THE STRUCTURE AND OXYGEN EQUILIBRIUM PROPERTIES
OF THE INTRACELLULAR HEMOGLOBINS FROM THE
BIVALVE MOLLUSC BARBATIA REEVEANA

by

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The red blood cells of the bivalve mollusc Barbatia reeveana

contain two hemoglobins. One, a tetramer (M_r = 60,000), is typical

of the other intracellular hemoglobins. The other is unique among

any intracellular hemoglobin studied so far. It is very large with

a molecular weight of 430,000, and has a subunit with a molecular

weight of 32,000 consisting of two oxygen binding domains. The

structure and oxygen equilibrium properties of these intracellular

hemoglobins are presented in this thesis.

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INTRODUCTION

Hemoglobin is the most ubiquitous respiratory protein found in vertebrate and invertebrate species (Prosser, 1973). Hemoglobin molecules reversibly bind oxygen and serve primarily to transport oxygen from the external environment to the organism's respiring tissues. Tissue hemoglobin, or myoglobin, facilitates diffusion of oxygen into tissues and may also store oxygen for use during periods of hypoxia (Scholander, 1960; Hemmingsen, 1963; Wittenberg, 1970).

The structure of vertebrate hemoglobin has been the subject of numerous investigations. Kendrew (1963) showed by x-ray crystallographic studies that sperm whale myoglobin consists of a folded polypeptide chain of 153 amino acid residues containing a "pocket" for the heme prosthetic group. Perutz (1964) reported that the structure of vertebrate hemoglobin contains four polypeptide chains which each resemble the structure of vertebrate myoglobin, and that the heterotropic and homotropic properties of vertebrate hemoglobin are dependent on this quaternary structure.

The structure of invertebrate hemoglobins are less well known. Like the vertebrate pigments, many

invertebrate hemoglobins appear to be constructed of multiples of 17,000 daltons as hypothesized by Svedberg (Svedberg, 1933; Svedberg and Pederson, 1940). Study of the tertiary structure of an annelid (Glycera) monomeric hemoglobin showed the typical myoglobin-like fold characteristic of vertebrate hemoglobins (Padlan and Love, 1974). A few invertebrate hemoglobins, however, have remarkably different structures. These invertebrate hemoglobins are unusual in that they are polymeric proteins which have subunits constructed of a linear series of covalently linked oxygen binding domains (Waxman, 1975; Wood and Mosby, 1975; Terwilliger et al, 1976; Terwilliger and Terwilliger, 1977; Terwilliger et al, 1978; Terwilliger and Terwilliger, 1978a and 1978b; Dangott and Terwilliger, 1979). Bivalve molluscs are one group of invertebrates that possess both these unusual domain series hemoglobin structures as well as the more typical vertebrate-like hemoglobins.

Many bivalve molluscs possess hemoglobin or myoglobin as a respiratory protein. When the molecules are intra-cellular, they appear as monomeric molecules with one heme and a molecular weight of 15-17,000, or dimeric, or tetrameric aggregates of this monomer. The red color of adductor muscle in Mercenaria mercenaria is the result of monomeric myoglobin of 16-17,000 molecular

weight (Koppenheffer and Read, 1969). Gill tissue

of _Phacoides pectinatus_ contains hemoglobin of 14,500

molecular weight, as well as some tetrameric and possible

octomeric aggregates (Read, 1965). Circulating cells

of the arcid bivalve _Noetia ponderosa_ have a dimeric

hemoglobin of 34,000 molecular weight (Freadman and Mangum,

1975). The intracellular hemoglobins of _Anadara_

transversa have molecular weights of 30,000 and 58,000

(Yang, 1974), similar to the intracellular hemoglobins

of _Anadara broughtonii_ (= _A. inflata_), which are

putative dimers and tetramers with molecular weights of

34,000 and 68,000 (Saskawa and Satake, 1967). The dimer

is composed of 15,500 molecular weight electrophoretically

indistinguishable subunits; the tetramer can be resolved

into two dissimilar polypeptide chains, designated $\propto$

and β , similar to the vertebrate hemoglobins (Furuta

_et al_, 1977).

The hemocoelic fluid of some clams contain large

extracellular hemoglobins (Manwell, 1963). The extra-

cellular hemoglobin of the clam _Cardita_ is 12×10^6

molecular weight and contains one heme per 16-22,000 g

protein (Waxman, 1975; Terwilliger _et al_, 1978). Upon

denaturation of _Cardita borealis_ extracellular hemoglobin,

subunits of 290-300,000 molecular weight are produced;

when digested with subtilisin this subunit yields

15-17,000 molecular weight fragments that reversibly bind oxygen (Terwilliger and Terwilliger, 1978a). Extracellular hemoglobins from the bivalves Astarte castanea and Cardita affinis are of similar construction (Terwilliger and Terwilliger, 1978b). A structure for these clam polymeric hemoglobins has been suggested similar to that proposed for the planorbid snail Helisoma hemoglobin (Terwilliger et al, 1976). These polymeric molluscan hemoglobins may be aggregations of subunits that consist of a covalently linked series of oxygen binding domains (Terwilliger et al, 1976; Terwilliger et al, 1978; Terwilliger and Terwilliger, 1978a and 1978b).

The structures of intracellular and extracellular clam hemoglobins appear to differ from one another strikingly. The low molecular weight bivalve myoglobins and hemoglobins are contained within cells, whereas the large clam hemoglobins, which consist of an aggregation of subunits composed of linked oxygen binding domains, appear to be extracellular.

Oxygen equilibrium studies have been carried out on myoglobin and hemoglobin from several bivalve species. Myoglobin of Mercenaria mercenaria exhibits high oxygen affinity (P_{50} = 0.55 mm Hg) and shows no cooperativity or Bohr effect (Manwell, 1963). These characteristics

are shared by other molluscan monomeric myoglobins

(Read, 1966), as well as by myoglobin from humans

(Rossi-Fanelli and Antonini, 1958), horse (Theorell,

1934) and hagfish (Manwell, 1958).

Clam hemoglobins located within circulating cells

have lower oxygen affinities than myoglobins. Djangmah

et al (1978) found that for the arcid Anadara senilis,

the dimeric hemoglobin has a P_{50} = 4.2-6.7 mm Hg and that

the tetrameric hemoglobin has a P_{50} = 8.0-13.0 mm Hg;

both show cooperativity and have small Bohr effects.

Anadara satowi dimeric hemoglobin has a P_{50} = 9.0 mm Hg,

shows no Bohr effect, and exhibits cooperativity with

a Hill coefficient of N = 1.5; the tetramer has a

P_{50} = 11.0 mm Hg and shows both a slight reverse Bohr

effect and strong cooperativity with N = 2.1 (Ohnoki

et al, 1975). Oxygen equilibrium studies on unseparated

hemoglobin solutions from Anadara broughtonii indicate a

P_{50} = 10 mm Hg with N = 1.15 at 20 $^{\circ}$C (Kawamoto, 1928).

Both A. broughtonii dimeric and tetrameric hemoglobin

components show strong heme-heme interactions with N = 1.8

and 2.8-3, respectively; however, with a change in pH

there is no concomitant change in the oxygen binding

properties of these hemoglobins (Furuta et al, 1977).

This lack of a Bohr effect for A. broughtonii

hemoglobin is in marked contrast to the heterotropic

properties exhibited by vertebrate hemoglobins, particular-
ly since both the tetrameric structure of this clam and
vertebrates share a $\alpha_2 \beta_2$ structure. Perutz (1970)
suggested a mechanism for the Bohr effect of human
HbA hemoglobin requiring, in part, a critical role for
the carboxy terminal histidine residues in the β chains.
The lack of a Bohr effect for A. broughtonii tetrameric
hemoglobin may be attributed to the substitution of
leucine residues in place of histidine as the carboxy
termini of the α and β chains (Furuta et al, 1977).

Oxygen equilibrium studies by Manwell (1963) on
hemoglobin from another arcid clam, Noetia ponderosa,
show a P_{50} = 13.5 mm Hg and a Hill coefficient of N = 1.1
at 22-24 $^{\circ}$C, with no Bohr effect in the pH range 6.68-
8.24. Freadman and Mangum (1976) reported a P_{50} = 5.30
at 10 $^{\circ}$C and 6.25 mm Hg at 23 $^{\circ}$C for Noetia ponderosa
hemoglobin within intact red cells in the pH range 6.6-
7.4. These half-saturation values givin by Freadman
and Mangum (1976) are similar to the values given by
Yang and Parkurst (1973) for N. ponderosa hemoglobin
extracts. This similarity in oxygen affinity of
hemoglobin in intact cells and hemoglobin extracts of
N. ponderosa suggests that the oxygen binding properties
of this hemoglobin are not modified by intracellular
constituents (Freadman and Mangum, 1976). This same

finding has been reported for other invertebrate hemo-
globins (Mangum and Carhart, 1972; Mangum et al, 1975).

Collett and O'Gower (1972) have reported the
unusual temperature dependent oxygen binding behavior
of hemoglobins from the red cells of Anadara granosa,
Anadara maculosa, and Anadara trapezia. These authors
found that the P_{50} values for hemoglobin extracts from
these three clams increases with temperature over the
range 15-20 ^{O}C (at 20 ^{O}C the P_{50} for A. granosa and A.
maculosa is 5.6-5.9 mm Hg, that for A. trapezia is 10.5
mm Hg). Remarkably, however, at the higher temperature
range (20-30 ^{O}C), there is an increase in oxygen
affinity with increasing temperature. This unusual
sinistral shift in the oxygen equilibrium curve at
high temperatures may serve as a physiological adaptation
to the decreased availability of oxygen in warm water
(Collett and O'Gower, 1972).

The functions of large extracellular clam hemoglobins
have not been as extensively characterized as the other
bivalve pigments. The extracellular hemoglobin of
Cardita floridana at 22-24 ^{O}C has a P_{50} value of 11 mm Hg,
independent of pH in the range 7.03-7.5, and a Hill
coefficient slightly less than 1 (Manwell, 1963). The
lack of any homotropic or heterotropic interactions for
this large complex molecule is vexing. The 16,000 dalton

heme-containing domains generated by enzymatic digestion share the same oxygen binding properties of the intact pigment (Terwilliger and Terwilliger, 1978a). The oxygen affinities of clam circulatory hemoglobins, both within cells and extracellular (in the range 10 - 20 mm Hg) are lower than most other molluscan hemoglobins (Read, 1966), although not as low as some vertebrate hemoglobins (human HbA has a P_{50} = 26 mm Hg under physiological conditions) (Manwell, 1963).

The purpose of this thesis is to examine the structure and function of the hemoglobins from the bivalve mollusc Barbatia reeveana. I collected this arcid from the wave-exposed intertidal rocks in the Gulf of California and noticed that they contained abundant hemoglobin which seemed to be intracellular. As this species was previously unstudied and since it was found in a semi-tropical environment, its respiratory physiology might be interesting, especially in light of the unusual temperature effects exhibited by the arcid clams studied by Collett and O'Gower (1972). One of the most remarkable findings of this study discussed below is the occurrence and structure of a polymeric (M_r = 430,000) hemoglobin contained within cells, as well as a smaller, more typical tetrameric pigment. The structural and functional characteristics of these hemoglobins were

studied and the results are presented in this thesis.

MATERIALS AND METHODS

Barbatia reeveana (Orbigney) was collected from the
wave-exposed rocky intertidal, near El Coyote, in
Conception Bay, Baja California, Mexico, and identified
according to Keen (1971). The animals were transported
to the laboratory where they were kept in aerated aquaria
at room temperature (19-23 ^{O}C) with daily fresh change of
seawater (32°/oo).

Blood was collected by inserting a pipette into the
clam's pallial sinus. Blood from 1-5 animals was pooled
and washed three times with ice-cold 3 % NaCl. The
final cell pellet was resuspended in 1 mM Tris·HCl
(pH 8.0) and placed on ice for 1 hr. The resulting
solution was centrifuged at 12,000 x g for 10 min. and
the supernatant was applied to a column (1.9 x 100 cm)
of Sephadex G-100 equilibrated with 0.05 I Tris·HCl
buffer (pH 8.0) 0.1M in NaCl. The column was previously
calibrated with Blue Dextran, bovine serum albumin,
ovalbumin, α-chymotrypsinogen A, and sperm whale
myoglobin (Sigma Chem. Co.).

Hematocrit values were obtained from blood freshly
collected and immediately taken up in a micro-hematocrit
capillary tube and centrifuged at 3,000 rpm until no

further packing of the cells occurred (5 min.). The blood hemoglobin concentration was determined by converting the pigment in the cell lysate to the cyanmet-derivative and using the millimolar extinction at 540 nm of cyanmethemoglobin E = 11.0 (van Assendelf, 1975). A heme to protein ratio of 1:16,000 was used in calculating hemoglobin concentrations.

The Hb-H hemoglobin component was concentrated by vacuum dialysis and dialyzed versus either 0.05 I Tris·HCl (pH 8.0) 0.1 M in NaCl or, 0.05 I Tris·HCl (pH 7.0) 0.1 M in NaCl, and 0.01 M in $MgCl_2$. The hemoglobin was applied to a column (1.9 x 100 cm) of Bio-gel A-5M, 200-400 mesh, (Bio-Rad Lab.) previously equilibrated with either of the above buffers. The Bio-gel column was calibrated with Eudistylia vancouverii chlorocruorin (M_r = 2.8 x 10^6) (Terwilliger et al, 1975), Cancer magister hemocyanin (M_r = 9.0 x 10^5 and 4.5 x 10^5) (Ellerton et al, 1970) and bovine heart lactate dehydrogenase (M_r = 150,000) (Sigma Chem. Co.).

Sedimentation velocity experiments were carried out on hemoglobin in 0.05 I Tris·HCl (pH 8.0) 0.1 M in NaCl with a Beckman-Spinco Model E ultracentrifuge equipped with a RTIC temperature control unit and scanning optics. The temperature was near 20 $^\circ$C and the rotor speeds were 36,000 rpm for the 13.6 S material

(1.5 mg/ml) and 44,000 rpm for the 9.7 S material

(0.7 mg/ml). Sedimentation coefficients were calculated

by the method of least squares from the plot of ln r vs.

s and corrected to the viscosity and density of water

at standard conditions (Svedberg and Pederson, 1940).

The Hb-H hemoglobin was dialyzed against 0.01 M

ammonium bicarbonate and applied to a column (1.9 x 12 cm)

of DEAE (diethylaminoethyl)-cellulose (Sigma Chem. Co.)

equilibrated with the same buffer. The column was washed

with a large volume of this buffer, and the pigment was

eluted by establishing a linear gradient (250 ml each

chamber) between 0.01 M ammonium bicarbonate and the

same buffer 0.5 M in NaCl. Samples purified in this

manner were used in amino acid composition, heme content

and extinction coefficient determinations.

The Hb-L hemoglobin was dialyzed versus 1 mM

sodium phosphate buffer (pH 6.6) and applied to a column

(1.9 x 12 cm) of CM (carboxy methyl)-cellulose, medium

mesh, (Whatman) previously equilibrated with the same

buffer. The column was washed with a large volume of

this buffer and the hemoglobin was eluted by developing

a linear gradient (250 ml each chamber) between 1 mM

sodium phosphate buffer (pH 6.6) and the same buffer

0.1M in NaCl. The Hb-L$_1$ hemoglobin fraction obtained

from this treatment was used in determination of heme

content and extinction coefficient.

The Hb-H hemoglobin was concentrated by vacuum dialysis and then dialyzed versus 0.04 M sodium glycinate buffer (pH 9.5), 0.01 M in EDTA (ethylenediaminetetra-acetic acid) and applied to a column (1.9 x 90 cm) of Sephacryl S-200 equilibrated with the buffer, 0.01 M in NaCl. The column was calibrated with Blue Dextran, bovine serum albumin, ovalbumin and sperm whale myoglobin.

Isoelectric focusing of Hb-H previously dialyzed against 0.01 M Tris·HCl (pH 8.0) 0.01 M in NaCl, and 1 % in glycine and converted to the cyanmet derivative was carried out in tubes containing 5 % acrylamide gel which was 2 % in a 3:2 ratio of pH 3.5-10.5 and pH 4-6 ampholines (LKB Chem. Co.). The upper buffer reservoir contained 0.2 N NaOH and the lower buffer reservoir contained 0.01 M phosphoric acid. The gels were focused at 120 V for 12 hrs (4 $^{\circ}$C) and then removed and scanned at 415 nm with a Zeiss PMQ II Spectrophotometer. The pH gradient was determined by slicing the gel into 0.5 cm sections and measuring the pH of each section after 24 hrs in distilled water.

Electrophoresis of globin (Teale, 1959) was performed at pH 2.2 in the presence of 6.25 M urea (Panyim and Chalkey, 1969; Poole et al, 1974). Globins were incubated overnight at room temperature in a solution

of 10 M urea (deionized with Amberlite MB-1) 5 % in
acetic acid and 1 % in 2-mercaptoethanol. Pre-
electrophoresis of the gels and electrophoresis of the
sample were carried out at 2 mA per tube. The gels were
stained with Coomassie Blue and destained with 10 %
acetic acid.

