basis of a selective adsorption of BR onto biocompatible PVP that had been made water insoluble, but was still swellable, by light cross-linking.

MATERIAL AND METHODS

Solution of BR were prepared daily by dissolving bilirubin powder (from Bovine gallstones, Sigma) in 0.10 M NaOH and adjusting the volume with KH_2PO_4/NaOH buffer to achieve a final pH of 7.8 ± 0.1 Solutions were kept in the dark at 10°C All experiments were made in a dark room using a red-light.

The adsorbents, cross-linked PVP (Aldrich) and cholestyramine (Dowex 1X2, Aldrich) were pretreated in the following manner The PVP was washed overnight with buffer (50°C) to remove soluble polymer Subsequently, both cholestyramine (CA) and PVP were washed overnight

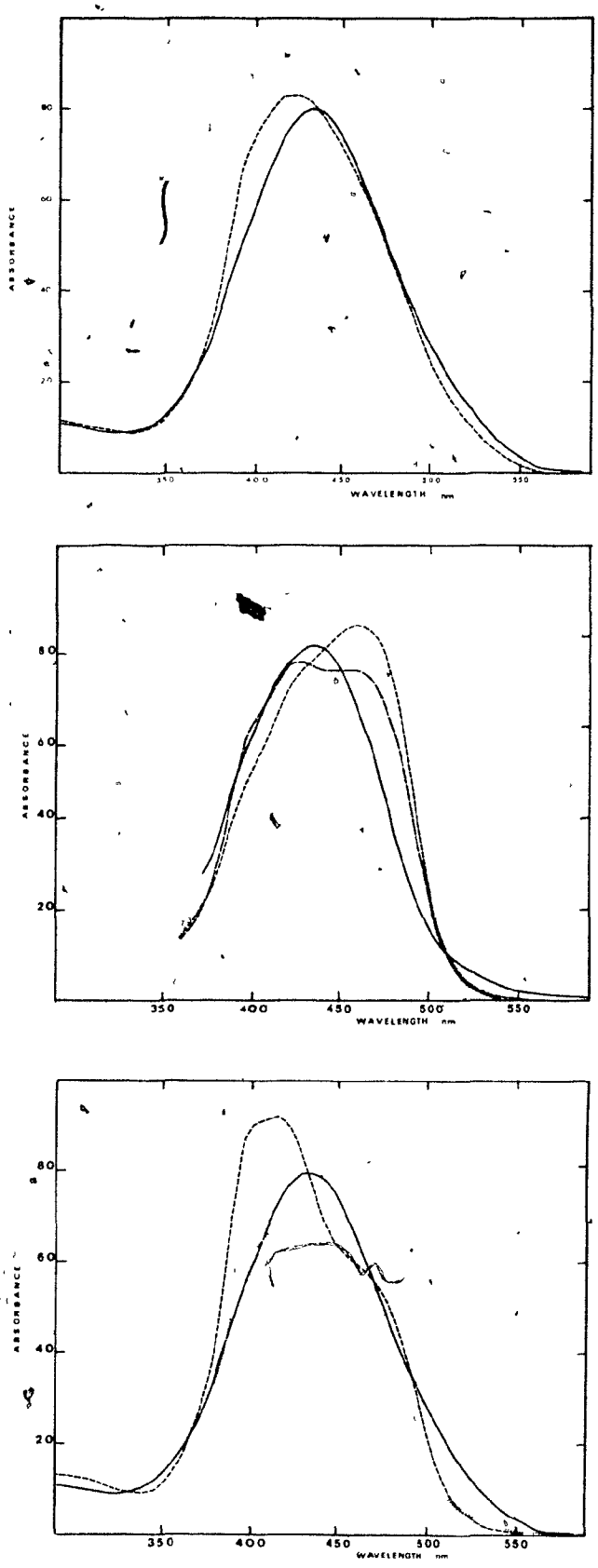


Fig 2 Effect of polyvinylpyrrolidone on the Bilirubin Spectrum
A ——— Bilirubin reference solution (10 mg/dl)
— — — Bilirubin to PVP ratio = 0 49
B ——— Bilirubin reference solution (1 mg/dl)
— — — Bilirubin to PVP ratio = 0 0046
— — — Bilirubin to PVP ratio = 0 0012
C ——— Bilirubin reference solution (10 mg/dl)
— — — Bilirubin to PVP ratio = 0.045, initial (BR)= 10 mg/dl
— — — Bilirubin to PVP ratio = 0,050, initial (BR) = 1 0 mg/dl

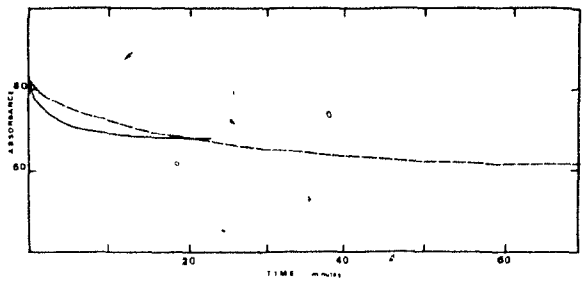
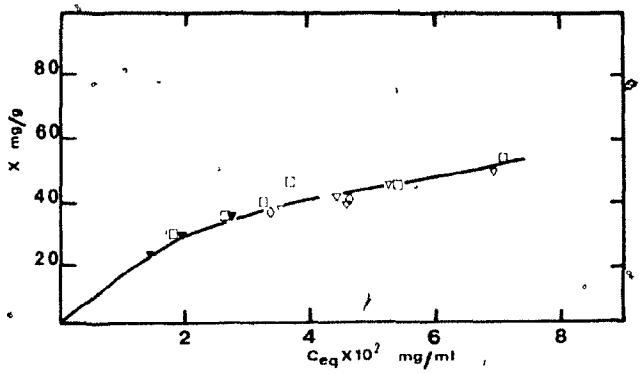


Fig. 3 Kinetics of Adsorption of Bilirubin at 10°C.
——— Polyvinylpyrrolidone
— — — Cholestyramine



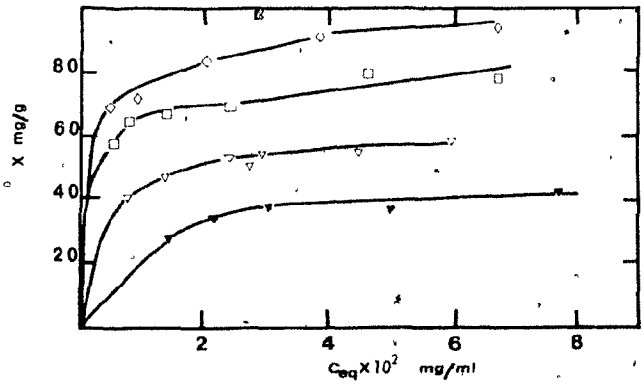


Fig 4 Isotherms for the adsorption of Bilirubin.
A Polyvinylpyrrolidone
B Cholestyramine
0°C, 10°C, 20°C, 25°C

with methanol, twice with 1.0M HCl (20 min), twice with 0.10M HCl (20 min), and twice with buffer (20 min). Finally, they were filtered, using a glass sintered filter, dried at 100°C under vacuum and the fraction with mesh size 100-140 was stored in a dessicator until use.

The adsorption studies were done in a specially designed apparatus, shown schematically in Fig. 1. To prevent formation of scratches, which encourage precipitation of BR, the glass stirrer was placed so that it did not touch the surface of the flask. In addition to the inlet neck for the stirrer the flask had two others necks, each of which was stoppered with a rubber septum to facilitate the purging of the flask with an inert gas and the withdrawal of solution during the experiment. The flask was coated with BR prior to use by stirring a BR solution (25 ml, 10 mg/dl) for 2 hrs. The solution was discarded and the flask rinsed with distilled water and dried.

The flask containing the BR solution (25 ml, 10.0 mg/dl) was placed in a thermostatted water bath and purged continuously with nitrogen. The mixture was stirred constantly. Adsorption was initiated by adding the appropriate weight of resin to the flask. Aliquots (1 ml) were withdrawn, at 15 min intervals for PVP and 30 min intervals for CA to determine the concentration of BR in the supernatant solution from its absorbance at 438 nm measured with a Beckmann model 25 double beam spectrophotometer using the buffer solution as the reference. Calibration experiments with BR solutions of known concentration yielded a linear Beer's law plot, in the concentration range of these studies, with a molar extinction coefficient of 4.44×10^4 l mole^{-1} cm^{-1}.

For studies of the kinetics of adsorption a continuous flow system was used. The solution was withdrawn from the adsorption flask, entering the bottom of 1 mm spectrophotometer flow cell, was withdrawn from the top of the cell by a piston pump and returned to the flask. Glass tubing was used throughout except at the connections to the cell, pump and flask. Corrections were made for the rate of loss of BR in the absence of asdsorbent.

RESULTS

Experiments were carried out to verify the previous report by (Ploussard et al. 1972) of a «red-shift» in the visible spectrum of BR upon addition of water-soluble, i.e., non-crosslinked PVP. When PVP (Matheson, Coleman and Bell, average molecular weight of 360,000) was added to aqueous BR solution, pH = 7.8, at a weight ratio of BR to PVP of 0.5 the absorbance maximum, λ_{max}, shifted from 435 to 420 nm, i.e., a «blue-shift rather than the reported «red-shift» was observed (Fig. 2). However, when the weight ratio of BR to PVP was decreased to 0.05 a shoulder appeared at 460 nm on the peak with λ_{max} at 420 nm. A further decrease in BR/PVP ratio to 0.005 resulted in a relative decrease in the absorbance at 420 nm and an increase at 460 to give two peaks. Finally at a BR/PVP ratio of 0.0012 the peak at 420 nm is seen

only as a shoulder on the main peak that appears at λ_{max} = 460 nm, corresponding to a «red-shift» of aqueous BR. This is in agreement with the report of Ploussard et al. (1972) since their studies were made at a BR/PVP ratio of 5×10^{-4}, i.e., under conditions where the peak at 420 is no longer observed.

