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The first reported observation of hemoglobin association with a plasma protein was made by Polonovski and Jayle in 1938. Since that time, countless investigators have contributed to the characterization of the hemoglobin-binding plasma protein, haptoglobin. Haptoglobins have been found in many mammals, including humans and in various birds. However, very little research has been applied to reptilian and amphibian systems regarding haptoglobin presence. This study investigated the amphibian Taricha granulosa with regard to the presence of haptoglobin in its plasma and the overall

ability of the animal to exhibit hemoglobin conservation upon hemolysis. Results of this research indicated that although the newt is void of haptoglobin, it is capable of binding free hemoglobin and demonstrates hemoglobin and iron conservation upon induced hemolysis. *In vitro* experiments suggested that two serum albumins as well as a slightly larger plasma glycoprotein associate with free hemoglobin in the newt. The stoichiometry of interaction of the albumins to hemoglobin or the glycoprotein to hemoglobin is apparently one hemoglobin-binding protein to one-half hemoglobin (one $\alpha\beta$ dimer). The hemoglobin of the newt was also observed to lack affinity for human haptoglobin.

Since bacteriostasis has been suggested to be another function of haptoglobin, the hemoglobin-plasma protein complexes of *Taricha* along with the complex of mouse hemoglobin-human haptoglobin were used to supplement growth media for culturing two species of β-hemolytic bacteria. In each case, the bacteria were able to acquire the radiolabeled heme iron from the complexes as easily as from the respective free hemoglobins.

HEMOGLOBIN-BINDING PROTEINS IN THE AMPHIBIAN *Taricha granulosa*

by

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Typed by Nanette C. Cardon for Ralph T. Francis Jr.

Dedication

I dedicate this document to two people who have substantially influenced me and who greatly contributed to my successes.

First, to my beautiful mother who left this world far too early. Her academic excellence and scholarship along with selfless caring has, does and always will inspire me to excell; but all the while remain compassionate. She always will be much more than a memory.

And second, to my beloved friend, confidant and companion Lisa. We have traveled many miles together. She was always there for me and patient during those many trying times. She continues to let me bask in the warmth that is her intimate love.

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And last, but certainly not least, the source of the data, the newt Taricha granulosa. Many of those helpless, beautiful little ones gave up life so that certain proteins and physiologic functions could be compared across phylogenetic lines. Their sacrifice will always be remembered and revered.

Contribution of Authors

In Section IV, Tom Bailey performed the amino acid analysis of the proteins compared and helped with the interpretation of the data. In Section V, Joseph Booth prepared growth media and assisted with the bacterial transfers and preparation of the manuscript for publication. In Section VII, James Davie and Mike Sayre performed the histone protein purification. Elisabet Rocha and Georgia Riedel prepared reagents needed for the electrophoretic analysis. Forrest Zeimer provided technical support as well as aiding James Davie and myself in the preparation of the manuscript for publication.

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HEMOGLOBIN BINDING PROTEINS IN THE AMPHIBIAN *Taricha granulosa*

SECTION I

Introduction

The purpose of this study was to search for the existence of a haptoglobin (Hp) or another hemoglobin-binding protein in the plasma of an amphibian. Haptoglobins have been identified in most mammals investigated (1-5) and some birds (6,7). The fact that birds possess Hps has led to the belief that reptiles and probably amphibians also produce and use Hp (23). This assumption is not warranted and in fact no convincing evidence for the existence of Hp in either reptiles or amphibians has been reported. Thus, the question as to where in the phylogenetic perspective Hp originated remains unresolved.

Since the function of Hp is understood to be the binding of αβ dimers of hemoglobin (Hb) (8,9,10) (thus preventing the passage of the αβ dimers through kidney glomeruli and into subsequent excrement), the general assumption is that all vertebrates possessing a

glomerular kidney also possess Hp (23). All vertebrates are believed to possess a tetrameric Hb that is confined to an erythrocyte, and all land vertebrates (amphibians to mammals) have a kidney that has a glomerulus and a Bowman's capsule as the primary nephric unit (11). With these two properties in common, an assumption of phylogenetically ubiquitous Hp presence would not seem unreasonable. However, as was stated above, Hps have not been conclusively shown to exist in reptiles or amphibians. On the contrary, evidence indicating that the amphibian Taricha granulosa was void of Hp is provided in this thesis (see section II and reference 12). This animal was chosen for study for two reasons. First, it is a caudatan amphibian thus one of the most primitive of all land vertebrates, and second, it is prevalent in this part of Oregon and collection was relatively easy and inexpensive.

Since the evidence gathered in our research pointed to an absence of Hp in Taricha, attention was then given to any possible interactions between Taricha Hb and its plasma proteins. The first set of experiments performed involved the use of nondenaturing or "native" polyacrylamide gel electrophoresis (PAGE). The patterns of hemolyzed Taricha plasma were significantly different from those of human hemolyzed plasma. The most mobile protein band of human plasma in a native gel is serum

albumin. It is also the largest band due to the fact that albumin represents 60% of the total plasma protein in mammals (78). The component of hemolyzed Taricha plasma that had the greatest electrophoretic similarity to human albumin was significantly slower in mobility and constituted not one protein but a pair of protein bands only slightly separated in the gel. Together, the proteins of this "split pair" represented approximately 30% of the total plasma protein of Taricha. Although the Taricha plasma sample was visibly red from Hb, a protein band that would correspond to free Hb was lacking. And upon staining the gel for peroxidase activity (21) (thus heme presence) the split pair of proteins stained intensely, suggesting that Hb might be complexed with other proteins. Along with the peroxidase-active protein pair were two other bands that reacted for heme presence. When the components of hemolyzed Taricha plasma were separated using gel filtration and subsequently purified, it was found that the plasma contained two types of Hb-binding proteins. One has been identified as a glycoprotein approximately 75,000 daltons in molecular weight composed of a single polypeptide chain with an isoelectric point of approximately 6.3. The split-pair proteins representing the second type are also single polypeptide chains of approximately

68,000 daltons with no apparent glycosylation and with isoelectric points of approximately 4.7 and 4.8. These smaller proteins were found to possess characteristics typical of serum albumins (see section IV). This finding was of great interest to this study due to the fact that reptilian, avian or mammalian albumins do not demonstrate the ability to bind to their respective Hbs (13,14). Also, multiple albumins have not been observed in mammals and birds, and only rarely seen in reptiles (15).

Since the function of Hp is to bind extracellular Hb and prevent the passage of Hb into the glomerular filtrate (hemoglobinuria) (16) which results in nephritis and undesirable losses of iron, the question arose: Does Taricha conserve Hb and/or free iron upon induced hemolysis? That question was addressed as will be discussed later in this section.

The last aspect of this thesis research centers on the question: Are Hb-binding proteins bacteriostatic? A previous study (17) reported that a culture of pathogenic E. coli isolated from a human infant afflicted with peritonitis grew well in media supplemented with Hb. The same strain however, did not grow when the Hb was replaced by the HpHb complex. This fact was interpreted to mean that the bacteria were unable to assimilate the

heme or heme iron of Hb complexed with Hp. We decided to assess the ability of two β-hemolytic pathogenic species, Staphylococcus aureus and Streptococcus pyogenes to take up radiolabeled iron from Hb and the HpHb complex (in vitro). Radiolabeled Taricha Hb and its Hb-protein complexes were also compared using the two pathogenic bacteria species.

Background

In 1938, Polonovski and Jayle (18) observed that a horse serum protein exhibited strong peroxidase activity only after horse Hb was added to the serum. Shortly thereafter, the protein was identified as a complex of Hb and an Hb-binding protein which was then named haptoglobin (haptein meaning to fix or seize in Greek). Hp was subsequently found in human serum and in the serum of other mammals (1-5) and some birds (6,7). Humans have been seen to possess ambiently by far greater levels of Hp over other mammals (23). Initially, Hp was thought to be nonexistent in birds (6,24). However by injecting turpentine in chickens thereby initiating what has been termed the "acute-phase" response (52), researchers were able to detect levels of Hp high enough to allow characterization of the Hp molecule (6,7). Turpentine has also been used to stimulate production of Hp in mammals (26,27). The acute-phase response is characterized by a significant increase in the concentration of various α_2 plasma proteins. Haptoglobin represents approximately 70% of the increased protein content (28). Elevated Hp levels are found in mammals afflicted by inflammatory conditions such as burns, trauma, collagen diseases, scurvy, amyloidosis, kidney disease, Hodgkin's disease, lymphosarcoma and reticulum cell sarcoma (29).

Attempts were made to elicit Hp synthesis by attempting to produce the acute-phase response in the amphibian Taricha granulosa. After repeated injections subcutaneously, intramuscularly or intraperitoneally in the same amounts administered to mammals and birds in previous studies (24-27), no detectable increase in any plasma protein of Taricha was detected. This evidence suggests that the acute-phase response is a mechanism associated with higher vertebrate physiology.

Hp Structure

Human Hp is an assemblage of four polypeptide chains (30-32). Two heavy chains (molecular weight of 40,000) each containing a polysaccharide chain were found to be covalently bonded via disulphide bridges to two nonglycosylated light chains (molecular weight of 9,300). The arrangement of the Hp molecule is often designated HLLH to describe the order of intersubunit attachment, which is similar to the arrangement of the heavy and light chains of immunoglobulins (31,32). The Hp subunits have been sequenced (34-36), giving rise to observations that the heavy chain has serial homology to serine proteases (35) while the light chain has homology to the light chains of the immunoglobins (33). This combination of relatively unrelated proteins has proved to be difficult to explain.

Hp genetics

It was noted in 1955 that there were three major patterns of peroxidase-active components in electrophoretic analysis of human hemolyzed plasma (37). Work that followed revealed the presence of three allelic forms of Hp in humans. Hp 1-1, a single tetramer (molecular weight (MW) of 100,000) was described as were the polymeric forms of Hp 2-1 (average MW of 200,000) and Hp 2-2 (average MW of 400,000) (38,39). The latter two forms exist as polymers linked by disulphide bonds that produce a mixture of polymers increasing in size (see Figure I.1). It was believed that all other animals with the exception of a goat (40) possess only a monomeric Hp (a single tetramer) similar to Hp 1-1. That notion was shown to be in error however (3,4), with animals such as sheep and cow exhibiting polymeric Hps. There also have been various genetic variants of human Hp identified from electrophoresis (25,136).

The nature of the binding of Hb to Hp is unique in physiologic systems. The complexation was determined to be dependent on the dissociation of the Hb tetramer into $\alpha\beta$ dimers and that each dimer bonded to a site on each heavy Hp chain (137). The resulting stoichiometry of Hb to Hp is 1:1, that is one Hb tetramer to one Hp tetramer. This is the case for all three genetic forms of

Hp. The binding of Hb to Hp is thought to be similar to antibody-antigen binding, except that the HpHb association is essentially irreversible (41,42).

Hp synthesis and HpHb complex degradation

The site of Hp synthesis is believed to be the parenchymal cells of the liver (43). Tissue cultures of these isolated cells have given rise to Hp production (43,44) and with the use of immunofluorescence, Peters and Alper (45) localized Hp in liver tissue during rapid Hp synthesis.

The degradation of the HpHb complex begins with its recognition by amoeboid macrophage cells of the reticuloendothelial system, followed by transport to the liver where specific receptors bind the complex (46). The Hb heme is then cleaved by the hepatic enzyme α-methenyl oxygenase, thus liberating the iron for assumption by the liver (47). The Hp-globin complex is then proteolytically degraded (46), which makes Hp a nonrecyclable protein unlike antibodies (46).

Interspecies Hp-Hb interactions

When the Hbs from other mammals, birds, reptiles, one amphibian and a fish were introduced to human Hp, only the amphibian and fish Hbs failed to exhibit strong binding to Hp (48). In another study (49), the strength of interaction was followed by the measurement of peroxidase activity increase produced by the HpHb complex over that of the free Hb. The resulting data suggested that mouse Hb associated with human Hp in a manner far more similar to the human Hp-Hb interaction than those of canine, horse, rat and bovine Hbs. Another study reported that when Hb solutions of chicken, guinea-fowl or turtle were introduced to chicken serum, there resulted complex formation in each case as observed from gel filtration (7). When human, horse, cow, rabbit, frog or wallaby Hbs were added to the chicken serum however, no complex formation was observed in any of the cases (7). That evidence suggested that chicken Hp has a range of interaction with Hb which excludes mammalian and amphibian Hbs. Consequently, the question was raised: Does chicken Hb possess as narrow a range of interaction with Hp? Two studies have offered an answer. Using fluorescence quenching, Cohen-Dix *et al.* (48) found that chicken Hb complexed with human Hp with similar affinity as human, rabbit and sheep Hbs. And when Delers *et al.* (50) devised an affinity chromatographic method for

specifically removing Hb-binding proteins from plasma using chicken Hb bound to Sepharose 4BCL resin, they reported that along with chicken Hp they were able to isolate human and rat Hps. The results of these two studies indicate that although chicken Hp has a relatively narrow range of interaction with other Hbs, its Hb possesses a wider range and complexes with mammalian Hp. It would seem then that with regard to molecular evolution, the Hp-binding character of Hb has not changed as much as the Hb-binding site of the Hp molecule.

There has been little study of amphibian plasma proteins regarding structure and function. Some studies that have examined amphibian plasma have reported proteins similar to those in higher vertebrates, such as albumin (51,52). Liang (53) observed frog plasma to contain a protein electrophoretically similar to serum albumin that bound Hb upon hemolysis. That study was the first to suggest that a serum albumin could bind Hb and that amphibians were the only group to possess such an interaction. The studies contained in this thesis support that idea.

Overviews of subsequent sections:

Section II

This manuscript has been published in Comparative Biochemistry and Physiology (London) (see reference 12). The plasma of Taricha granulosa is examined for Hb-binding proteins and the resulting Hb-protein complexes are characterized. The lack of Hp in T. granulosa plasma is discussed as well as is the inability of its Hb to bind to human Hp.

Section III

This manuscript is currently in press with Comparative Biochemistry and Physiology (London). The ability of the newt T. granulosa to conserve Hb and iron upon induced hemolysis was established in this study. Hemolysis was induced using the hemolytic agent phenylhydrazine and the excretion of iron followed using the radioisotope iron-59. Injection of purified T. granulosa ^{59}FeHb into its circulation was also performed with similar results as the induced hemolysis. The synthesis of ^{59}FeHb was also monitored in this study.

Section IV

This manuscript was submitted for publication to Archives of Biochemistry and Biophysics. The emphasis of this study is on the comparison of T. granulosa Hb-binding proteins to serum albumins of a snake, chicken and four mammals including human. Results of the work support the contention made in Section II that T. granulosa serum albumin is involved in Hb-binding. The properties of the different species' albumins compared include: molecular weight, isoelectric character, immunocrossreactivity, amino acid composition and the binding of dyes and hemin.

Section V

This manuscript was submitted to the Journal of Bacteriology for publication. The acquisition of iron-59 from mouse and T. granulosa Hbs by two β-hemolytic bacterial species is the subject of this paper. Staphylococcus aureus and Streptococcus pyogenes were each grown in media containing: mouse ^{59}FeHb, mouse ^{59}FeHb-human Hp, T. granulosa ^{59}FeHb and T. granulosa ^{59}FeHb-albumin and 75K protein complexes. The purpose of these experiments was to determine if Hp or other Hb-binding proteins function as bacteriostats (17). The results indicate that in the cases of the two pathogenic strains, no retardation of growth or ^{59}Fe uptake was

exerted as a result of Hp complexing with Hb. On the contrary, in the case of S. pyogenes, the HpHb complex appeared to give up ^{59}Fe easier than did free mouse Hb. In the cases of growth in T. granulosa media, no significant differences of iron uptake was noted between the cultures grown in free newt Hb or its complexes.

Section VI

This paper has been published in Analytical Biochemistry (see reference 118). It provides a method of hemoprotein detection in sodium dodecyl sulfate-polyacrylamide gels. Significant in this report is the comparison of three different peroxidase-specific stains currently used, along with the description of a "double-staining" technique involving dimethoxybenzidine (DMB) and Coomassie blue R-250. The double-staining allows smaller amounts of heme to be detected in gels compared to detection by (DMB) alone. This study benefited the thesis work by allowing small amounts of T. granulosa hemoproteins to be identified on second dimensional SDS gel analysis.

Section VII

This manuscript has been accepted for publication in the Journal of Chromatography. Its emphasis is on

protein purification and preparation for further characterization. The advantages of using boric acid instead of glycine in the electrophoresis buffer for both denaturing SDS gels and nondenaturing "native" gels, and of using the fluorescent dye 8-anilino-1-napthalene sulfonic acid (ANS) instead of Coomassie blue for visualization of proteins in polyacrylamide gels are discussed in this paper. The methodology involved here was of great use to this thesis research regarding T. granulosa plasma protein purification.

Section VIII

Summary.

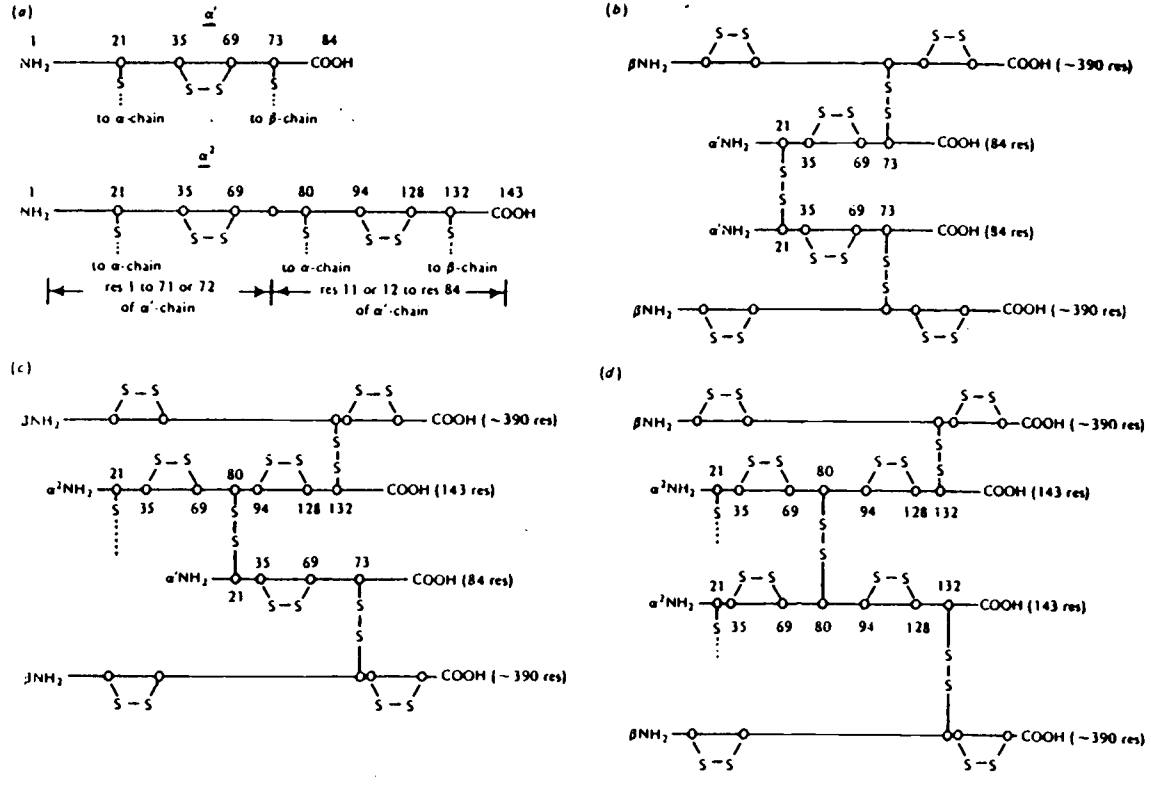

Fig. I.1 Schematic representation of human haptoblobins.
(a) alpha chains; (b) subunit structure of Hp 1-1;
(c) subunit structure of Hp 2-1; (d) subunit structure of Hp 2-2.
Taken from Principles of Biochemistry by A. White, P. Handler, E. Smith, R. Hill and I.R. Lehman. (1978) Sixth edition, p.912. McGraw-Hill Book Company, New York.

SECTION II

TWO HEMOGLOBIN-BINDING PROTEINS IDENTIFIED IN THE PLASMA OF THE AMPHIBIAN *Taricha granulosa*

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Abstract

1. The plasma of the amphibian Taricha granulosa has been shown to be void of haptoglobin.
2. Upon hemolysis, Taricha albumin and another protein associate with hemoglobin.
3. The acute-phase response to inflammation observed in birds and mammals appears to be absent in Taricha.
4. Taricha hemoglobin failed to bind to human haptoglobin.
5. Taricha hemoglobin not only failed to dissociate into αβ dimers as did human Hb, but formed αβ octamers.

Introduction

The hemoglobin-binding glycoprotein haptoglobin (Hp)[1] was first reported by Polonovski and Jayle (18) and has since been observed in the plasma of most mammals (3,4,9), certain birds (6) and snakes (13). However, the study of snakes failed to show that the hemoglobin-binding protein revealed was analogous to mammalian Hp either in structure or mechanism. And to this date, no convincing evidence for the existence of Hp in amphibians has been presented.

This study has examined hemolyzed plasma of the newt Taricha granulosa and has found no indication of the presence of Hp. However, through the use of various analytical methods such as gel filtration, affinity and ion exchange chromotography, polyacrylamide gel electrophoresis and analytical ultracentrifugation, two plasma proteins have been observed to associate with Taricha hemoglobin (Hb) upon hemolysis. One of these proteins has been tentatively identified as albumin on the basis of its abundance relative to the other plasma proteins, its molecular

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[1]Abbreviations: Hp, haptoglobin; Hb, hemoglobin; EDTA, ethylenediamine tetraacetic acid; DEAE, diethylaminoethyl; α and β, subunits of Hb; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

weight and electrophoretic mobility, as described in "Results". In addition, Taricha Hb was shown to lack affinity for human Hp and does not freely dissociate into $\alpha\beta$ dimers (a requirement for Hb binding to Hp; 54,55).

The so-called acute-phase response (52) which is indicated by a dramatic rise in the α_2 plasma glycoproteins, has been elicited in such animals as rabbits (26) and chickens (6) in order to increase Hp levels for study. The most widely-used means of acute-phase stimulation are the subcutaneous, intramuscular or intervenous injections of turpentine. When Taricha was injected either by subcutaneous or intramuscular means, there was no detectable rise in the α_2 or any other plasma proteins.

Materials and Methods

Animals: Taricha granulosa newts were captured wild in Benton County, Oregon. They were in their yearly aquatic phase and were found in ponds. They averaged 17 cm in length and 20 gm in weight.

Plasma: As with most aquatic amphibians, Taricha has low plasma protein levels when in its aquatic phase and osmoregulation is presumed to be the reason (51). These low protein levels require the use of

greater plasma volumes compared with those investigations of terrestrial vertebrate plasmas. The newts were bled from the aorta into heparinized pasteur pipettes. The blood was placed into 0.5 ml capped centrifuge tubes and the erythrocytes pelleted using a microcentrifuge for 2 minute spins at 10,000 rpm. The plasma supernatant fluid was removed and stored at 0°C.

It should be mentioned here that obtaining unhemolyzed Taricha plasma has been almost impossible. Different saline and buffer solutions were tried, with the best results coming from simply excluding the use of a saline solution and centrifuging the erythrocytes in the plasma alone. Even then, a slightly-reddish hue was present. Taricha erythrocytes are among the largest in vertebrates (56) and seem to be physically fragile compared to mammalian erythrocytes. For the acute-phase response experiment, each newt was injected with 0.01 ml of turpentine and left to incubate at 21°C. At 6 hour intervals thereafter, two newts were bled and the plasma electrophoresed to detect any increase in protein levels. The total time period involved was 72 hours.

Human serum was supplied by the Red Cross of Portland, Oregon. Human met-Hb (Sigma) was added to the serum in an amount sufficient to saturate the Hp

present. When hemolyzed newt plasma was desired, the blood was frozen at 0°C, thawed and the cellular debris pelleted using the microcentrifuge.

Gel Filtration: The hemolyzed human serum and Taricha hemolyzed plasma were each eluted over a 1.5 x 90 cm column of Sephadex G-100 equilibrated with a 0.05 M Tris-HCl buffer, pH 7.2 with 0.05 M NaCl; 1.5 ml fractions were taken. These fractions were read for absorbance at both 415 and 280 nm for heme and protein presence, respectively.

Polyacrylamide Gel Electrophoresis (PAGE): Nondenaturing PAGE was performed on a mini gel apparatus (8 x 10 cm) obtained from Idea Scientific of Corvallis, Oregon, according to Davis (29) with certain modifications. The tray buffer was made of 0.05 M Tris, 0.06 M boric acid with 1.0×10^{-3} EDTA and riboflavin was replaced with 0.1% ammonium persulfate for the acrylamide polymerization. The nondenaturing or "native" gels were run with either a stacking phase or made of an acrylamide gradient (3 to 20%) without the stacker. Sodium dodecyl sulfate (SDS) PAGE was used for dissociation of plasma components and estimation of their molecular weights. The SDS PAGE procedure followed Laemmli (57) except that the Tris-boric acid tray buffer used in the native PAGE was used here also (with added 10% SDS). R-250 Coomassie blue was used

to stain both native and SDS gels for general protein (58) and o-dianisidine was used to reveal heme-containing components of native PAGE samples (21).

Affinity Chromatography: Blue Sepahrose (Sigma) was used for isolation of albumin (59). A 1.0 x 15 cm column was equilibrated with a 0.05 M Tris-HCl buffer, pH 7.0 with 0.10 M KCl. The material bound to the column was eluted (after washing the unbound material off) by increasing the KCl concentration to 1.5 M in one step. Stripping the resin was performed by washing with 0.05 M Tris-HCl, pH 7.0 with 1.5 M KCl and 6 M urea.