Sodium dodecyl sulfate polyacrylamide electro-
phoresis with or without the presence of 8 M urea was
carried out on 1.5 mm slab gels (Studier, 1973) with a
discontinuous buffer system (Laemmli, 1970). A gel
concentration of 12.5 % and a constant ratio of acrylamide
to bisacrylamide of 30 : 0.8 was used. Globin or
performic acid oxidized globin (Hirs, 1967) was first
denatured in boiling incubation buffer (with or without
8 M urea) containing 2 % sodium dodecyl sulfate, 5 %
2-mercaptoethanol, and 1 mM PMSF (phenylmethylsulfonyl
fluoride) for 1.5 min. Calibrants were ovalbumin, $\propto$ -
chymotrypsinogen A, sperm whale myoglobin, and lysozyme
(Sigma Chem. Co.). The gels were stained in Coomassie
Blue according to Fairbanks et al (1971). Globin was
also denatured in boiling 6 M guanidine hydrochloride
with 2-mercaptoethanol, incubated for 2 h at 37 ^{o}C· and
dialyzed extensively against the sodium dodecyl sulfate
incubation buffer prior to analysis by sodium dodecyl
sulfate electrophoresis. Apparent molecular weight of

globin denatured in 6 M guanidine hydrochloride and
reduced with 5 mM dithiothreitol was determined on
Sephacryl S-200 (1.4 x 83 cm) equilibrated with 6 M
guanidine hydrochloride, 0.05 I Tris·HCl (pH 7.0) and
0.5 mM dithiothreitol according to Fish et al (1969).
The column was calibrated with Blue Dextran, bovine serum
albumin, ovalbumin, ∝ -chymotrypsinogen A, and sperm
whale myoglobin under identical denaturing conditions.

Hemoglobin was digested with subtilisin (Carlsberg
Type VIII Sigma Chem. Co.) in 0.04 M sodium glycinate
buffer (pH 9.5) at 24 ^{o}C for 2 h and the reaction stopped
by addition of phenylmethylsufonyl fluoride. The
concentration of enzyme used relative to hemoglobin
for separation of the digestion products was 1 mg
enzyme per 25 mg protein. A control aliquot of the pH
9.5 hemoglobin was treated in the same way without the
addition of subtilisin. The digestion products were
separated on a Sephadex G-100 column (1.9 x 114 cm)
equilibrated with 0.05 I Tris·HCl (pH 8.0) buffer 0.1 M
NaCl. The column was previously calibrated with Blue
Dextran, bovine serum albumin, ovalbumin, ∝ -chymotryp-
sinogen A and sperm whale myoglobin.

Extinction coefficients were determined by measuring
the absorbance at 280 nm of a hemoglobin solution of
known volume and weighing the lyophilized sample. The

heme content of hemoglobin was determined as the
cyanmet-derivative according to the method of Drabkin
and Austin (1935) using a 540 nm millimolar heme
extinction coefficient of 11.0 for the cyanmethemoglobin
(van Assendelf, 1975). The amino acid composition
was carried out on purified hemoglobin according to
Spackman et al (1958). Cysteine and cystine were
determined as cysteic acid on performic acid oxidized
globin after Hirs (1956).

Absorbance maxima were determined using a Zeiss
PMQ-II spectrophotometer on freshly prepared hemoglobin.
Hemoglobin was deoxygenated by addition of a small amount
of sodium dithionite and carbonmonoxyhemoglobin was
prepared by bubbling a hemoglobin solution with carbon
monoxide gas (Matheson Co.) after the addition of a
small crystal of sodium dithionite.

Oxygen binding of both the intact hemoglobins and
the digested hemoglobin separated by gel chromatography
were studied spectrophotometrically (Benesch et al,
1965) with a Zeiss PMQ II spectrophotometer equipped
with a temperature controlled cell holder. Samples
were dialyzed versus the appropriate buffer before
analysis.

RESULTS

The hemoglobin of _Barbatia reeveana_ is found within circulating cells in the clam's vascular system. The average blood volume is 0.35 ml $\pm$ 0.03 (S.D.) (N = 9) per g wet weight tissue. The average hematocrit is 6.1 vol percent with a range of 2.6 to 13.1 vol percent. The hemoglobin concentration varies among clams; the hemoglobin concentration of blood pooled from 6 animals is 0.54 g per 100 ml. After 4 months in aquaria, most of the clams appeared anemic.

Barbatia hemoglobin chromatographs on Sephadex G-100 at pH 8.0 as two peaks (Fig. 1). The heavy hemoglobin fraction, Hb-H, elutes in the column void volume and accounts for approximately 40 % of the total hemoglobin eluent. The light hemoglobin fraction, Hb-L, has an apparent molecular weight of 60,000, obtained from the plot of log molecular weight versus elution volume (Fig. 2). The heavy hemoglobin fraction, Hb-H, after concentration and dialysis against either pH 7 or 8 buffer, chromatographs on Bio-gel A-5M equilibrated with the appropriate buffer as two symmetrical peaks (Fig. 3). The major Hb-H component (approximately 70 %) has an apparent molecular weight

of 430,000 and the minor Hb-H component (approximately 30 %) has an apparent molecular weight of 220,000 (Fig. 4). A similar elution pattern is observed when the major 430,000 molecular weight Hb-H component is rechromatographed on the Bio-gel column, except that the 220,000 dalton Hb-H component is reduced to approximately 15 % of the hemoglobin eluent.

After Bio-gel chromatography at pH 8.0 of the Hb-H hemoglobin fraction, sedimentation velocity studies were performed on both the 430,000 and 220,000 molecular weight components separately. From the plot of ln r vs. sec, where r is the distance of the boundary from the center of rotation at time t, the Hb-H 430,000 molecular weight component has a sedimentation coefficient of 13.6 $S_{20,w}$, and the Hb-H 220,000 molecular component an $S_{20,w}$ = 9.7. The Hb-H 430,000 molecular weight component also shows the presence of an additional boundary corresponding to approximately 10 % of the material having a sedimentation coefficient of ca 9 S. The sedimentation velocity results obtained for the Hb-H 220,000 molecular weight component suggest that this component is homogeneous. These sedimentation results corroborate the results from gel chromatography which suggest the Barbatia Hb-H hemoglobin contains a 430,000 molecular weight component (13.6 $S_{20,w}$) that tends to

dissociate into 220,000 molecular weight (9.7 $S_{20,w}$) material. There is, however, no evidence for reassociation of the 9 S component into a larger structure.

Subsequent to Sephadex G-100 chromatography, isoelectric focusing of Hb-H cyanmethemoglobin indicates that the pigment appears heterogeneous. Scans of gels at 415 nm show a major peak corresponding to a pI = 5.7 with a shoulder near 5.8 and a minor peak with a pI = 5.4 (Fig. 5).

Barbatia Hb-H hemoglobin was dialyzed against 0.01 ammonium bicarbonate buffer and chromatographed on a DEAE-cellulose column equilibrated with the same buffer. The pigment eluted in the presence of a linear NaCl gradient as a single asymmetric peak (Fig. 6). The ratio of absorbances at 280 and 540 nm was nearly constant across the peak. The 280 : 540 ratio for the cyanmethemoglobin derivative from the pooled fractions under the bar in Figure 6 was the same as that found for Hb-H cyanmethemoglobin before passage through the DEAE-cellulose column. Hb-H hemoglobin purified in this way contains 1 heme per approximately 16,000 g protein based upon a millimolar extinction coefficient of 11 at 540 nm for the cyanmet-derivative and an extinction coefficient of 3.3 for a 1 mg/ml solution of Hb-H hemoglobin at 280 nm.

After the Sephadex G-100 chromatography, the Hb-L
hemoglobin component was dialyzed against 1 mM sodium
phosphate buffer and chromatographed on a CM-cellulose
column equilibrated with the same buffer. The pigment
eluted in the presence of a linear NaCl gradient as 3
symmetrical peaks of successively decreasing size
(Fig. 7). Spectrophotometric scans of hemoglobin from
the bars under each peak as shown in Figure 7 indicate
that the major peak, Fraction Hb-L$_1$ contained little
methemoglobin. Scans also showed that the second peak,
Hb-L$_2$, contained more methemoglobin than Hb-L$_1$, and
that the third peak, Hb-L$_3$, possessed more methemoglobin
than either the first or second peaks. The ratios of
absorbances at 280 nm and 540 nm for the cyanmethemo-
globin derivatives of the fractions under the bars in
Figure 7 were slightly reduced from that found for Hb-L
cyanmethemoglobin before passage through the CM-cellulose
column. Hb-L$_2$ hemoglobin purified in this manner
contains 1 heme per 15-17,000 g protein based upon a
millimolar extinction coefficient of 11 at 540 nm for
the cyanmet-derivative and an extinction coefficient of
2.8 for a 1 mg/ml solution of Hb-L$_1$ hemoglobin at 280 nm.

Subsequent to the G-100 chromatography, isoelectric
focusing of Hb-L cyanmethemoglobin shows one major band
with a pI = 8.15 and a minor band with pI = 7.85, along

with a trace band with pI = 7.4 (Fig. 8).

The absorbance maxima of <u>Barbatia</u> Hb-H and Hb-L hemoglobins is given in Table 1.

Urea gel electrophoresis at pH 2.2 of purified Hb-L$_1$, Hb-L$_2$, and Hb-L$_3$ globins as shown in Figure 7 illustrate that each fraction consists of two sharp staining bands (Fig. 9a). When the Hb-L$_1$, Hb-L$_2$, and Hb-L$_3$ globin fractions are electrophoresed together in urea gels, two staining bands are apparent; this suggests that all three fractions may consist of the same two polypeptides.

Sodium dodecyl sulfate slab gel electrophoresis of globin or hemoglobin from the three Hb-L fractions obtained according to Figure 7 shows two staining bands corresponding to molecular weights of 15,000 and 16,700 (Fig. 10).

Tube gel electrophoresis of purified Hb-H globin in 6.25 M urea at pH 2.2 shows one sharp band (Fig. 9b). The amino acid composition of <u>Barbatia</u> Hb-H hemoglobin is given in Table 2.

The subunit structure of <u>Barbatia</u> Hb-H was examined under several conditions of denaturation. Sodium dodecyl sulfate slab gel electrophoresis was used to analyse protein treated in the following ways: 1. globin 2. performic acid oxidized globin 3. pigment which

had been incubated in 6 M guanidine hydrochloride at
100 ^{O}C in 2-mercaptoethanol and dialyzed versus the
sodium dodecyl sulfate incubation buffer 4. Protein
which had been treated with sodium dodecyl sulfate and
reducing agent in the presence of 8 M urea and electro-
phoresed in sodium dodecyl sulfate and urea. After
each treatment the protein electrophoresed as a single
band with an apparent molecular weight of 32,000
(Fig. 11 a-d). If the protein was incubated in 6 M
guanidine hydrochloride with 5 mM dithiothreitol for 2 h
at 37 ^{O}C and chromatographed on a column of Sephacryl
S-200 in equilibrium with 6 M guanidine hydrochloride
with 0.5 mM dithiothreitol, the protein elutes as a
single component with a molecular weight of about 37,000
(Fig. 12).

Barbatia Hb-H hemoglobin was dialyzed versus 0.04 M
sodium glycinate buffer (pH 9.5) with 0.1 M NaCl and
0.01 M EDTA and chromatographed on a Sephacryl column
equilibrated with the same buffer (Fig. 13). The major
peak eluted in the void volume along with a shoulder
peak too close to the void volume for determination
of its molecular weight.

Barbatia Hb-H hemoglobin was dialyzed against 0.04 M
sodium glycine buffer (pH 9.5) and digested for 2 hrs
at 24 ^{O}C with enzyme to protein ratios (w/w) of 1/5,

1/10, 1/25, 1/50, 1/100, 1/250, 1/500, and 1/1000.

The digestion products were analyzed by sodium dodecyl

sulfate slab gel electrophoresis as shown in Figure 14.

The major product obtained after digestion of the Hb-H

hemoglobin with variable enzyme concentrations has an

apparent molecular weight of 16,500. There appears to

be a greater quantity of this 16,500 dalton material

produced from Hb-H samples incubated with enzyme

concentrations from 1/25 to 1/250 than for hemoglobin

incubated with the higher or lower enzyme concentra-

tions. The control hemoglobin incubated under the same

conditions but without subtilisin shows one band with an

apparent molecular weight of 32,000. The digested Hb-H

hemoglobin samples also show 32,000 dalton staining

bands corresponding to putative undigested hemoglobin.

The quantity of this undigested hemoglobin is observed

to decrease with increasing enzyme concentration. A

staining band with an apparent molecular weight of

29,000 corresponding to the enzyme control is evident

in some of the hemoglobin digests. Minor bands with

molecular weights of 26,500, 23,500 and 22,500 are

observed from the digested aliquots. The staining

intensity of these minor bands appears to decrease with

increasing enzyme concentrations. Trace bands with

molecular weights less than the major 16,500 dalton

digested hemoglobin product are present near the dye front of the gel and may represent digested polypeptide fragments.

Barbatia Hb-H hemoglobin digested under the previous conditions with a 1/25 enzyme concentration produced fragments which chromatograph on Sephadex G-100 at pH 8.0 as shown in Figure 15. The major peak (Fraction I) elutes in the void volume and the apparent molecular weights of Fraction II and Fraction III are 57,000 and 30,000, respectively. When the Hb-H hemoglobin is incubated with a 1/10 enzyme concentration, the amount of 30,000 and 57,000 molecular weight materials is increased. Analyses by sodium dodecyl sulfate slab gel electrophoresis of controls and fractions under the bars as shown in Figure 15 are presented in Figure 16. Fraction I (void volume) consists of a major band with an apparent molecular weight of 32,000 along with 2 trace bands with molecular weights of 27,000, 23,000 and a minor 17,000 dalton band. Fraction II shows a major band with a molecular weight of 17,000 and a trace band of 32,000 daltons. Fraction III shows only one band corresponding to a molecular weight of 17,000. When 2-mercaptoethanol is excluded from the sodium dodecyl sulfate incubation buffer there is no apparent change in the sodium dodecyl sulfate slab gel

electrophoresis results from those presented in Figure 15. The ratio of absorbances at 280 nm and 415 nm for Fractions I, II and III from Figure 14 are similar to that of the intact Hb-H pigment suggesting that each fraction contains 1 heme per approximately 16,000 g protein.

Barbatia Hb-H hemoglobin that has been purified by Sephadex G-100 chromatography has a pH independent oxygen equilibrium curve in the range pH 7.2-8.0, with a value $P_{50} = 36 \pm 3.0$ mm Hg at 20 $^\circ$C (Fig. 17a). Below pH 7.2 the oxygen affinity of Hb-H hemoglobin appears variable. The Hb-H hemoglobin exhibits moderate cooperativity that is independent of pH in the range pH 6.85 to 8.0 with a Hill coefficient of $N_{\frac{1}{2}} = 1.8 \pm 0.3$ at 20 $^\circ$C (Fig. 17b). Spectrophotometric scans before and after oxygen equilibrium experiments showed that little methemoglobin was formed.

The effect of temperature on the oxygen binding behavior of Hb-H hemoglobin at pH 8.0 and pH 7.2 is shown by van't Hoff plots in Figure 18. At pH 8.0 the plot appears linear; the overall heat of oxygenation, including the heat of solution of oxygen, for the 15-35 $^\circ$C temperature range is $\Delta H = -4.5$ kcal/mole. At pH 7.2, the plot is nearly linear from 15 to 25 $^\circ$C, and the heat of oxidation is also $\Delta H = -4.5$ kcal/mole.

However, above 25 $^{\circ}$C the oxygen equilibrium of Hb-H hemoglobin at pH 7.2 shows little temperature sensitivity: from 25 to 30 $^{\circ}$C the value $\triangle$H = -1.6 kcal/mole and for the temperature range 30-35 $^{\circ}$C the value $\triangle$H = +0.1 kcal/ mole. The amount of cooperativity is observed to increase slightly ($N_{\frac{1}{2}}$ = 1.6 at 15 $^{\circ}$C to $N_{\frac{1}{2}}$ = 2.1 at 35 $^{\circ}$C) for oxygen binding studies on Hb-H carried out at pH 8.0. Temperature has no apparent effect on cooperativity of Hb-H at pH 7.2 (Fig. 19).

The oxygen equilibrium of Barbatia Hb-L that has been purified by Sephadex G-100 chromatography is pH independent in the range pH 6.8-7.6 with a P_{50} = 17.5 $\pm$ 0.6 mm Hg at 20 $^{\circ}$C (Fig. 20a). A slight reverse Bohr effect with ϕ = +0.2 is suggested from pH 7.6 to 8.0. There is no effect of pH on the strong cooperativity of Hb-L with $N_{\frac{1}{2}}$ = 2.2 $\pm$ 0.2 (Fig. 20b).

The effect of temperature on the oxygen equilibrium half-saturation values of Hb-L hemoglobin at pH 7.2 and pH 8.0 is shown in Figure 21. At pH 7.2 for the temperature range 15-25 $^{\circ}$C the heat of oxygenation, including the heat of solution of oxygen, is $\triangle$H = -1.5 kcal/mole: for 25 to 35 $^{\circ}$C the value $\triangle$H = -8.7 kcal/mole. At pH 8.0 oxygen equilibrium studies on Hb-L indicate that in the temperature interval 15-30 $^{\circ}$C the heat of oxygenation value $\triangle$H = -7.3 kcal/mole. The oxygen

binding behavior of Hb-L for the temperature interval
30-35 ^{O}C at pH 8.0 has a value $\triangle H = +0.3$ kcal/mole.
The amount of cooperativity of Hb-L hemoglobin at pH
7.2 appears to be independent of temperature. At pH
8.0 in the lower temperature range 15-25 ^{O}C, the
cooperativity of Hb-L also appears independent of
temperature, but, is observed to decrease from $N_{\frac{1}{2}} =$
2.3 at 25 ^{O}C to $N_{\frac{1}{2}} = 1.46$ at 35 ^{O}C (Fig. 22).

Oxygen equilibrium determinations were performed
on the Hb-H subtilisin digested hemoglobin Fraction III
isolated according to Figure 14. Fraction III has a
$P_{50} = 12.8$ mm Hg with a $N_{\frac{1}{2}} = 1.7$. The oxygen affinity
of this digested hemoglobin is much greater than the
intact pigment, although the amount of cooperativity
remains unchanged under the same oxygen binding
conditions.