It is noteworthy that the two spectra at a BR/PVP ratio of 5×10^{-3} (Fig. 2c) are essentially identical inspite of a 10 fold change in initial concentrations. This suggests that some equilibrium, or equilibria, exists between BR and PVP. Indeed, since there are two shifts in λ_{max} of BR, (435 → 420 and 435 → 460), depending on the relative amounts of BR and PVP, it is possible that two different complexes are formed. At high BR to PVP ratios BR may occupy mainly one binding site on the PVP, giving to the peak at 420 nm At the lower BR/PVP ratios it could occupy two binding sites, changing its conformation and giving rise to the peak at 460 nm. At the intermediate ratios both complexes would coexist.

The substantial effect of PVP on the BR spectrum suggested that adsorption studies were warranted. This study was begun with an investigation of the relative rates of adsorption onto the two different resins. The kinetic studies at 10.0°C showed that PVP adsorption is complete by 20 min while at least 60 min are required for CA (Fig. 3). When PVP is added to the BR solution a sharp decrease in the absorbance occurs and is followed by a levelling off at about 20 min The completion of adsorption is clearly defined. In contrast, the rate of decrease in absorbance for CA is less pronounced and extends over a longer period of time. Although the point at which adsorption is complete is difficult to assess it is clear that the capacity is greater for CA than for PVP.

On the basis of the rate studies adsorption isotherms were obtained as the amount of BR adsorbed per g of resin added at several temperatures (Fig 4). The amount of BR adsorbed, calculated from the decrease in BR concentration, was taken after 30 min for PVP and after 90 min for CA. The most striking features of these isotherms is the temperature independence of the adsorption onto PVP and the increase in the amount of BR adsorbed onto CA with increase in temperature.

Attempts were also made to desorb the BR. However, only 8% of the BR was desorbed from a PVP sample that had 55 mg of BR adsorbed/g of resin after stirring in buffer solution for 1 hr. This suggests that the adsorption process is in fact a chemisorption, or at least involves a relatively strong interaction, as is also suggested by the increase in BR adsorbed on CA with increase in temperature.

The adsorption isotherms were replotted according to the Langmuir equation

$$\frac{C}{x} = \frac{1}{ab} + \frac{C}{a}$$

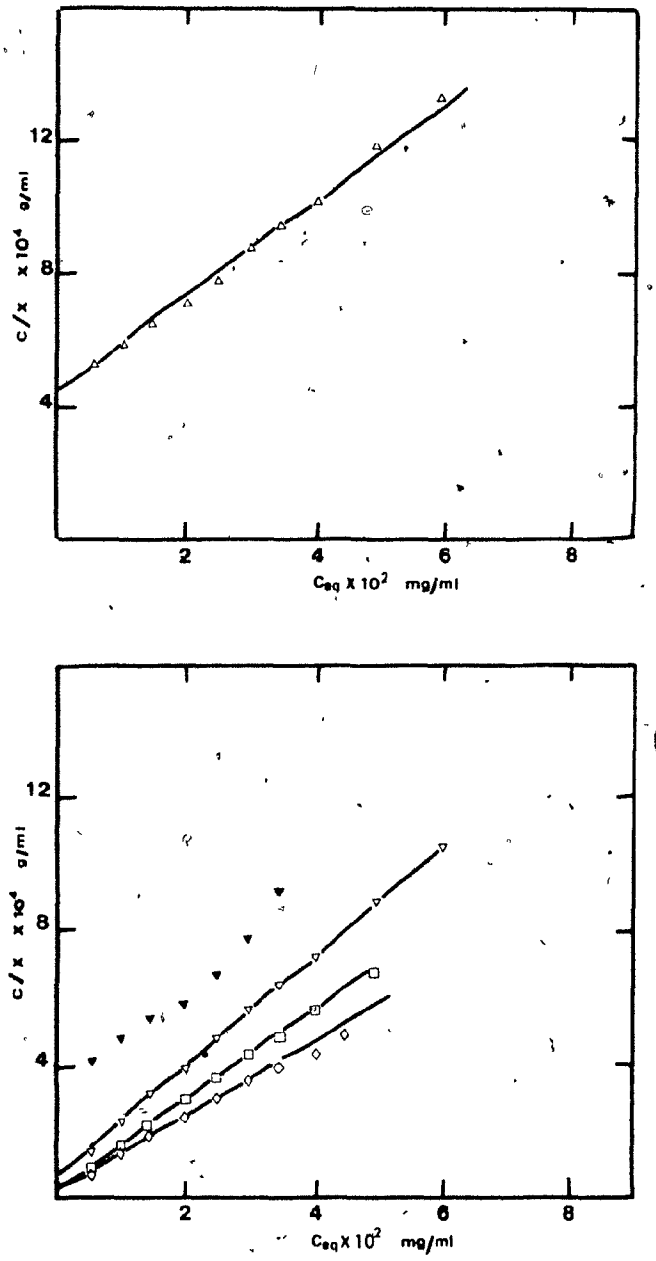


Fig 5 Langmuir Isotherms for the Adsorption of Bilirubin
A Polyvinylpyrrolidone
B Cholestyramine
▼ 0°C
▽ 10°C
□ 10°C
◇ 25°C

where x is the amount of BR adsorbed per g of adsorbent, C is the equilibrium concentration and a and b are constants. The linear relationship obtained by plotting C/x as a function of C (Fig. 5) shows that BR adsorption follows Langmuir behaviour although some discrepancy is evident for adsorption onto CA at 0°C. Solid-solute in-

TABLE I - ADSORPTION CHARACTERISTICS OF CHOLESTYRAMINE AND OF POLYVINYLPYRROLIDONE FOR BILIRUBIN FROM AQUEOUS BUFFER AT pH = 7.8

Resin	Temperature (°C)	Molecules of BR adsorbed: g of resin ($\times 10^{-19}$)	Resin repeat units: Molecules of BR adsorbed
PVP	0-40 0	7 9	67
CA	10 0	6 4	38
CA	20 0	7 9	30
CA	25 0	9 4	25

teractions are strong enough to compete with solid-solvent interactions in the first layer but not thereafter. The number of molecules in the monolayer bound per g of adsorbent, calculated from the Langmuir equation can be related to the number of binding sites if it is assumed that one BR molecule occupies on binding site (Table I) The number of binding sites are comparable for these two resins on a weight basis Taken on a repeating unit basis (ignoring cross-linking) PVP has one BR molecule for every 67 repeat units On the basis of the manufacturer's claim of 4×10^{-3} equivalents of quaternary ammonium ions per g of CA, it binds one BR molecule for every 25 ions (at 25°C). For CA the number of binding sites effective in adsorption apparently increases with increase in temperature.

DISCUSSION

In any adsorption process four major different steps can be involved

1 Adsorbate diffusion to the adsorbent surface;
2 Desorption of solvent molecules from the adsorbent surface (an endothermic process),
3 Desolvation of the adsorbate (an endothermic process),
4 Adsorption of the adsorbate onto the adsorbent surface (an exothermic process)

In these experiments the solution were always stirred Thus, diffusion is not expected to have a significant effect on the rate of the overall process The increase in BR adsorbed onto CA with increasing temperature indicates that the effect of the endothermic processes (2 and 3) is greater than that of the exothermic process

However, it could also mean that a chemical reaction requiring an energy of activation or which is endothermic is involved in the adsorption process

In the case of adsorption onto PVP, the magnitude of the endothermic and exothermic processes are evidently identical, i e , the adsorption process appears to be athermal. Previous studies (Clifford 1974; Olsson and Samuelson 1974) strongly indicate that binding to PVP is generally the result of a H-bond formation, presumable to the C=O function on the PVP.

It is also of interest that the number of binding sites on CA increases with an increase in temperature. It is possible that the conformation of adsorbed BR changes with change in temperature thus altering the binding capacity of the resins

If this were the case, however, then similar behaviour would be expected for both resins. Hence, a change in the nature of the BR — resin interaction is implied. It seems possible that the reactivity of the quaternary ammine of the CA is temperature dependent Certainly, a previous report clearly shows that the adsorption of BR onto the CL^- form of the resin is quite different from the adsorption onto the HCO_3^- form (Sideman et al 1982)

However, it is also possible that the physical accessibility of the resin surface is quite different for these two resins. In these experiments, adsorption was initiated by adding dry resin to the BR solution It is possible that the wetting and swelling characteristics of the styrene base resin (CA) would be poorer than those of PVP which in its uncross-linked form is water soluble Further experiments are planned to test this matter

Although CA will bind more BR than PVP, adsorption onto the latter is favoured kinetically Adsorption of BR onto PVP reaches saturation in a few minutes At short times PVP adsorbs more BR than CA This could be an important factor if the resins were used in a column for perfusion.