A wheat germ agglutinin - Sepharose-4BCL resin was packed into a 0.8 x 8 cm column equilibrated with a 0.05 M Tris-HCl buffer, pH 7.0 with 0.1 M NaCl. The elution of bound material was performed using a linear gradient of 0.0 to 0.25 M N-acetyl-D-glucosamine. This resin has shown affinity for plasma glycoproteins that contain sialic acid and/or N-acetyl-D-glucosamine as do Hp and hemopexin (60).

A Taricha Hb - 4BCL Sepharose column was made and the elution performed according to Delers et al.(50).

Ion Exchange Chromatography: DEAE Bio-Gel A (Bio Rad) agarose was employed with a 0.02 M Tris-HCl buffer, pH 7.2 with an accompanying linear NaCl gradient of 0.0 to 0.5 M. This resin (packed into a 1.0

x 20 cm column) was used to separate the components of the 100K dalton region of the G-100 elution of Taricha hemolyzed plasma.

Analytical Ultracentrifugation: Experiments were performed on a Beckman Model E ultracentrifuge. Human and newt Hbs were converted to the cyanomet form (61) and each solution was diluted to 5.0×10^{-6} M using an extinction coefficient of 11.5×10^{3} at 540 nm. The samples were spun at 40,000 rpm and scanned for 415 nm absorbance at 8 minute intervals. Sedimentation coefficient distributions were calculated from the scanner data by the method of van Holde and Weischet (62).

Results

The G-100 elution profiles of human and Taricha granulosa hemolyzed plasmas are shown in Figure II.1. The G-100 column was calibrated with five proteins of known sizes before the plasma elutions. The molecular weight estimation of each peak is noted on each profile. Peak no. 1 in the human profile has a substantial Soret absorbance (415 nm). This material eluted at the void volume of the column and is composed of material 150K daltons or larger. This Soret peak is the Hp-Hb complex having the molecular weight of 160K daltons. Elution peaks no. 3 and no. 4 are methemalbumin and hemoglobin, respectively.

The profile of Taricha hemolyzed plasma is significantly different, however. First of all, in peak no. 1, there is very little Soret absorbance. Peak no. 2 in the 95-110K dalton range reveals substantial amounts of 415 nm absorbing material. There are no 415nm or 280 nm peaks in the 68K region where albumin would normally be, but there is a hemoglobin peak (no. 3) afterward. It would seem from the comparison that Taricha plasma, upon hemolysis, possesses no large complex involving Hb and has no serum albumin. In order to identify the components responsible for the major differences between the two G-100 profiles, SDS and native PAGE were both utilized. Since peak no. 2 of the Taricha elution indicated the most abundant 415 nm absorbance outside of the free Hb peak, the mid-fraction of peak no. 2 was sampled and applied on both SDS and native PAGE (see Figure II.2). The native gel was stained for peroxidase activity with o-dianisidine (21) and the SDS gel was stained with R-250 Coomassie blue. The presence of Hb subunits in the SDS sample explains the high Soret yield. However, two other protein bands were also revealed with molecular weights of 68K and 75K daltons. The native gel demonstrated the mobility pattern of those proteins at pH 8.2. After the native gel was stained for peroxidase activity, it too was stained with

Coomassie blue with no additional bands appearing. The most mobile band of the native pattern has been identified through second-dimensional SDS PAGE (63) analysis as being composed of Hb and the 68K protein thought to be Taricha serum albumin.

In order to fractionate the peak no. 2 components further, a Bio-Gel A DEAE anion exchange column was used. Figure II.3 shows that profile and the SDS PAGE separation of each peak's components. The 75K protein eluted with the majority of Hb, while a small portion of Hb came off with the 68K albumin. When a densitometric scan of the Hb - albumin SDS gel lane was run, the R-250 Coomassie blue yield (at 550 nm) of the Hb subunits compared to the albumin yield suggested a possible one-half Hb to one albumin stoichiometry. That would explain why the 75K and 68K proteins eluted in the 100K region of the G-100 profile. A 30K Hb dimer bound to either of those proteins would result in a complex in a 98K to 105K dalton range. Since the binding of mammalian Hb to Hp involves the concommitant dissociation of the Hb tetramer into dimers, the idea of Taricha Hb dimers binding to the 68K and 75K proteins would seem plausible. However, as will be noted shortly, Taricha Hb does not appear to dissociate into $\alpha\beta$ dimers, thus another mechanism would be indicated.

Since the presence of Taricha albumin had not been conclusively shown, a procedure for its purification was sought. Blue Sepharose (cibacron blue) is known to exhibit great affinity for albumin (59). To test the affinity of Blue Sepharose for Taricha Hb under the elution conditions, Hb from the G-100 separation was passed through the column. All of the Hb eluted in the unbound wash. However, when the Taricha G-100 peak no. 2 fraction was passed through, most of the Hb and the 75K protein came off in the unbound wash while the 68K material and some Hb eluted in the 1.5 M KCl step. The fact that the 68K protein showed affinity for the cibacron ligand is strong evidence that it is indeed serum albumin. The presence of Hb in the high KCl fraction provides additional evidence for an association between Taricha albumin and Hb.

In attempting a form of reconstitution of Hb to albumin and the 75K protein, a Taricha Hb-4BCL Sepharose affinity column was made (50) and slightly hemolyzed Taricha plasma was placed on the column and eluted. All of the plasma with the exception of most of the 75K protein and albumin came off in the unbound wash. However, upon the introduction of 8 M urea to the column, the remaining 68K albumin and the 75K protein eluted, suggesting that they were bound to the immobilized Hb.

Since Hp is 5.3% sialic acid (64) and has affinity for the wheat germ agglutinin-Sepharose resin, it was decided to pass the G-100 peak no. 2 fraction through this resin also. The result was that neither the 68K albumin nor the 75K protein showed affinity for the lectin column.

To find out if human Hp binds Taricha Hb, the cyanomet form of the Hb was incubated with human serum for 12 hours at 21°C and 37°C in separate trials. Figure II.4 shows a native gradient gel stained with o-dianisidine. Densitometric scans of the gel (at 470 nm) revealed no Hp-Taricha Hb complex formation at either temperature. Since requirement for formation of the complex is that tetrameric Hb must first dissociate into αβ dimers, human and Taricha cyanomet Hb sedimentation coefficients were compared using the Beckman Model E analytical ultracentrifuge. The sedimentation coefficient distribution in each sample is shown in Figure II.5. The distributions were obtained according to van Holde and Weischet (62). The 2.8S value represents an αβ dimer, the 4.5S value a tetramer and the 6.9S value an octamer. The concentration of each sample was 5.0×10^{-6} M or approximately a 1000-fold dilution of the intracellular concentration. The human Hb exhibited the presence of dimers while Taricha did not. Not only did Taricha

fail to form dimers, but there was evidence of tetramers pairing into octamers. The octamerization of amphibian Hbs has been reported in previous studies (65,66). Whether Taricha Hb would bind to human Hp if there were dimer formation is not presently known.

After the turpentine-treated newt plasma samples were electrophoresed on native PAGE and stained with R-250 Coomassie blue, each 6 hour interval sample was scanned densitometrically at 550 nm. There was no visible trend of any protein amounts increasing.

Discussion

The hemolyzed plasma of Taricha granulosa was shown to lack a 150K or larger Soret-absorbing peak similar to human plasma after elution through Sephadex G-100 (although there was a small amount of 415-absorbing material at the void volume probably resulting from Hb aggregation, which is known to occur in hemolyzed newt plasma). However, it possessed a large Soret peak in the 100K dalton range and also showed no peak for albumin. Instead, Taricha albumin was found in fractions from the 100K peak along with Hb and a 75K protein. When fairly clear newt plasma (minimally hemolyzed) was eluted through the G-100 column, the profile revealed a small, broad peak in the 70K region that was shown by gel electrophoresis to contain free

75K and albumin proteins (data not shown). Since the dimers of either the 68K albumin or the 75K protein would elute close to and in the void volume respectively, these two proteins had to be associated with another component that would result in a complex between 95 and 110K daltons in molecular weight. Hb subunits were seen on SDS gels from the G-100 peak no. 2 demonstrating that Hb existed in a form (or forms) approximately 100K daltons in size. That would suggest that the Taricha Hb eluting at that volume must be either in the form of an octamer, an $\alpha\beta$ dimer associated with other proteins, or both. In order to distinguish these possibilities, two chromatographic methods were employed.

First, the cibacron blue Sepharose resin was used to selectively separate and identify albumin from that G-100 fraction of Taricha hemolyzed plasma. The 68K protein showed affinity for that resin and as it eluted from the column under high salt, a portion of the Hb present was shown to coelute (as seen on SDS PAGE). As a control, purified Taricha Hb was passed through the column with none of it sticking to the cibacron ligand. Native PAGE revealed only one component from the G-100 peak no. 2 high salt elution. Those results suggested an albumin-Hb association.

Secondly, DEAE ion exchange chromatography was used to separate the peak no. 2 components. As is shown in Figure II.3 this method also revealed some Hb copurifying with albumin. The 75K protein was seen to elute with most of the Hb of the sample. In order to establish any affinity of the 75K protein for Taricha Hb and further demonstrate an albumin-Hb association, the Taricha Hb-4BCL Sepharose affinity resin was used. The 75K protein and albumin were the only plasma proteins to bind to the column. The combined results of the G-100 gel filtration, affinity and ion exchange chromatographic procedures are good evidence for the Hb-binding nature of Taricha albumin and 75K protein.

Since these two plasma proteins exhibited Hb-binding behavior, did they resemble Hp regarding carbohydrate content? In order to answer that question, the wheat germ agglutinin-Sepharose affinity resin was used. Although mammalian Hp has shown specificity for that lectin, neither the 75K protein nor albumin of Taricha did. That evidence indicated that at least with regard to glycosylation, the Hb-binding proteins in Taricha do not resemble mammalian Hp.

Taricha Hb demonstrated the inability to bind to human Hp and did not dissociate into αβ dimers. That, along with the lack of a Soret peak in the G-100 void

volume plus the lack of affinity of the plasma proteins associated with Hb for the lectin column, is strong evidence for the absence of Hp in Taricha plasma. The absence in Taricha of the acute-phase response, which is made up of α_2 plasma glycoproteins (the majority of the protein increase is Hp) further supports the conclusion that this animal is ahaptoglobinemic.

An interesting point here is that even though Taricha Hb failed to show dissociation into dimers, it is suggested by the G-100 filtration and PAGE analysis that the 75K and albumin proteins are associated with an Hb dimer. It would seem then, that these two proteins may induce the splitting of the 60K newt Hb tetramer into 30K dimers. Since the dissociation of tetrameric Hb into $\alpha\beta$ units (or dimers) is required for Hb binding to Hp, it was believed and has been reported that the $\alpha_1\beta_1$ surface contains the Hb residues that are involved in the strong Hp-Hb bond (55). However, Taricha Hb has been shown to stay associated in the tetrameric form and failed to bind to human Hp even after prolonged incubation.

Since the evidence points to Taricha Hb complexing with the 68K and 75K plasma proteins resulting in molecular weights of approximately 100K daltons, the Hb must be in a form of about 30K in size. This

would suggest that although Taricha Hb was not observed to freely dissociate into αβ dimers, it can be induced to dissociate by interacting with the 68K albumin and the 75K protein. That would infer that instead of the binding site existing on the inside of the Hb tetramer as with birds and mammals, the site on Taricha Hb might be on the outer surface of the molecule. An alternative hypothesis would be that Taricha Hb exists with a very small amount of αβ dimers, an amount at equilibrium that would not be detectable during analytical ultracentrifugation. Complexing of these dimers with the Hb-binding proteins would then drive this reaction to yield significant amounts of complexed dimers. This idea would however require the additional assumption that Taricha Hb dimers do not bind to human Hp.

Finally, the function of Hp in higher vertebrates has been presumed to be the binding of Hb dimers which alone can pass through the glomerulus of the kidney and be excreted (resulting in the loss of vital iron and renal damage). The Hp-Hb complex is too large to pass into the glomerular filtrate. Since Taricha Hb as with other amphibian Hbs associates into octamers instead of splitting into dimers upon hemolysis, the octamerization may very well serve the same renal filtrate exclusion function of Hp. The albumin and 75K

protein bind the smaller Hb forms, thereby also contributing to Hb conservation.

It is then an attractive consideration that evolutionarily the responsibility for Hb (thus iron) conservation upon hemolysis started with Hb polymerizing into larger forms such as octamers. From that point, as evolution proceeded, albumin may have become a scavenger of Hb dimers, a phenomenon not seen in birds and mammals. The 75K protein in Taricha plasma may represent a further advancement in Hb conservation on the way towards mammalian haptoglobin.

Attempts to purify the 75K protein and albumin of Taricha granulosa are currently in progress. If success is achieved, efforts will be made to complex Taricha Hb to each protein. The stoichiometry of binding could then be established along with other characteristics of the resulting complexes.

Acknowledgements:

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Figure II.1. Sephadex G-100 elution profiles: (A) Human hemolyzed serum and (B) Taricha hemolyzed plasma. The column was calibrated previous to the elutions and the estimated molecular weight of each peak is designated. The human sample showed three 415 nm peaks: the Hp-Hb complex in peak no. 1, methemalbumin in peak no. 3 and Hb in peak no. 4. The Taricha sample showed only two significant 415 nm peaks: the 100K dalton peak no. 2 and the Hb in peak no. 3. Also, there was a lack of absorbance at either 280 nm or 415 nm in the 70K region where serum albumin would normally elute. Peak no. 1 in both profiles represents the void volume material that is 150K or larger in size.

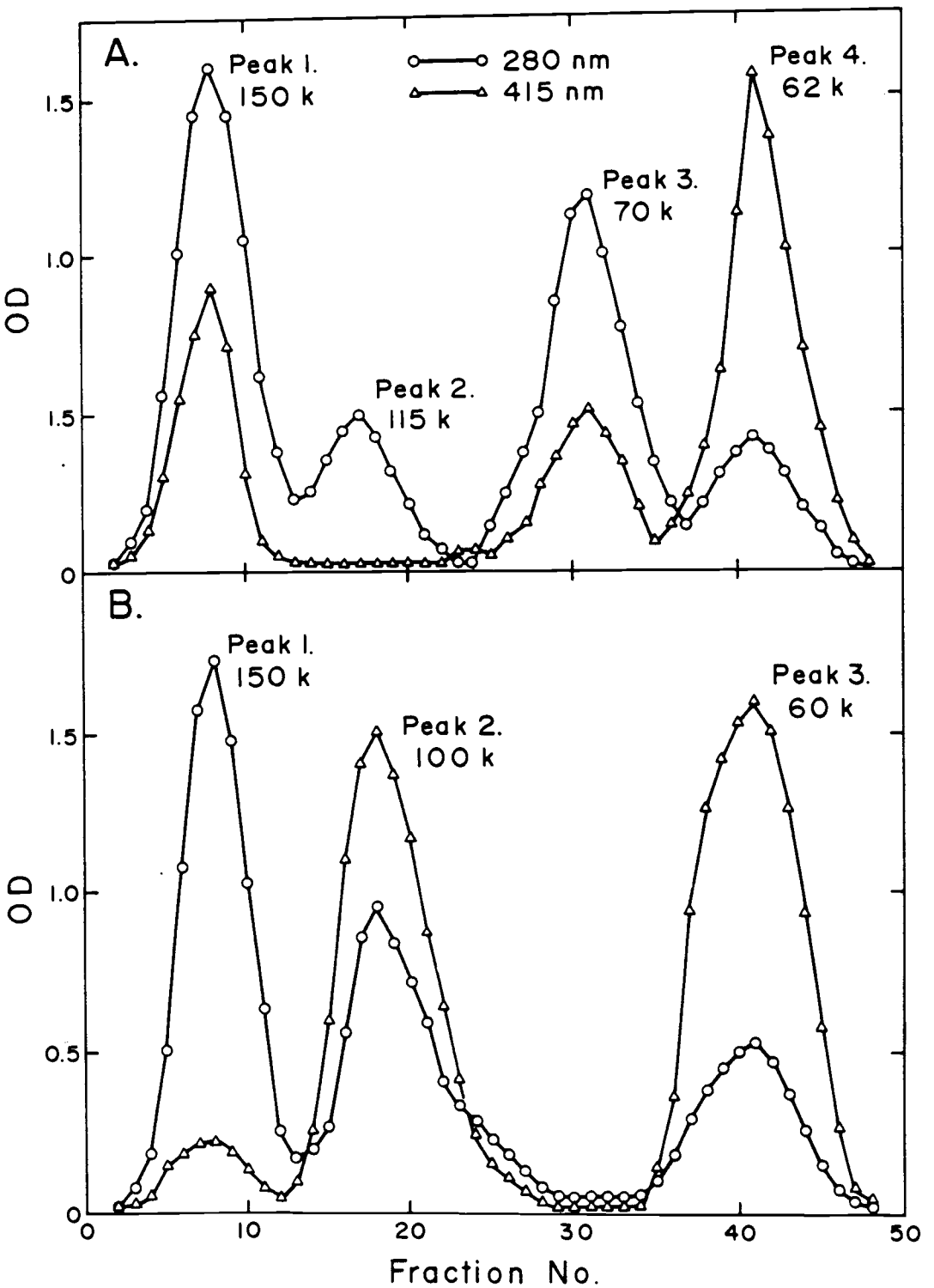
A.
Peak 1.
150 k
280 nm
415 nm
Peak 4.
62 k
Peak 3.
70 k
Peak 2.
115 k
OD
1.5
1.0
1.5
0
B.
Peak 1.
150 k
Peak 2.
100 k
Peak 3.
60 k
OD
1.5
1.0
0.5
0
0
10
20
30
40
50
Fraction No.

Fig. II. 1

Figure II.2. Two modes of PAGE separation of a Taricha G-100 100K mid-peak sample. (A) 3 to 20% acrylamide gradient native PAGE and (B) SDS PAGE of 12% acrylamide. When the most anodal band (Alb-Hb) on the native gel was excised, treated according to O'Farrell (1975) and electrophoresed on SDS PAGE as the second dimension, the 68K albumin band and the two 15K Hb subunit bands seen in B were observed. The native PAGE (A) was first stained with o-dianisidine for presence of heme, then with R-250 Coomassie blue for protein; no additional bands appeared. Although the position of the 75K-Hb complex on the native gel pattern (A) has not been established, it is believed to be the band slightly anodal to the Hb octamer, designated 2(Hb).

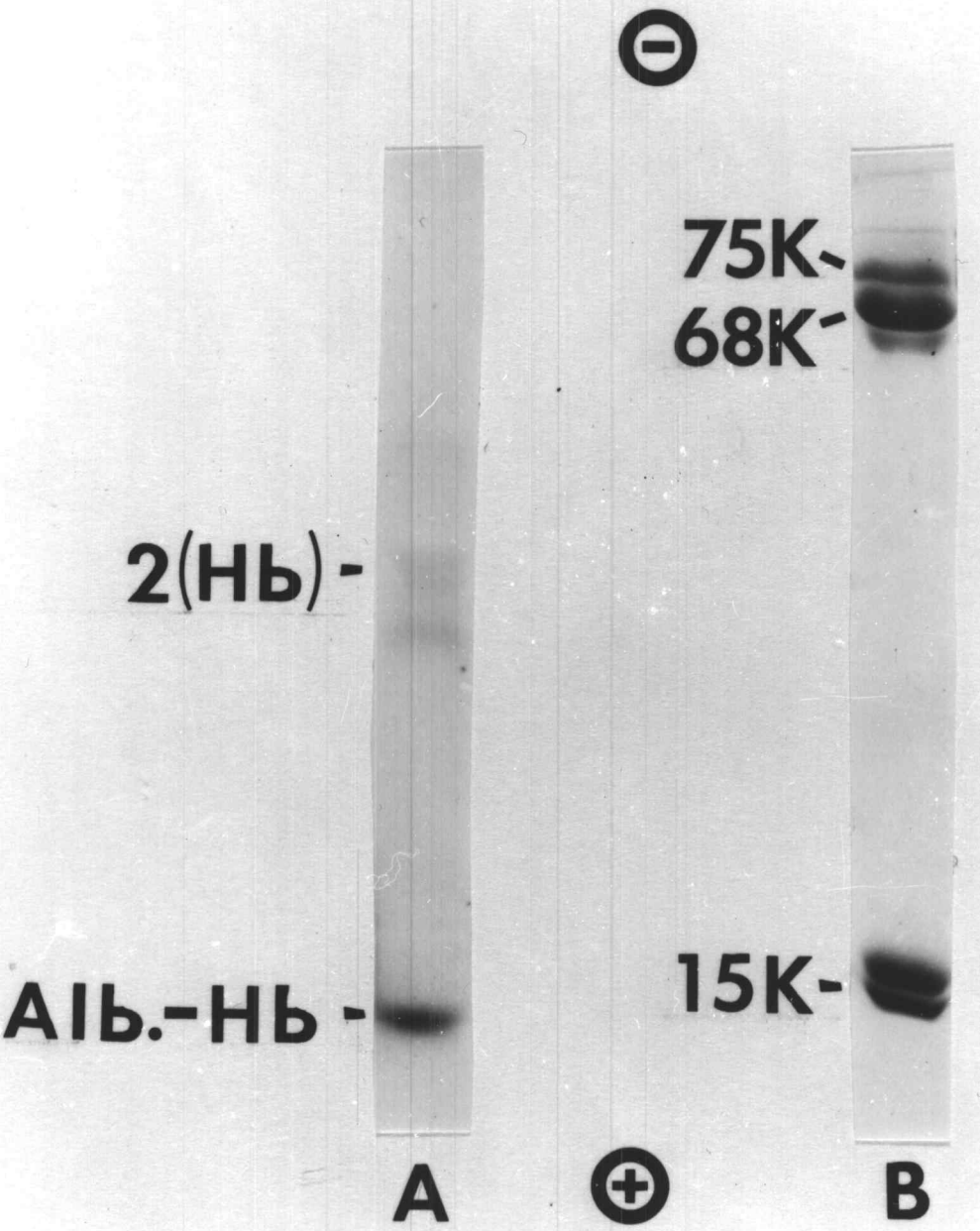

Fig. II.2

Figure II.3. DEAE-agarose anion exchange elution of the Taricha G-100 peak no. 2 sample. A 0.02 M Tris-HCl buffer, pH 7.2 with a 0.0 to 0.5 M NaCl gradient. Pictured adjacent to each peak is the SDS PAGE separation (12% acrylamide) of the peak's components. The 75K protein eluted with the majority of the Hb (15K subunits) of the sample shortly after the NaCl gradient began. Along with the 68K albumin was a smaller portion of Hb. Taricha Hb was also seen to elute off the Blue Sepharose column with albumin.

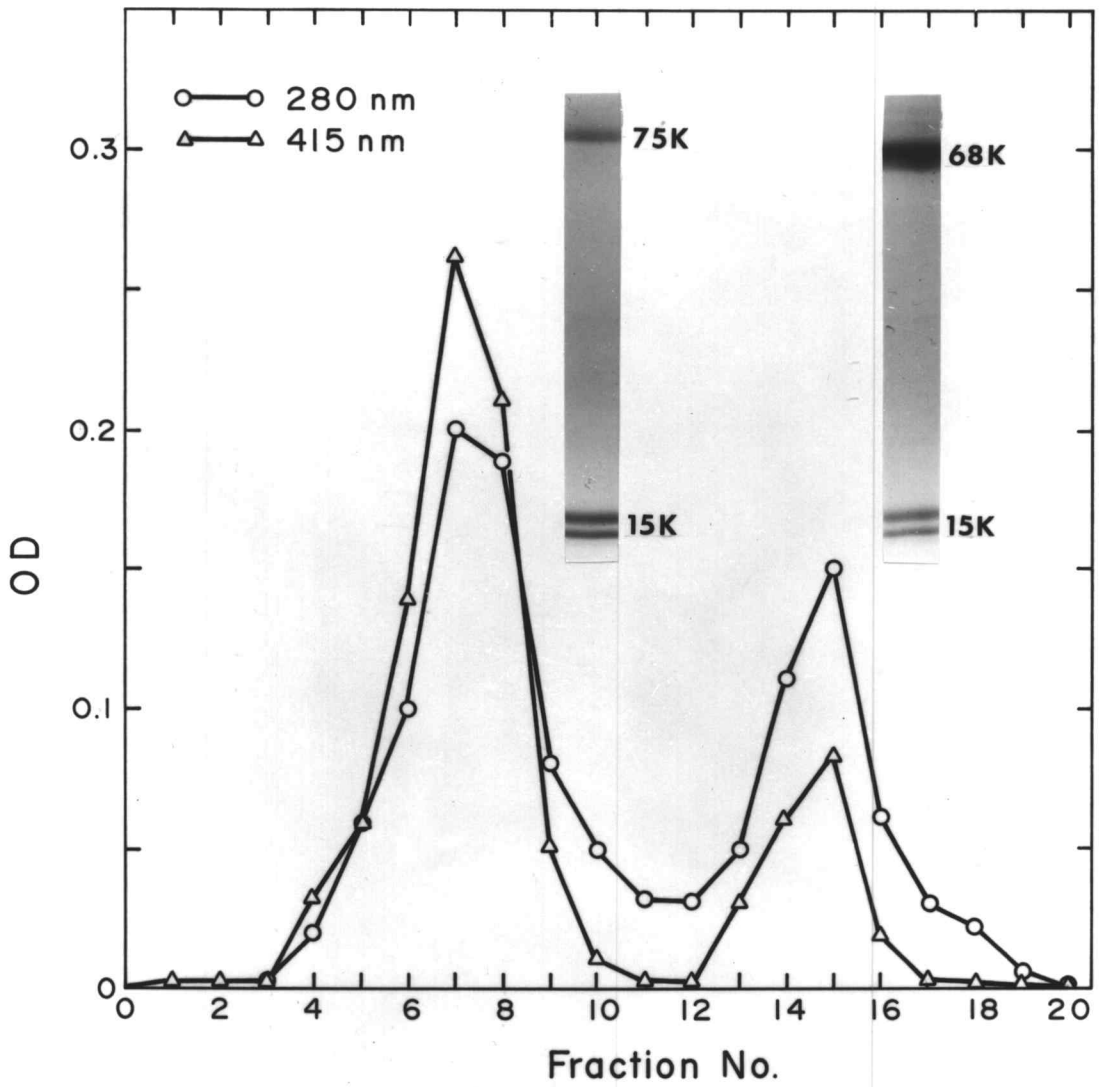
280 nm
415 nm
75K
15K
68K
15K
OD
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
18
20
Fraction No.