DISCUSSION

The bivalve mollusc *Barbatia reeveana* (Orbigney)
has a zoogeographical range from Manuela Lagoon, Baja
California to Zorritos, Peru. This species is found
from the rocky intertidal to depths of 120 m (Keen,
1971). The clam is common in aggregates between rocks
in the intertidal where the shell is often deformed
from constriction by the substrate. *Barbatia* attaches
to the substrate by a thick lamellar byssus that is
secreted from glandular grooves in the foot (Kusakabe
and Kitamori, 1948). In the intertidal and aquaria,
I observed that *Barbatia* actively responds to changes
in illumination by rapidly retracting its foot and
closing the valves. Similar photosensitivity has been
observed in other arcid species (Braun, 1954). Altough
there is little known concerning the autecology of
Barbatia, Bretsky (1967) reported that *Barbatia
domingensis* was most abundant in turbulent environments
and, on the Bermuda Platform, appeared to be limited
by suitable coral or rock substrate for attachment.

The erythrocytes of arcid clams are generally
thin, slightly biconvex, nucleated cells, containing
hemoglobin throughout the cytoplasm (Dawson, 1933).

Freadman and Mangum (1976) reported that the red cells

of Noetia ponderosa are 17 microns in diameter and

possess a single nucleus. Ochi (1979) found that the

erythrocytes of Barbatia viresceas are 20 microns in

diameter and in the electron microscope show mitochondria,

endoplasmic reticulum, lysozomes, golgi, and occasion-

ally more than two nucleii.

The mean hematocrit of Barbatia reeveana reported

here is 6.1 vol %, which is similar to the mean value

of 6.5 vol % reported for Anadara inflata blood (Nicol,

1966). Both Barbatia and Anadara have slightly lower

blood cell volumes than Noetia ponderosa with a mean

hematocrit of 8.11 vol % (Freadman and Mangum, 1976).

The concentration of hemoglobin in the blood of

Barbatia was observed to vary among individuals. Some

individuals exhibited severe anemia, particularly clams

held in aquaria for a considerable time (4 months).

Fox (1955) reported that the hemoglobin concentrations

of some gastropod molluscs depends upon the ambient

oxygen tension. The hemoglobin content of large

Anadara inflata individuals was found to be greater

than that of smaller animals (Kawamoto, 1928). A size

versus hemoglobin content relationship was not determined

for Barbatia.

The hemoglobin concentration of Barbatia blood

pooled from 6 clams is 0.54 g/ 100 ml. This quantity

is low compared to the values of 3.49 g/100 ml given

by Freadman and Mangum (1976) for Noetia ponderosa

and 1.9 g/ 100 ml reported by Sato (1931) for Anadara

inflata. The concentration of intracellular hemoglobin

in the blood of Barbatia is also lower than that reported

by Manwell (1963) for the extracellular hemoglobin

concentration of Cardita floridana (1-2 g/ 100 ml)

and that given by Terwilliger et al (1978a) for the

extracellular hemoglobin of Cardita borealis (1.9 mg/ml).

Barbatia hemoglobin can be fractionated by Sephadex

G-100 chromatography into two molecular weight components.

The absorbance maxima of these two hemoglobins, Hb-L

and Hb-H, are similar to the spectra of other hemo-

globins (Lemberg and Legge, 1949).

The light hemoglobin fraction, Hb-L has an apparent

molecular weight of 60,000. Urea gel electrophoresis

of this 60,000 molecular weight pigment showed two sharp

staining bands which suggests that the molecule consists

of two polypeptides. Sodium dodecyl sulfate slab gel

electrophoresis results showed that Hb-L is apparently

constructed to two subunits with molecular weights of

15,000 and 16,700. The native 60,000 dalton pigment

was found to contain 1 heme per 15-17,000 g protein.

These findings suggest that Barbatia Hb-L is probably

a tetramer with a $\alpha_2 \beta_2$ structure. A hemoglobin with
similar structure was reported by Furuta et al (1977)
for Anadara broughtonii. Tetrameric hemoglobins with
$\alpha_2 \beta_2$ structures are distributed widely among verte-
brates, but there are few reports of these structures
found within invertebrates. The arcid bivalves appear
unusual in this regard since, with the exception of
Noetia ponderosa (Freadman and Mangum, 1976), they
generally possess a tetramer component with two
dissimilar polypeptide chains (Furuta et al, 1977;
Djangmah et al, 1978).

Barbatia Hb-L was separated by cation-exchange
chromatography into three heme-containing fractions.
These proteins appeared identical by urea gel and
sodium dodecyl sulfate slab gel electrophoresis.
Absorbance measurements, however, indicated that these
hemoglobins differed from each other by the quantity
of methemoglobin present. This implies that Hb-L may
have been separated by cation-exchange chromatography
according to heme oxidation state differences among the
Hb-L molecules. Isoelectric focusing of Hb-L cyanmethemo-
globin, however, also suggests that the pigment is
heterogeneous. These results suggest that the quaternary
structure of Hb-L may exhibit conformational heterogeneity.

Perhaps the most interesting aspect of the

respiratory pigment of Barbatia is the nature of Hb-H. The heavy hemoglobin fraction, Hb-H, consists of a major 430,000 molecular weight component with a sedimentation coefficient of $S_{20,w} = 13.6$. This 430,000 molecular weight polymer seems to dissociate into 220,000 dalton heme-containing material (9.7 $S_{20,w}$). The 220,000 molecular weight structure does not appear to reaggregate into the larger molecule. This 430,000 molecular weight hemoglobin is strikingly larger than the molecular weight of any intracellular hemoglobin previously studied.

Barbatia Hb-H chromatographs on an anion-exchange column as a single asymmetrical peak suggesting micro-heterogeneity. Isoelectric focusing of Hb-H cyanmethemoglobin indicates that the pigment appears heterogeneous. This heterogeneity may be due to the concurrence of the 430,000 dalton polymer with its putative dissociation product.

Urea gel electrophoresis of Hb-H showed one sharp band which implies that the heavy hemoglobin component of Barbatia consists of one polypeptide. This poly-peptide of Hb-H is distinguished by urea gel electrophoresis from the polypeptides constituting the tetramer (Hb-L). From these findings it seems unlikely that the occurrence of the large Hb-H hemoglobin is the

result of aggregation of the more typical Hb-L tetramer, or its subunits. Instead, as discussed below, the subunit of Hb-H is unlike that seen for any other intracellular hemoglobin studied so far.

Although Hb-H contains 1 heme per approximately 16,000 g protein, the smallest subunit produced by a number of harsh denaturing reagents has a molecular weight of 32,000 by sodium dodecyl sulfate slab gel electrophoresis. Additionally, the Hb-H subunit appeared as a 37,000 dalton fraction by Sephacryl S-200 chromatography in 6 M guanidine hydrochloride with reducing agent. These results are consistant with the hypothesis that the 32,000 dalton subunit is constructed of two covalently linked oxygen binding domains.

If the intact Hb-H pigment is digested with the protease subtilisin prior to sodium dodecyl sulfate slab gel electrophoresis, a major staining band corresponding to 16,500 daltons is observed. The molecular weight of this digestion product resembles the molecular weight of one Hb-H heme containing unit (M_r = 16,000). The digested hemoglobin also showed three minor bands with molecular weights between those of the intact subunit (M_r = 32,000) and the putative domain (M_r = 16,500). These minor bands may correspond to polypeptide fragments produced by digestion of the

subunit at sites other than the link connecting the two domains. The subunit appears to be most susceptable to digestion by subtilisin at the region linking the two domains.

Chromatography by Sephadex G-100 of the digested hemoglobin yielded a 30,000 dalton heme-containing fraction that, when analyzed by sodium dodecyl sulfate electrophoresis, was shown to consist of a 17,000 dalton polypeptide. One possible explanation for this is that the covalent bond linking the domains was cleaved by the enzyme, but that the denaturing agent (sodium dodecyl sulfate) was required to dissociate this cleaved subunit into monodomains. Gel filtration of the digested hemoglobin also yielded a 57,000 dalton heme-containing fraction; sodium dodecyl sulfate slab gel electrophoresis of this fraction showed mostly 17,000 dalton with some 32,000 dalton materials. This 57,000 dalton column isolated fraction of the digested hemoglobin probably consists of dimers of cleaved or undigested subunits. The void volume fraction of the chromatographed digested hemoglobin was shown by sodium dodecyl sulfate slab gel electrophoresis to consist mostly of the 32,000 dalton subunit. From these analyses of the digested hemoglobin, it appears that the covalently linked oxygen binding domains of the subunit may be

additionally held together by non-covalent bonds,

possibly similar to those bonds that stabilize a typical

dimeric hemoglobin.

Each 16,500 dalton oxygen binding domain of

Barbatia Hb-H is similar in molecular weight to the

typical myoglobin like subunit of many invertebrate

and all vertebrate hemoglobins. The didomain structure

proposed for the subunit of Hb-H Barbatia hemoglobin

is similar to the subunit structure reported for the

extracellular hemoglobin of the branchiopod crustacean

Lepidurus bilobatus (Dangott and Terwilliger, 1979).

Lepidurus extracellular hemoglobin has a molecular

weight of 680,000 with a 33-34,000 molecular weight

subunit composed of 14,800 and 16,500 molecular weight

oxygen binding domains (Dangott and Terwilliger, 1979).

The bivalve mollusc Cardita borealis contains a large

($M_r = 12 \times 10^6$) extracellular hemoglobin that has a

.300,000 dalton subunit consisting of 15-17,000 dalton

oxygen binding domains (Terwilliger and Terwilliger,

1978a). The planorbid snails Helisoma and Planorbis

contain large extracellular hemoglobins ($M_r = 1.7 \times 10^6$)

with subunits of 175,000 daltons (Waxman, 1975; Wood

and Mosby, 1975; Terwilliger et al, 1976) composed of

15,000 molecular weight domains (Terwilliger et al,

1978). It has been suggested (Terwilliger et al, 1978)

that these large extracellular molluscan hemoglobins
are composed of subunits which contain linearly linked
oxygen binding domains similar to the molluscan
hemocyanins (Brouwer, 1976). The structure of Barbatia
Hb-H, while resembling some of these extracellular
pigments, is unique among any intracellular hemoglobin
previously studied. It is very large for an intra-
cellular hemoglobin (M_r = 430,000), and has a subunit
of 32,000 daltons consisting of two oxygen binding
domains.

The oxygen equilibrium of Barbatia Hb-L shows
strong cooperativity that is independent of pH with
$N_{\frac{1}{2}}$ = 2.2 at 20 ^{O}C. The oxygen affinity of this tetramer
has a P_{50} = 17.5 mm Hg at 20 ^{O}C that is unaffected by
pH in the range pH 6.8 - 7.6. At pH 8.0 the oxygen
half-saturation value is P_{50} = 21.5 mm Hg; this apparent
reverse Bohr effect has a value ϕ = 0.2.

Several other arcid species have tetramers that
display similar cooperativities but higher oxygen
affinities than those displayed by the tetramer of
Barbatia. The tetramer of Anadara senilis has a
P_{50} = 8.0 - 13.0 mm Hg at 28 ^{O}C (Djangmah et al, 1978),
whereas the tetramer of Barbatia has a P_{50} = 27.7 - 35.5
mm Hg at 30 ^{O}C, depending on pH. Ohnoki et al (1973)
reported that Anadara satowi tetrameric hemoglobin has

a P_{50} = 11.0 mm Hg with $N_{\frac{1}{2}}$ = 2.1 at 20 $^{\circ}$C; this tetramer displays a reverse Bohr effect similar to Barbatia, but at acid pH values. Although the tetramer of Anadara broughtonii exhibits no heterotropic binding properties, it shows strong cooperativity with $N_{\frac{1}{2}}$ = 2.8 - 3.0 (Furuta et al, 1977).

The oxygen binding properties of Barbatia Hb-L varied depending upon the experimental pH and temperature. At pH 7.2 the cooperativity of Hb-L was observed to decrease slightly with increasing temperature above 25 $^{\circ}$C. A similar decrease in cooperativity with increasing temperature was reported for hemoglobin extracts from three Anadara species by Collett and O'Gower (1972). The oxygen affinity of Barbatia Hb-L at pH 8.0 decreased with increasing temperature in the range 15 - 30 $^{\circ}$C (ΔH = -7.3 kcal/mole). Similarily, the heat of oxygenation of Hb-L at pH 7.2 for the temperature interval 25 - 35 $^{\circ}$C is ΔH = -8.7 kcal/mole. These thermal characteristics of Barbatia Hb-L are like those seen for vertebrate hemoglobins, that is, the oxygen affinity decreases with increasing temperature (Antonini, 1965). The heats of oxygenation for Hb-L given above are also near the range (-9 to -14 kcal/mole) reported for other hemoglobins (Rossi-Fanelli et al, 1964). However, under some experimental conditions

the oxygen equilibrium of Hb-L displayed low temperature sensitivity; at pH 7.2 from 15 - 25 $^{\circ}$C the heat of oxygenation ΔH = -1.5 kcal/mole. Freadman and Mangum (1976) suggested that the low temperature dependence of Noetia ponderosa hemoglobin at 10 $^{\circ}$C (ΔH = -2.12 kcal/mole) may permit this pigment to operate at high blood PO_2's, which would be expected to occur at low environmental temperatures. A possible physiological role for hemoglobin with temperature independent behavior at high environmental temperatures is discussed below.

The large molecular weight fraction of Barbatia hemoglobin (Hb-H) has a very low oxygen affinity (P_{50} = 36 mm Hg at 20 $^{\circ}$C pH 7.2 - 8.0) compared to other intracellular and extracellular hemoglobins. The oxygen affinity of Hb-H is independent of pH in the range 7.2 - 8.0, but may be slightly variable below pH 7.2. The pigment displays moderate cooperativity ($N_{\frac{1}{2}}$ = 1.8 at 20 $^{\circ}$C) that is independent of pH at 20 $^{\circ}$C.

Oxygen equilibrium studies of Barbatia Hb-H were also carried out at a variety of temperatures. At pH 8.0 the cooperativity of Hb-H was observed to increase slightly with increasing temperature above 25 $^{\circ}$C. The heat of oxygenation for the pigment at pH 8.0 in the temperature range 15 - 35 $^{\circ}$C has a value ΔH = -4.5 kcal/mole. At pH 7.2 in the temperature range 15 - 25 $^{\circ}$C

the heat of oxygenation is the same as that observed
for the pigment at the higher pH. These heats of
oxygenation suggest that the oxygen affinity of the
pigment under the above conditions show a lower
sensitivity to changes in temperature than most other
hemoglobins. Weber (1972) reported that the low value
of ΔH = -5.3 kcal/mole for hemoglobin from the lugworm
Arenicola marina indicates that the oxygen affinity
of this pigment is relatively insensitive to changes
in environmental temperature.

Remarkably, at pH 7.2 and above 25 ^{O}C, Barbatia
Hb-H displays very little sensitivity to temperature:
ΔH = -1.6 kcal/mole from 25 to 30 ^{O}C and, ΔH = +0.1
kcal/mole from 30 to 35 ^{O}C. This unusually low tempera-
ture sensitivity of Barbatia Hb-H hemoglobin at high
temperature was observed to occur at pH 7.2, within the
range (pH 7 - 7.3) given by Freadman and Mangum (1976)
for the intracellular pH of the erythrocyte of the clam
Noetia ponderosa. This suggests that the relatively
temperature independent oxygen binding behavior of
Barbatia observed experimentally at pH 7.2 may serve as
a physiological adaptation in a thermally fluctuating
environment. Data concerning the oxygen content of the
clam's blood at different ambient temperatures is
necessary in order to lend support to this hypothesis.

However, some speculative discussion concerning this hypothesis is presented below.

Barbatia is subject to wide seasonal variations in environmental temperatures with some habitat areas in the Gulf of California reaching over 30 ^{O}C (Robinson, 1974). During these periods of high temperatures the clam would be expected to tolerate a reduction in available dissolved oxygen while its metabolic demand for oxygen concomitantly increases. The low temperature sensitivity of Barbatia Hb-H at high environmental temperatures may be important in subtidal habitats where low levels of available oxygen are likely to occur during summer. Collett and O'Gower (1972) found that hemoglobin extracts from three Anadara bivalve species show an increase in P_{50} values with increasing temperature from 15 to 20 ^{O}C, but that in the range 20 to 30 ^{O}C, the oxygen affinity varied inversely with temperature. Collett and O'Gower (1972) proposed that this unusual thermal characteristic of Anadara hemoglobin is an adaptation to increase the oxygen gradient across the ctenidia when the clam is in warm oxygen depleted water.

In Barbatia, the low temperature sensitivity of Hb-H hemoglobin may facilitate the complete oxygenation of this low affinity pigment, especially when the

clam is subjected to high ambient temperatures. The striking temperature insensitivity of Barbatia hemoglobin may function to facilitate the uptake of oxygen by the clam and be responsible, in part, for the animal's ability to be eurythermal.

The results of oxygen equilibrium studies show that the isolated 30,000 dalton subtilisin-digested hemoglobin fraction combines reversibly with oxygen. The affinity and cooperativity of this digestion product appear consistent with its proposed structure; that is, the 30,000 dalton putative cleaved but undissociated didomain displays oxygen binding properties that appear to resemble those of a typical dimeric clam hemoglobin (Anadara satowi Hb I has a P_{50} = 10 mm Hg and N = 1.5) (Ohnoki et al, 1973). The oxygen affinity of the cleaved but undissociated didomain is greater than that of the undigested pigment while the amount of cooperativity is the same. Although the oxygen binding properties of the uncleaved didomain are not known, the intact subunit may be expected to display a relatively high affinity similar to that of the cleaved but undissociated didomain. The oxygen binding properties of the monodomain are not known.