During perfusion the contact time between the BR solution and the adsorbent would be relatively brief A resin adsorbing more rapidly could then well be more efficient than one with a higher capacity but slower kinetics This higher affinity also means that if other compounds were present in the plasma, .eg., bile acids, to compete for the binding sites, PVP might well be more efficient than CA

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Polymer resins with amino acid containing pendants for sorption of bilirubin. I. Comparison of Merrifield and polyamide resins

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KEY WORDS

Bilirubin
Merrifield resin
Polyamide resin
Arginine
Amino acids
Sorption

ABSTRACT

Merrifield resins with various amino acid containing pendants and a water swellable polyamide resin with the peptide alanine-alanine-alanine-arginine as the pendant group have been prepared by solid phase peptide synthesis. Merrifield resins with either arginine or lysine pendants are capable of sorbing bilirubin from aqueous solution (pH = 7.8) but those with other amino acid pendants gave no indication of sorption. The polyamide-arginine resin showed, on a functional group basis, a higher capacity for bilirubin than does cholestyramine. It is proposed that the formation of salt linkages causes a strong interaction of bilirubin with arginine and lysine.

INTRODUCTION

Hyperbilirubinemia, the accumulation of the bile pigment bilirubin (BR) in the blood, can cause serious tissue damage and may even result in death (1). Currently hyperbilirubinemia is being treated with considerable success by phototherapy, i.e., by illuminating the patient with visible light for periods of hours or days (2). However, since this treatment is not without side-effects (3), e.g., «bronze baby syndrome» (5), other methods of treatment are still being sought.

Hemoperfusion through a suitable sorbent is a possible alternative method for removing BR from plasma (4). Activated charcoal (6, 7), charged resins such as Dowex 1X (cholestyramine) (8, 9), and uncharged resins such as Amberlite XAD (8-10) have been used with some success in laboratory tests. Immobilized human serum albumin (HSA) is a somewhat more selective adsorbent, that is also biocompatible (11, 12), but its usefulness is limited severely by the fact that one molecule of HSA, with a molecular weight of 66,000 binds only two molecules of BR. Thus a large amount of HSA is required to remove significant amounts of BR.

Although the chemical structure of HSA has been fully established (13), the location and structure of the binding site for BR on HSA have not yet been clearly identified in spite of extensive studies (1). It seems likely, however, that the binding sites for BR constitute only small portions of the HSA molecule. Jacobsen showed that when the lysine, arginine, tyrosine, and histidine residues in HSA are modified chemically the binding constant for BR is markedly reduced (14, 15). This led him to postulate that these four amino acids must be at, or near, the binding sites. It is also possible that the binding sites are not preformed, but rather that the flexibility of HSA permits them to be formed by the folding of the HSA around the BR molecule (1).

These observations suggest that it should be possible to make a sorbent of high capacity for BR by attaching, as pendants, appropriate amino acid sequences, corresponding to the active sites on HSA, to a polymer backbone. This paper describes the preparation and preliminary testing of sorbent produced by attaching pendants consisting of a single amino acid unit to Merrifield resin using well known solid phase peptide synthesis techniques. The synthesis and adsorption behaviour of a sorbent composed of a water-swellable polyamide resin as the polymer backbone and arginine containing pendant groups is also presented.

EXPERIMENTAL

Preparation of resins

1. Merrifield resins. A series of sorbents was prepared by chemically attaching single amino acid residues to the Merrifield resin (a chloromethylated or hydroxymethylated polystyrene divinylbenzene resin) (16). For this synthesis the amino acids were protected at the α amino

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location and at the reactive side chains using suitable protecting groups' (bis-Bos for lysine, histidine and tyrosine, and mono-Boc for arginine) to ensure that the amino acid would be coupled to the resin at the carboxylic acid site The protected amino acids were attached to either the chloromethylated resin (Chemalog), in the case of lysine, arginine and tyrosine, or the hydroxymethylated resin (Biochem), in the case of histidine, using standard solid phase peptide synthesis techniques (17) Merrifield resins with proline, glycine, phenylalanine and aspargine pendants were purchased as the N-t-Boc amino acid resin ester The attached amino acids were deprotected by stirring the resin in trifluoroacetic acid (TFA) (40% in CH_2Cl_2, 40 min) and neutralized with triethylamine (10% in CH_2Cl_2, 5 min)

2 Polyamide resin A water swellable polyamide resin (Chemalog), which is a copolymer of dimethylacrylamide and N-acryl-1,6-diaminohexane reticulated with bisacrylyldiaminoethane was used as the polymer backbone (18) onto which the peptide Ala-Ala-Ala-Arg (where Ala is alanine and Arg is arginine) was synthesized. The Ala COOH group was attached directly to the resin by an amide bond to the resin NH_2 groups The trialanine portion was included to extend the Arg unit away from the polymer backone to make it more accessible for sorption

The complete sequence for the synthesis of the pendants on the polyamide resin is shown on Figure 1. The synthesis of the peptide pendants was achieved on a Vega model 250 automatic peptide synthesized by the symmetrical anhydride method (19) according to the schedule shown in Table I The resin was first swollen in CH_2Cl_2 and the hydrochloride salt was displaced with 40% diisopropylethylamine (DEA) in CH_2Cl_2 30 min Terbutyloxycarbonyl groups were used to protect the amino function of both alanine and arginine while nitro groups were used to protect the guanido function of arginine Both amino acids were obtained fully protected from Chemalog. Upon completion of the coupling step, as indicated by the ninhydrin test (20), the amino group was deprotected with 40% TFA and neutralized with 5% DEA After the last amino acid in the sequence had been introduced, the peptide was completely deprotected (α amino and R groups) by treatment with anhydrous HF at 0°C for one hour (21)

Adsorption studies

The techniques used in this study for studies of the adsorption of BR from buffer solution by the various resins were essentially as described previously (22). Solutions of BR were prepared daily by dissolving BR powder (from bovine gallstones, Sigma) in 0.10 M NaOH and adjusting to volume with KH_2PO_4/NaOH buffer to achieve

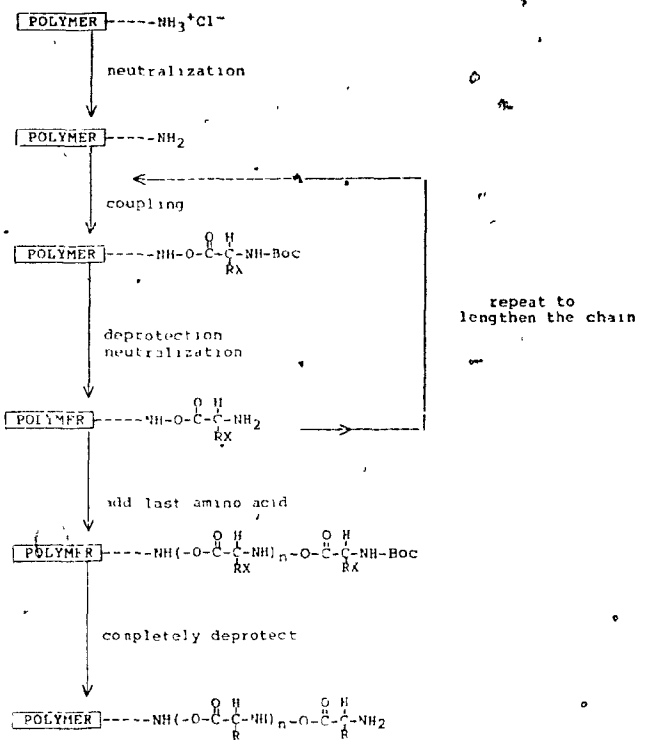

Fig 1 Synthesis scheme of the amino acid pendants onto the polyamide polymer backbone

TABLE I - PROGRAM FOR AUTOMATED SOLID PHASE PEPTIDE SYNTHESIS

Step	Operation	Reagent	Number of repetition	Time (minutes)
1	coupling	symmetrical anhydride	1	until completion
2	washing	CH_2Cl_2	4	2
3	deprotection	40% TFA in CH_2Cl_2	1	20
4	washing	CH_2Cl_2	2	0 75
5	washing	5% DEA in CH_2Cl_2	2	0 75
6	neutralization	5% DEA in CH_2Cl_2	1	5
7	washing	CH_2Cl_2	2	2
8	washing	DMF	2	2
9	washing	CH_2Cl_2	2	2
10	next coupling back to step 1			

TABLE II - AMINO ACID CONTAINING MERRIFIELD RESINS FOR SORPTION OF BR

Amino acid	Substitution mmol/g	R group status at a pH of 7 8	Colour
No acid (resin only)	—	no charge	white
Lysine	0 38	positive charge	yellow
Arginine	0 18	positive charge	yellow
Glycine	0 88	no charge	white
Histidine	0 25	no charge	white
Tyrosine	0 48	no charge	white
Asparagine	0 40	no charge	white
Phenylalanine	0 50	no charge	white
Proline a)	0 12	no charge	white

a) Proline is the only amino acid with the α NH_2 linked to the R group

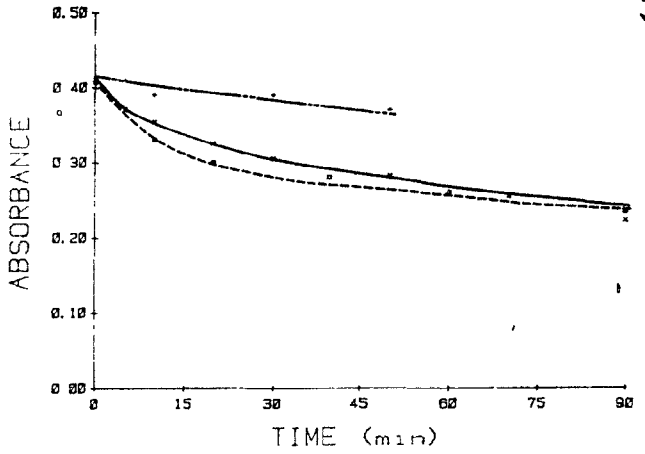

Fig 2 Kinetics of adsorption of bilirubin at 0°C
x Polyamide-Ala_3Arg_1
□ Cholestyramine
+ No adsorbent

a final pH of 7 8 ± 0 1 and a concentration of 10 mg/dl. This stock solution was diluted with KH_2PO_4/NaOH buffer (pH 7.8) as required Solutions were kept in the dark at 0°C and all experiments were made in a dark room using a red light

In a typical adsorption experiment with the Merrifield resins, the resin (≅60 mg) was added to 25 ml of stock BR solution in a stoppered flask. The suspended resin was mixed by inversion and left to stand for 30 minutes. The resin was filtered, washed first with buffer and then with CH_2Cl_2 Presence or absence of BR was judged from the colour of the resin.