Fig. II.3

Figure II.4. A 3 to 20% acrylamide gradient native gel stained with o-dianisidine. The samples are as follows: (A) Human serum (having Hp type 1-1) with human cyanomet-Hb added. The Hp-Hb complex is indicated (Hp-Hb), as is transferrin (TR), free Hb and methemalbumin (Alb). (B) The same human serum with Taricha cyanomet-Hb added. (C) The human serum of Hp type 1-1, alone. (D) Human serum having Hp type 2-1 with Taricha Hb added. (E) The same serum alone. Note the lack of Hp-Hb complex formed in lane B. There was no complex formation with Hp type 2-1 in lane D, either. The serum-Hb solutions were incubated at 37°C for 12 hours before electrophoresis. Samples incubated at 21°C showed the same electrophoretic patterns.

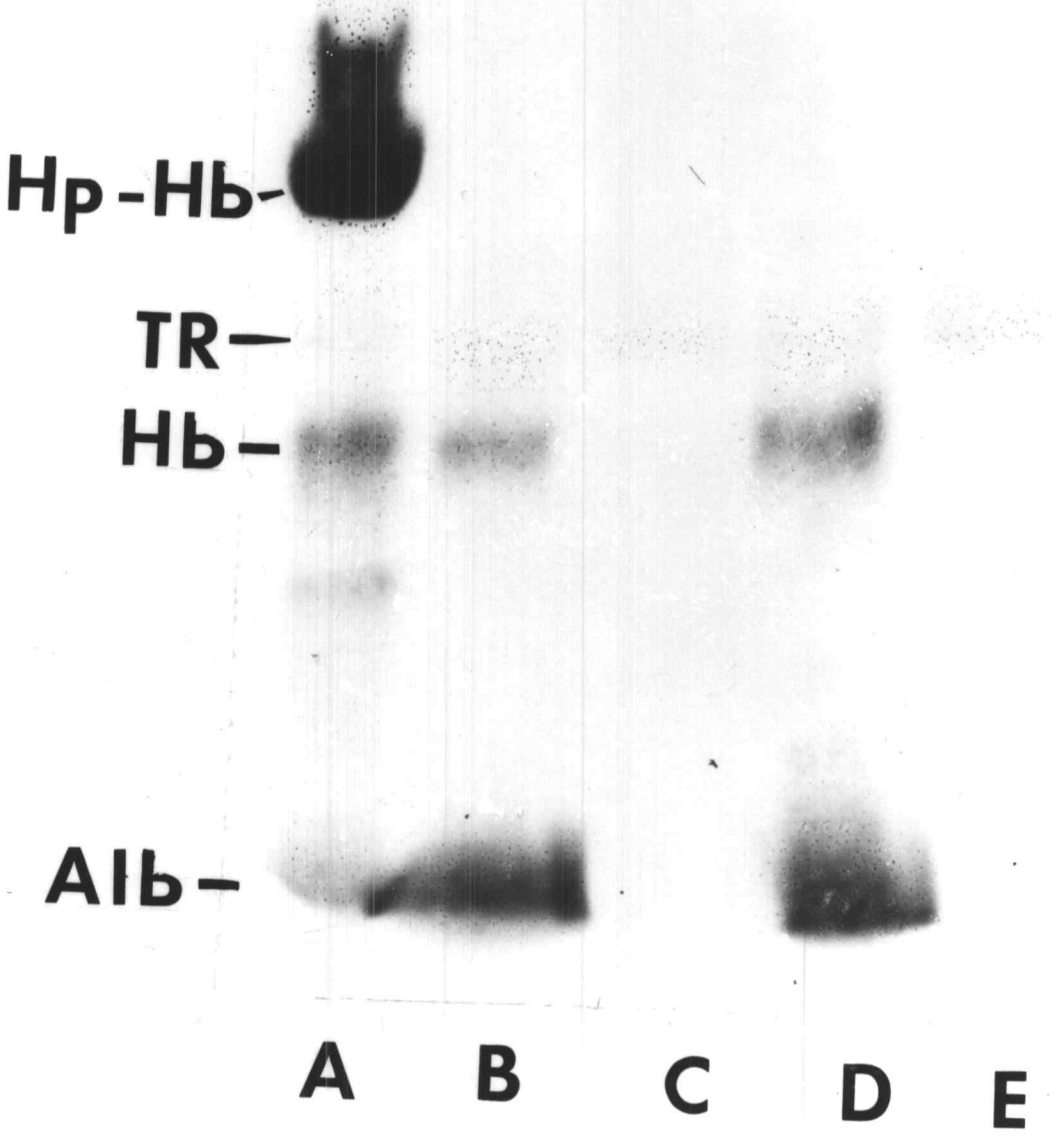

Fig. II.4

Figure II.5. The distribution of sedimentation coefficients in human and Taricha cyanomet-Hb solutions. This data describes the sizes of Hb molecules in each solution and the portion of the solution they make up. In the human sample, the 2.8S $\alpha\beta$ dimer makes up approximately 50% and the 4.5S tetramer about 35%; with transitional forms between the two. Taricha Hb showed no dimer (2.8S) form. The tetramer of 4.5S, at about 50%, was the smallest form observed. Along with the Taricha tetrameric form also existed a 6.9S octamer (paired tetramers) at about 30%; also with transitional forms between. Each Hb solution was 5.0×10^{-6} M or about a 1000-fold dilution of the intraerythrocytic concentration. The S or Svedberg unit is 1.0×10^{-13} sec.

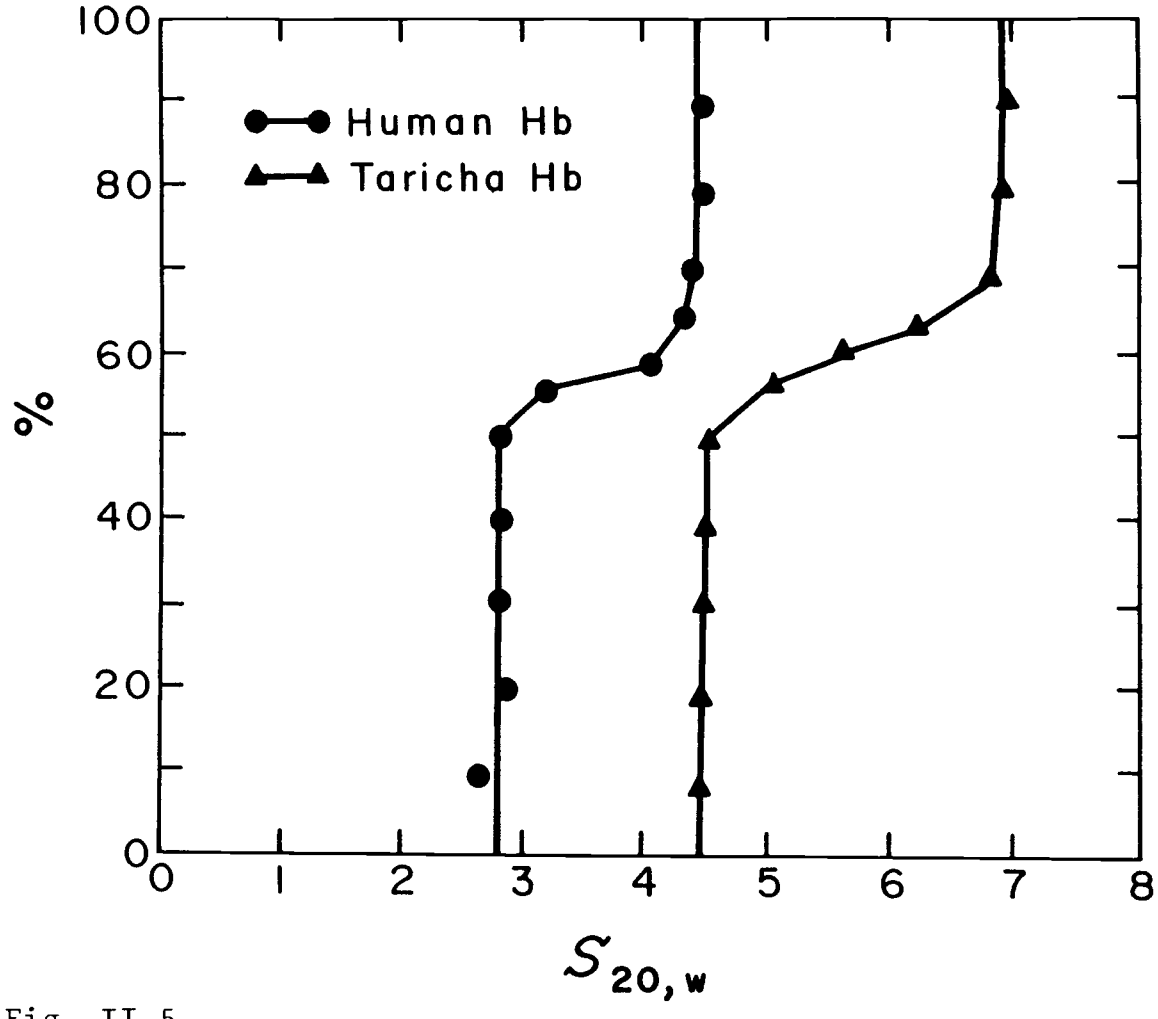

Fig. II.5

SECTION III

CONSERVATION OF IRON AND HEMOGLOBIN DURING INDUCED HEMOLYTIC STRESS IN THE AMPHIBIAN *Taricha granulosa*.

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Abstract

1. The capability of Taricha granulosa to conserve hemoglobin upon in vivo hemolysis has been investigated.
2. ^{59}Fe incorporation into Taricha hemoglobin was similar in rate to mammals and birds.
3. Phenylhydrazine-induced hemolysis resulted in comparatively low levels of ^{59}Fe and no discernible amounts of hemoglobin excreted after 10 days.
4. The addition of ^{59}Fe Hb to Taricha circulation resulted in relatively low levels of ^{59}Fe excretion and significant amounts of ^{59}Fe incorporation into new hemoglobin within 10 days.

Introduction

Hemolytic trauma and subsequent pathologic effects have been the focus of many studies for over fifty years. The fact that hemolysis results in a decrease of functional hemoglobin (Hb)[1], thus a decrease in the respiratory potential of an animal, makes it a serious condition. Loss of respiratory potential however, is not the only deleterious effect resulting from hemolysis. It has been observed in humans that once hemolysis reaches a certain degree, Hb passes through the glomerulus of the kidney and is excreted (16,76). This condition is termed hemoglobinuria and results in pathologic losses of iron. Since iron is required for the synthesis of many enzymes and oxygen carriers, and that the potential for hemolysis due to various causes exists, iron-conserving mechanisms would be anticipated to exist. Indeed, various iron-carrying proteins along with the organic iron chelator protoporphyrin IX and proteins that bind it have been identified. By far, the majority of research in the area of iron transport and storage mechanisms has been performed on humans.

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[1]Abbreviations: Hb, hemoglobin; Hp, haptoglobin; PBS, phosphate buffered saline; PHZ, phenylhydrazine; SDS, sodium dodecyl sulfate; BME, β-mercaptoethanol.

That the Hb-binding protein haptoglobin (Hp) firmly binds Hb and prevents its loss through the glomerulus, marks it as a significant iron-conserving protein. Hp not only prevents Hb loss, but also stabilizes the heme association with the globin (61,68,69) maintaining the intact complex for subsequent catalysis and iron recovery (46). Once the Hp levels are saturated with Hb however, the extra or free Hb loses a portion of its heme content and/or passes through the kidney into excrement (16,68). During normal breakdown of aged erythrocytes, Hb, heme and heme iron are adequately conserved and recycled. During pathologic hemolysis, whether induced by virulent bacteria, autoimmune response or by drug administration, hemoglobinuria has been detected and results in serious losses of iron (16,67,76).

Very little research has been lent to lower vertebrates such as reptiles or amphibians concerning post-hemolytic Hb recovery mechanisms. To the best of our knowledge, a plasma protein similar to human Hp has never been demonstrated to exist in either reptiles or amphibians. On the contrary, in a previous report, we observed an ahaptoglobinemic condition in the amphibian Taricha granulosa (12). We did however, observe Taricha albumin and another plasma

protein (not similar to human Hp) to bind to its Hb upon hemolysis.

Since we observed Hb-binding proteins in the plasma of Taricha, we wanted to know if Taricha exhibited Hb and iron conservation upon both drug-induced hemolysis and injection of Hb into their circulation.

Materials and Methods

Animals

Taricha granulosa were captured wild in Benton County, Oregon. They were in their aquatic phase and averaged 18 cm in length and 17 g in weight.

^{59}Fe labelling of Taricha Hb in vivo

Twenty-one newts were each injected intraperitoneally with 10 μCi of ^{59}Fe-citrate brought up to 0.1 ml injection volume with phosphate buffered saline (PBS). ^{59}Fe-citrate was supplied by ICN. The animals were not fed during the incubation period. Groups of three newts were bled beginning at two days after injection. The whole blood was spun in a microcentrifuge for 2 min. at 10,000 rpm. The plasma supernatant fluid was poured off and the erythrocyte pellet resuspended and then recentrifuged in PBS solution three consecutive times. The pellet was then resuspended

again in the PBS solution frozen at 0°C for 2 hr., thawed and the cellular debris separated out by centrifugation.

Gel filtration

The hemolyzate supernatant was eluted over a Sephadex G-100 column (1.5 x 90 cm) previously equilibrated with a 0.05 M Tris-HCl buffer, pH 7.2, with 0.05 M NaCl. The column had previously been calibrated with five proteins of known molecular weights. Two ml fractions were taken, read for absorbance at 415 nm and 280 nm for heme and protein respectively, and were placed in a Packard gamma counter for evaluation of ^{59}Fe levels.

Drug-induced hemolysis

Thirty-six newts were each injected intraperitoneally with 2 μCi of ^{59}Fe-citrate in a 0.1 ml volume injection. After 11 days stored at 21°C and without being fed, 11 groups of three animals each were injected with increasing amounts of phenylhydrazine (PHZ), supplied by Sigma. The PHZ was prepared as a stock solution of 10 mg to 1.0 ml with PBS. This solution was diluted with more PBS to make the various PHZ concentrations, each in 0.1 ml for injection. Three newts were kept as controls and received no PHZ. The remainder of the animals in groups of three, were each

administered a dose per group of the following series: 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.50, 0.60, 0.70, and 1.0 mg PHZ per animal. Each newt was placed in a plastic bag (with ventilation holes) containing a small amount of H_2O for moisture and the excrement collected. At 4 days and again at 10 days after PHZ injection, the excrement of each newt was removed from the bag, dried using a rotoevaporator, brought up in 1.0 ml of 2% sodium dodecyl sulfate (SDS), 0.0625 M Tris-HCl, pH 6.8 and counted for ^{59}Fe. For the assessment of any Hb present in each newt's excretion, β-mercaptoethanol (BME) was added to each sample to make 5% v/v and glycerol added to make 10%; the samples were boiled for 2 min. and loaded into a 15% acrylamide electrophoresis gel containing SDS according to Laemmli (57). Upon completion of electrophoresis, the gels were stained with Coomassie blue (58) and subsequently stained with silver (70) for protein indication. On the 10th day after PHZ injection, each newt was bled and the erythrocytes separated from the plasma by centrifugation. The erythrocyte pellet was counted for ^{59}Fe activity and the plasma supernatant fluid counted, diluted 30-fold and read for absorbance at 415 nm for Hb.

^{59}Fe Hb added *in vivo*

To observe the response of *Taricha* upon hemolysis that was not drug-induced, 3 groups of 3 newts each were given an intravenous injection of freshly prepared ^{59}FeHb. The doses were: 1.47×10^{-8}, 4.41×10^{-8} and 8.82×10^{-8} moles of Hb. Each newt was placed in a plastic bag and the excrement was removed at 3, 7 and 10 days after injection. The samples were again dried, brought up in the SDS solution and counted for ^{59}Fe. A portion of each sample was loaded on a 15% acrylamide SDS gel, electrophoresed and treated for protein presence as described above. On the 10th day, the animals were bled and the erythrocytes again separated from the plasma. The plasma was poured off and counted for ^{59}Fe as was the erythrocyte pellet.

Results

Figure III.1 illustrates the Sephadex G-100 elution of ^{59}Fe-injected *Taricha* hemolyzate. The first smaller peak represents void volume material with molecular weights of 150,000 or larger. It contained a small amount of aggregated Hb. The major peak eluted at a volume that indicated its size to be approximately 61,000. This material was the tetrameric Hb of *Taricha*. The G-100 gel filtration demonstrated that

the ^{59}Fe label was distinctly associated with Taricha Hb. At each time point afterward, hemolyzates from each group were eluted over the G-100 column with the amount of ^{59}Fe radioactivity increasing relative to the amount of absorbance for heme at 415 nm. Figure III.2 depicts the changes in ^{59}Fe in Taricha Hb amounts with time. At approximately 11 days after ^{59}Fe injection, there appeared a maximum of radiolabeled Hb. This is contrary to what Flores and Frieden (71) observed in bullfrog tadpoles that had been hemolytically stressed with PHZ and then injected with ^{59}Fe. They reported that there was little Hb synthesized even after 14 days. The tadpoles were kept at approximately 20°C as were the Taricha in this study. However, Meints (76) observed that in the frog Rana pipiens, there was significant recovery of Hb amounts within 10 days after PHZ injection. The rate of clearance of $^{59}FeHb$ between 11 and 17 days appeared to be similar to the rate of its synthesis.

Since the time course indicated that the maximum amount of ^{59}Fe existed at about 11 days, that period of time was used to allow maximal ^{59}Fe incorporation into Hb before the in vivo hemolysis study.

In order to facilitate in vivo hemolysis, PHZ was used. This drug has been used for studies of erythropoietic rates in amphibians (71,75,76.). Groups of

three newts were each given an increasing dose of PHZ and the excretion of each newt was collected. On the 4th day after PHZ treatment, the excretion was counted for ^{59}Fe activity. The same thing was done on the 10th day. Sixty to 70% of the total 10 day ^{59}Fe excreted was found in the 4th day collections. Much of this was ^{59}Fe that was still being cleared from the animals' systems by routes other than Hb metabolism. The total ^{59}Fe excreted at each PHZ amount is shown in Figure III.3. The increase of excreted ^{59}Fe with the increase of PHZ was relatively small until after the 0.5 mg per level. There was a 400% increase in excreted ^{59}Fe between 0.5 and 1.0 mg PHZ. The excrement samples were then loaded on a 15% polyacrylamide gel and electrophoresed. Upon staining the gel with Coomassie R-250, no Hb subunit bands were observed in any of the samples. The gel was then stained for protein with silver, with the same result (data not shown).

In order to be certain that the PHZ was effectively hemolyzing Taricha blood and to observe the effect of PHZ concentration on the degree of hemolysis, on the 10th day the animals were bled, the erythrocytes separated from the plasma and counted for ^{59}Fe as

was the plasma. The plasma was also read for absorbance at 415 nm for hemoglobin. Figure III.3 illustrates the effect of increasing amounts of PHZ on the amount of ^{59}FeHb in the plasma compared to the amount in the erythrocytes. The absorbance values at 415 nm for heme indicated that the increase of ^{59}Fe in the plasma was in the hemin form. In the high PHZ concentrations (0.5 to 1.0 mg) the Hb free in plasma was brown in color indicating it was in the metheme form. The oxidation of Hb to metHb in the presence of PHZ has been observed in previous studies (67,72).

The range of PHZ administration was established in reference to previous studies which used PHZ to cause extensive hemolysis *in vivo* (76,77). Those studies showed that PHZ at 25 µg/g body weight was adequate to cause complete hemolysis and oxidize the Hb present. The PHZ range used in this study was 0.0 to 1.0 mg PHZ per 14 g newt or 0.0 to 71 µg/g body weight. Meints (76) found that at 30 and 50 µg PHZ/g body weight of the frog *Rana pipiens*, there was weight gain of the frogs in the first 10 days after PHZ injection. It was deduced that the gain was by water retention due to reduced glomerular filtration thus kidney dysfunction. Since this study focused on whether hemin or Hb is lost through glomerular filtrate after hemolytic trauma, PHZ-induced kidney dysfunction would render

the results invalid. In order to insure reasonable results, each newt was weighed before PHZ injection and 3 more times throughout the experiment. The animals with PHZ doses up to 0.7 mg per 14 g newt (50 µg/g weight) not only showed no weight gain but had lost a small amount of weight. The volume of their excrement was also comparable to the control newts. The newts with 1.0 mg of PHZ injected did show weight gain however, but still were excreting more ^{59}Fe than the animals with lower PHZ doses.

The newts that were injected with ^{59}FeHb were kept in the same way that the PHZ-injected ones were and the excrements were removed, counted for ^{59}Fe and electrophoresed in the presence of SDS. Figure III.4 shows ^{59}Fe changes in the peripheral blood and excrement of Taricha during a 10 day period after having free Hb introduced into their circulation. There were small, gradually-increasing amounts of ^{59}Fe excreted with increasing ^{59}FeHb amounts injected. The amount of ^{59}Fe excreted was only 3 to 3.5% of the ^{59}FeHb injected. The majority of the excreted ^{59}Fe was found in the 3rd day samples. By the 10th day, there was no ^{59}Fe found in the excretions. On the 10th day, the newts were bled and their erythrocytes and plasma were each counted for ^{59}Fe. The plasma fractions were fairly clear and no absorbance readings

were made. The ^{59}Fe amounts in the plasma with increasing Hb loads did not increase significantly. The quantity of ^{59}Fe found in the erythrocytes was greater after 10 days than ^{59}Fe levels in the plasma or amounts that had been excreted within that period of time. The incorporation of ^{59}Fe into the erythrocytes was virtually linear with the increase of $^{59}FeHb$ injected into the newts. It appears that the majority of the observed ^{59}Fe was incorporated into new Hb and new erythrocytes and that the difference between erythorcyte ^{59}Fe and the ^{59}Fe in the plasma and excrement was greater with each increasing injection of $^{59}FeHb$. The ^{59}Fe level found in the erythrocytes was approximately 9 times the level in the plasma of the blood at the highest $^{59}FeHb$ dose and was approximately 3 times the ^{59}Fe amount found in the total excrement. The observed distribution of the ^{59}Fe (which accounted for only 15% of the total ^{59}Fe injected) illustrated in Figure III.4, suggests that after 10 days, the injected Hb was almost cleared from the plasma, approximately 9% of the injected ^{59}Fe incorporated into new Hb in the erythrocytes and only 3% excreted. Since no dissection of the animals was made after bleeding, it is not known where the remaining

^{59}Fe was stored. It is presumed that it was distributed primarily between the spleen, liver, and kidney (6,76,77).

Discussion

The *in vivo* incorporation of ^{59}Fe into Hb has been performed on mammals (80), birds (6), reptiles (77) and amphibians (76). In mice (80), a 10 to 15 day incubation period was used while in birds (6), the incubation was 7 days. This study revealed that *Taricha* had maximum ^{59}FeHb levels at around 11 days. The animals were kept at 21°C which is generally warmer than the temperature of the pond waters they frequent. The warmer environment of the laboratory would certainly affect poikiothermic animals and increase the rate of their overall metabolism including erythropoiesis. Since previous reports (71,74) indicated that amphibian hemopoietic responses are very slow compared to mammalian, it is believed that the elevated temperature in this study along with lack of food for the incubation period must have greatly influenced the rate of *Taricha* hemopoiesis.

Although several studies have followed erythropoiesis using ^{59}Fe after PHZ-induced hemolysis, in amphibians, knowledge of the loss of iron or Hb upon hemolysis seems to be absent or at least obscured.

The fact that iron losses can be detrimental to the organism and that haptoglobins have been found in mammals and birds to retard Hb losses after hemolytic stress, prompted the question: Do lower vertebrates such as amphibians have similar capabilities? Since they also possess Hb confined to blood cells and a kidney that includes the "Bowman's capsule" and glomerulus, one would anticipate some form of Hb, thus iron loss preventative mechanism to exist. From the PHZ treatment and the quasi hemolysis due to ^{59}FeHb injection into their circulation, Taricha granulosa were observed to exhibit iron and Hb retention upon hemolytic stress.