Oxygen equilibrium studies on Helisoma subtilisin-generated 17-19,000 dalton domains and linked domains

show, in contrast to the intact pigment, high oxygen affinity and no cooperativity or Bohr effect (Terwilliger et al, 1977). On the other hand, isolated domains of Cardita borealis extracellular hemoglobin show oxygen binding properties that are similar to the intact clam pigment (Terwilliger and Terwilliger, 1978a). Although the homotropic and heterotropic oxygen binding character- istics of the intact didomain of Barbatia are not known, the function of the pigment seems to depend upon its intact quaternary structure. In this regard Barbatia Hb-H hemoglobin behaves more like Helisoma hemoglobin than Cardita hemoglobin.

The erythrocytes of Barbatia contain two hemoglobins that have different molecular weights and oxygen affinities. The lighter fraction, Hb-L, has a $P_{50} = 17.5$ mm Hg at 20 ^{o}C in the pH range 6.9 - 7.6 and, the heavier fraction, Hb-H, has a $P_{50} = 36$ mm Hg at 20 ^{o}C in the range pH 7.2 - 8.0. The co-occurrence of these two hemoglobins with considerably different oxygen affinities suggest that the concomitant presence of the two hemo- globins in the clam may allow the transport of oxygen over a wide range of environmental oxygen tensions. Since Barbatia is abundant in the wave exposed rocky intertidal habitat where surface waters are probably well oxygenated, the low oxygen affinity of Hb-H seems

adaptive, and suggests that the pigment functions in oxygen transport rather than storage. The Hb-L hemoglobin component, with a higher oxygen affinity, may serve a role in oxygen transport or storage during periods of low tide. Alternately, Barbatia may utilize pathways of anaerobic metabolism during periods when it is unable to ventilate, as has been reported for other bivalves (de Zwaan and Wijsman, 1975). Further physiological studies are required before an actual physiological function can be prescribed for the hemoglobins of Barbatia.

Several of the arcid bivalves possess intracellular tetrameric and dimeric hemoglobins (Furuta et al, 1977). Barbatia contains a tetramer but lacks the typical arcid dimer. Instead, Barbatia erythrocytes contain a unique large molecular weight hemoglobin constructed of a didomain subunit. One is tempted to speculate that this subunit may be evolutionarily related to the dimer of other arcids derived, perhaps, from the tandem duplication of a globin genome as has been suggested for other large domain structures (Terwilliger and Terwilliger, 1978a). In this respect, sequence studies comparing the domain of Barbatia and the polypeptide of a typical arcid dimer would be interesting. The large molecular weight intracellular hemoglobin found

in the red cells of _Barbatia_ is unique, and appears to be an excellent pigment for further study of domain structure and synthesis.

Figure 1. Chromatography of <u>Barbatia</u> <u>rceveana</u> hemoglobin

on Sephadex G-100. Buffer 0.05 I Tris·HCl (pH 8.0)

0.01 M in NaCl. ● = absorbance 540 nm. Protein

calibration markers: (a) Blue Dextran, (b) bovine

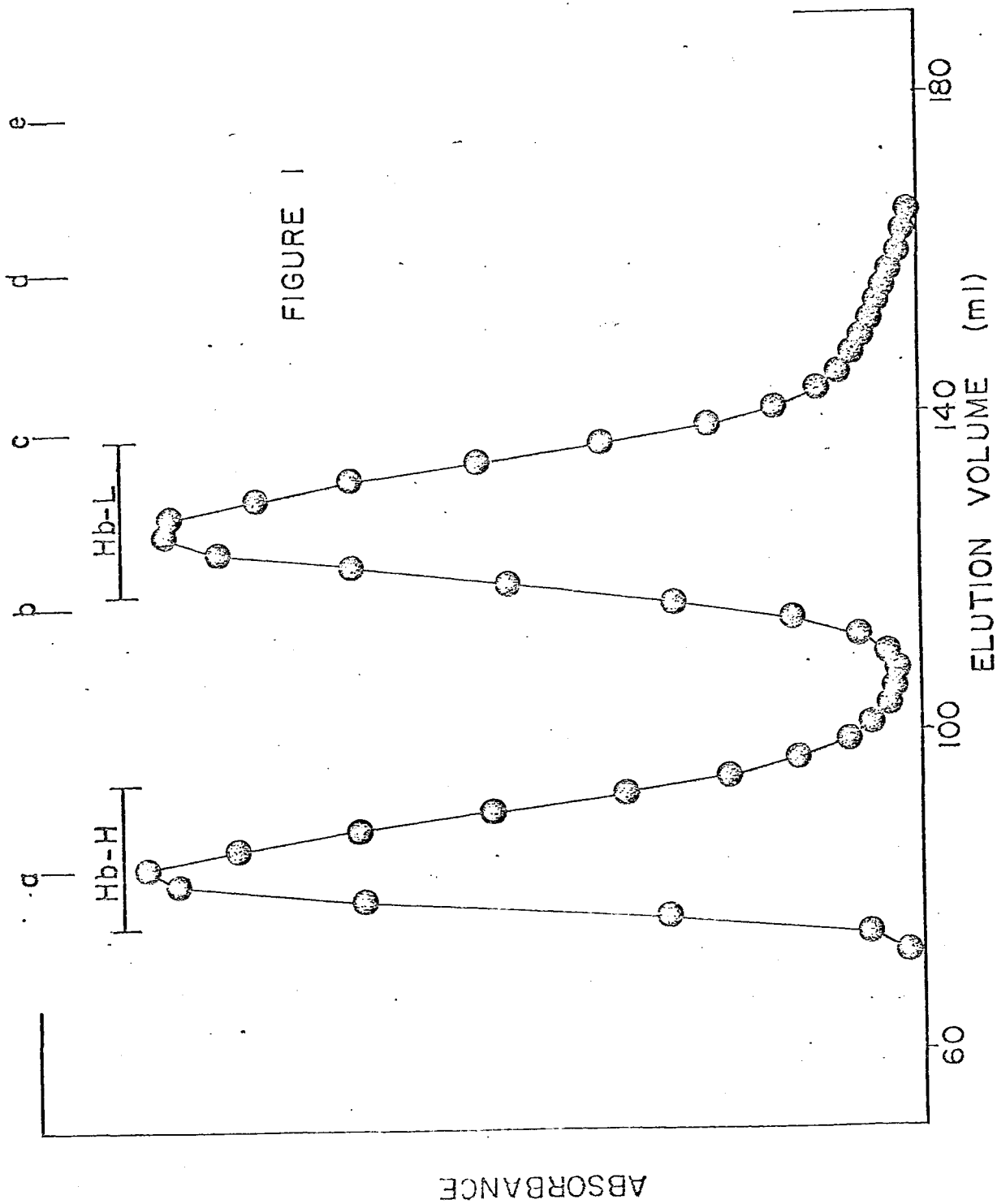serum albumin, (c) ovalbumin, (d) $\propto$ -chymotrypsinogen A,

(e) sperm whale myoglobin.

FIGURE I

Figure 2. Molecular weight of <u>Barbatia reeveana</u> hemoglobin on Sephadex G-100. Data is from Figure 1. Protein calibration markers are (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) $\propto$ -chymotrypsinogen A, (e) sperm whale myoglobin. The arrows indicate the volume at which the hemoglobins elute from the column.

FIGURE 2

ELUTION VOLUME (ml)

MOLECULAR WEIGHT (X 10^4)
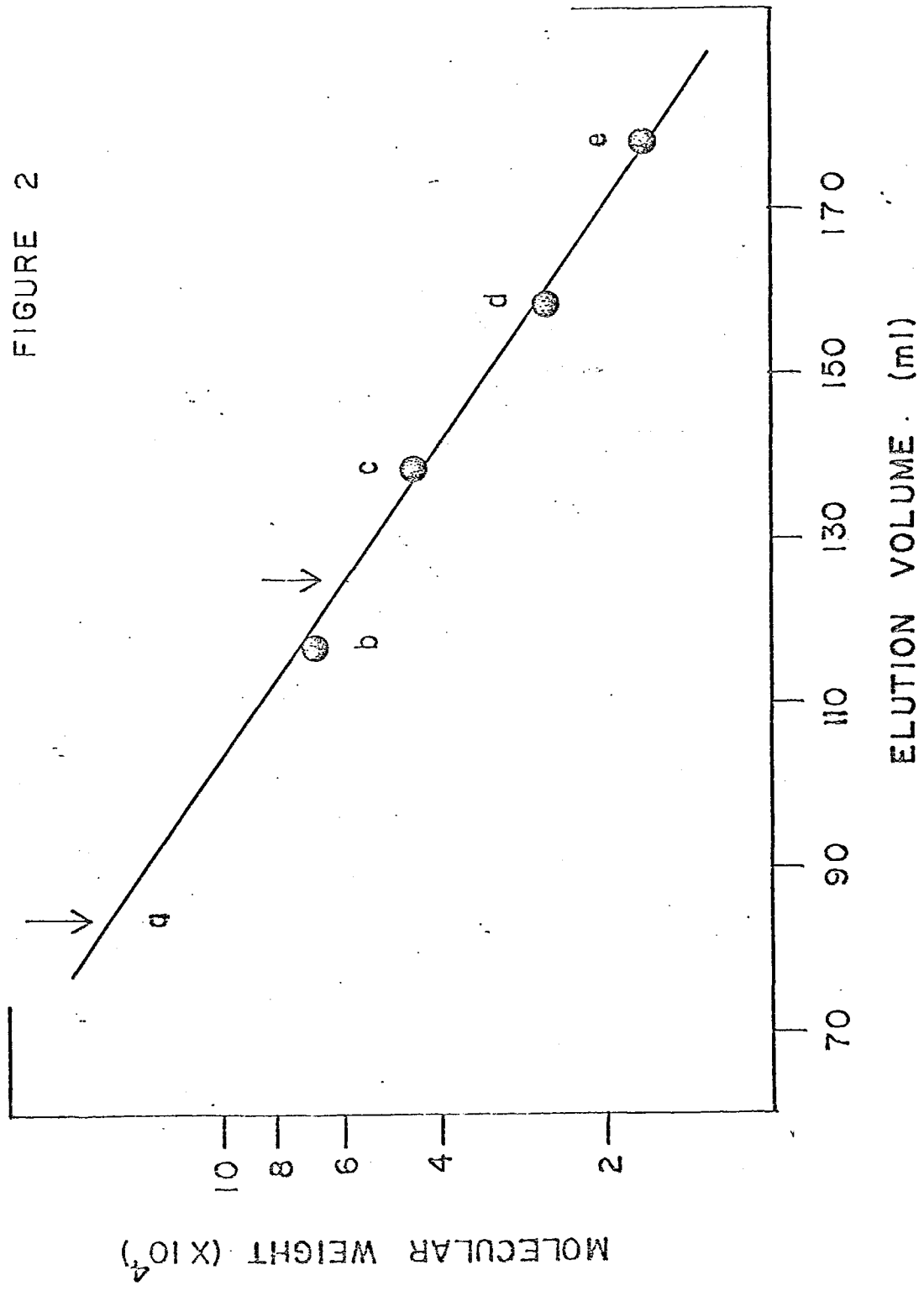
Figure 3. Chromatography of <u>Barbatia</u> <u>reeveana</u> Hb-H
hemoglobin fraction on Bio-gel A-5M. ◐ = Absorbance
at 415 nm. Buffer: 0.05 I Tris·HCl (pH 7.0) 0.01 M
NaCl, 0.01 M MgCl$_2$. Protein calibration markers are
(b) <u>Cancer</u> <u>magister</u> hemocyanin, (c) <u>Cancer</u> <u>magister</u>
hemocyanin, (d) bovine heart lactate dehydrogenase.
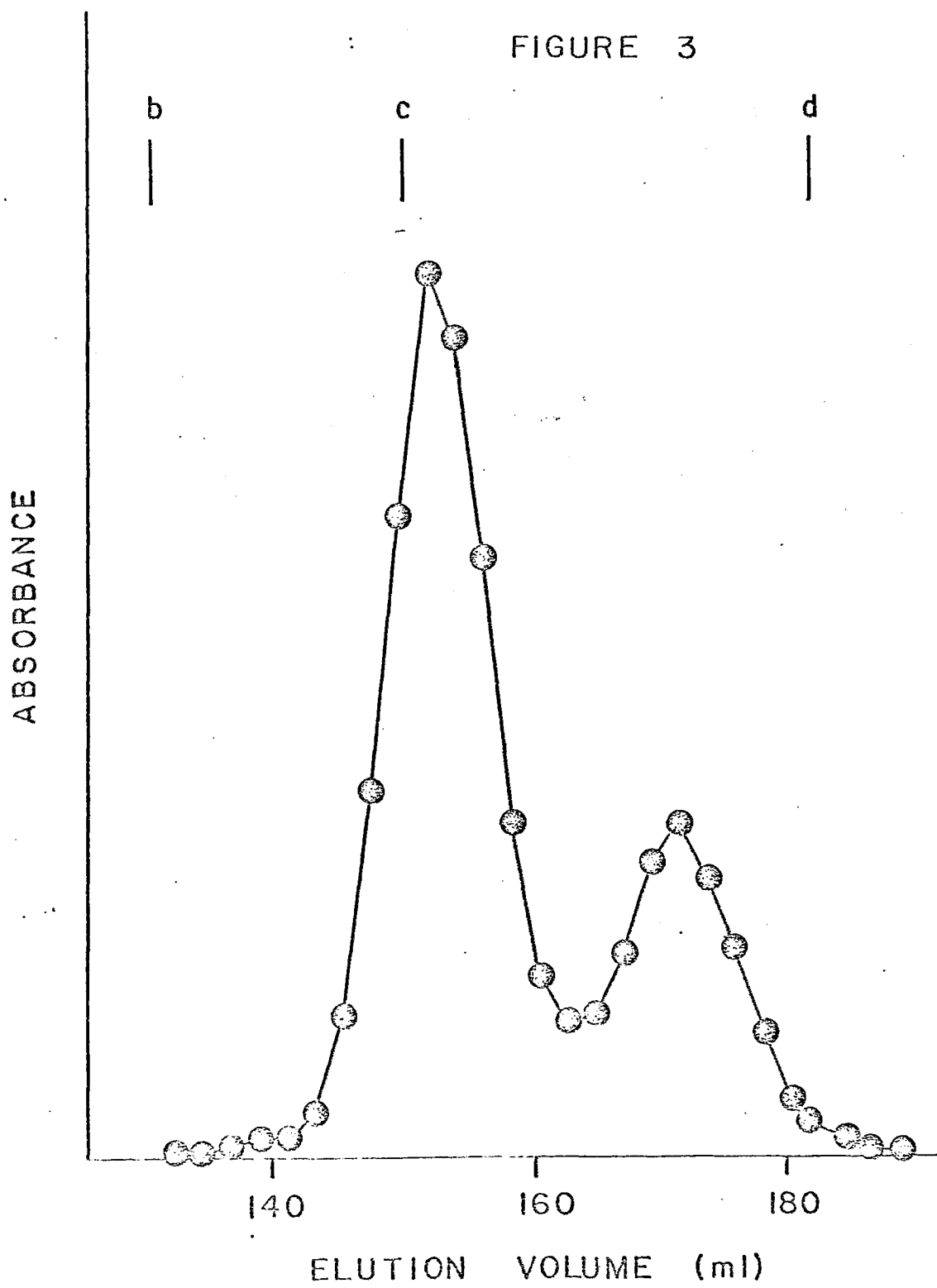
FIGURE 3

Figure 4. Molecular weight of <u>Barbatia</u> <u>reeveana</u> Hb-H
hemoglobin fraction on Bio-gel A-5M. Data is from
Figure 3. Arrows indicate the volume at which the
hemoglobins elute from the column. Calibration
proteins are (a) <u>Eudistylia</u> <u>vancouveri</u> chlorocruorin,
(b) <u>Cancer</u> <u>magister</u> hemocyanin, (c) <u>Cancer</u> <u>magister</u>
hemocyanin, (d) bovine heart lactate dehydrogenase.