For the study of the adsorption of BR by the polyamide resin, 25 ml of BR solution at an appropriate concentration was added to the resin contained in the adsorption flask The flask was placed in an ice-water bath and purged continuously with nitrogen. For the study of the kinetics of adsorption, aliquots (0.5 ml) were withdrawn at desired times. To obtain the adsorption isotherms only one aliquot was withdrawn at 60 minutes

The concentration of BR was determined from the absorbance at 438 nm measured with a Beckmann model 25 double beam spectrophotometer using the buffer solution as the reference Calibration studies with BR solutions of known concentration yielded a linear Beer's law plot, in the concentration range of these studies, with a molar extinction coefficient of 4.44×10^4 l mol^{-1} cm^{-1}

RESULTS

1 Merrifield resins: For none of the Merrifield resins to which a single amino acid residue had been attached was there sufficient adsorption to cause a measurable decrease in the concentration of BR in the adsorbate solution. However, since BR is highly coloured qualitative observations, based on the colour of the resin after washing with CH_2Cl_2, were possible (Table II). A yellow colour was taken as an indication that sorption of BR had occurred while a white resin indicated a lack of or a weaker interaction Two resins, one with lysine and the other with arginine pendants, showed colour with the latter being more intense It is of interest to note that lysine and arginine are two of the four amino acids proposed by Jacobsen to be located at the binding sites of HSA for BR (14, 15).

These qualitative observations indicate that the presence of suitable amino acid pendants on the resin can cause strong interaction with BR. Both the lysine and the arginine containing resin retained a significant amount of yellow colouration even after washing with CH_2Cl_2. The failure to adsorb larger quantities of BR seemed to result from poor contact with the adsorbate solution. None of the resins listed in Table II were wetted by water and, hence, also did not swell in the adsorbate solution. Attempts to achieve better wettability by increasing the length of the amino acid pendants were unsuccessful

2 Polyamide resin. The polyamide resin with the Ala-Ala-Ala-Arg pendants attached at the functional sites showed a greatly increased capability to adsorb BR as compared to the similarly substituted Merrifield resins. Figure 2 shows that the rate of adsorption of BR onto the polyamide-Ala-Ala-Ala-Arg resin is similar to that of cholestyramine (22). For further studies to derive the

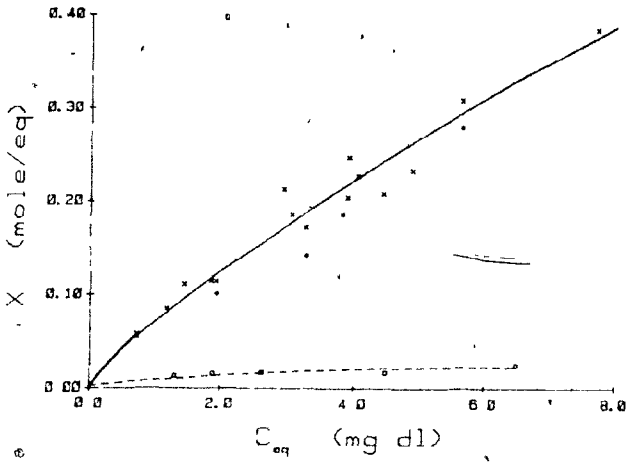

Fig 3 Isotherms for the adsorption of bilirubin
x Polyamide Ala_3Arg_1 at 0°C
* Polyamide Ala_3Arg_1 at room temperature
□ Cholestyramine at 0°C

isotherms, adsorption was considered to be complete after 60 minutes although, due to continuous degradation of BR caused by environmental factors, the point of completion cannot be defined unambiguously An adsorption isotherm, showing the amount of BR adsorbed (in mg) at 0°C per gram of resin added at various final concentrations of BR is shown in Figure 3 The amount of BR adsorbed is apparently not altered significantly by increasing the temperature to room temperature.

An attempt to desorb BR from the resin into buffer solution resulted in only 14% of the adsorbed BR returning back into solution after one hour. Hence, at the resulting equilibrium concentration of the desorbed solution, the amount of BR that remained adsorbed per gram of resin does not fall back onto the isotherm This suggests that the BR molecule is tightly adsorbed to the resin and that reversibility is achieved only with difficulty, if at all Nonetheless, a linear relationship was obtained when the adsorption isotherm was replotted according to the Langmuir equation

$$x = \frac{1}{ab} + \frac{1}{c}$$

where x is the amount of BR adsorbed per gram of adsorbent, c is the equilibrium concentration and a and b are constants[4]

This indicates that the adsorption follows a Langmuir behaviour (monolayer adsorption) The number of molecules bound in the monolayer per gram of adsorbent, calculated from the Langmuir equation, can be related to the number of binding sites if it is assumed that one molecule of BR occupies one binding site. The polyamide-Ala_3Arg_1 resin adsorbed 1.1×10^{20} molecules of BR in the monolayer

DISCUSSION

The failure of the Merrifield-glycine resin to adsorb detectable amounts of BR suggests that BR does not interact significantly with the α amino group. Only lysine and arginine containing resins are active and, of the amino acids tested, these are the only two that have a positively charged R group at a pH of 7 8 Furthermore, the resin with arginine appeared to be a more potent adsorbent. This might be expected since arginine is more basic than lysine (pK_R(Arg) = 12 48, pK_R(Lys) = 10.55).

Bilirubin is known to exist as a dianion in aqueous solution at pH of 7.8 (1). It is possible, therefore, that a salt linkage between the carboxylic acid groups of BR and the positively charged R group of the amino acids is responsible for the observed interaction. The other amino acid pendants are not ionized at a pH of 7 8 and, consequently, are unable to form salt linkages.

It is of particular interest that the resins containing the amino acids tyrosine and histidine, previously postulated to be at or near the binding sites for BR on HSA (14, 15), did not show a detectable interaction with BR Although it is still possible that these amino acids may be near the binding sites on HSA for BR, their role in the binding would seem to be of a secondary nature.

The observed differences in interaction that result when different amino acids form the side chains provide strong evidence that it should be possible to prepare a sorbent that has high capacity and perhaps even specificity, for BR by appropriate selection of the backbone of the polymer and of the amino acid or peptide pendants The adsorption behaviour of the polyamide-Ala-Ala-Ala-Arg resin offers additional evidence On a weight basis this resin has an adsorption capacity similar to that of cholestyramine at BR concentration below 10 mg/dl (22). However, the polyamide resin has a lower substitution, 0 20 meq/g rather than 3.5 meq/g for cholestyramine. If the arginine substitution were increased to the same level as that of cholestyramine it would adsorb approximately 20 times as much BR, provided that the BR molecules did not interfere with each other at this higher density. Moreover, the polyamide-Ala_3Arg_1 resin does not reach saturation as rapidly as does cholestyramine. For a complete monolayer as determined from the Langmuir plot, the arginine resin binds one molecule of BR for every active site while cholestyramine has one in every 35. Thus, on a repeating unit basis, the polyamide resin has a greater capacity. Furthermore, the adsorption of BR onto the polyamide resin does not show any temperature dependence

The adsorption kinetics of the polyamide-Ala_3Arg_1 resin are similar to those of cholestyramine which suggest that the same type of interaction is involved in both

cases The slow kinetics and the limited desorption from the polyamide-Ala_3-Arg_1 indicate that the adsorption is due to chemisorption, with formation of chemical bonds, rather than physisorption with only weaker physical interactions Since both sorbents have charged side chains, salt linkages can be formed and are likely for the binding of BR to proteins

A formation of specific interactions between biliprotein and histidine immobilized on Sepharose gel has been reported by Rabier et al (23). These interactions occurred at a pH of 5 which is above the isoelectric point of the protein and below that for histidine. These biliproteins also formed an interaction with lysine and arginine, the other positively charged amino acids Although a proton exchange or charge transfer mechanism was proposed, salt linkages seem equally possible.