The PHZ study revealed that the lysing of Taricha erythrocytes by PHZ appeared to be implemented via a cooperative effect by the drug (Figure III.3). It also demonstrated that even with severe levels of hemolysis, comparatively low amounts of ^{59}Fe were excreted. SDS electrophoresis revealed that the excreted iron was for the most part not Hb iron, for the gels showed an absence of Hb even though ^{59}Fe amounts were more than high enough to allow detection of ^{59}FeHb. Although Hb levels too low to be indicated might have been present, the portion of the excreted ^{59}Fe associated with the Hb would have been very slight. Since the ^{59}Fe-citrate had been injected 11

days before the PHZ injection and the newts were not fed during that time, the ^{59}Fe excreted during the PHZ experiment could have been from spleen and liver stores which might have been agitated by PHZ. Another possibility is that since Taricha Hb was observed in this laboratory to lose a small portion of its heme during nondenaturing PAGE, the excreted ^{59}Fe might have been (at least in part) free hemin dissociated from the oxidized Hb. It is probable that the excreted ^{59}Fe upon PHZ treatment is comprised of both stored and hemin ^{59}Fe. The fact that it was not primarily $^{59}FeHb$ was apparent from the gels. Even if it was ^{59}Fe in the Hb form, the fact that the radioactivity increase in the excrement did not follow the increase of $^{59}FeHb$ free in plasma suggested that Taricha possesses the ability to conserve Hb and retard iron losses upon hemolytic stress.

In order to determine the fate of free Hb introduced into Taricha circulation, purified $^{59}FeHb$ was injected into the newts. The ^{59}Fe that was excreted during the 10 day period was only 3.5% of the injected amount and approximately one-third of the ^{59}Fe that was found incorporated into the erythrocytes. It is not known whether all of the ^{59}Fe in the erythrocytes in prophyrin-chelated and found in the Hb.

Since the affinity of porphyrin for iron is very high (81), enzymatic degradation of the porphyrin ring is required for the recovery of iron during catalysis of Hb in humans (78). The incorporation of ^{59}Fe into Taricha Hb within 10 days could occur via porphyrin degradation in either the spleen or liver, or through incorporation of the intact ^{59}Fe hemin directly into the newly synthesized Hb. Since in this experiment there was no loss of functional Hb as in the PHZ treatment, it is not a surprise that there were greater amounts of ^{59}Fe excreted (although probably due to the lack of food during the incubation period, the ^{59}Fe excrement levels were still relatively low). In the PHZ experiment, only 1% of the injected ^{59}Fe was excreted in 10 days while almost 4% of the total was excreted in the $^{59}FeHb$ injected study.

Both the PHZ-induced hemolysis and the $^{59}FeHb$-injection experiments demonstrated the Hb and iron conserving capability of Taricha granulosa. The results of this in vivo work indicate that Taricha Hb binding to two plasma proteins or its octamerization observed in a previous study in this laboratory (12,73) might very well represent the mode of Hb retention similar to the function that has been suggested for haptoglobin in higher vertebrates.

Acknowledgements:

The authors wish to thank the Medical Research Foundation of Oregon for support of this research. We are also grateful to Dr. D. Buhler for the use of his Packard gamma counter and to Cindy Romo for the graphical interpretation of the data.

Figure III.1

Sephadex G-100 gel filtration of ^{59}Fe injected Taricha hemolyzate (11th day). The smaller peak eluted at the void volume of the column and represents aggregated Hb. The larger peak contained Taricha tetrameric Hb at 61,000 daltons in molecular weight. The ^{59}Fe profile indicates its specific association with Hb.

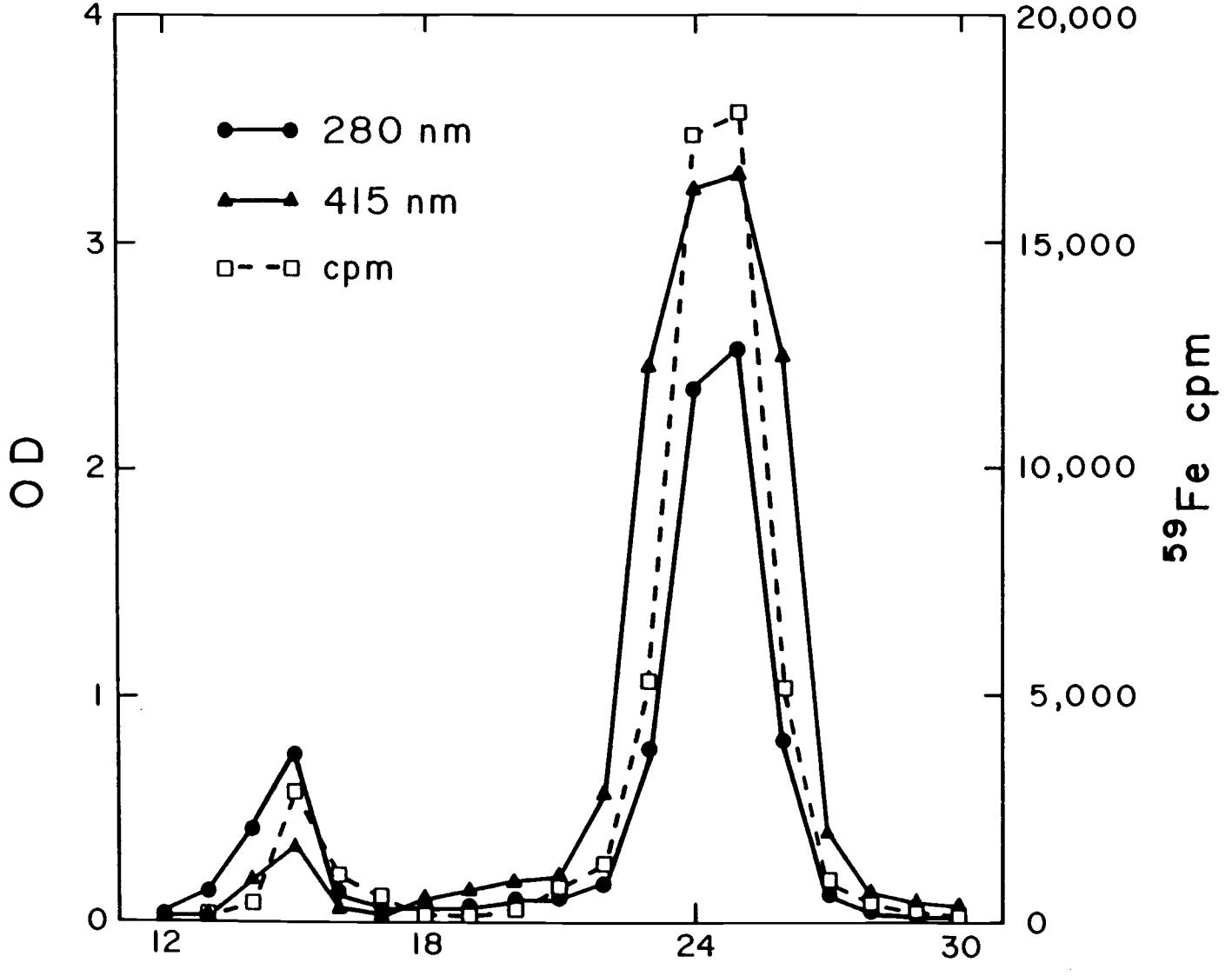
280 nm
415 nm
cpm
OD
59Fe cpm
fraction no.
0
1
2
3
4
5,000
10,000
15,000
20,000
12
18
24
30

Fig. III.1

Figure III.2

The changes in ^{59}Fe/µmole of Taricha Hb with time. The greatest amount of $^{59}FeHb$ existed at 11 days after injection and the rate of $^{59}FeHb$ increase was similar to the rate of decrease (up to 17 days). After 17 days, the rate of decrease appeared to slow significantly (average values are shown).

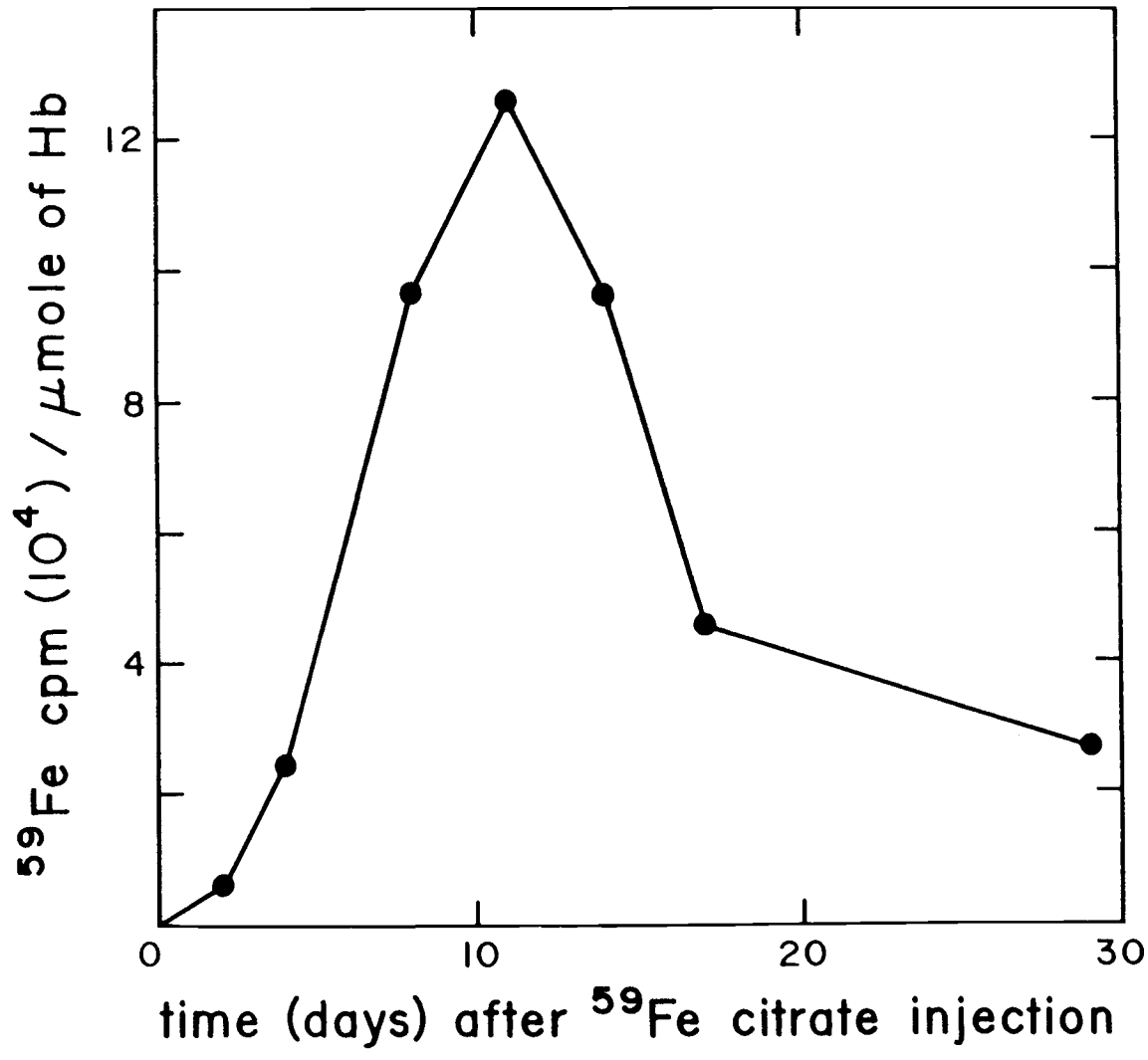
^{59}Fe cpm (10^4) / μmole of Hb
12
8
4
0
10
20
30
time (days) after ^{59}Fe citrate injection

Fig. III.2

Figure III.3

The ratio of ^{59}FeHb in the plasma to ^{59}FeHb in red blood cells (rbc) 10 days after phenylhydrazine (PHZ) injection. Hemolysis was increased with increasing PHZ doses. The absorbance at 415 nm also demonstrates the increase of hemolysis with increasing amounts of PHZ. Also shown are the amounts of ^{59}Fe excreted compared to increasing PHZ doses (■-■). Even with extensive hemolysis, relatively little ^{59}Fe was excreted. No Hb was found in the excrements. (Average values are shown).

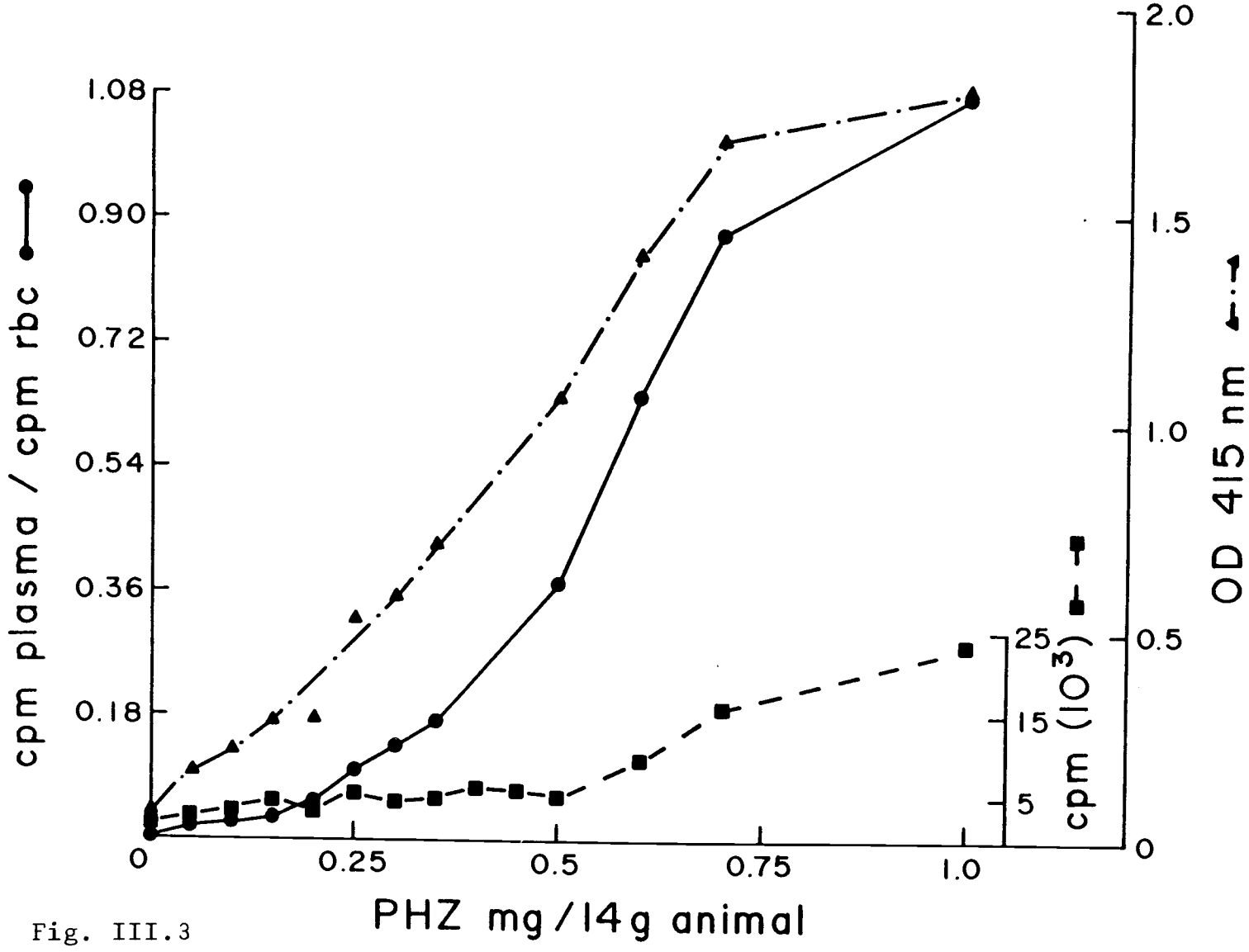
cpm plasma / cpm rbc
1.08
0.90
0.72
0.54
0.36
0.18
0
0.25
0.5
0.75
1.0
PHZ mg/14g animal
2.0
1.5
1.0
0.5
0
OD 415 nm
25
15
5
cpm (10³)

Fig. III.3

Figure III.4

The observed distribution of ^{59}Fe 10 days after $^{59}FeHb$ injection. No Hb was found in the excrements (average values are shown).

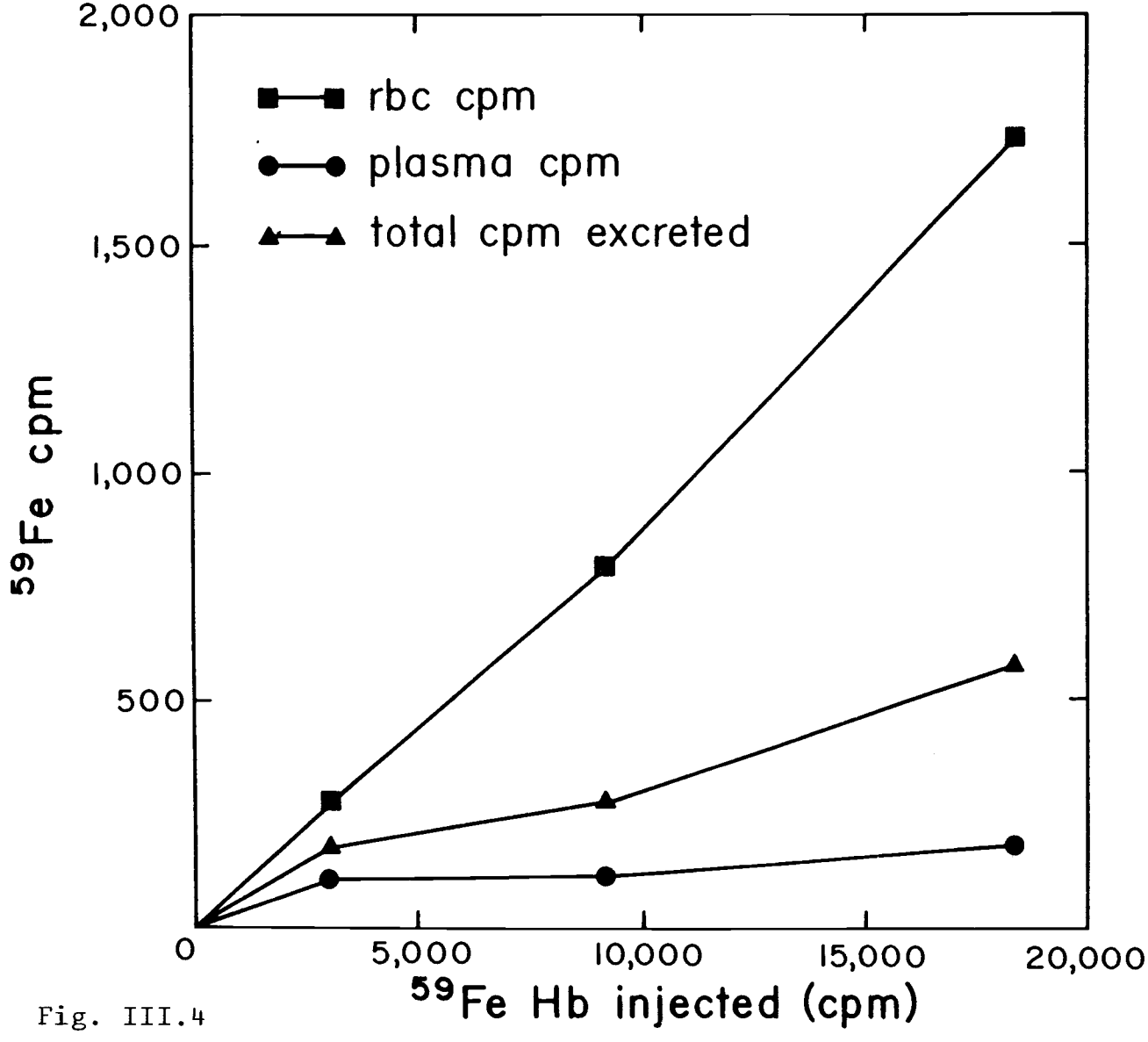
2,000
1,500
1,000
500
0
rbc cpm
plasma cpm
total cpm excreted
59Fe cpm
5,000
10,000
15,000
20,000
59Fe Hb injected (cpm)

Fig. III.4

SECTION IV

BISALBUMINEMIA IN AN AMPHIBIAN

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Abstract

In a previous study, we observed two hemoglobin-binding plasma proteins in the amphibian newt Taricha granulosa; one of them resembled serum albumin of higher vertebrates. Improved electrophoretic resolution has revealed two albumin-like proteins in Taricha plasma. Since the binding of hemoglobin by serum albumins has not been observed in higher vertebrates, it was decided to compare directly the amphibian's proteins to mammalian, avian and reptilian albumins. Properties such as molecular weight, amino acid composition, isoelectric character, immunoreactivity and the binding of hemin and dyes were used to assess similarities among the proteins. Results indicated that although they possess unusual affinity for hemoglobin, the two newt proteins are definitely serum albumins. This comparative study has demonstrated that although certain traits that govern tertiary structure have been conserved in serum albumins, other properties such as composition, possession of determinants for the same antibodies and affinity for certain ligands have varied considerably.

Introduction

Human and bovine serum albumins remain two of the most-studied proteins after many years. Early studies identified serum albumins as a major source of osmoregulation in mammalian blood (100, 101). This function is performed by virtue of albumin's relatively low molecular weight, high surface charge and high ion-binding capacity (102). Serum albumins have also been identified in birds (103) and reptiles (15), but evidence for their existence in amphibians is limited.

Human serum albumin (HSA)[1] was observed to bind and transport hydrophobic ligands such as fatty acids, bilirubin, hemin, tryptophan, steroids, thyroxine, drugs and dyes (101,104). Humans and other primates are the only species believed to possess hemin-binding serum albumins (68). Bromophenol blue (BPB), a commonly-used tracking dye for gel electrophoresis

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[1] Abbreviations: HSA, human serum albumin; BSA, bovine (cow) serum albumin; OSA, ovine (sheep) serum albumin; PSA, porcine (pig) albumin; CSA, chicken serum albumin; SSA, snake (garter) serum albumin; TSA, Taricha serum albumin; BPB, bromophenol blue; CB, cibacron blue; Hb, hemoglobin; BME, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; Con A, Conconavalin A; SDS, sodium dodecyl sulfate; CbR-250, Coomassie blue R-250; 68K, Taricha 68,000 molecular weight protein; 75K, Taricha 75,000 molecular weight protein.

(29) has been observed to bind to HSA and bovine serum albumin (BSA), as has the dye cibacron blue (CB) (105). The binding of the two dyes by albumin proved to be a valuable comparative tool in this study. And since the proteins believed to be albumins in Taricha were observed to bind its hemoglobin (Hb) upon hemolysis (12), other serum albumins were assayed for their Taricha Hb-binding capabilities.

Another property albumins have in common is a structural organization of 9 loops cross-linked by 17 disulfide bridges resulting in 3 domains (106). The only exception noted so far is chicken albumin, which lacks one disulfide bridge (107). Since to our knowledge no reptilian or amphibian albumins have been sequenced, structural similarities between them and albumins of higher vertebrates have not been reported.

Since albumin can be unfolded by denaturants and the disulfide bonds reduced (108,109), it was decided to compare Taricha proteins to other albumins with regard to structural changes upon reduction with β-mercaptoethanol (BME) (determined by denaturing polyacrylamide gel electrophoresis (PAGE)).

Another characteristic of serum albumins is the absence of glycosylation, and albumin is the only major protein plasma component that lacks associated saccharide (101,110). The Taricha proteins were

assayed for glycosylation using saccharide-specific gel staining (111,93) and affinity chromatography (5).

The amphibian's proteins were further compared to other albumins using isoelectric focusing (112), Ouchterlony immunodiffusion (113) and amino acid analysis (114,115). The amino acid compositions were compared for their degrees of relatedness using statistical analysis recently formulated for assessing evolutionary relationships among proteins (116).

Materials and Methods

The albumins of human (HSA), sheep (OSA), cow (BSA), pig (PSA) and chicken (CSA) were purchased from Sigma. Sigma also supplied hemin, bromophenol blue, Reactive Blue 2-Sepharose (Cibacron blue) and Concanavalin A-Sepharose (Con A). Rabbit antiserum to HSA was purchased from Accurate. Sodium dodecyl sulfate (SDS) was obtained from BDH and the electrophoretic apparatus was purchased from Idea Scientific of Corvallis, Oregon.

Plasma Fractionation

The serum albumin of garter snake (SSA) was isolated using ammonium sulfate fractionation according to Keller and Block (117). The plasma of Taricha was first fractioned by elution through a Sephadex G-100

column (12). The fractions that contained material of 65 to 75K daltons in molecular weight were pooled, placed in dialysis tubing and concentrated 20-fold against polyethylene glycol 6000 (Sigma). The concentrate was then either treated with $(NH_4)_2SO_4$ as was snake serum or passed through a Blue Sepharose column (described later in this section).

Polyacrylamide Gel Electrophoresis (PAGE)

Nondenaturing or "native" PAGE was performed according to Davis (29) except that 0.06 M boric acid replaced the 0.38 M glycine in the tray running buffer (12). Denaturing SDS-PAGE was implemented according to Francis and Becker (118). The gel electrophoresis was carried out in mini-slabs 8 x 10 x .08 cm. Staining with Coomassie blue R-250 (CbR-250) and destaining were performed according to Davie (58).

Protein Composition

The compositions of the albumins and Taricha proteins were determined using a Beckman 120B amino acid analyzer according to Spackman et al. (114), with the cystine amounts determined according to Spencer and Wold (115). The composition data were subjected to analysis by the statistical method of Cornish-Bowden (116).

Immunodiffusion

The reactivities of the albumins and *Taricha* proteins with rabbit antihuman albumin serum were determined with the use of agarose micro films (MCI) according to Ouchterlony and Nilsson (113). Twelve µl of each albumin solution (1.0 mg/ml) were placed in each of the 6 peripheral wells of the swollen agarose film and the same volume of the antihuman albumin serum was placed in the center well.