FIGURE 4
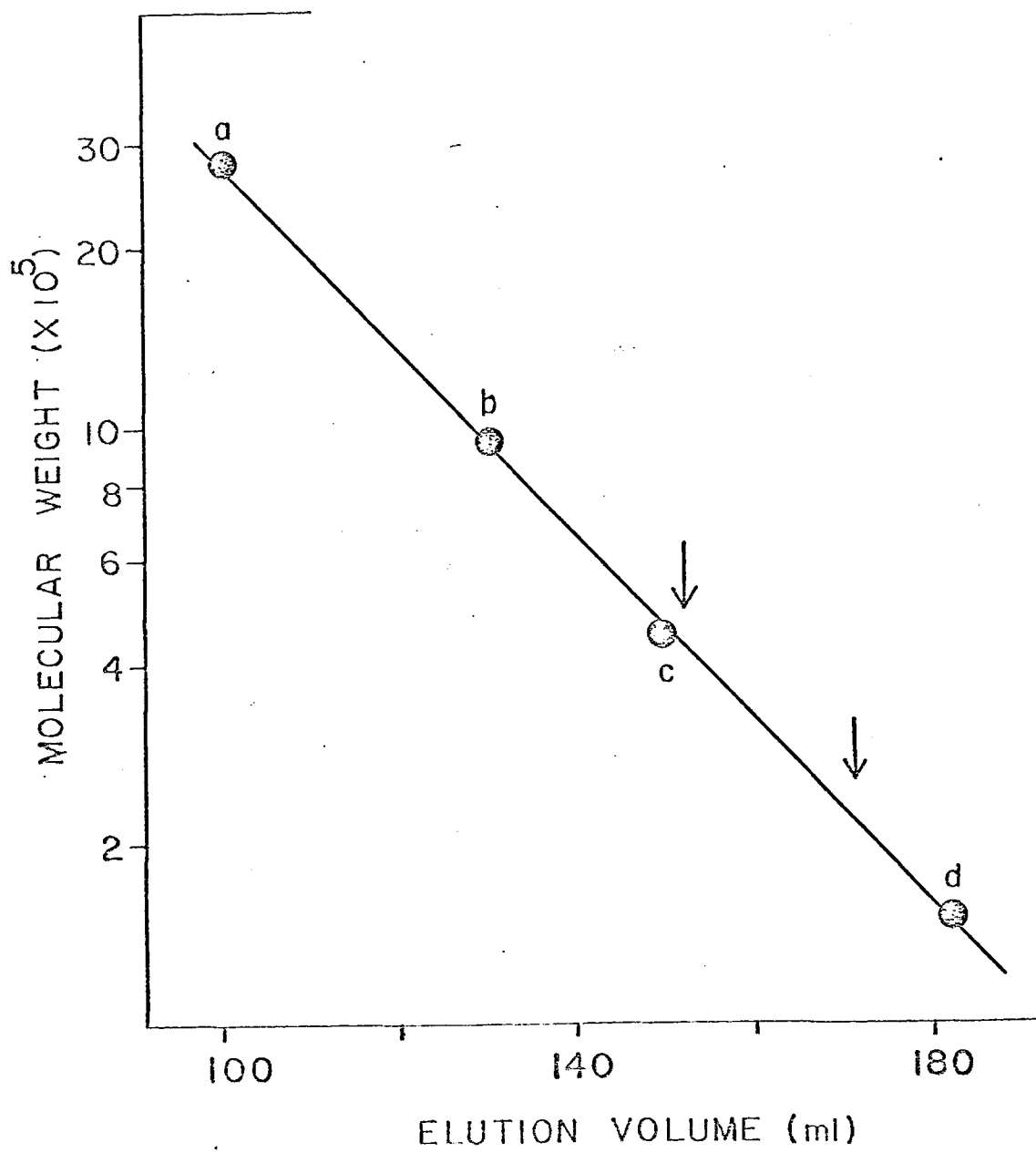
Figure 5. Spectrophotometric scan of isoelectric focused _Barbatia_ _reeveana_ Hb-H cyanmethemoglobin at 415 nm. Gel concentration 5 % acrylamide.
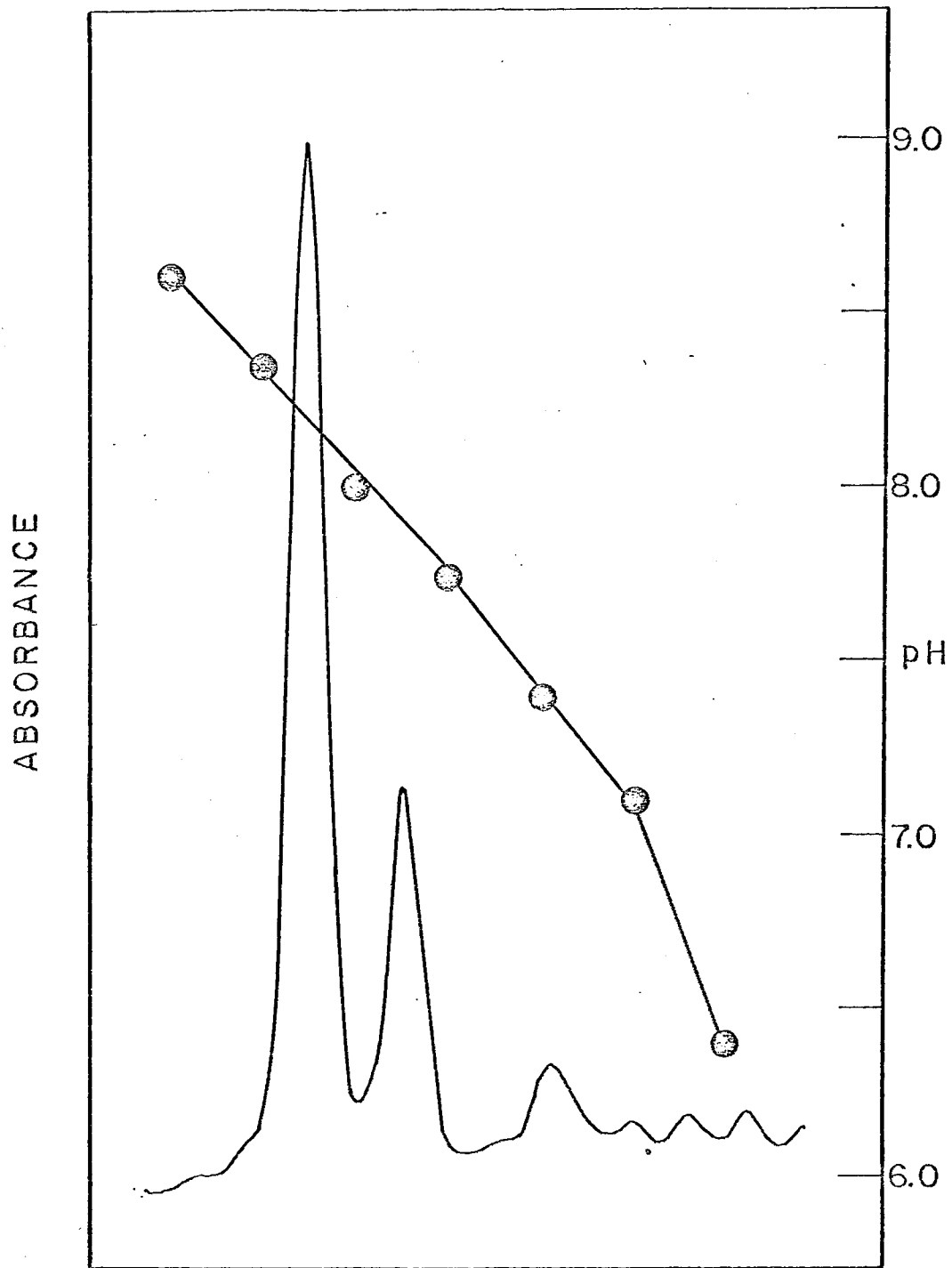
FIGURE 5
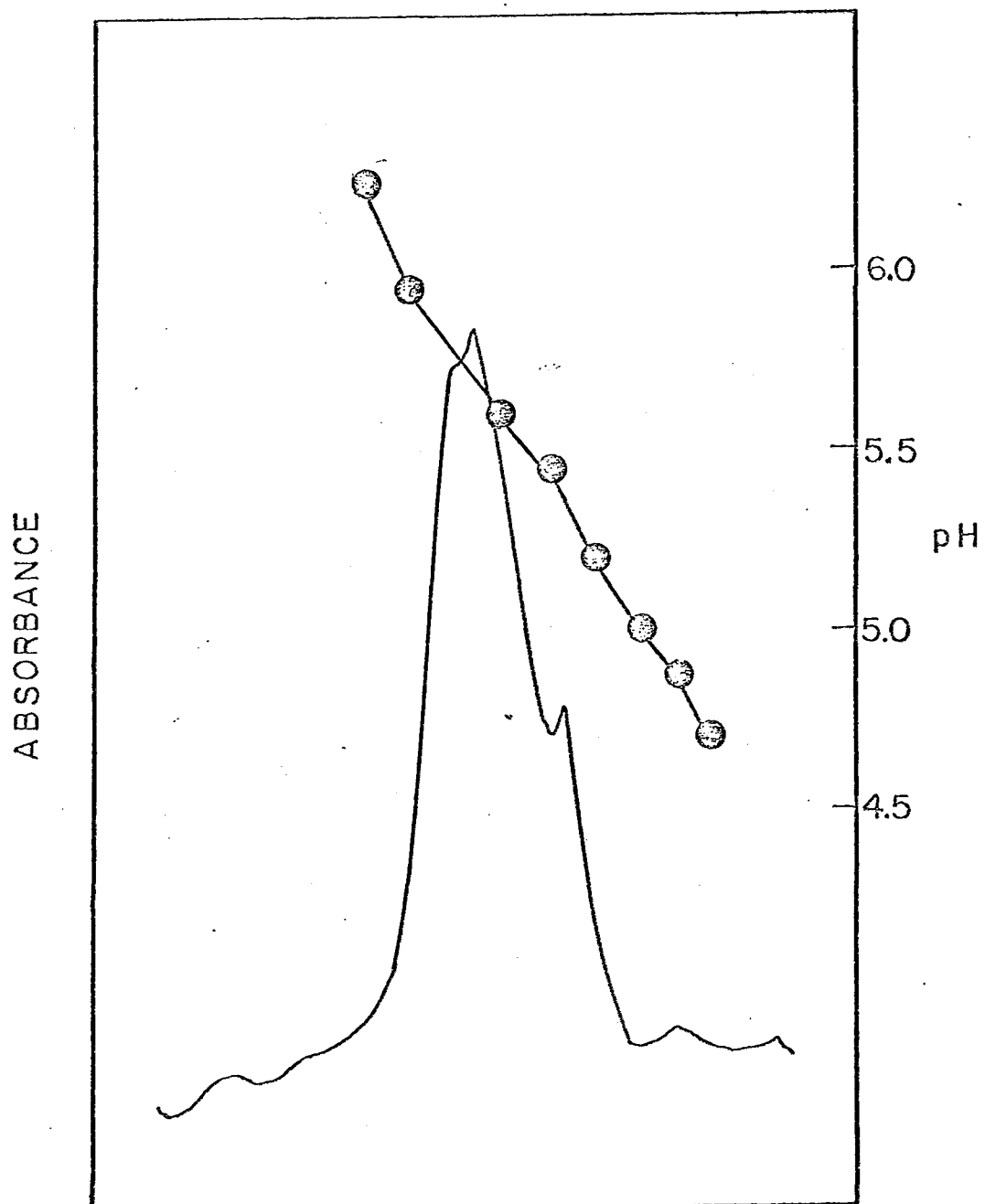
Figure 6. DEAE-cellulose chromatography of <u>Barbatia</u> <u>reeveana</u> Hb-H hemoglobin. $\triangle$, Absorbance at 540 nm, $\bigcirc$, Absorbance at 280 nm. Buffer: 0.01 M NH_4HCO_3. The pigment elutes in the presence of a 0-0.5 M NaCl linear gradient.

FIGURE 6

56

ELUTION VOLUME (ml)
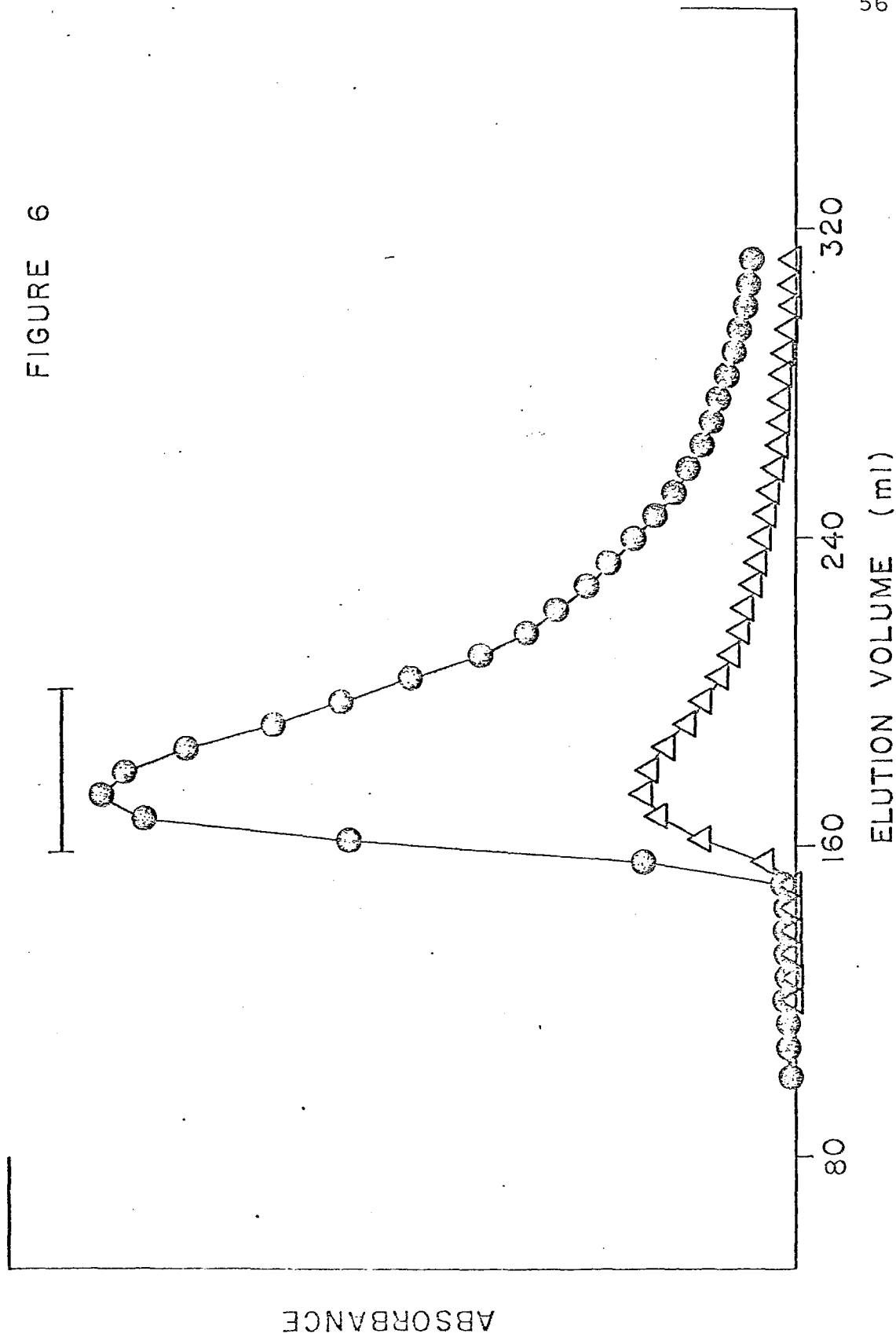
ABSORBANCE

Figure 7. CM-cellulose chromatography of <u>Barbatia</u>
<u>reeveana</u> Hb-L hemoglobin. ▲, Absorbance 540 nm,
◉, Absorbance 280 nm. Buffer: 1 mM sodium phosphate
(pH 6.6). The pigment elutes in the presence of a
0-0.1 M NaCl linear gradient.

FIGURE 7

58
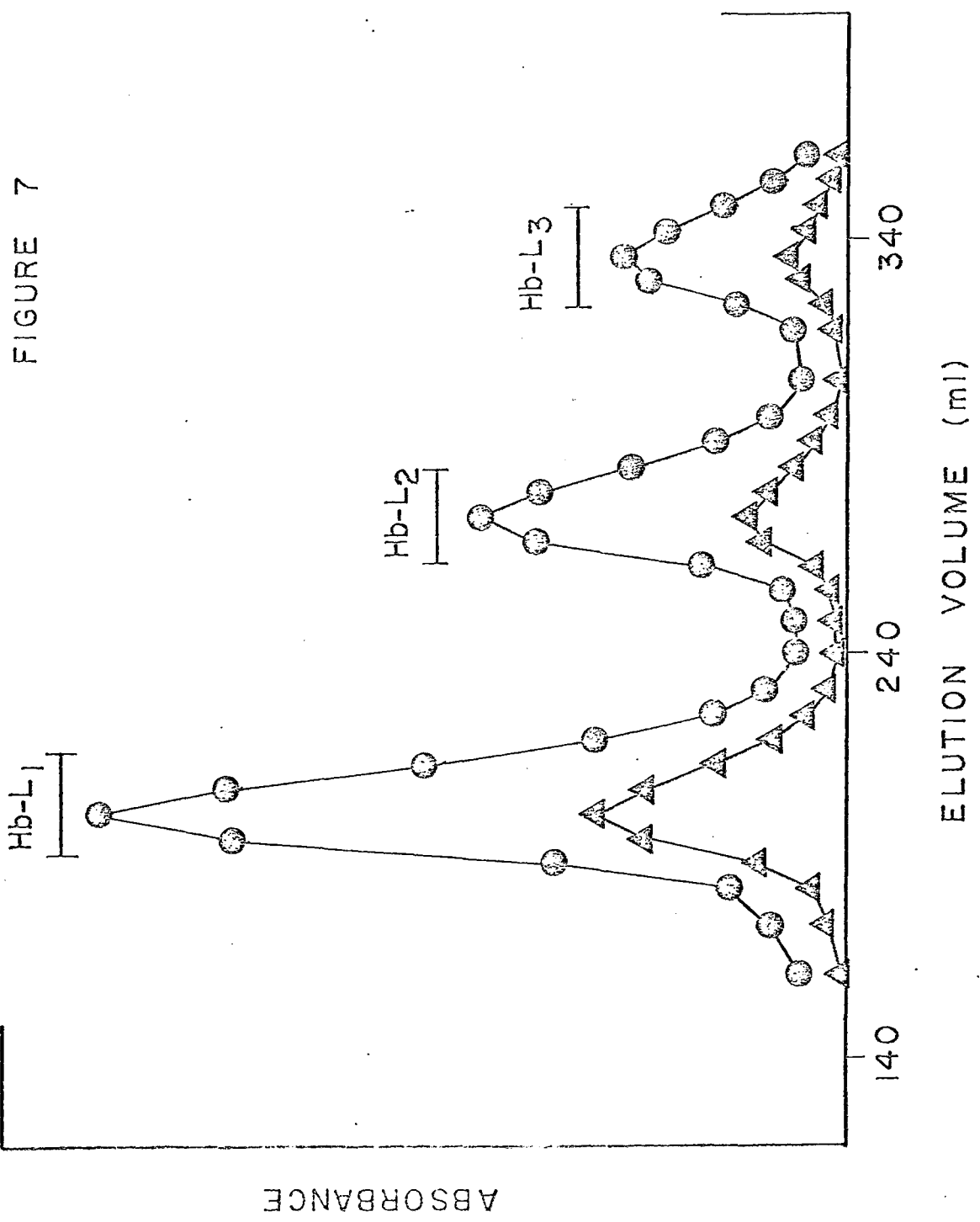
Figure 8. Spectrophotometric scan of isoelectric focused _Barbatia_ _reeveana_ Hb-L cyanmethemoglobin. Gel concentration 5 % acrylamide.