On the other hand, Van der Eyk et al (24) reported that although BR does not interact with protonated polyhistidine (pK = 9 4) it can do so with the unprotonated polyhistamine (pK = 5 2) Hydrogen bonding between the weakly acidic pyrrolic nitrogen and the weakly basic protons of the BR lactam rings, rather than salt linkages, was proposed However, if such interactions occur to a significant extent, an interaction would also be expected between BR and the amino acids capable of hydrogen bonding, e.g histidine, proline and asparagine. This study indicates that such interaction apparently does not occur to a degree sufficient to cause sorption of BR by these amino acids when they are present as pendants on a Merrifield backbone, and that salt linkages rather than H bonding are responsible for the binding of BR to proteins

A detailed study of the characteristics of the adsorption of BR by other amino acid pendants on the polyamide resin is underway Studies are also being made to determine the effect of increasing the number of active amino acid units in each side chain and to investigate the possibility of producing pendants that mimic the active sites for BR on HSA

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Polymer resins with amino acid containing pendants for sorption of bilirubin. II. Polyamide resins with various basic amino acids.

by

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KEYWORDS

ADSORPTION

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AMINO ACID PENDANTS

ABSTRACT

Short peptides, three to eight amino acids in length, containing various combinations of alanine, arginine, lysine, histidine and tyrosine have been synthesized onto water-swellable polyamide resin by the solid phase peptide synthesis method. The amount of bilirubin adsorbed from aqueous buffer solution (pH = 7.8) by these resins increases with increasing basicity of the amino acids in the pendants. As the number of basic amino acids on the pendant is increased from one to five a 4.7 fold enhancement in the adsorption capacity is seen for arginine while a 9.3 fold enhancement is obtained for lysine. A corresponding increase in length for the non-basic histidine results in a 6 fold enhancement. With alanine the adsorption capacity is uneffected by an increase in pendant length.

INTRODUCTION

Immobilized serum albumin is an effective sorbent for the removal of bilirubin by hemoperfusion (1,2). However, each albumin molecule which has a molecular weight of 66,000, binds only two molecules of bilirubin tightly (3). Thus, a large amount of sorbent is required for removal of a significant amount of bilirubin. It seems most probable that only a small portion of the albumin molecule is active in binding bilirubin. A sorbent with a higher capacity should result from the immobilization of the active part only of the human serum albumin, e.g., by grafting onto a suitable polymer backbone an amino acid sequence that would mimic the binding site.

A serious difficulty in preparing such a polymer arises from the fact that, although the chemical structure of albumin is well established (4), the exact nature of its binding sites for bilirubin has not yet been determined. Attempts have been made to identify it by cleaving bovine serum albumin (BSA) or human serum albumin (HSA) and testing the binding characteristics of the fragments. For example, Reed et. al. (5) reported that three fragments all containing the residues 186 to 235 retain an affinity for bilirubin comparable to that of native HSA. Geisow and Beaven (6) showed that the CD spectrum of fragments containing residues 1-386 and 49-307 bound to bilirubin is similar to that of bilirubin-HSA as reported by Blauer and Wagniere (7,8). Chemical modification of specific amino acids of HSA led Jacobsen to conclude that the four amino acids tyrosine, histidine, arginine and lysine are at, or near, the binding site

(9,10). By fragmenting C^{14}-labelled bilirubin bound covalently to HSA he later showed that the residues 240-258 are involved in the binding (11). Since only one lysine, lysine 240, is located in that sequence it was assigned to the binding site.

A previous report from this laboratory compares the adsorption behaviour of bilirubin by various synthetically prepared sorbents with pendants consisting of a single amino acid on a Merrifield resin to that of a water-swellable polyamide resin with an arginine-alanine-alanine-alanine pendant (12). Although the adsorption capacity of the Merrifield resin in aqueous buffer is severely limited by its hydrophobic nature, the results clearly indicated a strong interaction of bilirubin with resins containing either a lysine or an arginine pendant group. By comparison, the water-swellable polyamide resin with the arginine containing pendants adsorbed bilirubin efficiently with a complete monolayer capacity 35 times greater than that of the commonly used resin cholestyramine (Dowex 1X2) (15).

The large capacity of this polyamide Arg_1Ala_3 resin suggests that the pendant groups do not necessarily have to mimic precisely the binding sites for bilirubin on HSA to create a favourable environment for interaction. This paper presents a systematic study of the effect of changes in the amino acid composition of the pendant, both type and number, on the adsorption behaviour by bilirubin on the resins.

EXPERIMENTAL

Preparation of the resins: A water swellable polyamide resin (Chemalog), which is a copolymer of dimethylacrylamide and N-acryl-1,6-diaminohexane reticulated with bisacryldiaminoethane, was used as the polymer backbone (16) onto which the peptide pendants were synthesised. The first alanine of the pendant is attached directly to the resin NH_2 groups via the COOH group thus forming an amide bond. A trialanine "spacer" portion was always included next to the polymer backbone to extend the active unit and so make it more accessible for sorption.

Grafting was achieved through a modification of the solid phase peptide synthesis technique as described previously (12). Completion of the coupling and deprotection steps was checked by the ninhydrin method which can detect less than 1% of free NH_2 (13) so that 99% completion of each coupling step can be achieved. Hence, for the longest pendant that consists of 8 amino acids 93.2% of the pendants should have the complete sequence (14). Fully protected amino acids, N-α-t-BOC-L-alanine, N-α-t-BOC-2-bromo-CBZ-L-tyrosine, N-α-t-BOC-N-ω-nitro-L-arginine, N-α-t-BOC-N-ϵ-2,4-dichloro-CBZ-L-lysine and N-α-t-BOC-N-im-tosyl-L-histidine were obtained from Chemalog. The sorbents were synthesized in different batches. To obtain the various pendant lengths in a given batch, part of the resin was withdrawn when a given sequence length was complete. The synthesis was resumed with the remaining sample.

Adsorption studies: Solutions of bilirubin were prepared daily by dissolving the powder (from bovine gallstones, Sigma) in 0.010 M NaOH and adjusting to volume with KH_2PO_4 buffer to

achieve a final pH of 7.8 ±0.1 and a concentration of 10.0 mg/dl. This stock solution was diluted with KH_2PO_4/NaOH buffer (pH = 7.8). Solutions were kept in the dark at 0°C and all experiments were made in a dark room using a red light.

The adsorption was initiated by adding 25 ml of bilirubin solution, at an appropriate concentration, to approximately 10 mg of polyamide resin contained in the adsorption flask described previously (15). The flask was placed in an ice water bath and purged continuously with nitrogen. An aliquot was taken for analysis at 60 minutes. Duplicate experiments were reproducible to ± 5%.

The concentration of bilirubin was determined from the absorbance at 438 nm measured with a Beckmann model 25 double beam spectrophotometer using the buffer solution as the reference. Calibration studies with bilirubin solutions of known concentration yielded a linear Beer's law plot, in the concentration range of these studies, with a molar extinction coefficient of 4.44×10^{4} l mol^{-1} cm^{-1}.

RESULTS AND DISCUSSION

As mentioned previously, all of the sorbent resins in this study had the Ala_3-polyamide structure as the starting point for further resins. To establish a reference point for comparison of sorption characteristics isotherms were determined for the adsorption of bilirubin by the unsubstituted polyamide resin and the Ala_3-polyamide resin at 0°C. Figure 1 shows the amount of bilirubin adsorbed, per equivalent of pendants, plotted as a function of the final equilibrium concentration. Also included

on this figure is the previously reported isotherm (15), determined under similar conditions, for the adsorption of bilirubin by cholestyramine. It is of interest to note that on an equivalent of active sites basis both the polyamide and the Ala_3-polyamide resins have the ability to adsorb bilirubin in amounts that significantly exceed that adsorbed by cholestyramine. Because of the higher substitution of cholestyramine it has a greater capacity expressed on a per gram of sorbent basis. The ability of the Ala_3-polyamide to adsorb bilirubin is reduced to approximately one half by acetylation of the α-amino groups of the Ala_3 pendants.

A series of experiments was made to determine the relative adsorption ability of the polyamide resin to which were attached pendants with the four amino acids proposed by Jacobsen to be at or near the binding site for bilirubin on human serum albumin. Figures 2 to 5 show isotherms for resins with the structure X_nAla_3-polyamide, X being one of the four amino acids and n varying from 1 to 5. It is apparent from these isotherms that for a given n the adsorption capacity of these resins for bilirubin, expressed on a moles of bilirubin per equivalent of pendant basis, follows the order arginine > lysine > histidine > tyrosine, i.e., decreasing with decreasing basicity. At pH = 7.8 it is expected that both arginine and lysine pendants would be positively charged while histidine and tyrosine would be uncharged. This is based on the assumption that the pK_a of the amino acids in the pendants are similar to those of free amino acids, as is known to be the case for small peptides in solution (17).

Doubling the number of active amino acids in the pendant generally results in an increased capacity for bilirubin when X is a charged amino acid with a basic pK_a, such as arginine or lysine (Figs. 4 and 5), but a smaller increase is obtained when X consists of the pendants with uncharged tyrosine and histidine (Figs. 2 and 3). This suggests that charge density is an important factor in the adsorption of bilirubin by these resins. A confirmation of the importance of charge density is demonstrated by experiments involving resins with pendants containing an even higher number of arginine, lysine or histidine units in the pendants (Figs. 3 to 5). In the case of arginine, increasing the number of charged amino acids from two to three has no apparent effect on the adsorption isotherm but a further increase to five enhances the adsorption capacity by a factor of 4.7 as compared to that of the resin with pendants containing only one amino acid. For lysine the adsorption is enhanced by a factor of 9.3 when the pendant length is increased from one to five while for histidine the enhancement is about 6. A summary of the isotherms, based on the amount of bilirubin adsorbed at high equilibrium concentration where the change in adsorption with concentration is small, is given in Table I.