Assays for Glycosylation

The *Taricha* proteins were tested for saccharide presence using two gel staining methods and an affinity chromatographic procedure. The periodic-acid-Schiffs (93) and Alcion blue (111) reagents were used to stain both native and SDS gels specifically for glycoprotein, and a Conconavalin A-Sepharose resin was used to bind proteins that are glycosylated (5). The Con A column (1.0 x 10 cm) was equilibrated with a 0.05M phosphate buffer, pH 7.0. Each protein sample was introduced to the resin and then 4 volumes of starting buffer were washed through the column. A step gradient elution of 0.10, 0.20 and 0.30 M α-D-methylmannoside followed and 1.0 ml fractions were read for absorbance at 280 nm.

Albumin Binding of Dyes, Hemin and Hemoglobin

Bjerrum (119) noted that HSA and BSA bound bromophenol blue (BPB) during gel filtration. Since BPB is normally used as a tracking dye for PAGE (29), a native gel run was prepared including the albumins and Taricha proteins. The samples were prepared by adding the lyophylized protein to a solution of 0.0625 M Tris-HCl, pH 6.8, 10% glycerol and 0.001% BPB. Approximately 20 µg of each protein were loaded onto the gel and electrophoresed for about 1.5 hr at 120V at 4°C. During the run, the gel was illuminated against a white background so visualization of any BPB migration could be detected.

Following the observation that the sulfonated aromatic dye Cibacron blue (CB) bound to HSA (105), the dye has been coupled to column supports such as Sepharose for the purpose of selectively removing serum albumins from plasma (105). A 1.0 x 15 cm column was filled with Blue Sepharose and the resin was equilibrated with a 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M KCl. The samples were equilibrated in the starting buffer and each applied to the column in separate runs. The resin was washed with 4 volumes of starting buffer and then with the same buffer with the KCl concentration increased to 1.5 M. Stripping the resin for regeneration was done by

washing with the elution buffer with 6 M urea added. One ml fractions were collected and the entire elution was monitored for protein using absorbance at 280 nm.

For assessment of the hemin-binding abilities of the albumins and Taricha proteins, the procedure of Daniels et al. (69) was followed. One mg of hemin was dissolved in 1.0 ml of 0.10 N NaOH and the pH rapidly lowered to 8.4 by adding 0.1 M Tris to the solution. Twenty mg of each protein were added and the solution incubated for 30 min at room temperature. The excess hemin was removed by dialysis against 0.05 M Tris-HCl, pH 8.0, overnight at 4°C. Aliquots of the samples were taken, made to 10% glycerol and electrophoresed in a 7% native gel. The gel was then stained for peroxidase activity (heme presence) (118). Densitometry of the gel lanes was performed using a Gilford 220 scanning spectrophotometer at 490 nm.

In order to determine albumin affinity for Taricha Hb, 10 mg of each albumin and Taricha protein were dissolved in 1.0 ml of a 20 μM Taricha cyanomet-Hb solution buffered by 0.05 M Tris-HCl, pH 7.5. The samples were incubated for one hr at 21°C, each was loaded onto a 7% native gel and the proteins were resolved. Adjacent to each albumin-Hb sample was a sample of the same albumin without the Hb. The gel

was stained for peroxidase activity and subsequently stained for protein with CbR-250. The staining patterns were recorded and compared.

Isoelectric Focusing

The proteins were compared regarding isoelectric character in mini-slab gels according to Hoyle (112). The proteins were stained with CbR-250 and destained (58).

Results

After the Sephadex G-100-fractioned Taricha plasma material was fractioned further with increasing $(NH_4)_2SO_4$ concentrations (Fig. IV.1), SDS-PAGE revealed a single band with a molecular weight of 68K daltons which remained soluble in 3 M $(NH_4)_2SO_4$, as is characteristic of serum albumins (15). When an aliquot of the same G-100 fraction was electrophoresed in native-PAGE, 3 proteins were resolved; a single band and a slightly-separated pair with twice the mobility. The 3 M $(NH_4)_2SO_4$ fraction revealed only the high-mobility pair of proteins. Second-dimensional SDS-PAGE analysis (63) of that G-100 fraction demonstrated the low mobility protein to be the Hb-binding 75K protein of Taricha, previously reported (12). The high-mobility pair of proteins migrated as one band

in SDS-PAGE, indicating a molecular weight of 68K daltons (see Fig. IV.2). Densitometry of the native gel showed that the pair of 68K proteins consisted of an anodal protein that was approximately 4-fold more abundant than the more cathodal protein (Fig. IV.3). This was the case in the plasmas of other Taricha specimens, indicating this "bisalbuminemia" (120) was not a polymorphic condition. Since bisalbuminemia in humans is caused by either chemical modification of the protein (121) or the partial saturation of albumin by certain ligands (120), the gel protein bands were examined further. The proteins were electroeluted from excised gel slices, hydrolyzed and analyzed for amino acid composition. Results demonstrated the two gel bands represented distinct proteins. The data will be listed later in this section.

Since the structure of serum albumins has been highly conserved (104,106,122) and disulfide bridges are responsible for much of the distinctiveness of albumin structure, SDS-PAGE was employed to compare the Taricha proteins to the albumins regarding structural changes upon reduction with β-mercaptoethanol (BME). Figure IV.2 shows various albumins with the Taricha 68K proteins. The molecular weights of all of the proteins were approximated using the mobilities

of the molecular weight markers. It appears that CSA undergoes the greatest change of SDS extension of the molecule after reduction of the disulfide bonds. SSA exhibited the smallest change after reduction. Both of those proteins appear to be the same size as the other albumins, as indicated by their reduced forms. The 68K Taricha proteins displayed sizes of both forms that were virtually indistinguishable from HSA, OSA and BSA. In a previous SDS-PAGE run (12) the 75K of Taricha had been prepared with and without BME, and run along with the 68K proteins. The 75K protein failed to show any change in apparent molecular weight upon reduction with BME.

The Ouchterlony immunodiffusion (113) method performed on agarose films revealed BSA and OSA cross-reactivity with rabbit antihuman albumin serum (Fig. IV.4). CSA, SSA and the Taricha proteins however failed to react.

Isoelectric focusing in gels (113) indicated that the albumin and the Taricha 68K proteins have similar pI values. The isoelectric points observed are as follows: HSA, 4.7; BSA, 4.6; OSA, 4.6; CSA, 4.7; SSA, 4.6; Taricha 68K proteins, 4.7 and 4.8. The 75K protein of the newt has a pI of 6.3 (not shown).

After the albumins and newt proteins were resolved in both native and SDS-PAGE systems, the gels were

stained for carbohydrate presence (93,111) and subsequently with CbR-250 for protein (58). Both carbohydrate-specific stains produced the same results. HSA used as a control failed to stain as did Taricha 68K proteins. The 75K protein did stain however, as did control proteins ovalbumin and transferrin (not shown). As controls, HSA and BSA were eluted through the Con A-Sepharose column individually, resulting in neither binding to the resin. When the 65-75K Sephadex G-100 fraction of Taricha plasma was eluted, both 68K proteins failed to bind to the resin. The 75K protein however was eluted only in the 0.10 and 0.20 M α-D-methylomannoside washes, as was transferrin in a separate run.

The dyes BPB and CB have been reported to be bound by HSA and BSA in the case of BPB (119) and by HSA in the case of CB (105). As the albumins and newt proteins were electrophoresed, HSA, BSA, OSA and PSA exhibited an association with BPB. CSA, SSA and newt proteins showed no such interaction. The Blue Sepharose column was shown to bind mammalian albumins and Taricha 68K proteins, but was unable to retain CSA, SSA and the 75K protein of Taricha. The mammalian albumins eluted with 1.5 M KCl as did one of the newt 68K proteins, but the 6 M urea wash dissociated more Taricha protein, 68K in size (see Fig. IV.5). The 1.5

M KCl and 6 M urea fractions of Taricha were dialyzed against H_2O overnight at 4°C, lyophylized, hydrolyzed and the amino acid compositions determined. The compositions agreed with the analyses of the two high-mobility proteins resolved in native gels (Fig. IV.3).

After the proteins had each been introduced to a hemin or cyanomet-Taricha Hb solution, incubated and resolved in native PAGE, the peroxidase activity-specific staining (118) revealed HSA to be the only protein to associate with hemin, and the newt proteins the only ones to bind Taricha Hb (Fig. IV.3). The Taricha sample lane of the native gel was separated, the peroxidase-active bands excised, treated according to O'Farrell (63) and inserted into wells of a 12% SDS gel for second-dimensional analysis. The gel was stained with CbR-250, destained and scanned densitometrically at 550 nm. The two high-mobility bands each had dissociated into a 68K and two 15K bands in the SDS gel, while the low-mobility native band dissociated into a 75K and two 15K bands. The densitometry revealed that after correcting for differences in molecular weights, each of the 68K proteins along with the 75K were found to be approximately equimolar with the pair of 15K proteins associated with them (Fig. IV.6). The 15K proteins are the α and β subunits of

Taricha Hb (12), so this evidence suggests a stoichiometry of Hb to either 68K or 75K proteins of one αβ dimer to one plasma protein. The quantitative peroxidase activity yields (123) of the bands in the native gel from duplicate lanes supported that interpretation (Fig. IV.6). The minor Hb subunit observed earlier by Coates et al. (158) was not resolved under the second-dimensional SDS-PAGE conditions used here.

The last property of the proteins compared was amino acid composition (Table I). The data were used to determine any phylogenetic relatedness among the proteins by implementing the method of Cornish-Bowden (116) which established the composition index:

$$S\Delta n = \tfrac{1}{2}\Sigma(n_{iA} - n_{iB})^2$$

where n_{iA} is the number of the i-th type amino acid in protein A and n_{iB} is the number of the same type amino acid in protein B (in residues/1000 in this case).

If $S\Delta n$ is less than 0.42 N (where N is the total number of residues per protein), it can be assumed that the proteins are almost certainly related (116). If $S\Delta n$ is greater than 0.42 N, but less than 0.93 N, there is weak indication that the proteins are related.

And if SΔn is greater than 0.93 N, the compositions do not support any suggestion of relatedness. The composition index values are listed in Table II. Table III lists the albumins and newt proteins in order of increasing composition indexes. From the SΔn values and the criteria established by Cornish-Bowden (116), the following observations can be made: 1) BSA, OSA and PSA indicate relatedness with HSA, while CSA, SSA and Taricha proteins exhibit indexes too large to assume a relationship with HSA based on composition alone; 2) Taricha 68K proteins indicate no greater relatedness to each other than do one of the mammalian albumins to another (e.g. OSA to PSA); 3) the 75K protein does not demonstrate relatedness to the newt 68K proteins and exhibits considerably greater numerical distance from the albumins than do the 68K proteins of Taricha.

Discussion

Peters (101) listed five criteria that if met would reasonably define a serum albumin. Maintaining solubility in half-saturated ammonium sulfate, possessing the greatest electrophoretic mobility of any major plasma component, having a molecular weight of 65 to 68K daltons, absence of carbohydrate along with comprising the main component of normal plasma or

serum are the properties that when combined, traditionally describe the uniqueness of serum albumin. All of those properties were included in this comparative study.

Since Masat and Dessauer (15) identified serum albumins in 15 reptilian species, it was decided to include a reptilian albumin with that of chicken and four mammals (including human) for the comparison with the Taricha proteins. Using the $(NH_4)_2SO_4$ fractionation procedure of Keller and Block (117), pure protein was isolated from garter snake that would be demonstrated to be a serum albumin. When Taricha plasma was subjected to the same $(NH_4)_2SO_4$ treatment (Fig. IV.1), protein which was later resolved into two components in native-PAGE was observed to have a solubility similar to the albumins of reptiles, birds and mammals (24). The two native-PAGE components have mobilities very similar to the albumins compared in Figure IV.3. Isoelectricfocusing of the Taricha high-mobility protein pair revealed them to have pI values of 4.7 and 4.8 which are within 0.2 pH units of the other albumins. Up to that point in the study, it appeared that Taricha did indeed possess at least one albumin if not two. Densitometric scans of the plasma samples in the gel revealed the two high-mobility proteins together constituted approximately 30% of the

total protein in Taricha plasma. That estimate was verified by comparing total lyophilized plasma protein mass to $(NH_4)_2SO_4$ fractioned protein mass. That 30% figure of Taricha does not compare with the amount of albumin in mammals which approaches 60%, but it is similar to the levels of serum albumins found in various reptiles (15). The more aquatic turtles in that study were found to have an albumin level of only 20% of overall plasma protein, while the totally terrestrial iguanid lizards had almost 50% of their plasma protein in albumin form. Those findings support the osmoregulative role given to serum albumin (100,102) and thus it was no surprise to find that Taricha, which were captured during their aquatic phase possessed lower albumin levels than those of more terrestrial animals.

Of the animals compared, Taricha was the only one to exhibit bisalbuminemia, a condition of two electrophoretic forms of albumin (120) (Fig. IV.3). In order to determine if the two "albumins" were different proteins or actually one protein with a portion of its population modified chemically or possessing mobility-altering ligand associations (120,121), second-dimensional SDS-PAGE was first employed (63). The two native gel bands of Taricha were found to have molecular weights of approximately 68K daltons. They

both appeared to be identical in size in the SDS gel, so the bisalbuminemia was not believed to be a result of different molecular weights. The next step taken in attempting to describe the reason for the bisalbuminemia in Taricha was the amino acid analysis of the protein in each gel band. The compositions indicated that the electrophoretic bisalbuminemia actually represented two different proteins. Whether or not differential ligand-binding contributed to the band splitting is not known. The existence of a pair of albumins in a snake had been previously reported by Masat and Dessauer (15), so the suggestion of multiple albumins in another lower vertebrate is not considered to be unreasonable.

Since the solubilities, electrophoretic mobilities, abundances in plasma along with molecular weights of the Taricha 68K proteins were very similar to those properties of the known serum albumins, it appeared that the amphibian possesses two serum albumins.

There remained one more criterion of classical albumin definition, and that was the absence of protein glycosylation (101,110). With one known albumin as a control, the Taricha proteins were subjected to two different saccharide-specific stains after resolution in polyacrylamide gels. Neither the

Schiff's reagent (93) nor Alcian Blue (111) stained HSA or the "albumins" of Taricha. The 75K Taricha protein did however react positively as did the glycoproteins transferrin and ovalbumin. The Con A-Sepharose affinity column produced results that agreed with the staining. The Taricha albumins showed no affinity for the lectin resin whereas the 75K protein exhibited specific binding to the column. The results of the staining and Con A affinity chromatography are strong evidence for the lack of glycosylation in the two albumins of Taricha, and the presence of carbohydrate in the 75K protein.

Weigle (124) tested the reactivites of various serum albumins with antiserum to BSA. HSA was observed to react with only about 14% of the intensity of BSA, while CSA and alligator albumin failed to react at all. Considering those results, it was not surprising to note a lack of reactivity of SSA and the Taricha albumins with antiserum to HSA (see Figure IV.4). The fact that a familiar albumin such as CSA lacks determinants for antibodies to a mammalian albumin exemplifies the variability of this class of proteins.

There are other properties that help distinguish serum albumins from other blood constituents. Certain

serum albumins have been reported to bind an impressively diverse array of ligands (104). Albumins have also been seen to possess a distinctive, conserved structural organization that includes disulfide bridges occuring at regular intervals (106,122). The ligand-binding behavior and structural conservation in many albumins were considered to be appropriate qualities to look for in the Taricha proteins.

The sizes of the amphibian's proteins had already been established, but it was not known if they would show significant structural change upon reduction with BME as had previously been observed with HSA and BSA in this laboratory (as seen in SDS-PAGE). To determine structural similarities among the albumins, SDS-PAGE was again employed. The results demonstrated a structural change in Taricha albumins after reduction that appeared virtually identical to that of the mammalian albumins (Fig. IV.2). CSA indicated a greater change in apparent size while SSA exhibited a noticably smaller change than mammalian or Taricha albumins. All of the albumins appeared to have almost identical sizes in their reduced forms. From the SDS-PAGE evidence, with regard to the disulfide nature of their structures, newt 68K proteins certainly appear to be "true" serum albumins.

With respect to the binding of certain ligands, the albumins compared, including those of Taricha exhibited a good deal of variability. When the binding of two different dyes by the albumins was assayed for instance, the resulting pattern was more erratic and complex than was anticipated. In the case of BPB, Bjerrum (119) observed the dye to interact with HSA and BSA. In this study, a visible amount of BPB was seen to associate only with mammalian albumins. There were no detectable amounts migrating with CSA, SSA, or Taricha albumins in native-PAGE.

The other dye included in this study, Cibacron blue had been reported to bind HSA (105). Rather than studying solution binding, immobilized CB in the form of Blue Sepharose was used in our experiments. Results of separate elutions of the various albumins duplicated the BPB results except for an interesting inconsistency (Fig. IV.5). Whereas BPB failed to exhibit association with Taricha albumins, CB showed affinity for one of them similar in strength to that of CB to HSA. The other newt albumin displayed such high affinity for the Blue Sepharose resin that at least in part, nonspecific binding is suspected. Nevertheless, as far as affinity for CB is concerned, at least one Taricha albumin appears to be more related to mammalian albumins than do CSA or SSA.

Human serum albumin has been reported to bind hemin (68) with certain histidine residues implicated in the interaction (125). Bunn and Jandl (68) reported that HSA along with other primate albumins bound hemin, while other mammalian albumins failed to do so. In this study, HSA was the only albumin to exhibit heme association. This result confirmed what had previously been observed concerning nonprimate mammals (68) and also indicated that the albumins of chicken, snake and Taricha also lack affinity for hemin. This evidence suggests that although hemin-binding by HSA is believed to be a crucial physiologic function (68,78), it has a narrow phylogenetic range and has evolved late in evolutionary time.

Hemoglobin has been known to be a ligand of the plasma protein haptoglobin for over 40 years (9). Hb has not however been demonstrated to bind to a known albumin. Liang (53) reported a frog's Hb interacted with an albumin-like protein in its plasma, but the protein was not demonstrated to be a true serum albumin. However, since in this study another amphibian was shown to possess an interaction suggested by Liang (53), it is probable that the frog then tested has an Hb-binding serum ablumin. As mentioned in "Results", the Taricha 75K protein seen previously to

also bind Hb (12), has properties that easily distinguish it from Taricha albumins. Its larger molecular weight, lack of structural change upon BME reduction, glycosylation, isoelectric character and solubility indicate it is not a serum albumin. Since the other albumins were observed to lack affinity for Taricha Hb, and newt albumins were seen to lack affinity for human and cow Hbs (from previous unpublished work in this laboratory), it is assumed that Taricha albumin-Hb interaction is very specific and requires unique structural and/or chemical properties of each protein.

The amino acid compositions of the albumins compared were also used to further assess any possible relatedness between the newt proteins and one if not more of the other serum albumins. Composition indexes calculated from the data in Table I are listed in Table II. Table III compares each albumin to the rest by listing the others in order of increasing composition index. From the theoretic limits stated by Cornish-Bowden (116) (see "Results"), the mammalian albumins indicate relatedness to each other while CSA, SSA and Taricha albumins do not show relatedness to any of the mammalian proteins. SSA has an index with CSA that is outside the upper limit, but one Taricha albumin has an index of 0.88 with SSA, indicating

possible relatedness. Taricha albumins displayed no greater relatedness between each other than did OSA and PSA together. The 75K protein indicated no relatedness with any of the albumins. From the comparison of albumin compositions, the proteins were seen to vary significantly; although residues that contribute to the conserved disulfide bridging (cysteine) and those that contribute to solubility and low isoelectric points (aspartic and glutamic acids) were found to be similar in amounts (Table I). It would seem that the albumin molecule has tolerated considerable change in composition without being grossly altered in its structural and chemical properties.

The combined results of this study suggest that the amphibian Taricha granulosa possesses two serum albumins. Although the two proteins lack certain ligand-binding abilities of HSA, so do the proteins in chicken and snake that are referred to as albumins. The Hb-binding nature of Taricha albumins is a unique property of that class of proteins that appears to have been lost in the reptilian through mammalian classes of vertebrates.

Acknowledgements

This work was supported by the Medical Research Foundation of Oregon. We wish to thank Jennifer Stephens and Carl Boswell for their invaluable help with the immunodiffusion runs. We are also grateful to Dr. Joseph Beatty for his guidance in the collection of the newts and supervision of their storage and feeding.

Figure IV.1

Flow chart depicting the progression of Taricha protein purification along with the various analytic procedures employed in this study. Snake albumin was purified by $(NH_4)_2SO_4$ fractionation and it, along with the other albumins included was also subjected to the assays shown here.

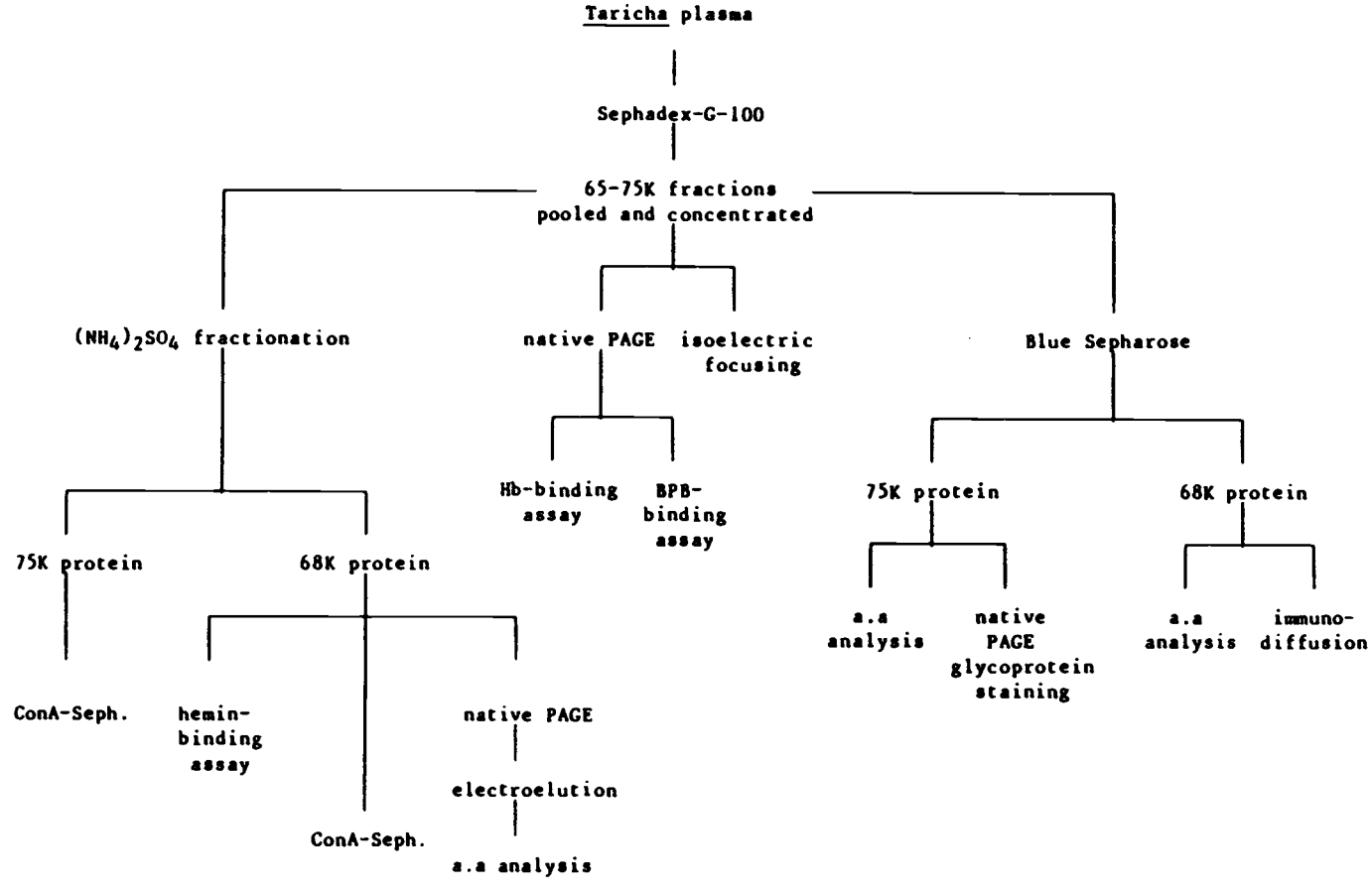
Taricha plasma
Sephadex-G-100
65-75K fractions pooled and concentrated
$(NH_4)_2SO_4$ fractionation
native PAGE
isoelectric focusing
Blue Sepharose
Hb-binding assay
BPB-binding assay
75K protein
68K protein
75K protein
68K protein
ConA-Seph.
hemin-binding assay
native PAGE
electroelution
ConA-Seph.
a.a analysis
a.a analysis
native PAGE glycoprotein staining
a.a analysis
immuno-diffusion

Fig. IV. 1

Figure IV.2

A 12% SDS gel that demonstrates the apparent molecular weight change resulting from reduction with BME in each of the following: 1) HSA; 2) HSA + BME; 3) OSA; 4) OSA + BME; 5) BSA; 6) BSA + BME; 7) CSA; 8) CSA + BME; 9) SSA; 10) SSA + BME; 11) TSA; 12) TSA + BME. The Taricha albumins (lanes 11 and 12) appear as one band with or without BME. The molecular weight markers are indicated in K daltons.