FIGURE 8

Figure 9. Urea gel electrophoresis patterns of

Barbatia reeveana hemoglobins. A. and B.:

A. Barbatia Hb-L hemoglobin fraction; B. Barbatia

Hb-H hemoglobin fraction.

FIGURE 9
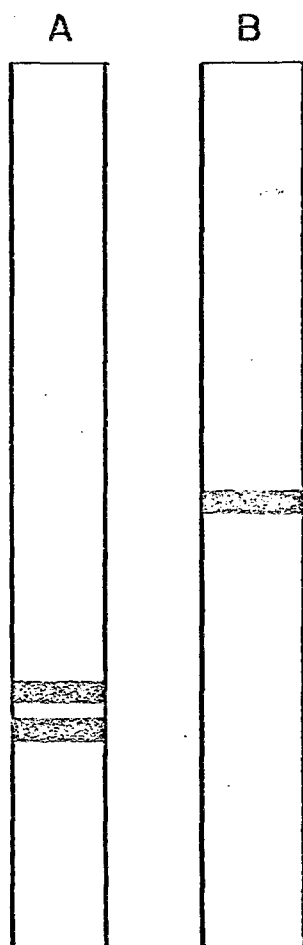
Figure 10. Sodium dodecyl sulfate slab gel electro-
phoresis of _Barbatia_ _reeveana_ Hb-L hemoglobin fraction.
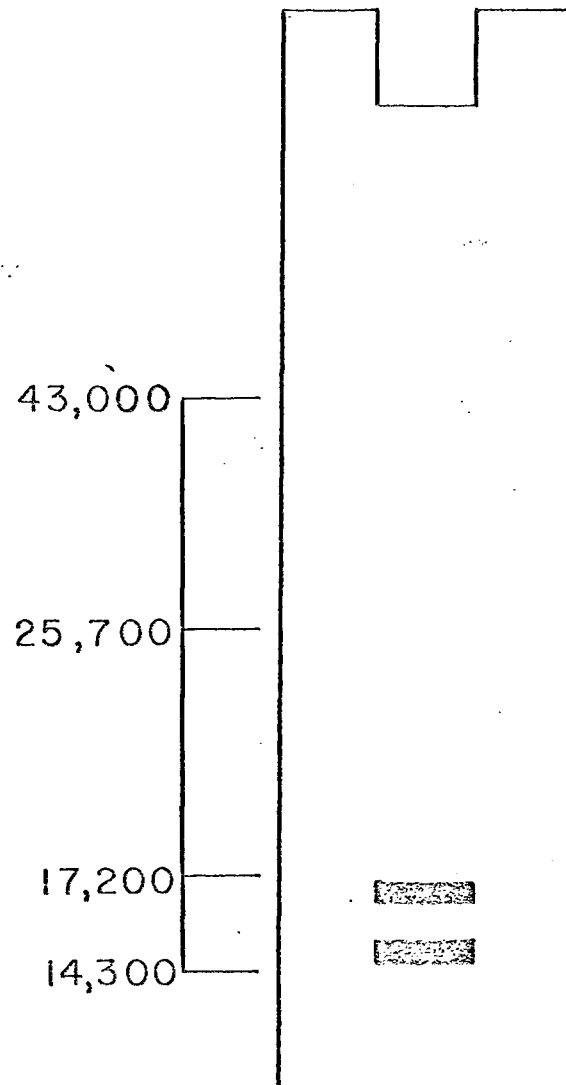Gel concentration 12.5 %.

FIGURE 10

43,000 —

25,700 —

17,200 —

14,300 —

Figure 11. Sodium dodecyl sulfate slab gel electro-
phoresis of Barbatia reeveana Hb-H hemoglobin fraction.
A-D: A. Barbatia Hb-H globin; B. Barbatia Hb-H
performic acid oxidized globin; C. Barbatia Hb-H
globin previously denatured in 6 M guanidine hydro-
chloride; D. sodium dodecyl sulfate slab gel
electrophoresis in 8 M urea of Barbatia Hb-H globin.
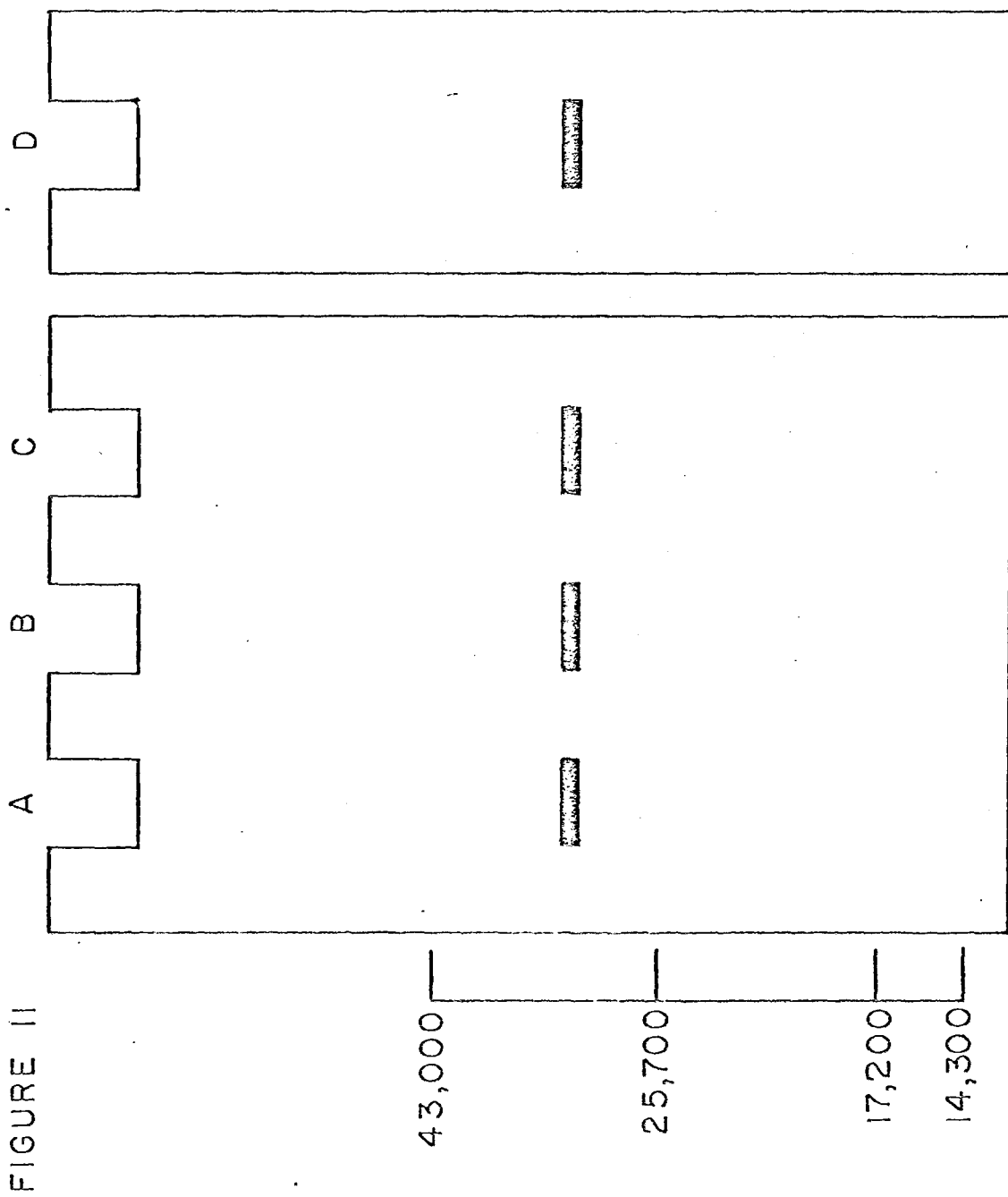Gel concentration 5 %.

FIGURE II

43,000

25,700

17,200

14,300

A   B   C   D

Figure 12. Chromatography of _Barbatia reeveana_ Hb-H hemoglobin fraction on Sephacryl S-200. ⊕ , Absorbance at 280 nm. Buffer 0.05 I Tris·HCl (pH 7.0) 6 M guanidine hydrochloride, 0.5 mM dithiothreitol. Hb-H globin previously reduced. Calibration markers are (a) bovine serum albumin, (b) ovalbumin, (c) α- chymotrypsinogen A, (d) sperm whale myoglobin, (e) potassium ferricyanide.
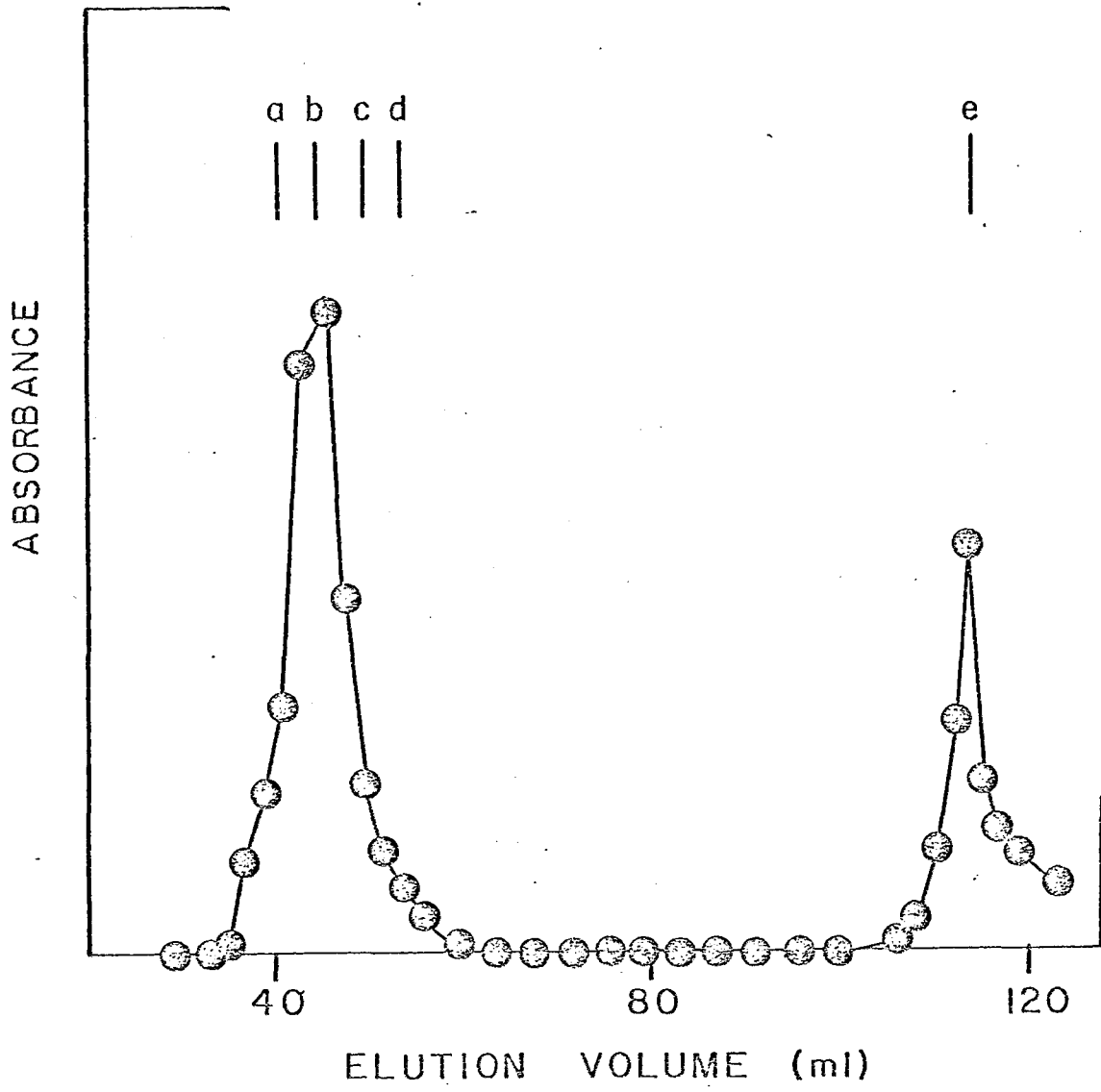
FIGURE 12

Figure 13. Chromatography of <u>Barbatia</u> <u>reeveana</u> Hb-H hemoglobin on Sephacryl S-200. ◉, Absorbance at 540 nm. Buffer: 0.04 M sodium glycinate (pH 9.5), 0.1 M NaCl, 0.01 M EDTA. Calibrant markers: (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) sperm whale myoglobin.
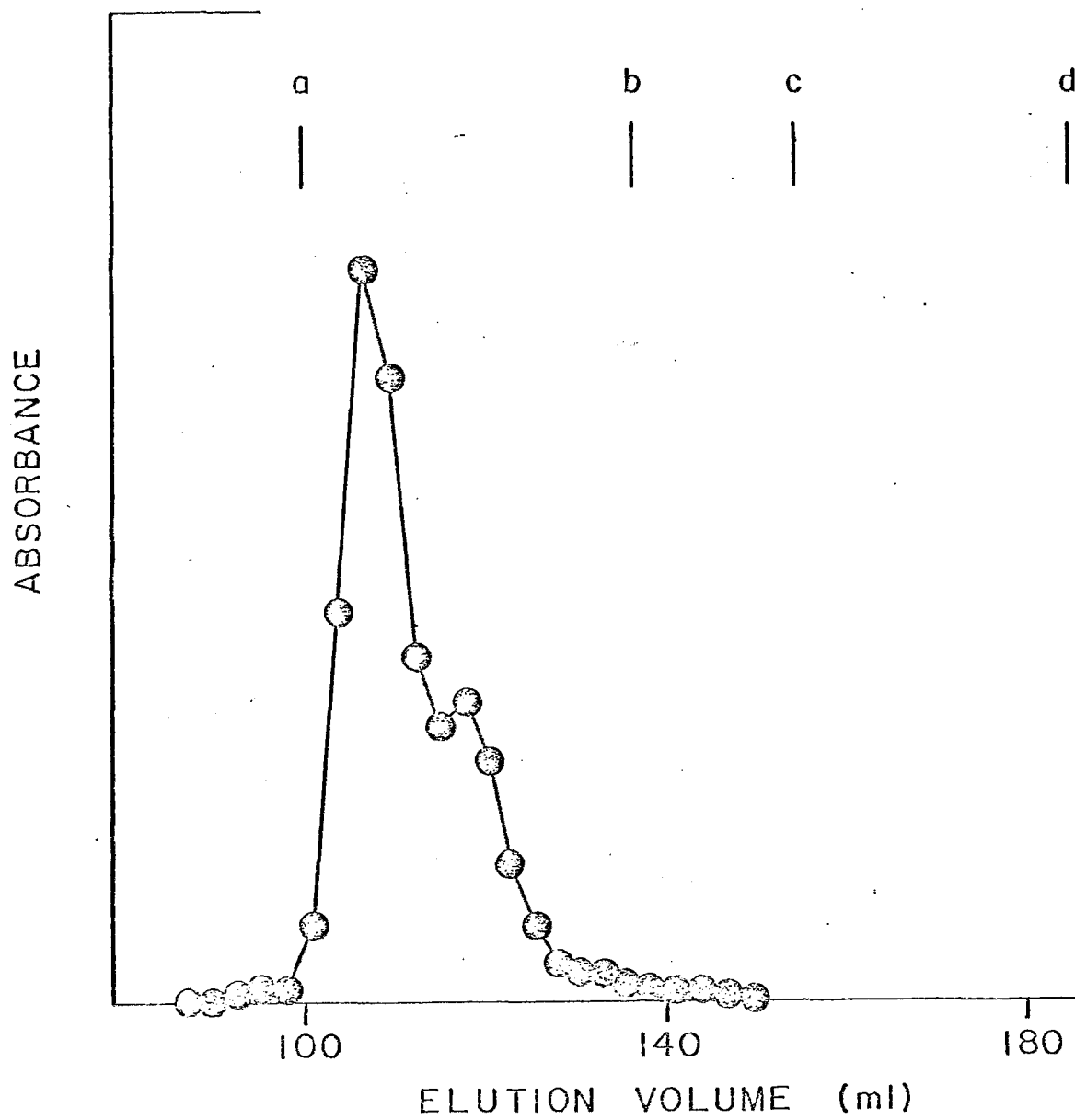
FIGURE 13