It is of interest to note that although the resin with the Arg_2Ala_3 pendants has a greater capacity for bilirubin per equivalent than does the Lys_2Ala_3 containing resin, the capacity of the Arg_5Ala_3 resin is somewhat less than that of the Lys_5Ala_3 resin. Apparently there is an optimum in charge density so that a further increase in basicity is not reflected in a larger adsorption capacity. Rather surprisingly, the resins with

Arg_2Ala_3 and Arg_3Ala_3 have approximately an equal capacity for bilirubin which suggests that factors other than charge density, perhaps conformation of the peptide chain, also play a significant role in the adsorption process. A further indication that conformation may be of importance is seen in the studies of the temperature dependence of the adsorption of the arginine series of resins. Of the resins with pendants consiting of one. two or five arginines only the last showed a temperature dependence, with increased capacity with increased in temperature (Fig. 4). It is quite probable that this is a result of a change in conformation of the longer peptide chain with increasing temperature.

The effect of the length of the pendants as opposed to its charge density was tested using arginine containing resins with extra alanine spacers either at the beginning of the chain, i.e., before the first arginine or between arginines, (Fig. 6). Each of these resins can be related to another resin with either the same number of amino acids or with the same number of arginines in the pendant. The 'Arg_1Ala_4' behaves like Arg_1Ala_3 which has the same number of arginines rather than like Arg_2Ala_3 which has the same number of amino acids in the pendants. Similarly $Arg_1Ala_3Arg_1Ala_3$ pendants behave like Arg_2Ala_3 and not like Arg_5Ala_3. The Ala_3 spacer separating the arginines apparently has minimal effect on the adsorption capacity that seems to be more dependent on the number of arginines in the pendants than on the length of the pendants.

The resin Arg_2Ala_3 was also tested with the two guanido groups still, protected by nitro groups but with the α amino

groups free. The adsorption capacity of this resin is reduced to one third of that of the Arg_2Ala_3 resin but it remains slightly more active than Arg_1Ala_3 resin (Fig. 7). This behaviour, like that if acetylated Ala_3, which retained a capacity one half that of Ala_3 (Fig. 1), gives strong evidence that the adsorption of bilirubin is not due solely to the interaction with the R group on the amino acid pendants. As shown by the adsorption behaviour of these "protected" resins, the α amino group is also active. This interaction was not detected with the Merrifield- glycine resins (12), possibly because of their lack of water swellability. The fact that the Merrifield-arginine and Merrifield-lysine resins adsorbed bilirubin shows that, as might be expected, the interactions with the charged R groups are more powerful.

SUMMARY

The charge density contributed by the R groups of the amino acids present in the pendants is a major contributing factor in the adsorption of bilirubin by the substituted polyamide resins. However, some adsorption also occurs at the α amino groups and even at the unsubstituted $-NH_2$ groups of the resin, both of which are charged at a pH of 7.8. This suggests that electrostatic interactions are the most important consideration for the strong adsorption of bilirubin. Effects due to other considerations, such as the conformation of the peptide chains, cannot be ruled out. A detailed study to determine the binding constants of the resins with the higher adsorption capacity is now under way.

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Table I

Relative Adsorption Capacities for Bilirubin by Substituted Polyamide Resins

amino acid	Number of amino acids attached to the Ala_3			
	1	2	3	5
arginine	3	10	10	14
lysine	1.5	4	-	14
histidine	1	2.5	-	6
tyrosine	1	2.5	-	-

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CAPTIONS

Figure 1 Adsorption of bilirubin onto the reference polyamide resins and cholestyramine (X = moles of bilirubin bound per equivalent of functional group).

□ Non substituted resin
△ Ala_3
○ Ala_3 acetylated
■ Cholestyramine

Figure 2 Adsorption of bilirubin onto the tyrosine containing polyamide resins (X = moles of bilirubin bound per equivalent of functional group).

---- Ala_3
△ Tyr_1Ala_3
○ Tyr_2Ala_3

Figure 3 Adsorption of bilirubin onto the histidine containing polyamide resins (X = moles of bilirubin bound per equivalent of functional group).

---- Ala_3
△ His_1Ala_3
○ His_2Ala_3
□ His_5Ala_3

Figure 4 Adsorption of bilirubin onto the arginine containing polyamide resins (X = moles of bilirubin bound per equivalent of functional group).

---- Ala_3
△ Arg_1Ala_3
▲ Arg_1Ala_3 20^oC
○ Arg_2Ala_3
▽ Arg_3Ala_3
□ Arg_5Ala_3
■ Arg_5Ala_3 20^oC

Figure 5 Adsorption of bilirubin onto the lysine containing polyamide resins (X = moles of bilirubin bound per equivalent of functional group).

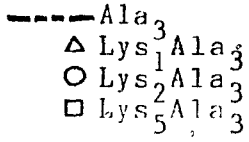

Figure 6 Effect of alanine spacers onto the adsorption of bilirubin by substituted polyamide resins (X = moles of bilirubin bound per equivalent of functional group).

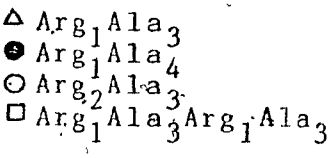

Figure 7 Adsorption of bilirubin onto polyamide resin with "protected" arginine containing pendants (X = moles of bilirubin bound per equivalent of functional group).