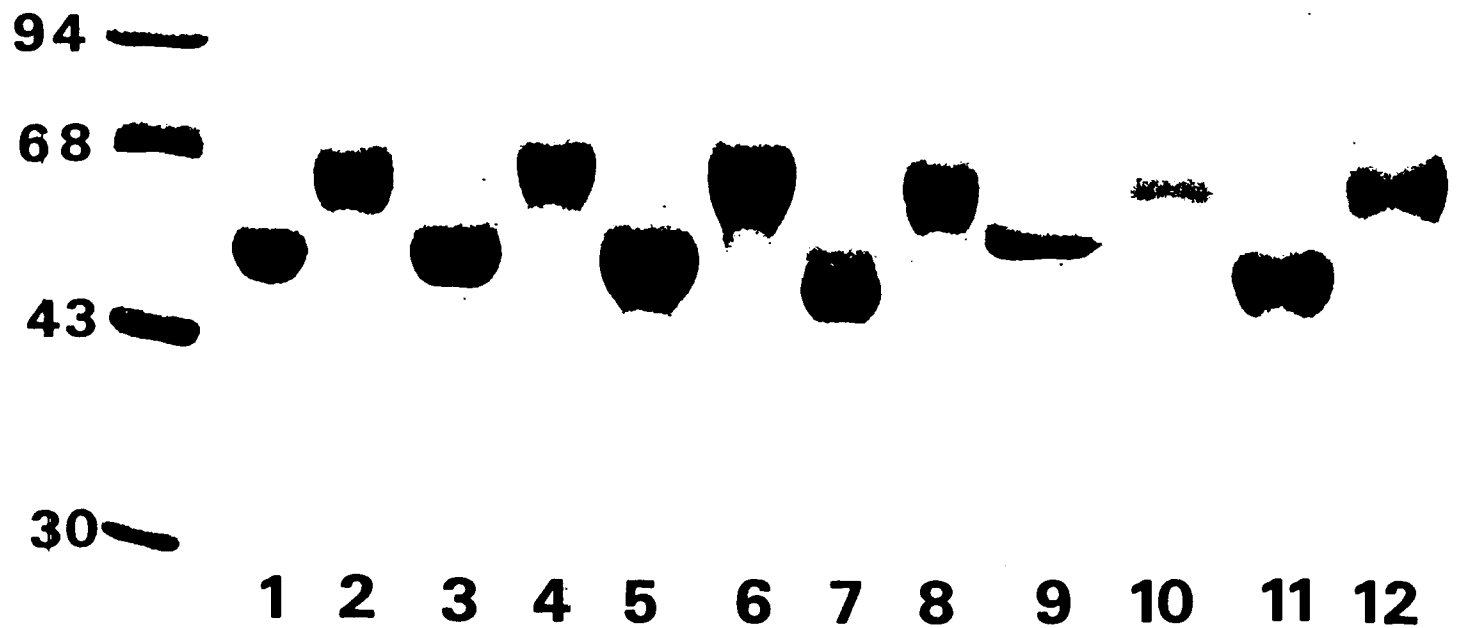

Fig. IV.2

Figure IV.3

a) A 7% native gel that illustrates the relative electrophoretic mobilities of: 1) HSA; 2) OSA; 3) BSA; 4) CSA; 5) SSA (arrow); 6) Taricha albumins; 7)Taricha albumin-Hb complexes. The other albumins (HSA through SSA) were revealed to lack affinity for Taricha Hb and their mobilities remained unchanged after incubation with the Hb.

b) A densitometric scan of Taricha albumins (lane 6 of the gel above) indicating their relative amounts revealed by CbR-250 staining.

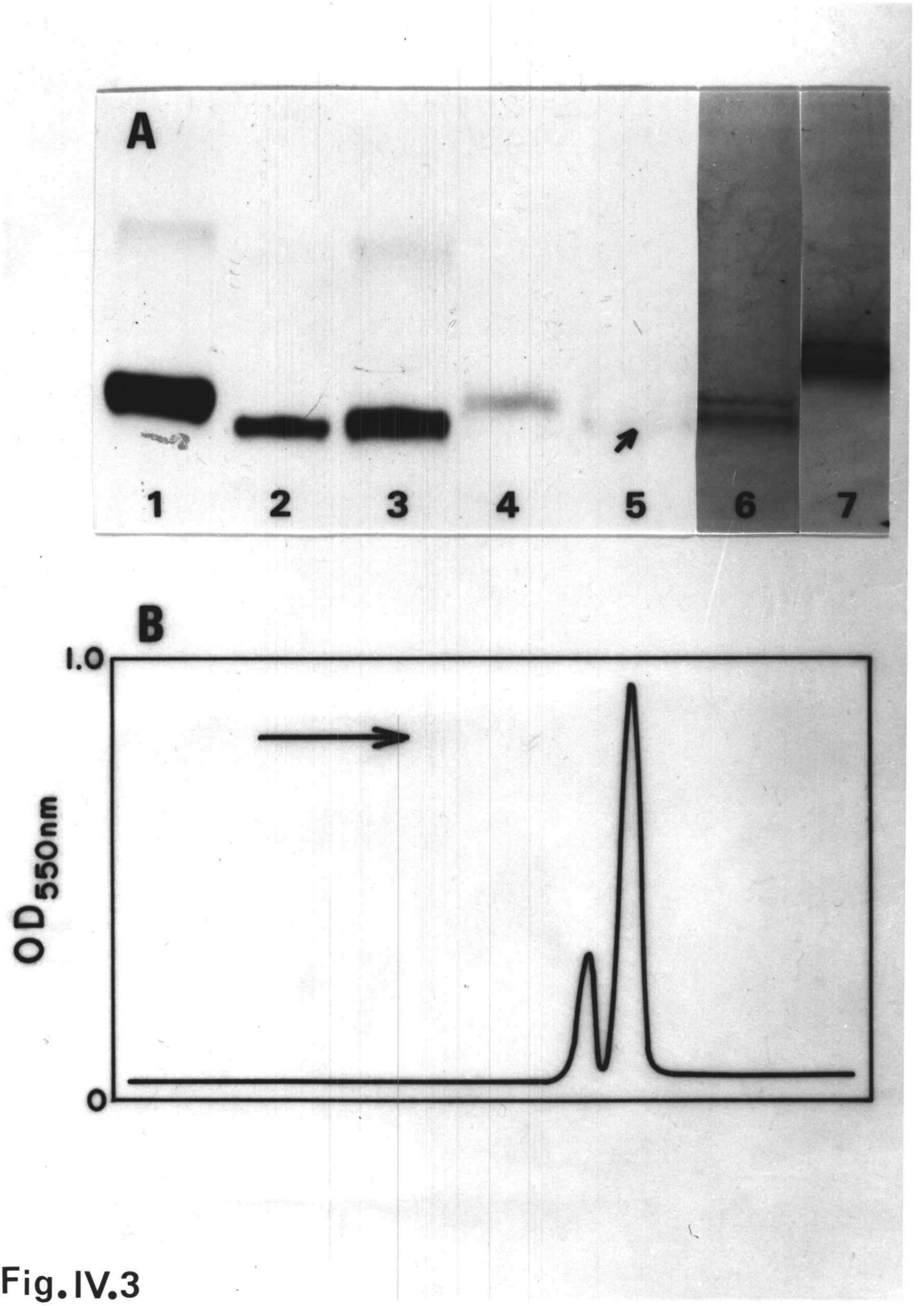

Fig. IV.3

Figure IV.4

An agarose micro film (3.5 x 3.5 cm) that has been stained with CbR-250 and destained after immunodiffusion of rabbit antihuman albumin serum into the following: 1) SSA; 2) OSA; 3) BSA; 4) CSA; 5) Taricha albumins isolated by $(NH_4)_2SO_4$ fractionation; 6) Taricha albumins eluted from the Blue Sepharose resin. While OSA and BSA reacted with the serum, there was no evidence of crossreactivity in each of CSA, SSA or Taricha albumins.

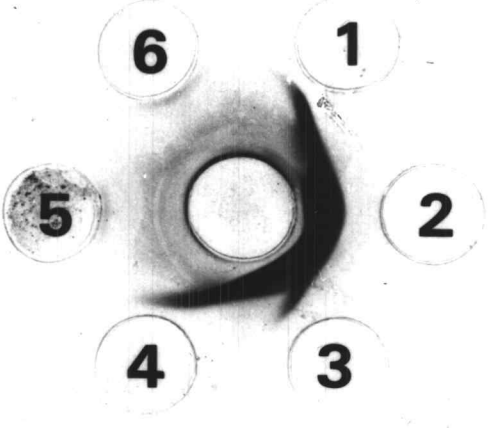

Fig. IV.4

Figure IV.5

An elution profile of the 65-75K dalton-Sephadex G-100 fractions of Taricha plasma eluted through the Blue Sepharose column. Peak 1 contained the 75K protein that failed to bind to the cibacron blue resin along with a small amount of albumin. Peak 2 contained one albumin that was eluted with 1.5 M KCl and Peak 3 contained the other albumin which was dissociated from the resin only after 6 M urea was introduced to the column. Amino acid analysis of the proteins in Peaks 2 and 3 confirmed them to be distinct proteins.

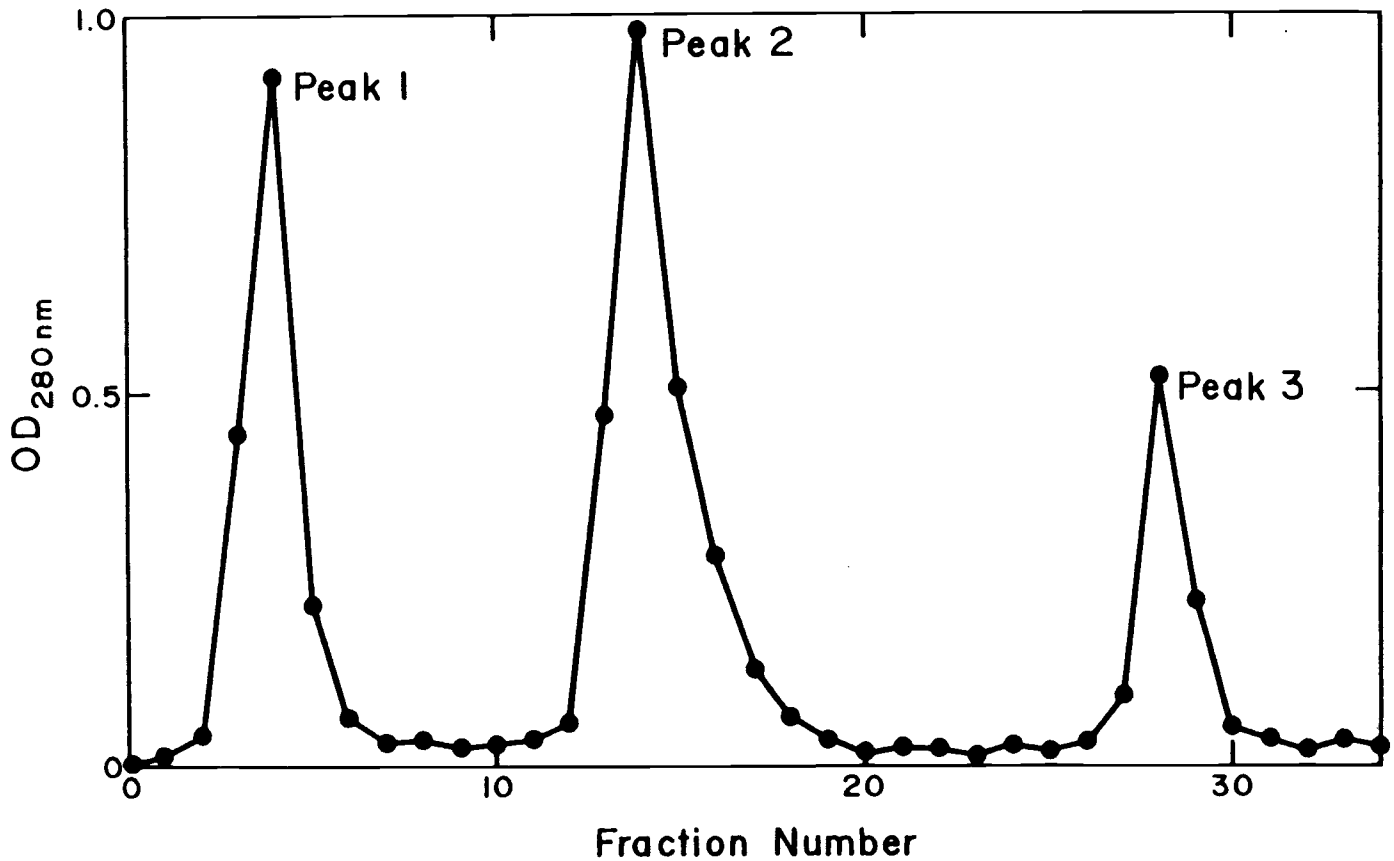

Fig. IV.5

Figure IV.6

Representative densitometric scans of the second-dimensional SDS-PAGE resolution of: A) native gel Taricha low-mobility peroxidase-active band; B) Taricha high-mobility peroxidase-active bands. The low-mobility band (a) was comprised of the 75K glycoprotein and the αβ subunits of Taricha Hb. The two high-mobility bands were each observed to dissociate into components of the same sizes (68K and 15K) in the same relative proportions. By using CbR-250 peak areas to estimate relative protein amounts (550 nm), a stoichiometry of one Hb-binding protein to one Hb dimer was revealed. The peroxidase activity peak areas (490 nm) from duplicated gel lanes supported that interpretation due to the quantitative nature of peroxidase staining yields.

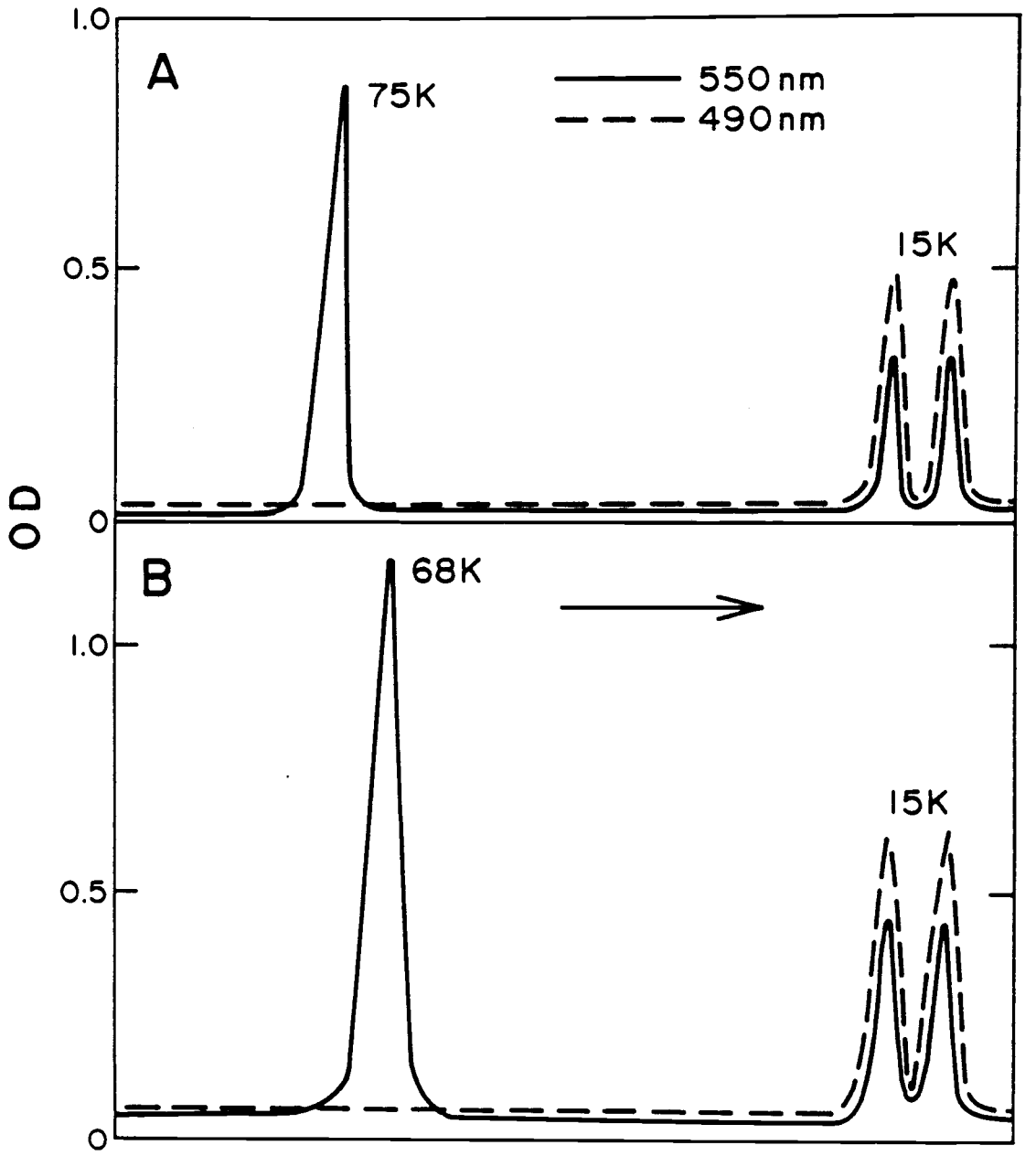

Fig. IV. 6

Table I. Amino Acid Compositions[a]

Amino acid	HSA	BSA	OSA	PSA	CSA	SSA	TSA(1)[c]	TSA(2)[d]	75K
Aspartic acid[b1]	88.1	91.4	98.2	82.9	96.9	108.3	99.4	86.1	110.5
Threonine	49.2	63.2	54.5	45.2	38.0	48.6	49.3	48.4	58.6
Serine	40.5	44.2	45.1	45.3	65.4	65.4	58.3	49.0	74.4
Glutamic acid[b2]	141.6	137.3	124.6	133.6	138.7	126.0	111.9	128.9	95.5
Proline	41.3	50.5	50.9	50.6	49.7	48.0	59.6	49.6	47.9
Glycine	21.3	27.5	33.7	29.1	43.0	54.9	49.9	41.0	83.0
Alanine	102.4	77.7	86.5	84.3	71.6	76.9	63.4	78.5	78.6
½ Cystine	60.6	60.2	56.8	62.3	57.4	48.5	48.1	45.4	37.6
Valine	67.1	59.9	63.1	57.5	61.7	55.4	57.0	55.6	60.4
Methionine	10.7	7.3	7.8	-	26.8	8.2	25.4	27.6	16.3
Isoleucine	16.0	22.0	23.7	33.1	51.6	53.2	46.1	48.5	40.0
Leucine	112.4	106.4	107.0	102.3	69.0	92.6	78.7	80.8	81.4
Tyrosine	30.1	30.4	35.8	35.0	35.0	31.9	31.1	34.2	38.3
Phenylalanine	52.1	48.2	47.0	45.2	45.9	34.1	40.1	36.7	37.0
Histidine	25.5	29.2	30.6	26.8	22.1	39.3	43.2	59.9	24.2
Lysine	96.4	101.7	93.2	117.6	76.9	76.1	96.0	95.8	81.4
Arginine	44.9	43.0	41.8	49.3	50.4	32.7	42.7	34.2	34.9

[a] Amino acid compositions as described in text (22 hr, 72 hr, dimethylsulfoxide oxidized hydrolysates).

[b] Values include prehydrolyzed: 1. aspargine and 2. glutamine.

[c] Taricha serum albumin eluted off the Blue Sepharose column with 1.5 M KCl.

[d] Taricha serum albumin eluted with 6 M urea.

Table II. SΔn Values Between Various Serum Albumins[a].

	HSA	BSA	OSA	PSA	CSA	SSA	75K[b]	TSA(2)	TSA(1)
TSA(1)	3.47	1.92	1.60	2.14	1.18	0.88	1.57	0.68	0
TSA(2)	2.75	1.79	1.62	1.93	1.60	1.17	3.01	0	
75K[b]	5.51	3.98	3.04	4.58	2.65	1.36	0		
SSA	2.99	1.97	1.47	2.45	1.19	0			
CSA	2.28	2.33	2.23	2.68	0				
PSA	0.89	0.46	0.66	0					
OSA	0.62	0.19	0						
BSA	0.61	0							
HSA	0								

[a] Values are in increments of N, where N is the total number of amino acids per protein.

[b] The 75K glycoprotein of Taricha.

Table III. Serum Albumins Listed in Order of Increasing $S\Delta n$ Values Against Each Other.

HSA	BSA	OSA	PSA	CSA	SSA	TSA(1)	TSA(2)
BSA	OSA	BSA	BSA	TSA(1)	TSA(1)	TSA(2)	TSA(1)
OSA	PSA	HSA	OSA	SSA	TSA(2)	SSA	SSA
PSA	HSA	PSA	HSA	TSA(2)	CSA	CSA	CSA
CSA	TSA(2)	SSA	TSA(2)	OSA	OSA	OSA	OSA
TSA(2)	TSA(1)	TSA(1)	TSA(1)	BSA	BSA	BSA	BSA
SSA	SSA	TSA(2)	SSA	PSA	PSA	PSA	PSA
TSA(1)	CSA	CSA	CSA	HSA	HSA	HSA	HSA

SECTION V

UPTAKE OF IRON FROM HEMOGLOBIN AND THE HAPTOGLOBIN-HEMOGLOBIN COMPLEX BY HEMOLYTIC BACTERIA

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Abstract

The abilities of Staphylococcus aureus and Streptococcus pyogenes to remove iron from mouse ^{59}Fe hemoglobin that was either in free form or complexed with human haptoglobin, have been evaluated. ^{59}Fe hemoglobin from the amphibian Taricha granulosa was also used in free form or complexed with the amphibian's hemoglobin-binding proteins. Contrary to what was reported from a study using pathogenic Escherichia coli, haptoglobin failed to exhibit bacteriostatic influence when complexed with hemoglobin. In our study, more ^{59}Fe was removed by the bacteria from the haptoglobin-hemoglobin complex than from free mouse hemoglobin. The hemoglobin and hemoglobin-plasma protein complexes of Taricha were stripped of ^{59}Fe at similar rates and extents by both species.

Introduction

Certain virulent bacteria have been shown to grow well in the presence of hemoglobin (Hb)[1] (139,140, 141). Originally, the globin protein was believed to be the nutritional source required by the bacteria for growth (143). However, it was later reported that Escherichia coli grew in the presence of Hb but not with apo-Hb (globin only) (142), thus the heme moiety was indicated to be the growth-promoting factor. That fact has been confirmed repeatedly with various species and strains of staphylococci and streptococci (144,145). It has also been reported that the simultaneous injection of Hb with E. coli into rats results in lethality compared to no lethal response observed after injection of E. coli alone (141). It is understood that in certain bacteria, virulence is dependent on higher levels of environmental iron than are required for normal metabolism (146).

The erythrocytic component with by far the greatest abundance is Hb. The remaining components

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[1]Abbreviations: Hb, hemoglobin; Hp, haptoglobin; HbP, hemoglobin binding protein; PBS, phosphate buffered saline; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

lack growth-promoting influence on pathogenic E. coli (141). It seems apparent then, that virulent bacteria such as Staphylococcus aureus and Streptococcus pyrogenes would exert energy-dependent hemolytic activity only to acquire the nutritional benefits offered by Hb heme.

Haptoglobin (Hp) when complexed with Hb has been reported to protect the Hb from denaturation by acid (61). Hp was also observed to prevent heme exchange from complexed Hb to free Hb (68), although Hb molecules were seen to readily exchange heme groups. And when the Hp-Hb complex was substituted for Hb in culture media, a lack of growth of a pathogenic strain of E. coli was reported, even though it had exhibited good growth in Hb-supplemented media (17). This bacteriostatic action of Hp was believed to result from the protection of Hb by Hp against bacterial proteolysis (17). In another study, however (147), Hb was reported to be virtually impervious to proteolytic attack by several proteases including staphylococcal protease. However the Hp-Hb complex was cut in the Hp component by the bacterial protease(147). Hb then, was not revealed to be protected by Hp, but rather Hp was spared proteolytic attack by various proteases (with the exception of staphylococcal protease) when complexed with Hb.

Our study has involved the use of radiolabeled mouse Hb and its complex with human Hp along with Taricha granulosa ^{59}FeHb and its complexes with the amphibian's hemoglobin-binding proteins (HbP) as media supplements. Clinical isolates of S. aureus and S. pyogenes (strains unknown) were grown separately in each of the different ^{59}Fe sources. The uptake of ^{59}Fe from each source by the bacteria was followed. Each bacterial species exhibited not only the ability to acquire ^{59}Fe from the Hbs, but appeared to assimilate more iron from the Hp-Hb complex. The supply of ^{59}Fe to the two species by Taricha Hb was observed to be essentially the same as that of the HbP-Hb complexes of the amphibian. To our knowledge, this is the first report of a study involving the uptake of Hb iron by hemolytic bacteria.

Materials and Methods

^{59}Fe Labeling of Mouse and Amphibian Hbs

Three male Swiss Webster mice (Simonson Laboratories) weighing between 27 and 30 g were each injected intraperitoneally with 5 μCi of ^{59}Fe-citrate (ICN) in 0.05 ml with phosphate buffered saline (PBS). Three large Taricha granulosa weighing between 19 and 23 g (captured in Benton County, Oregon) were injected with 5 μCi of ^{59}Fe-citrate in the same manner

as the mice. All of the animals were kept separately at room temperature for 12 days, after which they were anesthetized and bled from the aorta in heparized Pasteur pipettes. The blood was transferred to 0.05 ml capped centrifuge tubes and the erythrocytes pelleted using a microcentrifuge for 2 min. spins at 10,000 r.p.m. The plasma supernatant fluid of each sample was removed and the erythrocyte pellet resuspended and recentrifuged in PBS three consecutive times. The pellets were resuspended again in PBS, frozen at 0°C for 2 hr., thawed and the cellular debris collected by centrifugation.