Figure 14. Sodium dodecyl sulfate slab gel electro-
phoresis of _Barbatia_ _reeveana_ Hb-H hemoglobin digested
with subtilisin. A-J: A. control hemoglobin, B.
enzyme control, C-J. subtilisin concentrations of:
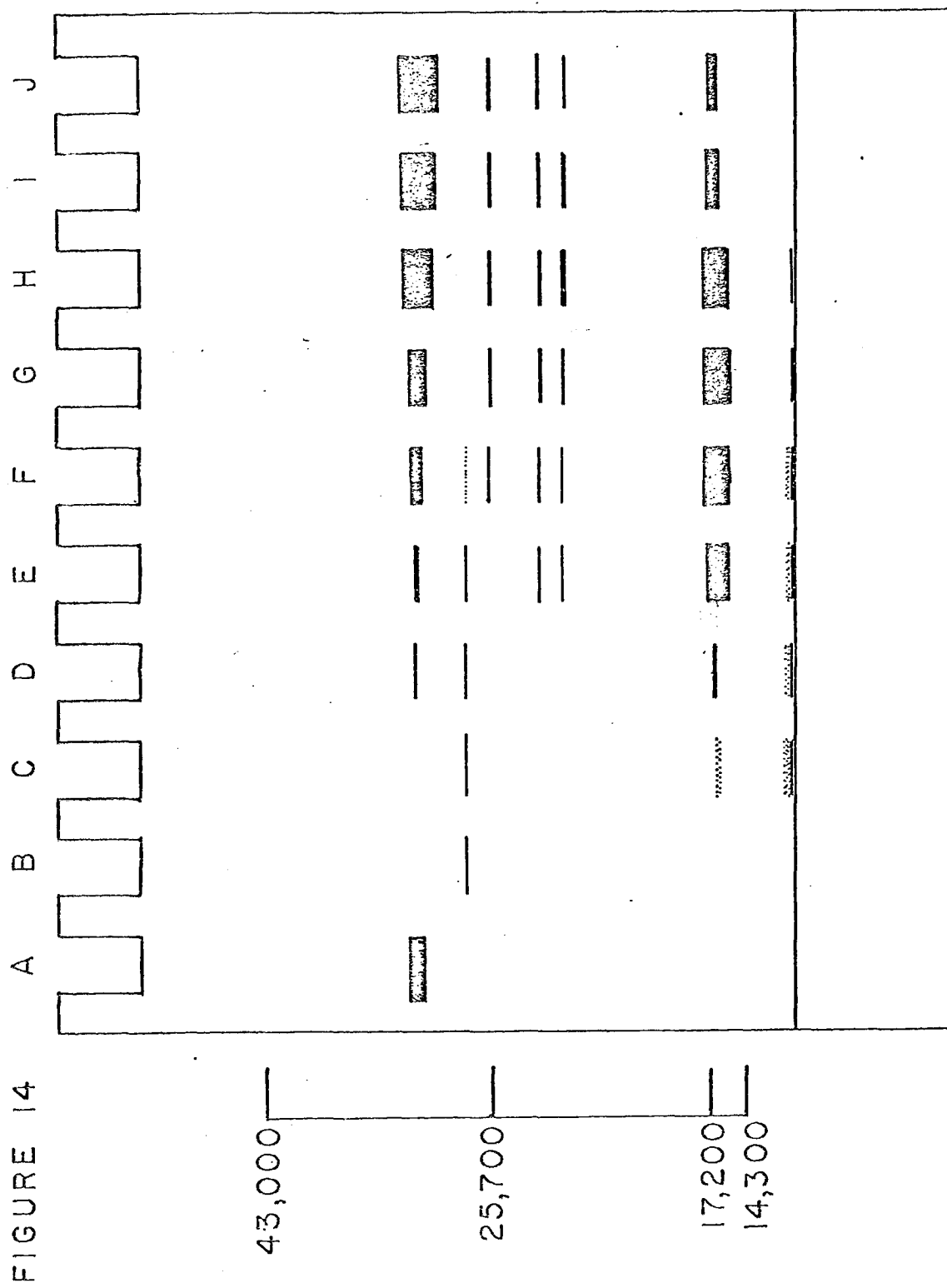C. 1/5, D. 1/10, E. 1/25, F. 1/50, G. 1/100,
H. 1/250, I. 1/500, J. 1/1000.

72

FIGURE 14

A B C D E F G H I J

43,000

25,700

17,200
14,300

Figure 15. Chromatography on Sephadex G-100 of 2 h subtilisin digested <u>Barbatia</u> <u>reeveana</u> Hb-H hemoglobin (1 mg enzyme to 25 mg protein, 24 $^{\circ}$C). $\triangle$, Absorbance at 415 nm. $\bigcirc$, Absorbance at 280 nm. Buffer; 0.05 I Tris·HCl (pH 8.0) 0.1 M NaCl. Calibration markers: (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) $\propto$ -chymotrypsinogen A, (e) sperm whale myoglobin.
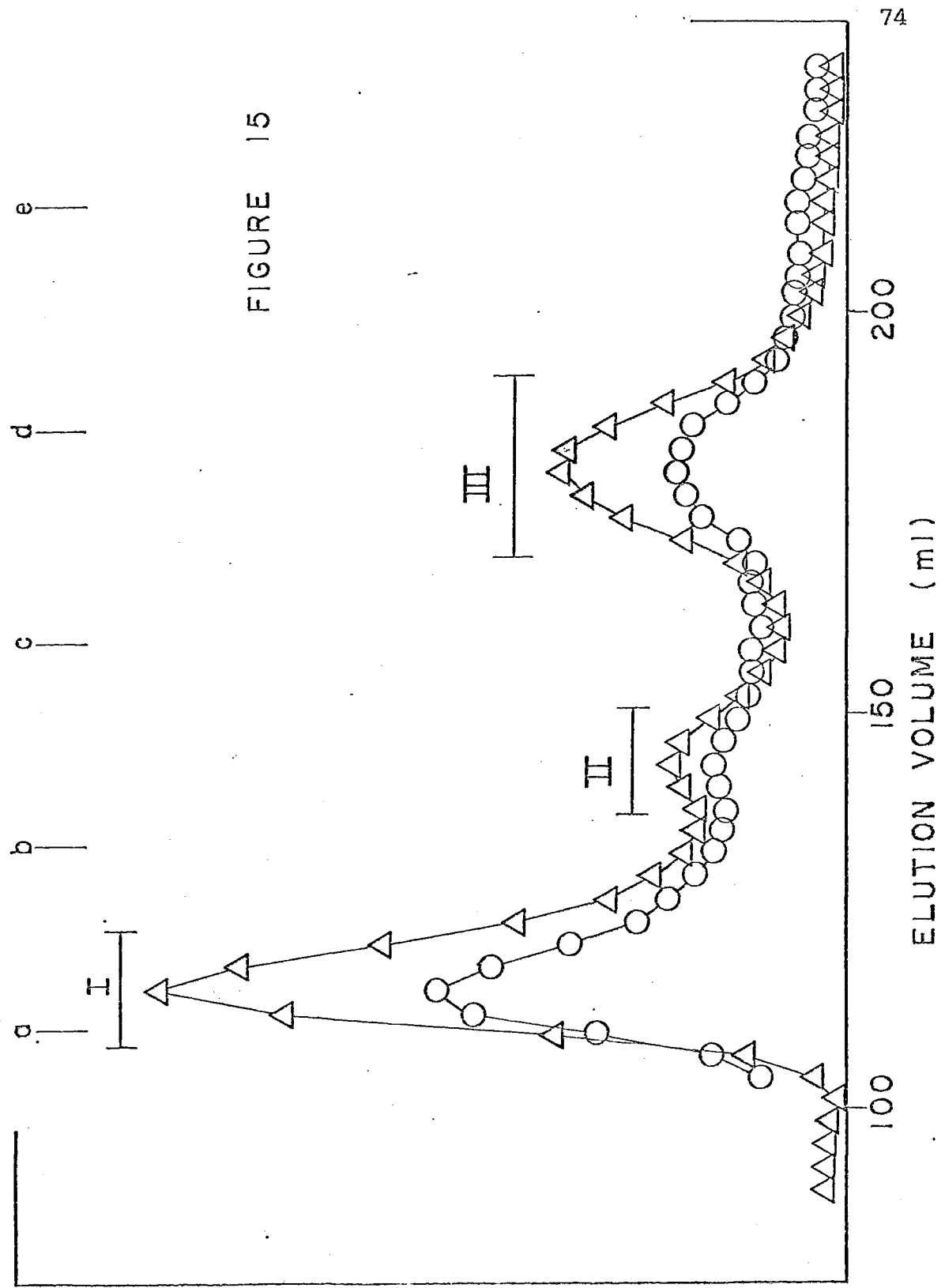
FIGURE 15

ELUTION VOLUME (ml)

ABSORBANCE

Figure 16. Sodium dodecyl sulfate slab gel electro-
phoresis of products isolated from subtilisin digested
Barbatia reeveana Hb-H hemoglobin. Gel concentration
12.5 %. A. hemoglobin control, B. whole digest
aliquot, C. Fraction I, D. Fraction II, E. Fraction
III. The fractions are from hemoglobin obtained as in
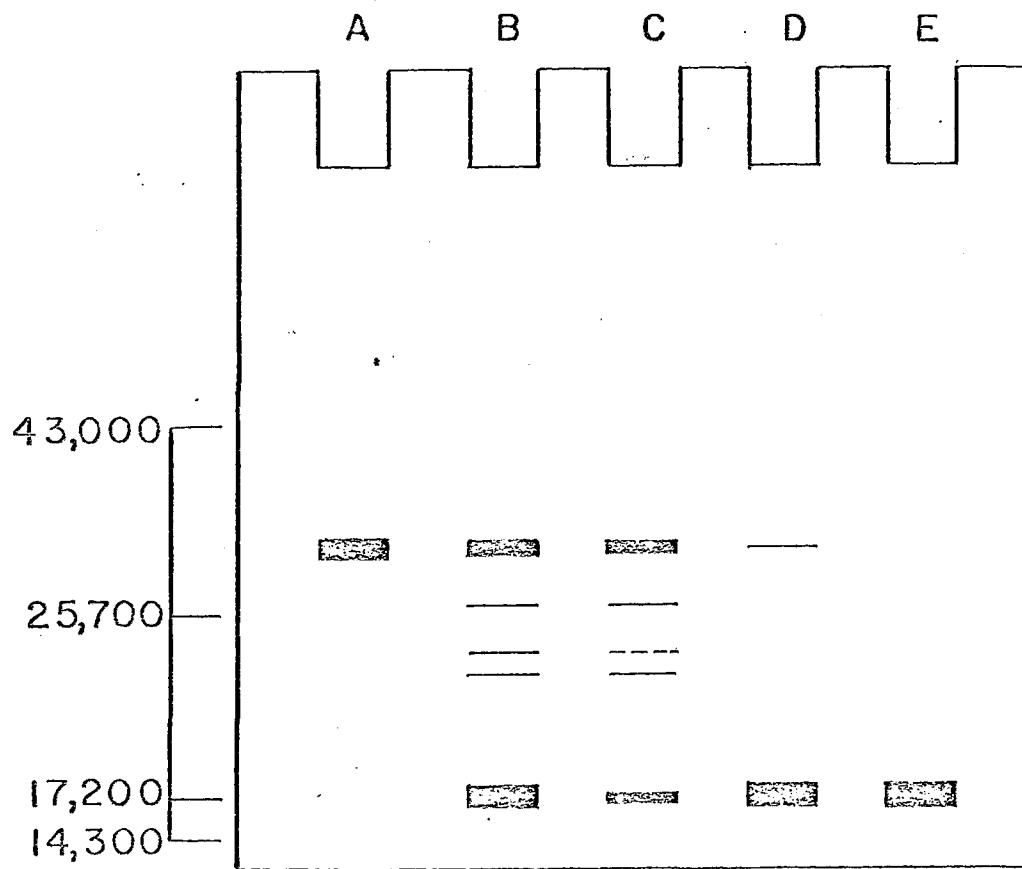Figure 15.

FIGURE 16

Figure 17. Effect of pH on the oxygen equilibrium of
Barbatia reeveana Hb-H hemoglobin at 20 ^{O}C: A and B.
A. Effect of pH on _Barbatia reeveana_ oxygen affinity,
B. Effect of pH on _Barbatia reeveana_ cooperativity.
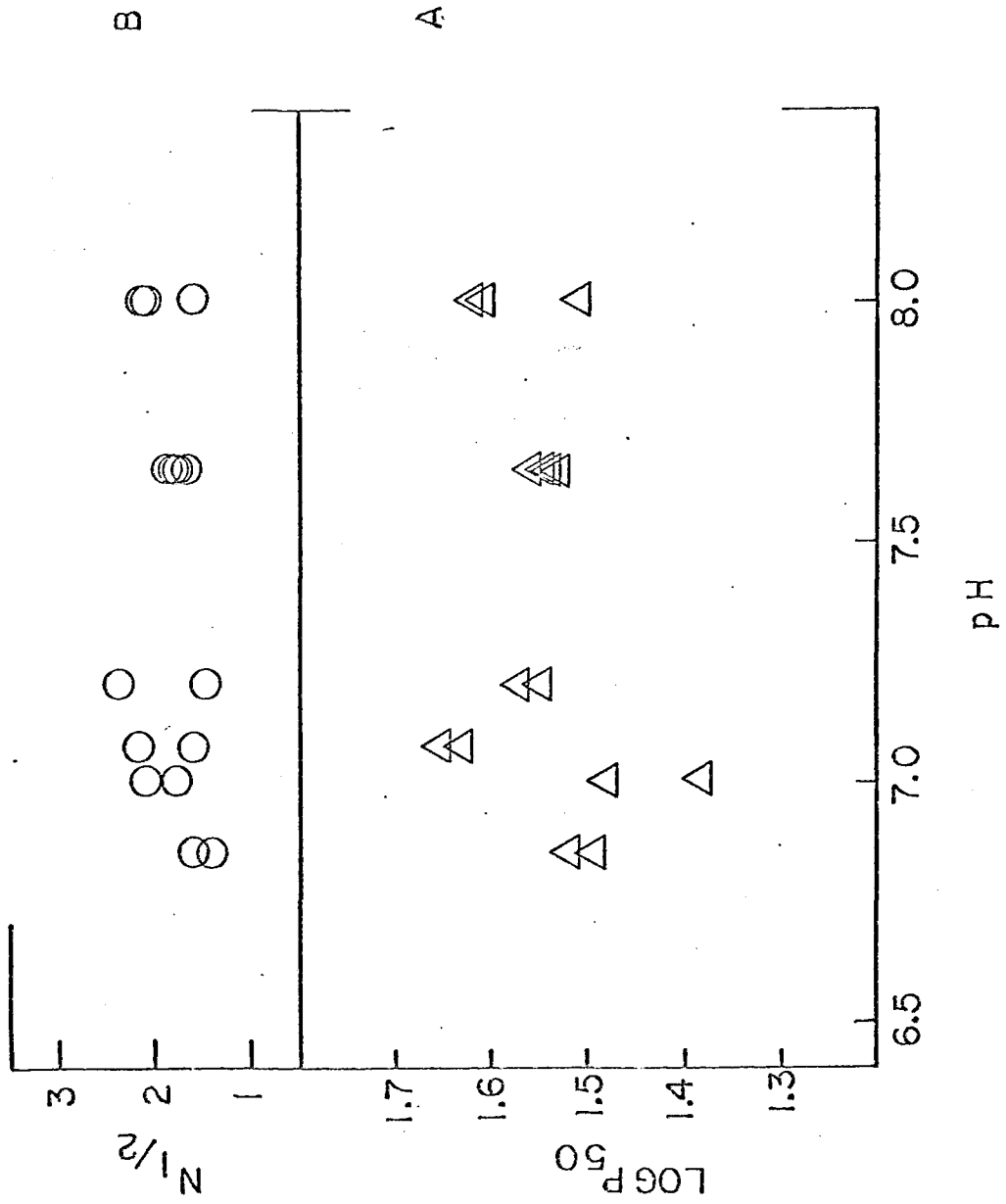
FIGURE 17