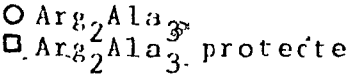

Fig 1

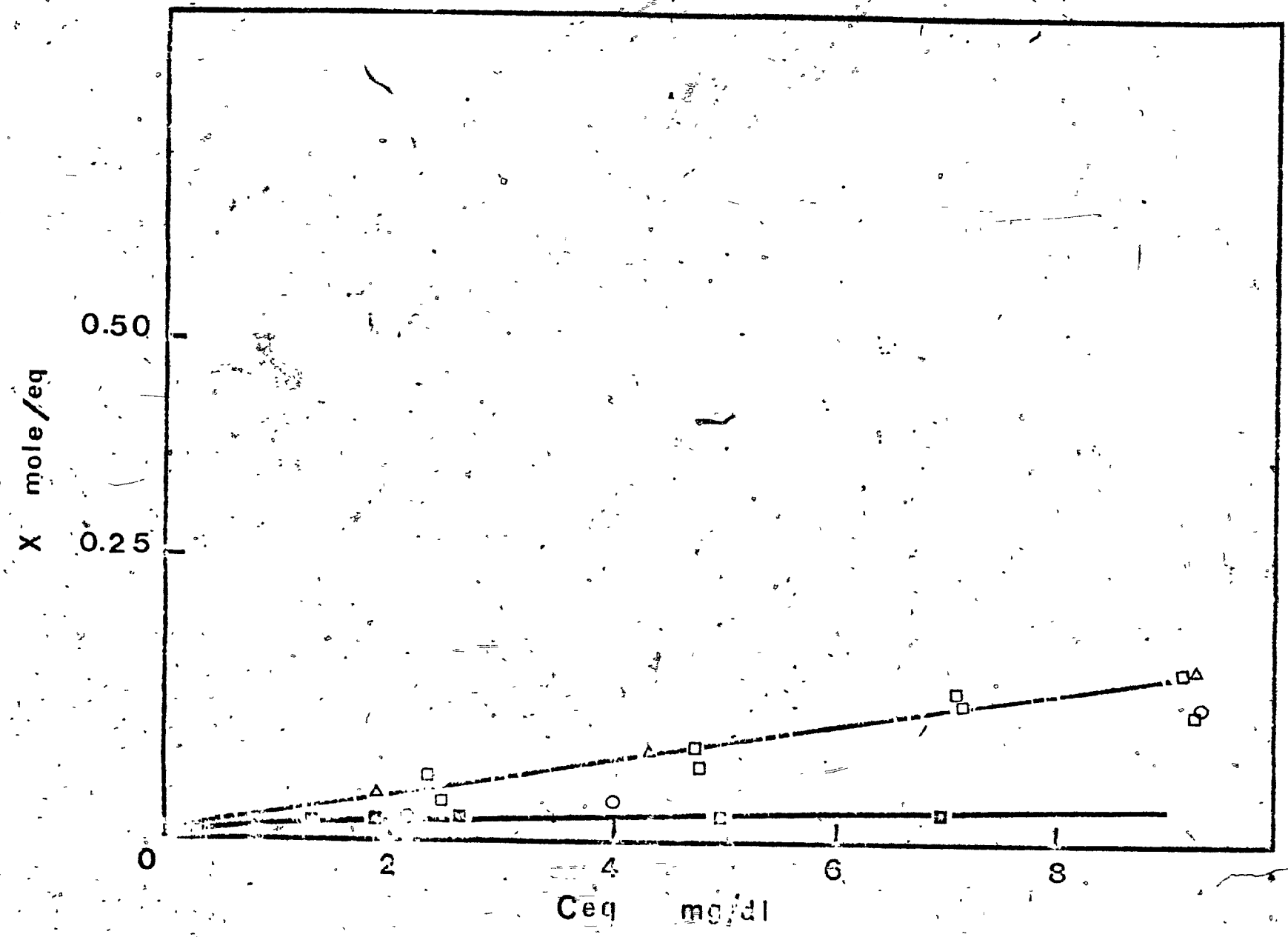

Fig 2

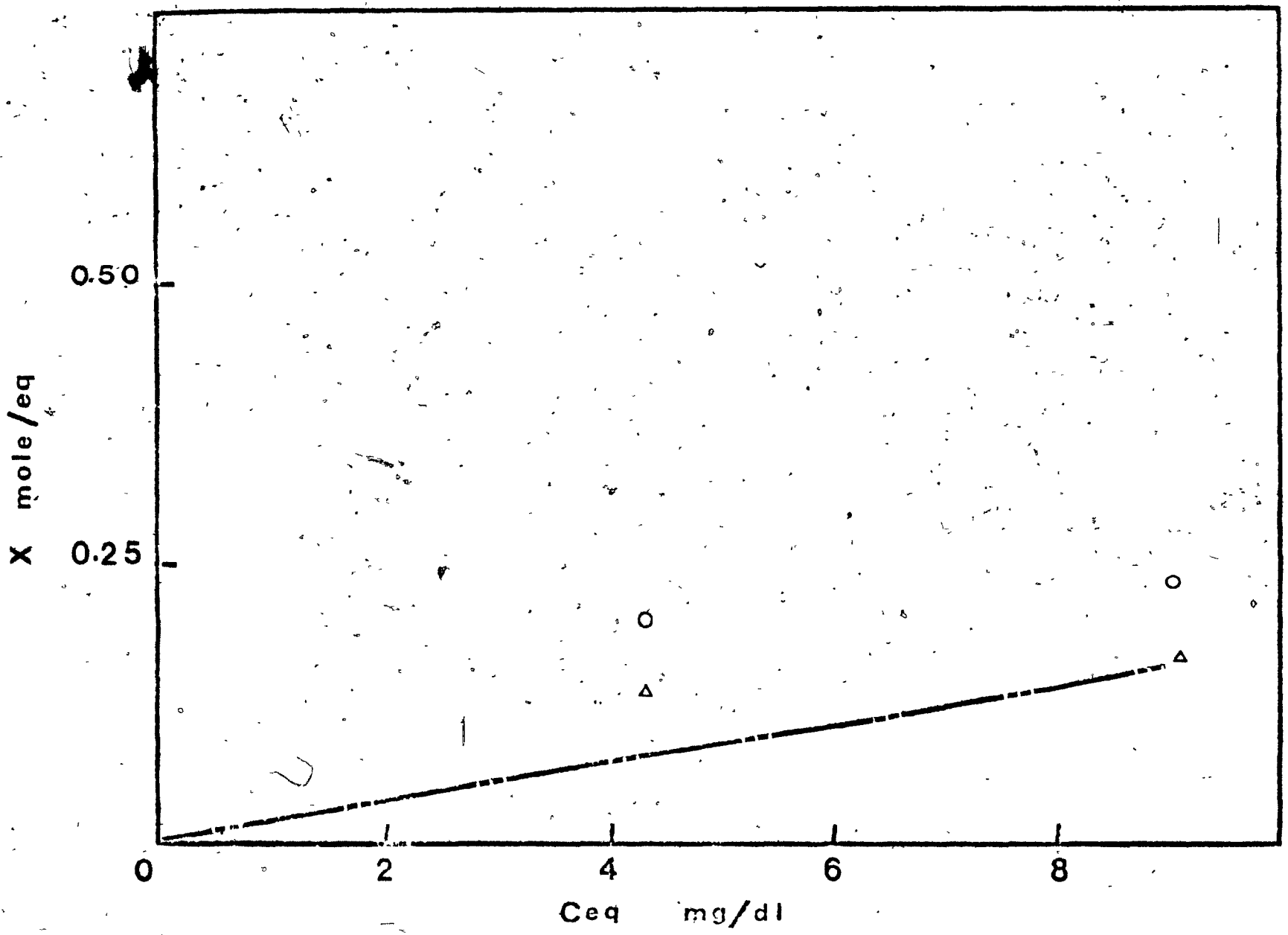

Fig 3

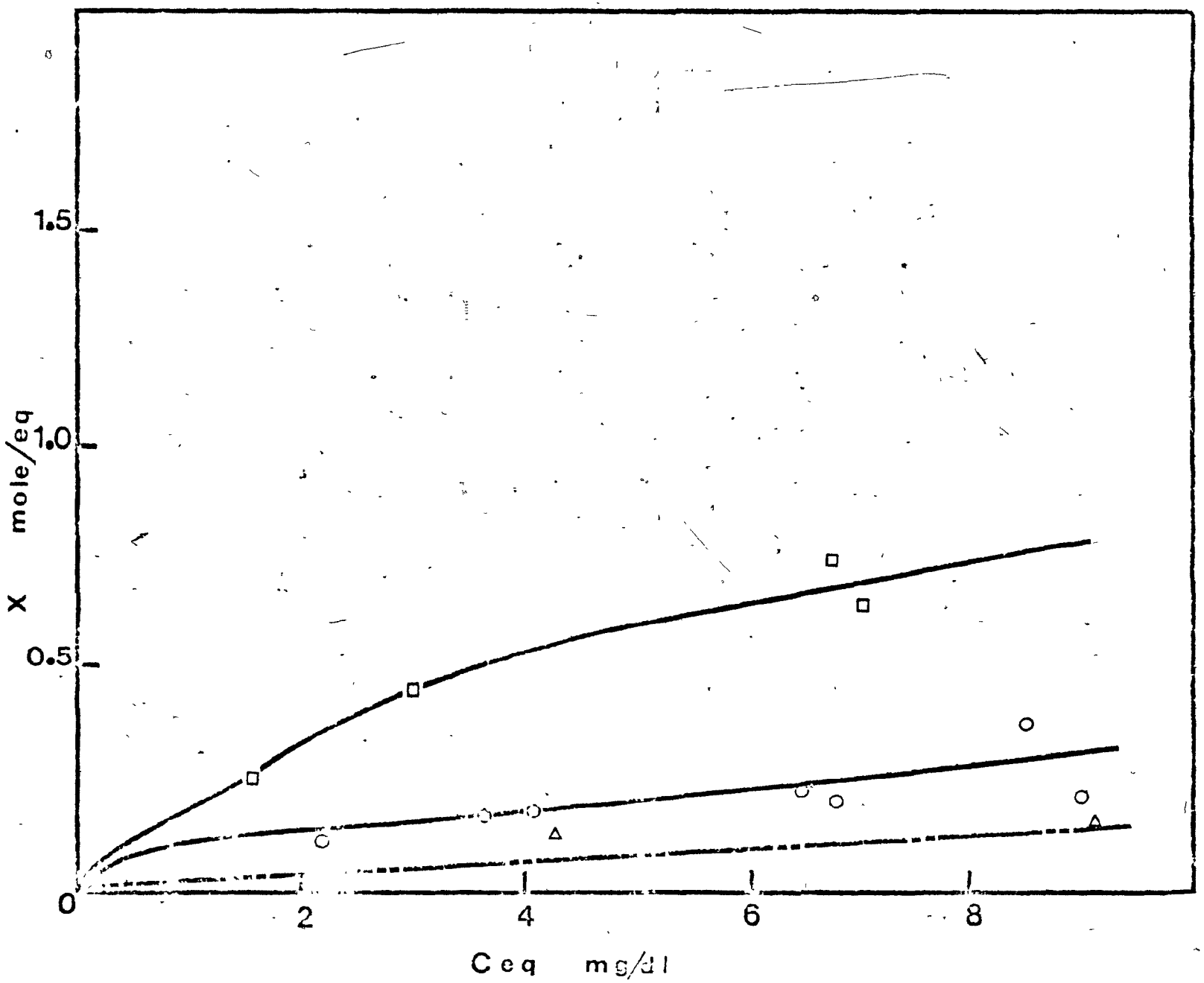