Gel Filtration

The mouse hemolyzate samples were pooled and eluted over a Sephadex G-100 column (1.5 x 90 cm) previously equilibrated with a 0.05 M Tris-HCl buffer, pH 7.2, with 0.05 M NaCl (12). The Taricha hemolyzates were treated in the same manner. The G-100 column had previously been calibrated with five proteins of known molecular weights. Two ml fractions were taken and read for absorbance at 415 nm. The Hb-rich fractions were pooled and concentrated in dialysis tubes against polyethylene glycol (PEG)-6000 (Sigma) to the point at which the absorbance at 540 nm was approximately 1.2 (1.7 ml volume). Using the

molar extinction coefficient of 1.4×10^4 at 540 nm (148) and the amount of radioactivity in each Hb sample determined by a Packard gamma counter, the specific activities were determined to be 9.63×10^4 cpm/μmole of mouse ^{59}FeHb and 1.12×10^5 cpm/μmole of Taricha ^{59}FeHb.

Preparation of ^{59}FeHb-Plasma Protein Complexes

Human plasma was furnished by the Red Cross of Portland, Oregon. Haptoglobin (Hp) was isolated from the plasma according to the method of Waks and Alfsen (31). The plasma (200 ml) was adjusted to pH 4.7 with HCl and dialyzed against a 0.01 M sodium acetate-acetic acid buffer (pH 4.7). After centrifugation at 15,000 g for 20 min., the plasma supernatant fluid was introduced to a DEAE-cellulose column (2 x 30 cm) equilibrated with a 0.01 M acetate buffer, pH 4.7. The column was then washed with ten volumes of a 0.02 M acetate buffer, pH 4.7. The Hp was eluted with 0.08 M acetate of the same pH. The Hp solution was then eluted through a Sephadex S-200 in a column 2 x 90 cm equilibrated with 0.05 M Tris-HCl, pH 7.4 with 0.05 M NaCl. The Hp fractions were pooled and concentrated in dialysis tubing against PEG-6000. The Hp was then stored at 0°C.

The pure Hp-Hb complex was obtained by combining the solutions of mouse ^{59}FeHb and human Hp so that there was a molar excess of Hb over Hp. The combination was incubated for 2 hr at 37°C with gentle shaking. The Hp-Hb complex was separated from free Hb by eluting the mixture through the Sephadex G-100 column used to purify the Hbs, under the same conditions. Two peaks were observed by monitoring the elution at 280 nm. The first peak represented material eluting in the void volume of the column. This was the Hp-Hb complex at approximately 160 K daltons in molecular weight. The second peak indicated material that eluted at a volume which suggested a size of 64 K daltons. It was the tetrameric mouse Hb. A sample of each peak was electrophoresed in a 7% polyacrylamide nondenaturing gel according to Francis and Becker (12) (Fig. V.1).

Taricha Hb-HbP complexes previously described (12) were obtained by adding an equal volume of the purified Taricha ^{59}FeHb solution to the plasma supernatant left from the separation of Taricha erythrocytes from whole blood. The mixture was incubated at room temperature for one hour. The complexes were separated from free Hb by elution through the G-100 column.

Media and Growth Conditions

Bacteria were grown in L broth consisting of (per liter): 10 g Bacto Tryptone and 5 g Bacto yeast extract (Difco Laboratories) with 10 g NaCl. The pH of the media was adjusted to 7.2. Bacteria were transferred from blood agar plates to flasks containing 100 ml of L broth with or without added rat Hb (100 μm) (Sigma). The cultures were incubated with shaking at 37°C. After 12 hr, the cultures grown in Hb-supplemented media had grown to stationary phase, while cultures innoculated into media without Hb showed no appreciable growth. The Hb-supplemented cultures were innoculated (1%) into flasks that each contained 100 ml of L broth supplemented with either mouse ^{59}FeHb, mouse ^{59}FeHb-human Hp, Taricha ^{59}FeHb or Taricha ^{59}FeHb-HbP complexes, each with 20,000 ± 200 cpm of ^{59}Fe activity. The cultures were incubated with shaking at 37°C. One-half ml samples of each culture were removed at various time points, placed in 1.5 ml capped centrifuge tubes and pelleted. The sample supernatants were removed and stored at 4°C, and the cell pellets were resuspended in fresh L broth containing no ^{59}Fe and repelleted. The supernatants were poured off and the tubes were placed in a Packard gamma counter for determination of ^{59}Fe activities.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to Laemmli (57) as modified by Francis and Becker (12). Mini-slab gels 8 x 10 x .08 cm were used in an electrophoretic apparatus purchased from Idea Scientific of Corvallis, Oregon. Gels were run at 4°C for approximately 1.5 hr at 140V.

Results

Both S. aureus and S. pyogenes were observed to be hemolytic while growing on the sheep blood-supplemented agar plates. Clearing of the red color in agar immediately surrounding the culture streak was extensive in both species, but was noticeably greater in the S. pyogenes plates.

Growth of either S. aureus or S. pyogenes that had been loop-transferred from blood agar into L broth without Hb was very slow for S. aureus and virtually nonexistant for S. pyogenes. After transfers were made to L broth supplemented with rat Hb (to 100 μM), growth of each species was greatly enhanced; Streptococcus growth appeared comparable to that of Staphylococcus (data not shown). The transfer of each culture into the various ^{59}Fe source-supplemented media appeared to grow equally well. Figures V.2 and V.3

illustrate the results of the ^{59}Fe uptake experiments. The iron uptake was slow in comparison to that observed in E. coli (149), but E. coli is far less fastidious in its growth requirements and has a shorter generation time than either S. aureus or S. pyogenes (150,157). After the first supernatant fluid of each time point sample was prepared and resolved in SDS-PAGE, stained for peroxidase activity (118) and for protein using Coomassie blue R-250 and ammoniacal silver (70), small amounts of Hb were detected. However, after the pellet wash (second supernatant) was analyzed by SDS-PAGE, no Hb was detected in any of the samples. After the sample pellets were counted for ^{59}Fe activity, some were prepared and resolved in SDS gels. Upon sensitive silver staining (70), no Hb was detected in any sample. The S. pyogenes pellets were observed to possess a distinct reddish hue in the latter two-thirds of the time point samples. The color of the S. aureus pellets remained light and unchanged throughout the time course.

The uptake of ^{59}Fe by S. aureus from either mouse Hb or the mouse Hb-human Hp complex depicted in Figure V.2A appeared similar in rate with the complex supplying more ^{59}Fe to the bacteria. S. pyogenes grown in the same ^{59}Fe-supplemented media exhibited a

increase of ^{59}Fe acquisition from the complex over that of free mouse Hb, at a higher rate. The rates and amounts of ^{59}Fe release by Taricha Hb and Hb-HbP complexes to both species appeared essentially the same (Fig. V.3).

Discussion

For years, hemolytic, virulent bacteria such as S. aureus and S. pyogenes have been observed to grow in the presence of Hb, although their growth was retarded in its absence (139,140). It was initally assumed that the protein degraded by bacterial proteases was the growth-stimulating factor. However, Davis and Yull (142) reported that apo-Hb failed to promote growth in pathogenic E. coli, where whole Hb stimulated growth. In other studies, hemin has been seen to promote growth of staphylococcal and streptococcal species when added to conventional growth media (144,145). It seems that S. aureus is capable of removing iron from transferrin in vitro and thereby, experience noticeable growth (150). It would appear then that it has the ability to synthesize protoporphyrin IX for iron coordination and construction of cytochromes required for aerobic metabolism (151). A streptomycin-resistant mutant of S. aureus was reported to require hemin for the synthesis of vital

hemoproteins such as catalase, cytochromes and cytochrome oxidase (144). It was noted however that this strain tends to regain its hemin-synthesizing ability if grown in hemin-free media (144).

In the case of S. pyogenes, it seems unclear whether or not it even uses hemoproteins for aerobic respiration. Although hemin has been observed to be necessary for the aerobic existence of streptococci such as S. faecalis and S. sanguis (140), S. pyogenes was reported in one study to lack hemin-induced NADH oxidase activity, thereby suggesting a lack of a cytochrome system (152). However, that same study indicated S. sanguis to be void of NADH oxidation. If the studies of S. sanguis are accurate, it appears that although this bacterial species does not require a cytochrome system for aerobic respiration, it still requires hemin for growth (probably to synthesize a catalase-like protein for protection against H_2O_2 formation).

Although Ritchey and Seeley (152) reported S. pyogenes to either facilitate NADH oxidation using flavin-like proteins or to lack the oxidation capacity altogether (depending on the strain), they did not indicate whether or not S. pyogenes required hemin for peroxidase activity. Since we observed S. pyogenes (strain unknown) to require Hb for growth, it is

likely that this species needs intact hemin for at least the formation of a catalase [since all streptococci are believed to be incapable of synthesizing protoporphyrin IX (152)]. The pellets of the S. pyogenes samples in this study were visibly red, while no such color was observed in comparably-sized S. aureus pellets. The color was not due to precipitated Hb as the SDS-PAGE analysis revealed. It is possible that the red color in the S. pyogenes pellets was due to hemin bound to bacterial outer membranes in free form and/or a secreted heme-binding protein.

Eaton et al. (17) reported that Hp, when complexed with media-supplemented Hb exerted a bacteriostatic effect on a pathogenic strain of E. coli. While the mechanism was not established, Hp protection of Hb against bacterial protease activity was suggested (17). In another study (149) E. coli that used the virulence-associated siderophore aerobactin, were able to remove iron from transferrin. E. coli used in the Hp-Hb experiment (17) acquired iron from heme, so the protease activity suggested was likely accompanied by that of a heme oxygenase [as protoporphyrin has a very high affinity for iron (81) and no siderophore has been shown to strip it of iron]. We did not assay the media for protease degradation of Hb so it is not

known whether or not S. aureus or S. pyogenes needed to degrade the globin component before acquiring the heme or heme iron.

Not only has Hp been reported to protect the complexed Hb from degradation by acid (61) but it also was seen to prevent heme exchange between the complexed Hb and free Hb or serum albumin (68). However, the hepatic enzyme α-methenyl oxygenase was reported to degrade the heme of the Hp-Hb complex almost as easily as heme of pyridine-hemichromogen (47), but had little effect on free Hb heme. The enzyme has been found in cow, guinea pig, rat and pig liver tissues (47,153), and is now believed to be the mechanism by which the liver recovers the complexed Hb iron (154). Since this enzymatic mechanism was shown to exist in various animals, it seems feasible that S. aureus and other Hb iron-utilizing bacteria might possess an enzyme with similar action. A bacterial heme oxygenase could degrade heme of the Hp-Hb complex and also Hb heme that was left more exposed due to protease attack of the globin. The liberated iron could then be chelated by a siderophore or similar compound as has been ascribed to S. aureus (150).

If S. pyogenes requires hemin for growth (as with other streptococci), a different interpretation has to be considered for a result of this study, however.

Bunn and Jandl (68) reported that while radiolabeled heme was seen to be exchanged between Hb molecules *in vitro*, it was observed to remain with Hb bound to Hp. Hemin has also been reported to exchange between horse Hb and myoglobin (155). The fact that Hb heme is not rigidly-fixed and can be transferred to other globins, albumin and hemopexin allows for the possibility of a protein with high affinity for hemin to be used by hemin-requiring bacteria. Or it is possible that hemin could be bound by specific membrane proteins.

S. pyogenes might therefore secrete a high heme-affinity protein such as hemopexin which would be bound by a membrane receptor when carrying heme. The heme could then be transferred into the cell interior. This overall mechanism has been reported to exist in mammalian systems where hemopexin removes heme from erythrocyte-free Hb or methemalbumin (78) and is transported to the liver, where parenchymal cell receptors bind it and transfer the heme into the cell. The receptor then releases the apohemopexin back into circulation (156).

However, since heme was not seen to be exchanged from the Hp-Hb complex to other proteins (68), the results of the *S. pyogenes* experiment in this study, (with the Hp-Hb complex as a ^{59}Fe media supplement) would be difficult to explain if not for the results

of a previous study. Lustbader et al. (147) reported that although Hb was relatively impervious to proteolytic cleavage by many enzymes, Hp was readily cut by trypsin, chymotrypsin, staphylococcal protease, thermolysin and plasmin. However, the Hp-Hb complex was cut only by the bacterial protease. This would contradict the suggestion by Eaton et al. (17) that upon complexation, Hp protects Hb against proteolytic attack by bacteria. It is important to this discussion that a bacterial protease was the only enzyme to degrade the Hp-Hb complex and that it was not shown to attack the Hb of the complex but rather cut the Hp in various places (147). This preferential attack of the complex Hp probably weakens the stabilization of the Hb allowing removal of heme.

The Taricha Hb-binding proteins failed to exert any overall influence on the iron or heme uptake from complexed Hb (Fig. V.3).

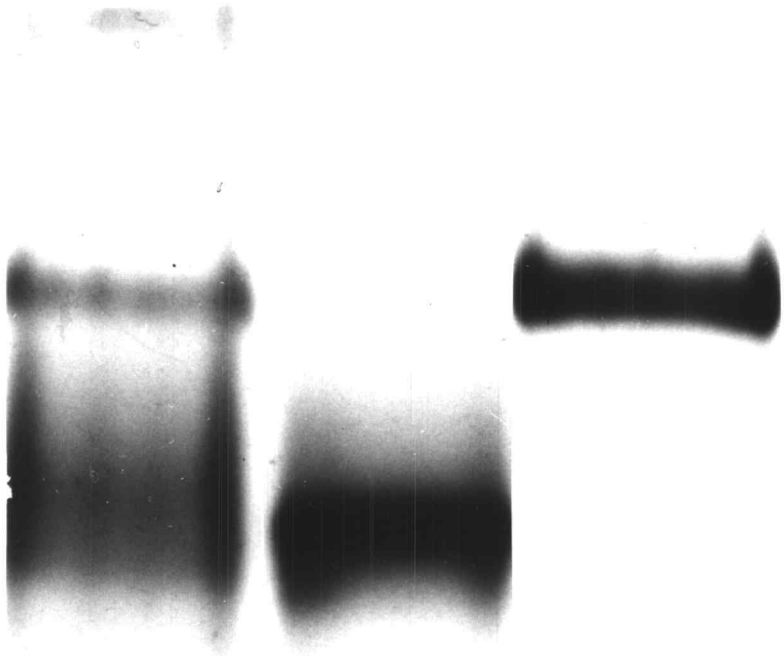
From the evidence produced in this study, human Hp and Hb-binding proteins of Taricha granulosa do not exert bacteriostatic influence regarding the uptake of iron or heme by Staphylococcus aureus or Streptococcus pyogenes. On the contrary, the two bacterial species assimilated more ^{59}Fe from the Hp-Hb complex than that from free Hb. The clinical importance of these two

virulent species warrants a study of the mechanisms of iron uptake from hemoglobin and its complex with haptoglobin.

Acknowledgements:

The authors wish to thank Dr. C.K. Mathews for use of the laboratory facilities and Dr. D. Buhler for use of his Packard gamma counter. We are also grateful to Ilsa Kaattari of the Microbiology department for supplying the bacteria, and to Dr. Gary Wright for providing medical background and significance relating to this study. This research was funded by the Medical Research Foundation of Oregon.

Figure V.1. A 7% nondenaturing gel that reveals the results of Sephadex G-100 factionation of the mouse Hb and human Hp mixture: 1) Prior to fractionation; 2) free mouse Hb; 3) human Hp-mouse Hb complex. The gel was stained for peroxidase activity.



1 2 3

Fig. V.1

Figure V.2. A. Iron uptake by Staphylococcus aureus from mouse Hb and the Hp-Hb complex. The rate of uptake from Hb and the Hp-Hb complex was similar, although more iron was removed from the complex.

B. Iron uptake by Streptococcus pyogenes from mouse Hb and the Hp-Hb complex. The rate of uptake from the complex was noticeably greater, as was the overall amount of iron removed from it.

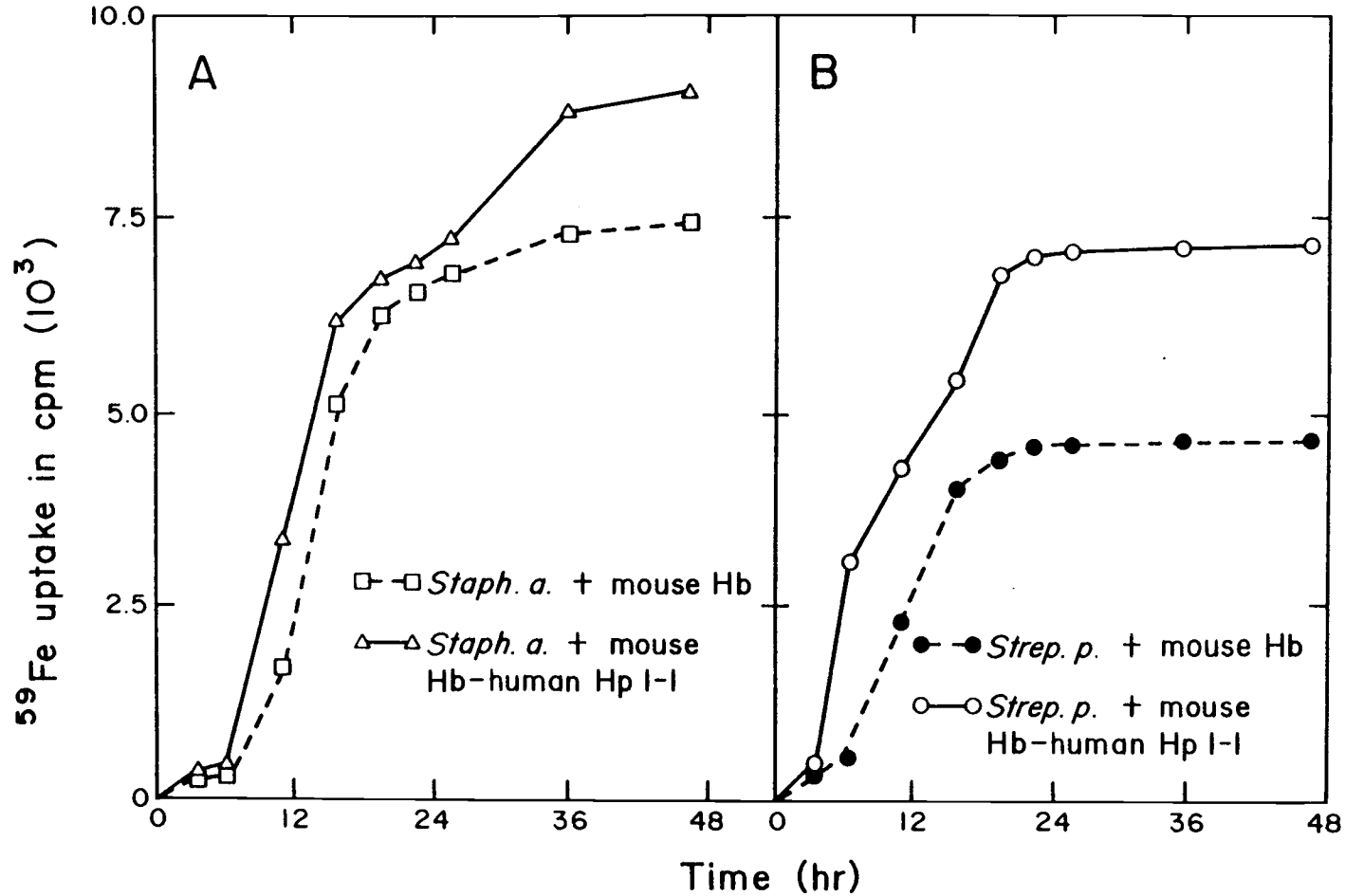

10.0
7.5
5.0
2.5
0
^{59}Fe uptake in cpm (10^3)
A
B
0
12
24
36
48
Time (hr)
Staph. a. + mouse Hb
Staph. a. + mouse Hb-human Hp I-I
Strep. p. + mouse Hb
Strep. p. + mouse Hb-human Hp I-I

Fig. V.2

Figure V.3. A. Removal of iron from Taricha granulosa Hb and HbP-Hb complexes by Staphylococcus aureus.

B. Removal of iron from the same by Streptococcus pyogenes. The rates and amounts of iron removed from the amphibian ^{59}Fe sources were similar with each bacterial species.

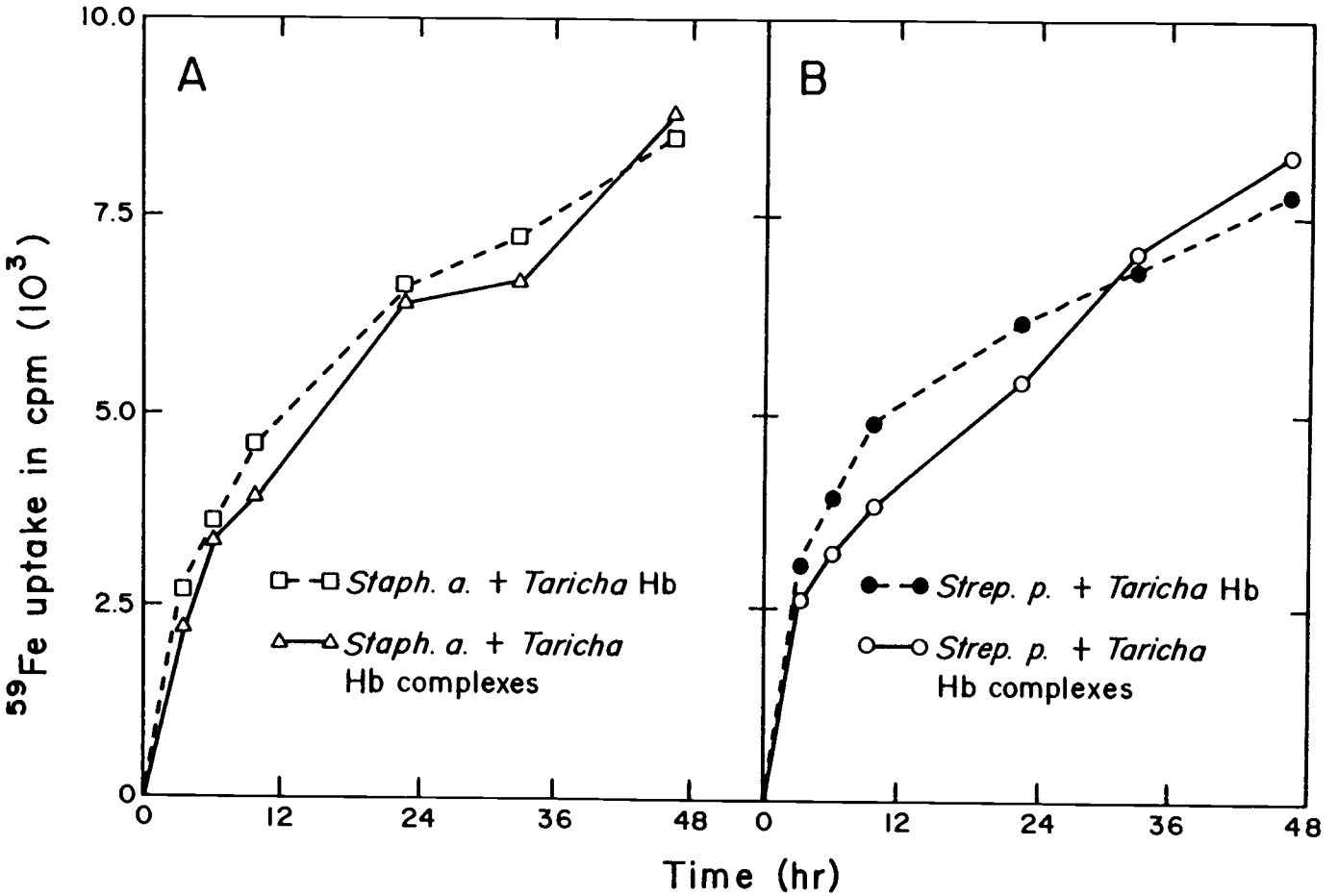

10.0
7.5
5.0
2.5
0
A
B
^{59}Fe uptake in cpm (10^3)
Staph. a. + Taricha Hb
Staph. a. + Taricha Hb complexes
Strep. p. + Taricha Hb
Strep. p. + Taricha Hb complexes
0
12
24
36
48
Time (hr)

Fig. V.3

SECTION VI

SPECIFIC INDICATION OF HEMOPROTEINS IN POLYACRYLAMIDE GELS USING A DOUBLE-STAINING PROCESS.

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Abstract

Hemoproteins were revealed in polyacrylamide gels in the presence of sodium dodecyl sulfate by staining with different benzidine derivatives. When the protein samples were treated with either β-mercapto-ethanol or dithiothreitol, a significant decrease in peroxidase activity of the proteins possessing non-covalently-bound heme led to diminished staining. However, when Coomassie blue R-250 staining followed the heme-specific stain it was observed that the hemoprotein bands stained more intensely than duplicate sample bands that had been stained only with the Coomassie blue R-250. This staining property allows the indication of hemoproteins in gels even after the peroxidase yield has been significantly depleted by reducing agents.

Introduction

The identification of hemoproteins in polyacrylamide gels by virtue of their peroxidase activity in the presence of H_2O_2 and a hydrogen donor such as benzidine, has been conducted for 20 years. However, those proteins were electrophoretically resolved under nondenaturing conditions which did not allow description of their size or subunit composition (2,13,21). With the advent of sodium dodecyl sulfate (SDS)[1] usage in polyacrylamide gel electrophoresis (PAGE) (57,82), came the assumption that the denaturing conditions specified by Laemmli (57), for example, brought about the dissociation of noncovalently-bound heme from a protein. Recently however, various studies (83,84,85) have demonstrated that while much of a protein's heme dissociates when SDS is present, a detectable amount remains bound, allowing for identification in gels.