Figure 18. Effect of temperature on the oxygen affinity of _Barbatia_ _reeveana_ Hb-H hemoglobin at pH 7.2 and pH 8.0. ●, pH 7.2. ▨, pH 8.0. Temperatures: 15 to 35 ^{O}C. Buffer: 0.05 I Tris·HCl, 0.1 M NaCl. Values represent the mean of two experiments.
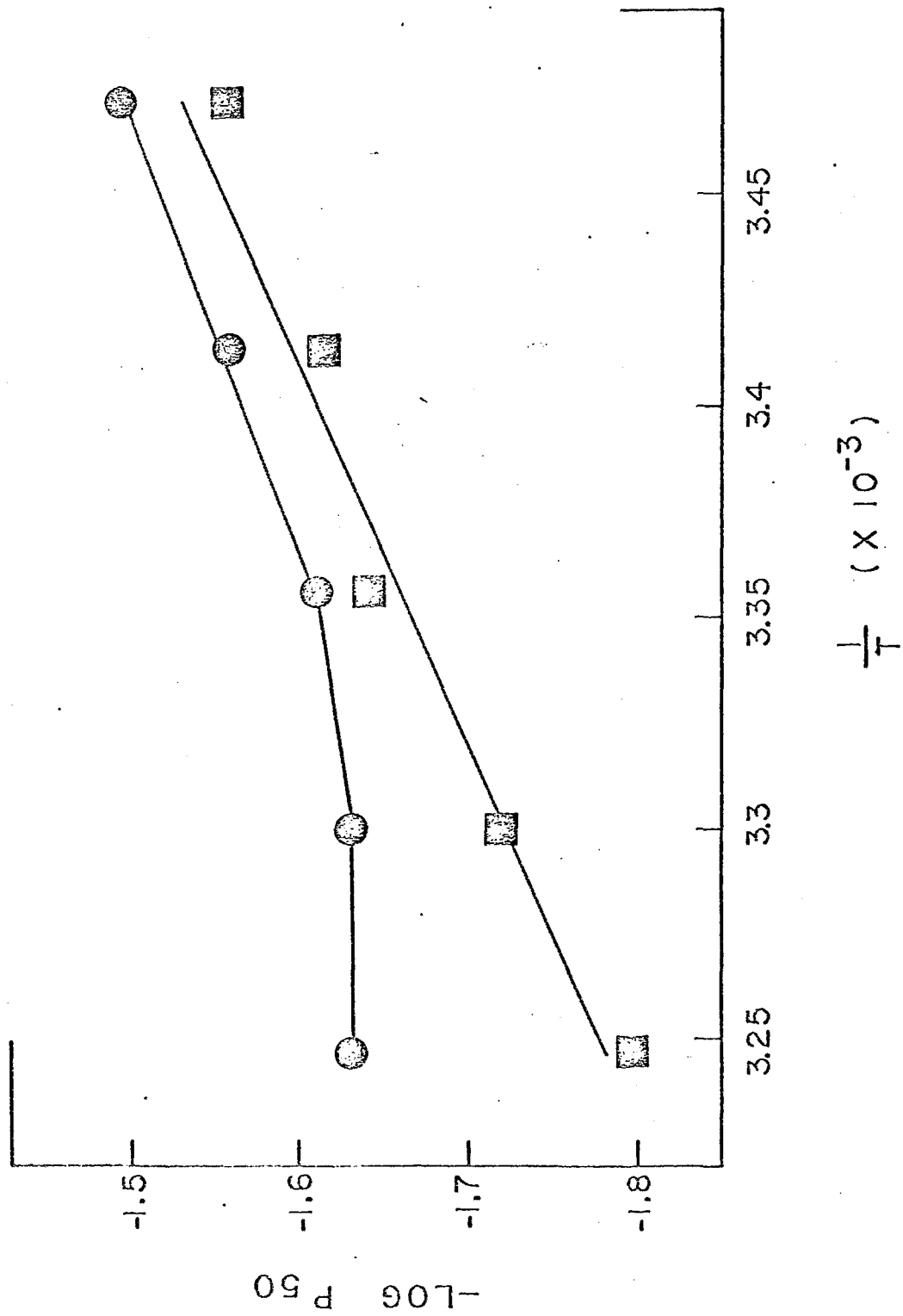
FIGURE 18

Figure 19. Effect of temperature on the cooperativity of _Barbatia_ _reeveana_ Hb-H hemoglobin at pH 7.2 and pH 8.0. O, pH 7.2. △, pH 8.0. Buffer: 0.05 I Tris HCl, 0.1 M NaCl. Temperatures 15-35 ^{O}C.
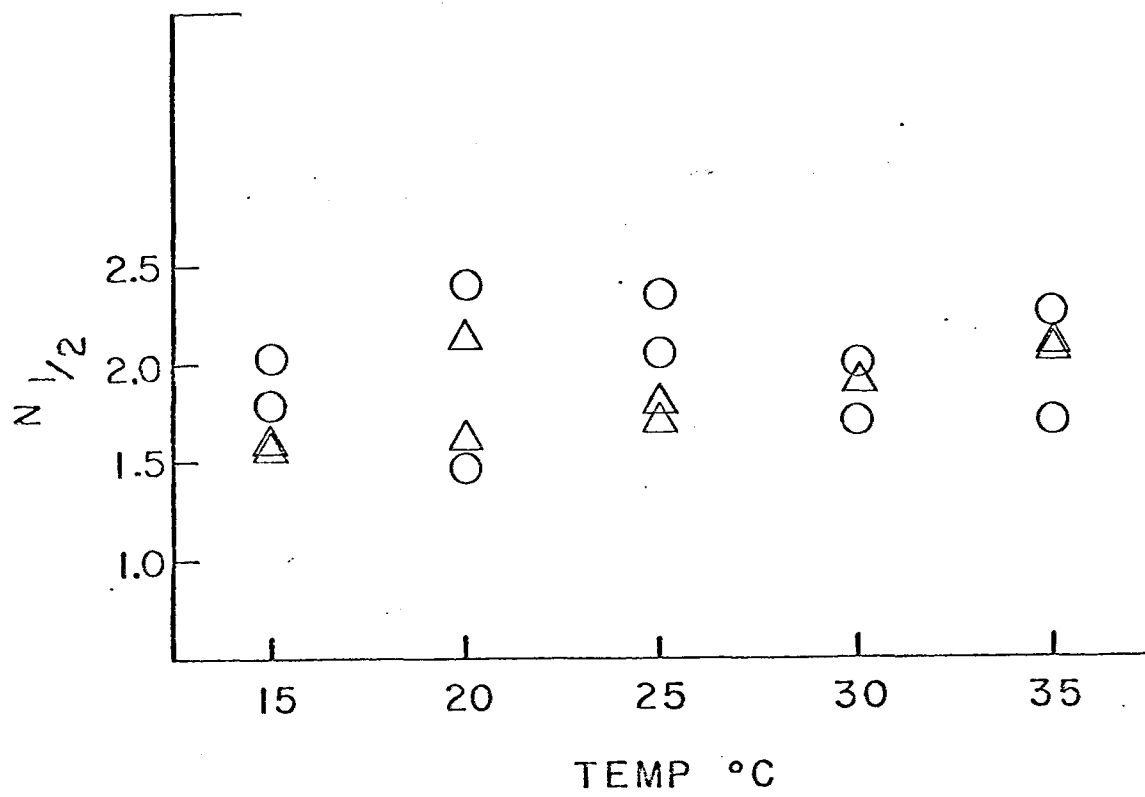
FIGURE   19

Figure 20. Effect of pH on the oxygen equilibrium of
Barbatia reeveana Hb-L hemoglobin at 20 ^{O}C: A and B.
A. Effect of pH on the oxygen affinity of Hb-L;
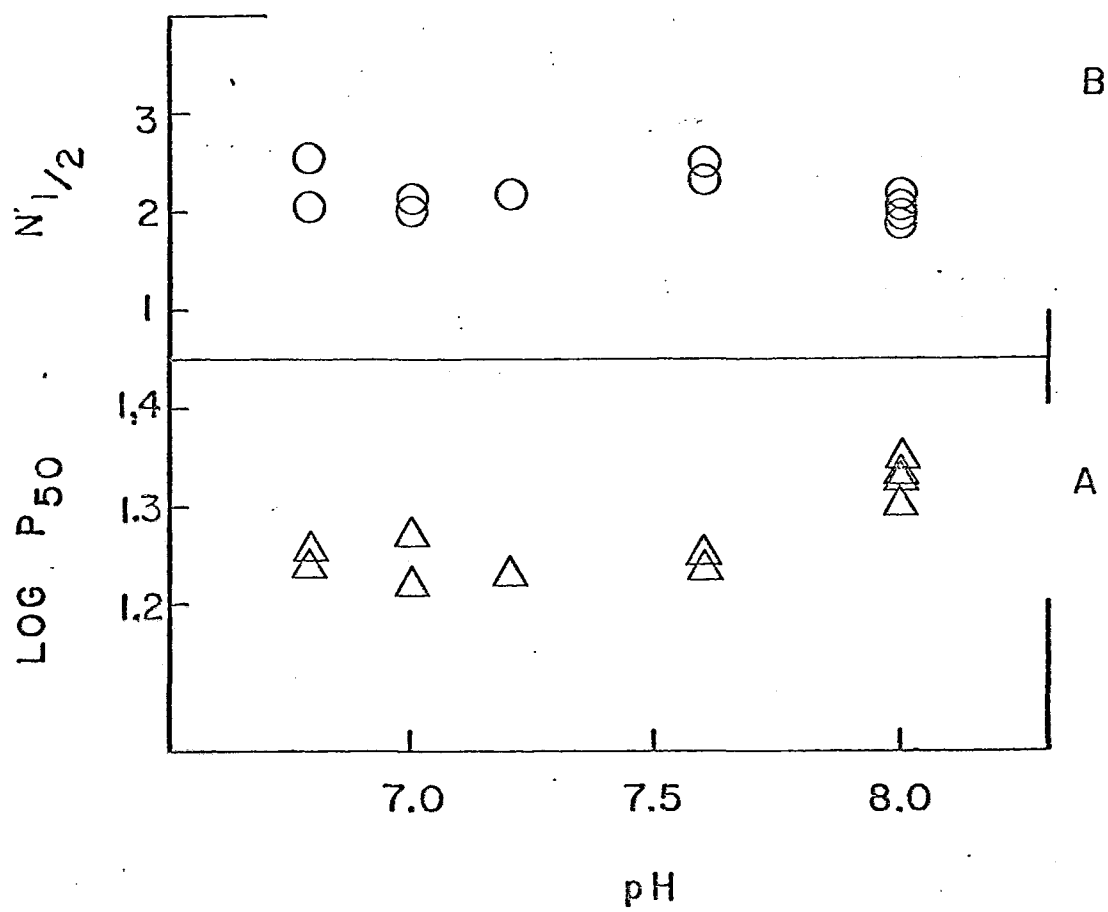B. Effect of pH on the cooperativity of Hb-L.

FIGURE 20

Figure 21. Effect of temperature on the oxygen affinity of _Barbatia_ _reeveana_ Hb-L hemoglobin at pH 7.2 and pH 8.0; ◐, pH 7.2. ▨, pH 8.0. Buffer: 0.05 I Tris·HCl, 0.01 M NaCl. Temperatures 15-35 $^\circ$C. Values represent the mean of two experiments.
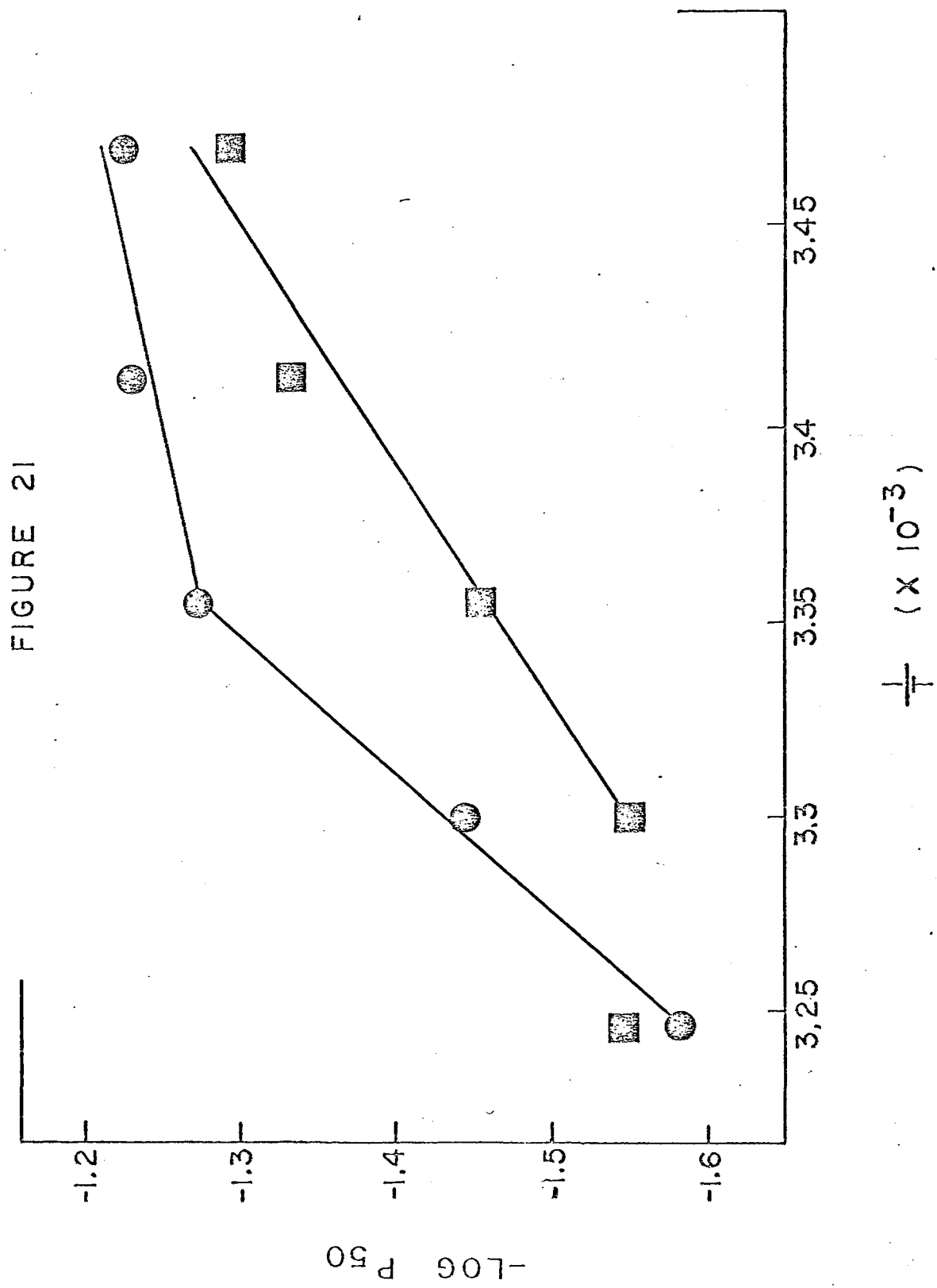
FIGURE 21

−LOG P50 vs $\frac{1}{T}$ ($\times 10^{-3}$)

Figure 22. Effect of temperature on the cooperativity
of _Barbatia_ _reeveana_ Hb-L hemoglobin at pH 7.2 and
pH 8.0. ○, pH 7.2; △, pH 8.0. Buffer: 0.05 I
Tris·HCl, 0.1 M NaCl. Temperatures 15-35 $^{\circ}$C.

FIGURE 22
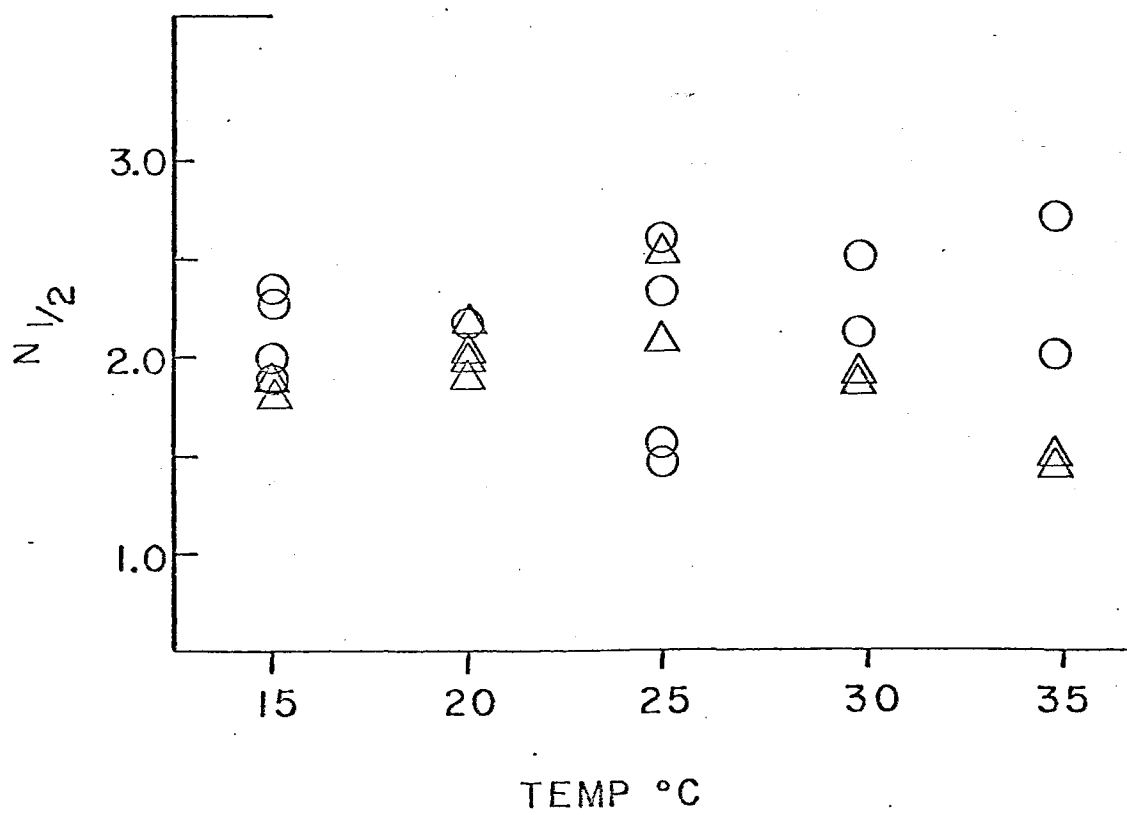
TABLE 1. ABSORBANCE MAXIMA OF <u>BARBATIA</u> <u>REEVEANA</u> Hb-H
AND Hb-L HEMOGLOBINS

		max		max	Soret max
Hb-H					
	Oxy	575		540	415
	Deoxy*		557		432
	CO	569		539	420
	Cyanmet			540	421
Hb-L					
	Oxy	574		540	414
	Deoxy*		555		430
	CO	568		539	420
	Cyanmet			540	419

*Deoxygenated with sodium dithionite

TABLE 2: AMINO ACID COMPOSITION OF <u>BARBATIA</u> <u>REEVEANA</u>
Hb-H HEMOGLOBIN

Amino acid	Mol %	Amino acid	Mol %
Lys	10.4	Ala	7.0
His	1.7	Half cys*	1.8
Arg	6.4	Val	8.0
Asp	16.0	Met	1.9
Thr	3.2	Ile	6.0
Ser	3.6	Leu	11.0
Glu	9.0	Tyr	0.8
Pro	3.2	Phe	4.2
Gly	6.5	Trp	**

* Determined as cysteic acid

** Not measured

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