fig4

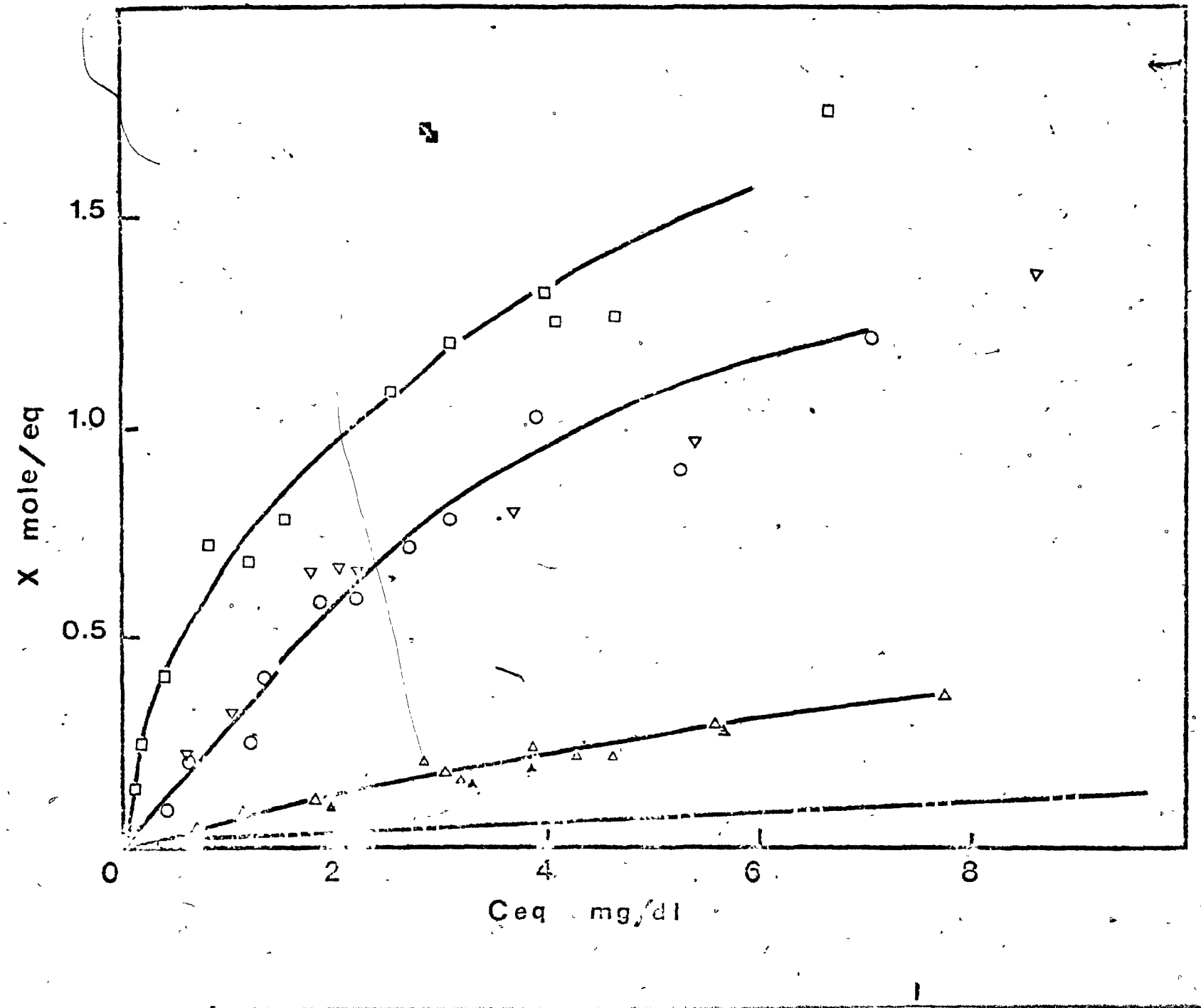

fig5

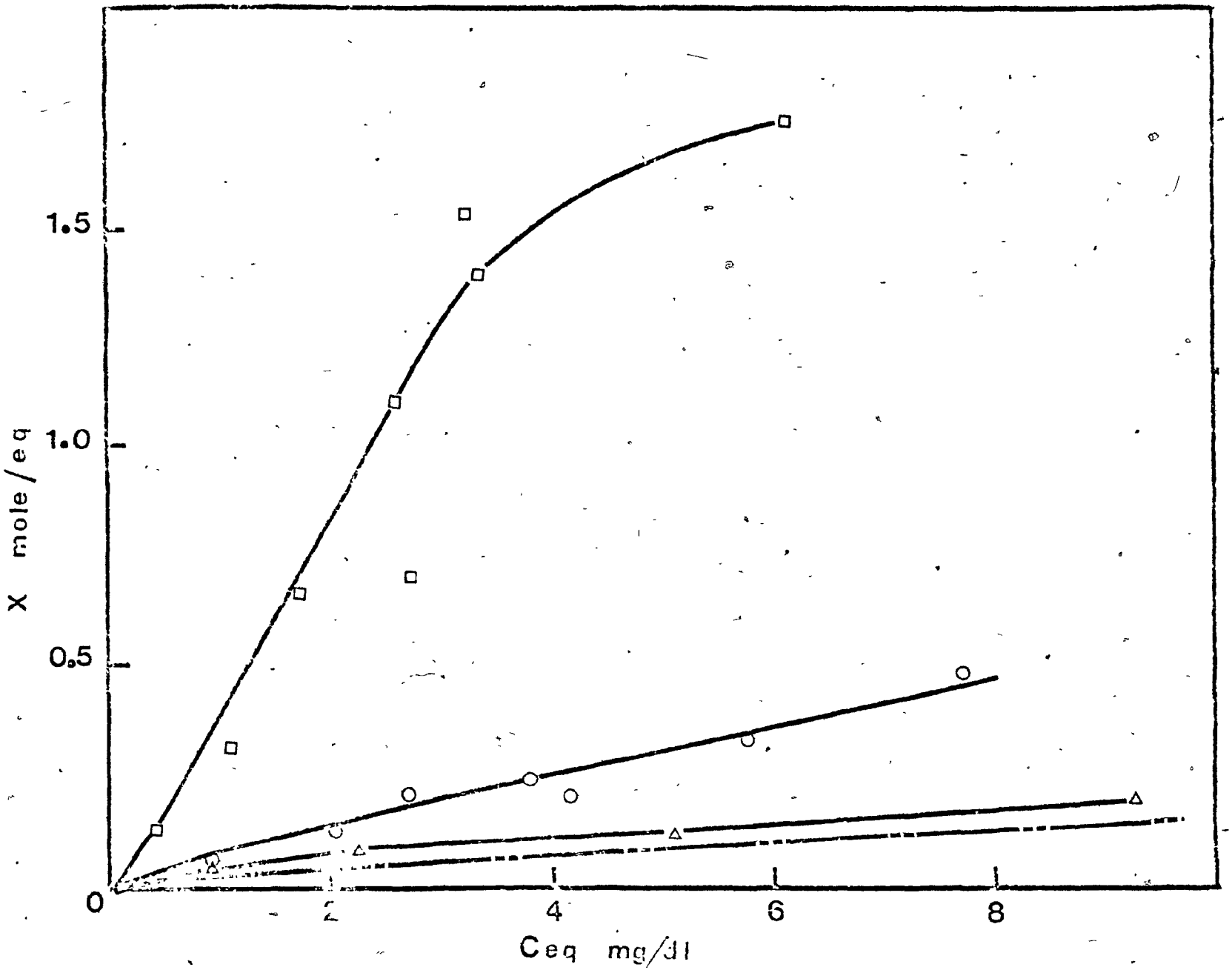

Fig 6

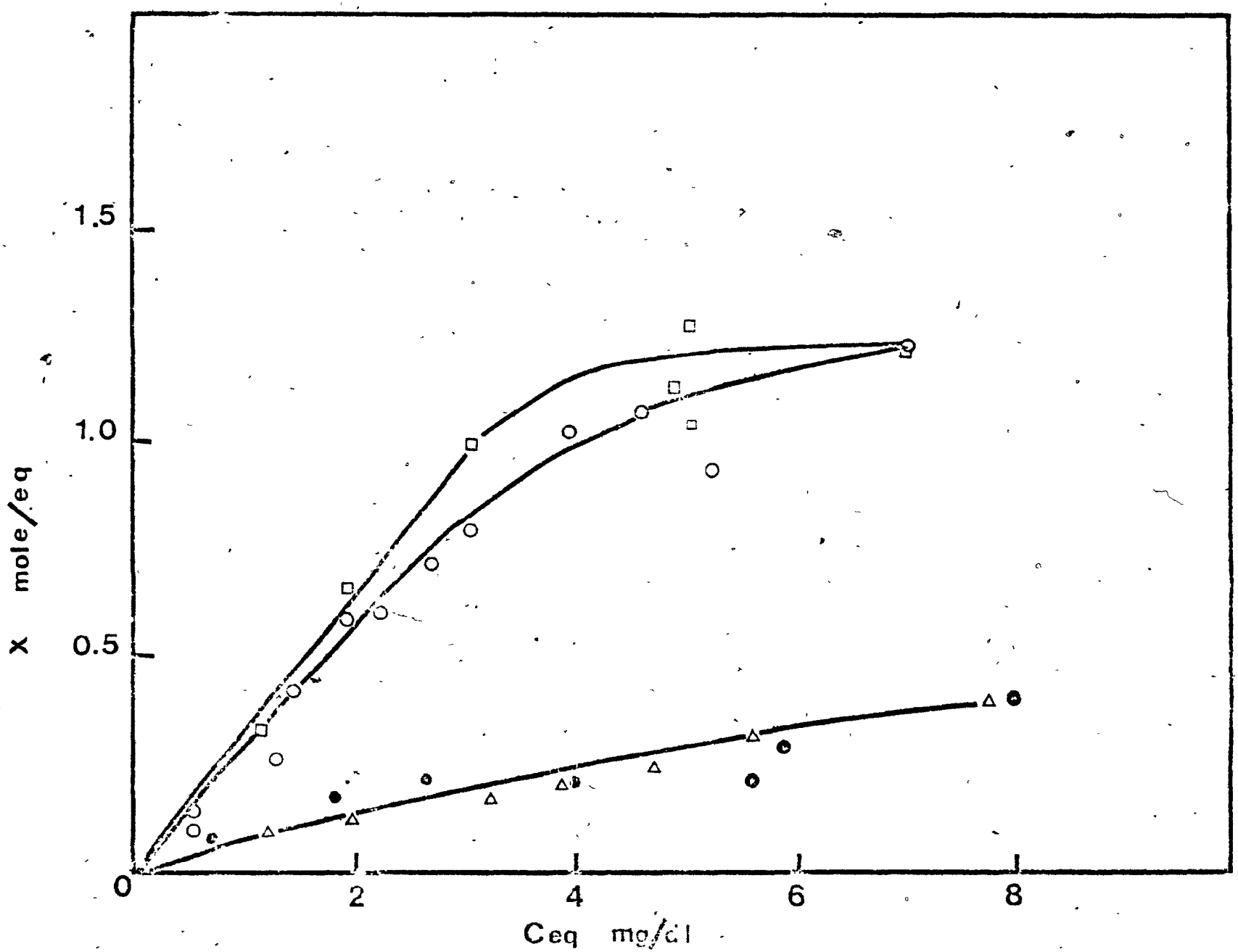

fig 7