In order to improve heme retention by proteins resolved in SDS PAGE, previous studies have either lowered the SDS concentration greatly (86) or replaced

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[1]Abbreviations: SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LDS, lithium dodecyl sulfate; BME, β-mercaptoethanol; DTT, dithiothreitol; DAB, diaminobenzidine; DMB, dimethoxybenzidine; TMB, tetramethylbenzidine; Hb, hemoglobin; cyto c, cytochrome c; CbR-250, Coomassie blue R-250; TCA, trichloroacetic acid.

SDS with the more expensive lithium dodecyl sulfate (LDS) (84). Both investigations studied cytochrome P_{450} in which the heme is noncovalently bound. Neither study included the use of a reducing agent such as β-mercaptoethanol (BME) or dithiothreitol (DTT) which are usually included in SDS PAGE sample preparations. It has been previously observed (87) and also seen in this laboratory, that in the presence of oxygen, a reducing agent facilitates the oxidation, thus destruction of the heme ring. This action leads to a proportional decrease in peroxidase activity of the total heme of the sample, resulting in weaker hemespecific staining in gels. Since the resolution of proteins in SDS PAGE often requires the use of a reducing agent for the cleavage of disulfide bonds (in order to free up individual polypeptides and allow estimation of their molecular weights), the resulting decrease in peroxidase activity of hemoproteins present in the reduced sample can render the identification of them very difficult.

Included in this study was the comparison of the peroxidase activity-staining properties of three previously-used benzidine derivatives. Diaminobenzidine (DAB) (83,88), dimethoxybenzidine (DMB) (also called o-dianisidine) (21,89) and tetramethylbenzidine (TMB) (90,91) were used to stain human hemoglobin (Hb) and

cytochrome c (cyto c) in gels. DMB was found to exhibit the most desirable staining and was subsequently used to stain horseradish peroxidase, catalase, sperm whale myoglobin and snake Hb. Also, that Coomassie blue R-250 (CbR-250) has affinity for oxidized DMB in gels was observed and this staining behavior was used to identify heme-containing bands on gels that were too faint to visualize after DMB staining.

Materials and Methods

Materials: 3,3'-Diaminobenzidine hydrochloride, 3,3'-dimethoxybenzidine dihydrochloride, 3,3'5,5'-tetramethylbenzidine, catalase, horseradish peroxidase, sperm whale myoglobin, human hemoglobin and cytochrome c were all purchased from Sigma. The SDS PAGE molecular weight kit was obtained from Pharmacia and the SDS was supplied by BDH.

Methods: The SDS PAGE analysis was based on the procedure of Laemmli (57) with two alterations. The PAGE running buffer was made of 0.05 M Tris, 0.06 M boric acid and 10^{-3} M EDTA, with no adjustment of pH. The sample preparation buffer was normally made of 0.0625 M Tris-HCl (pH 6.8), 5% BME, 2% SDS, 10% glycerol and 0.001% bromophenol blue. Certain samples were duplicated, with one dissolved in the buffer stated above and the duplicate dissolved in the same

buffer excluding the BME (or DTT). The sample proteins once dissolved in the buffers, were incubated at room temperature for 5 to 10 minutes.

The gels were composed of 7% polyacrylamide stacking and 15% separation phases in minislab form 8 x 10 x .08 cm. After electrophoresis for about two hours at 4°C, the gels were stained with each of the three peroxidase stains and/or CbR-250. Staining with DAB was performed according to McDonnel and Staehelin (83), TMB was used according to Lijana and Williams (91) and CbR-250 according to Davie (58). The DMB (o-dianisidine) was prepared as follows: 200 mg of DMB were dissolved in 180 ml of H_2O at room temperature and stirred vigorously for 15 minutes. Immediately before staining the gel, 20 ml of a 0.5 M sodium citrate buffer (pH 4.4), was added along with 0.4 ml of 30% H_2O_2. The gel to be stained with DMB was, after electrophoresis, immediately washed in 200 ml of 12.5% TCA for 30 min, followed by a 30 min H_2O wash. After one hour of staining in one of the peroxidase stains, each gel was washed in H_2O to clear any background. For assessment of relative heme amounts in different PAGE bands, densitometry using a Gilford 220 gel scanning spectrophotometer was performed. The gel strips were scanned at 490 nm.

Results

Figure VI.1 shows a comparison of the staining properties of the three benzidine compounds. Triplicate samples of human Hb and cyto c, each in reduced ($^{+}$BME) and unreduced ($^{-}$BME) forms, were applied to a slab gel. In all cases, the $^{+}$BME Hb sample bands were much fainter compared to the same sample without BME. The cyto c samples exhibited much less difference in staining intensities between the $^{+}$BME and $^{-}$BME forms. When BME was replaced by DTT (10mM), there was a slight increase in the staining of the reduced Hb sample.

Since the oxidation of the heme ring in the presence of a reducing agent and oxygen had been observed in a previous study (87) and had been repeatedly observed in this laboratory, it was decided to prevent or at least retard the rate of protoporphyrin methine-oxidation cleavage by purging nitrogen gas through the $^{+}$BME sample before and after addition of the sample protein. When these purged SDS sample solutions were run in SDS PAGE, the $^{+}$BME hemoproteins exhibited an ever greater increase in peroxidase activity over DTT treatment and stained more intensely. Table IV lists the relative amounts of peroxidase activities of the samples after different treatments. As can be noted, the combination of nitrogen purging

and DTT allowed the greatest peroxidase activity yield of the reduced samples.

Table V lists the relative amounts of free heme compared to heme-protein in the Hb and cyto c samples after SDS PAGE. These values were estimated from the densitometric measurements of peroxidase yields. The fact that almost 60% of the heme in the $^{-}$BME Hb sample remained associated with the globin subunits disputes the assumption that the noncovalently-bound heme is entirely dissociated upon treatment under standard Laemmli SDS conditions (54). As much or more heme remained associated with globin under these conditions than with the method of Sinclair et al. (84) which replaced SDS with LDS and lowered the sample buffer LDS concentration to 0.5% (whereas the Laemmli method calls for 2% SDS).

The three benzidine compounds exhibited different staining characteristics. TMB left faint bluish-green bands with a slight background. DAB stained the hemoproteins dark brown and left an intense undesirable background (Fig. VI.1). DMB stained the bands a dark reddish-brown, but left a clear gel background. Since DMB appeared to be the preferred compound, its sensitivity was tested in SDS PAGE with dilutions of Hb in $^{+}$BME and $^{-}$BME forms. The threshold for visualization of peroxidase-active Hb subunit bands

(unreduced), was approximately 40 picomoles of heme (data not shown). To test the peroxidase activities and staining of other hemoproteins in SDS PAGE, snake Hb, sperm whale myoglobin, catalase and horseradish peroxidase were all electrophoresed along with human Hb and cyto c and stained with DMB (Figure VI.2). All of the samples were left in $^{-}$BME form and polymerization of Hb is evident. Figure VI.2 also shows the same gel stained with CbR-250 for protein. There is obvious contamination of other proteins in both the peroxidase and catalase samples. This double-staining of the gel demonstrated this method's value as a purity check concerning hemoproteins in a sample preparation.

When a gel that had first been stained with DMB was then stained with CbR-250, the bands that had appeared peroxidase-active and had stained reddish-brown appeared purplish after the CbR-250 treatment. It was this color and its intensity that led to the discovery of a crucial point to this method. An SDS slab gel that contained duplicate Hb samples (one on each half of the slab) was electrophoresed and bisected. One half was stained with CbR-250 and the other was stained with DMB. The DMB-stained half was then treated with CbR-250. The gels were destained according to Davie (58) and each of the sample lanes

densitometrically scanned at 550 nm. The bands that had first been stained with DMB had higher absorbances at 550 nm than did the same bands that had been stained only with CbR-250. As a result of the measurements of the CbR-250 band absorbances, it was found that the DMB-CbR-250 staining resulted in a CbR-250 band peak increase of approximately 32% in -BME Hb and 8 to 18% in reduced Hb (depending on the reducing treatment).

Discussion

When compared to DAB and TMB, DMB (o-dianisidine), which has been used to identify hemoproteins in non-denaturing gels (21,89) was found to exhibit the greatest contrast between dark bands and clear gel background. That, coupled with its sensitivity, solubility in H_2O and ease of preparation, marked it as the peroxidase activity-staining compound of preference in our work (precautions should be taken when handling DMB as it is believed to be carcinogenic) (92). The sensitivity of DMB was tested using Hb in SDS PAGE and observed to be approximately 40 picomoles of heme. When the same Hb sample was treated with 5% BME, the peroxidase yield on gels was only 10% of the -BME Hb sample. In a previous study involving the indication of cytochrome P_{450} in SDS PAGE (84), DTT was left out of the sample preparation on the basis

that it facilitated the release of heme from the cytochrome protein. From the densitometric scans of the peroxidase activity of Hb in SDS PAGE performed in this laboratory, it is believed that it is not strictly a matter of increased heme dissociation that results in the decrease of heme staining of the cytochrome P_{450} protein, but rather the oxidation of the heme ring in the presence of DTT and O_2 (87). Hb was observed to behave in this manner. Cyto c, which has covalent bonds between the heme and protein components, was included in the method that used DAB as the peroxidase indicator (83). Five percent BME was included in that preparation of cyto c with substantial peroxidase activity remaining. When cyto c was run with Hb in SDS PAGE (Fig. VI.1) in $^{+}$BME and $^{-}$BME conditions, the decrease in its staining after addition of BME was slight compared to the decrease in Hb peroxidase yield (see Table IV). The stability imparted to the heme in cyto c by virtue of covalent attachment may aid in retarding oxidation of the heme ring.

It is evident that treating a hemoprotein that lacks covalent attachment of its heme to the protein with a reducing agent, results in significant depletion of intact heme in the sample. This then, causes problems when attempting to identify hemoproteins in

gels by using their peroxidase activities. When reduced hemoprotein sample bands in SDS PAGE were too faint to visualize, it was noted that the presence of oxidized DMB in a band caused a greater CbR-250 staining of that band compared with CbR-250 staining alone. Apparently CbR-250 has some affinity for oxidized DMB under these staining conditions, since in the absence of protein, hemin does stain when DMB is present (Fig. VI.2, lane #5). This has been observed in nondenaturing gels as well. This double-staining may then facilitate the identification of hemoprotein bands in SDS PAGE through a comparison of CbR-250 absorbance densitometric scans.

In attempting to maximize heme retention by a protein in SDS PAGE, a previous study (86) decreased the SDS concentration in the sample buffer to 0.1%. However, 0.1% is less than the critical micelle concentration of SDS (93) and it has been observed that this low SDS concentration failed to inhibit protease attack on other proteins (93). The boiling of an SDS PAGE sample solution has been observed to compensate for a low (e.g. 0.1%) SDS concentration regarding protease inhibition. However, it has been seen in our work that boiling tends to dissociate more heme in a sample than does a 10-fold increase in the SDS concentration. Consequently, low levels of

hemoproteins can be identified in SDS PAGE even with SDS concentrations high enough to inhibit proteolysis.

The results of this study show that even under the comparatively harsh denaturing conditions of the Laemmli method (57), about 60% of the noncovalently-bound heme of a hemoprotein sample (i.e. Hb) remains associated with the protein in SDS PAGE. Of the three benzidine derivatives tested, 3,3'-Dimethoxybenzidine (o-dianisidine) exhibited the most desirable staining properties. While the presence of a reducing agent diminishes the peroxidase activity yield of a hemoprotein, the DMB-CbR-250 staining method developed here has the sensitivity and specificity to allow identification of hemoproteins with low peroxidase activity yields.

Acknowledgement:

The authors wish to thank the Medical Research Foundation of Oregon for support of this work.

Figure VI.1 SDS PAGE slab gel that contains Hb and cyto c samples in triplicate. After electrophoresis, the gel was trisected with each section treated with one of three peroxidase activity stains. The samples are: (1) human Hb ($^{+}$BME), (2) human Hb ($^{-}$BME), (3) cyto c ($^{+}$BME), (4) cyto c ($^{-}$BME). The sections are separated by the vertical arrows and are left to right: TMB, DMB and DAB. The DAB background is extreme compared to those of TMB and DMB. The small arrow denotes the free heme in the unreduced Hb lane.

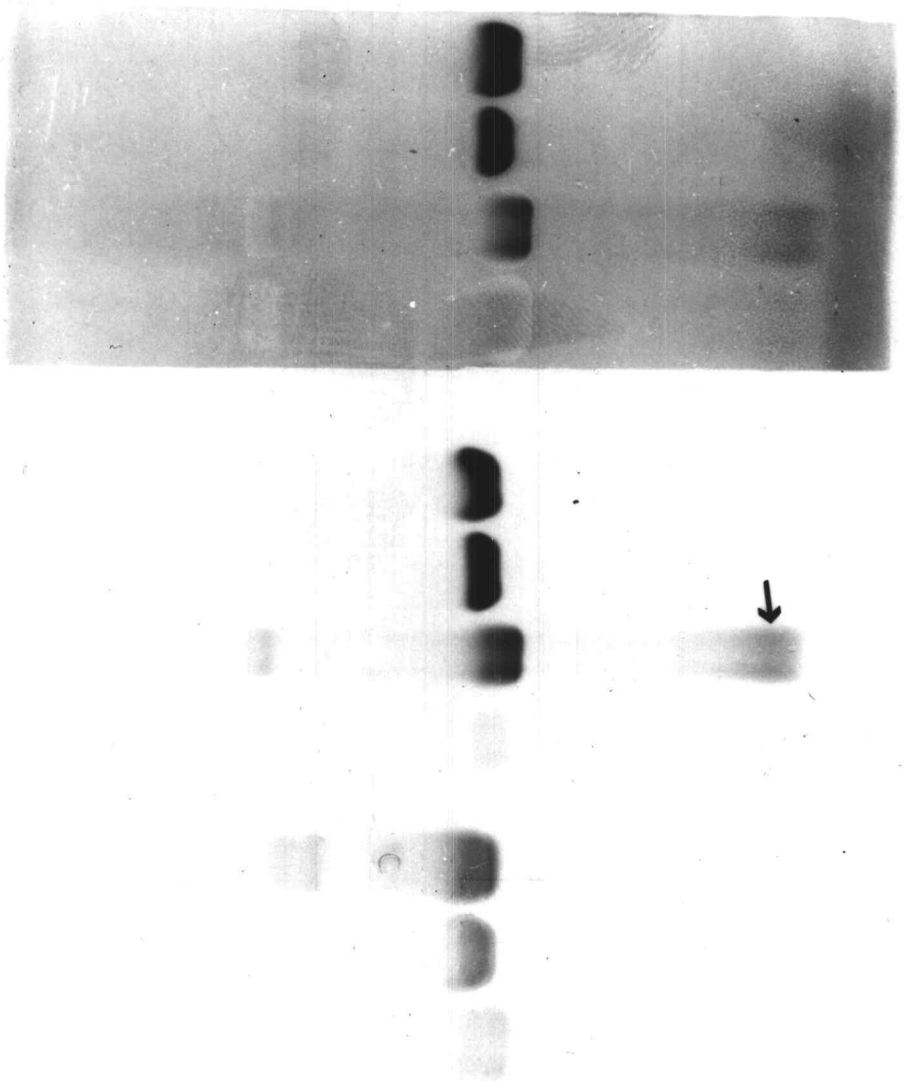

Fig. VI.1

Figure VI.2 The two photos are of the same SDS (15% acrylamide) gel at different stages of staining. The photo on the left represents the DMB indication of heme and hemoproteins. The photo on the right shows the CbR-250 staining of the same samples. The samples are: (1) blue racer snake Hb, (2) horseradish peroxidase, (3) catalase, (4) cyto c, (5) sperm whale myoglobin, (6) human Hb. The molecular weight standards are designated std and are in units of kilodaltons. Note the presence of Hb in the catalase sample and the significant protein contamination in both the peroxidase and catalase samples. The arrow in the left photo indicates free heme. None of the samples were reduced with BME or DTT and polymerization of some of them is apparent.

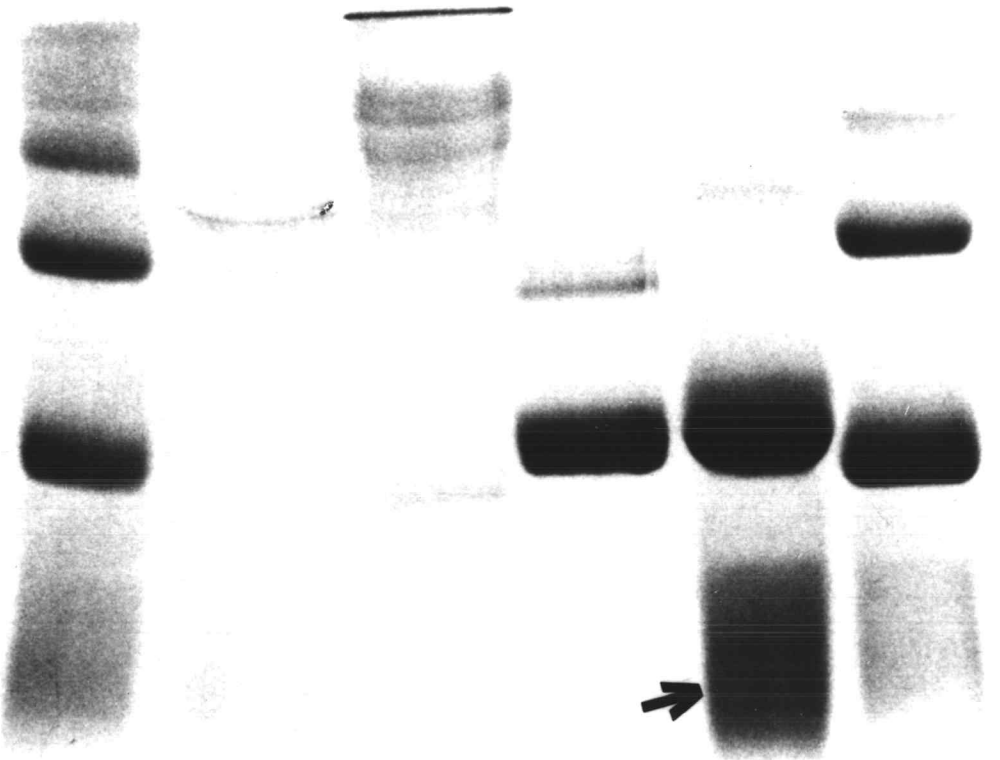

Fig. VI.2 - left photo

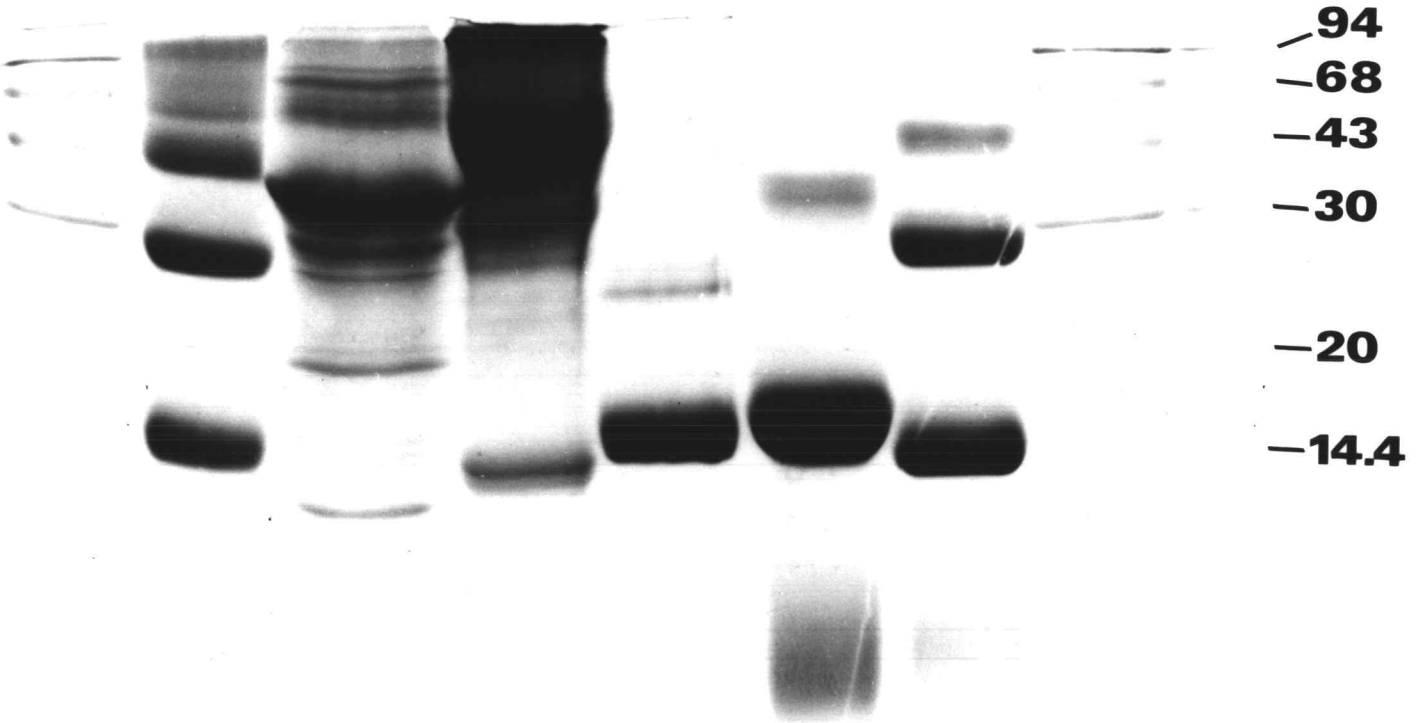

Fig. VI.2 - right photo

Table IV. Relative Peroxidase Activity from Densitometry (peak values in mm^2)

Sample	Unreduced	+5% BME	+10mM DTT	+10mM DTT + N_2
Hb	1,625	333	492	761
cyto c	428	401	408	411

Table V. Relative Free Heme and Heme-Protein Amounts (peak values in mm^2)

Sample	Free Heme	Heme-Protein	% of total Heme Remaining with Protein
Hb	1,164	1,625	58
cyto c	4	427	99

SECTION VII

AN EFFICIENT METHOD FOR VISUALIZATION AND ISOLATION OF PROTEINS RESOLVED IN POLYACRYLAMIDE GELS

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Summary

Polyacrylamide gel electrophoresis is a popular method used to purify proteins for reconstitution experiments, amino acid composition and sequence determinations. In this report we describe methods that will be of general use in the isolation and characterization of proteins and the benefits of substituting boric acid for glycine in the electrophoresis tray buffers. We also describe how proteins resolved in a variety of gel systems (including those containing sodium dodecyl sulfate) may be rapidly visualized with 8-anilino-1-naphthalene sulfonic acid and efficiently transferred to a second gel for two-dimensional gel analysis, or isolation by electroelution for subsequent characterization.

Introduction

In the course of attempting protein separation and purification, many problems may arise regarding adequate resolution. The use of gel filtration and ionic exchange column chromatography often leaves much to be desired when applied to a system with proteins of similar charge to mass ratios and/or sizes. The use of polyacrylamide gel electrophoresis (PAGE) systems for separation of proteins via electrophoretic mobility in either a nondenatured or denatured state [e.g. SDS (sodium dodecyl sulfate) gels] has been very popular and widely used. In most cases, the acrylamide gels offer significant enhancement of protein separation over the above-mentioned chromatographic methods.

We have used various electrophoretic techniques to purify and analyze various protein species. However, during the course of this work we have found some methods to be unsuitable regarding the purity of an isolated protein. For example, we have found that proteins resolved and isolated from polyacrylamide SDS

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[1]Abbreviations: SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-N,N'-bis (2-ethane sulfonic acid); ANS, 8-anilino-1-naphthalene sulfonic acid; HMG, high mobility group; AU, acid-urea.

gels prepared as described by Laemmli (57) contained a large amount of glycine when their amino acid compositions were determined. This high background of glycine resulted from the tray buffer. In this report we demonstrate that a relatively low concentration of boric acid may be substituted for glycine as the stacking anion without any loss of resolution of the proteins.

Hartman and Udenfriend (94) and Nerenberg et al. (95) have reported that ANS (8-anilino-1-naphthalene sulfonic acid) may be used to rapidly visualize proteins in nondenaturing polyacrylamide or agar gels. However, this staining technique has not been applied to gels containing SDS (99). In this report, we demonstrate that ANS staining does not interfere with the recovery of the protein by electroelution, or its migration into a second dimension gel such as a polyacrylamide acid-urea gel.

Experimental

Preparation of Human Plasma

Human plasma was prepared as follows: Whole blood was taken up in 0.9% NaCl-heparin solution and centrifuged at 2,000 g for 20 min at 4°C. The plasma supernatant (liquid) was removed and dialyzed against 0.10 M Tris-HCl, pH 7.4, for 24 hours at 4°C.

Preparation of Histones

Testes at intermediate stages of development were obtained from naturally maturing rainbow trout (Salmo gairdnerii) and stored at -80°C until use. The testes were homogenized in 10 volumes of Buffer A [1 M hexylene glycol, 10 mM piperazine-N-N'-bis (2-ethane sulfonic acid) (PIPES), pH 7.0, 2 mM $MgCl_2$, 1% thiodiglycol, 30 mM sodium butyrate]. The scissor-minced tissue was homogenized in a Virtis homogenizer at the lowest speed for one minute. The homogenate was filtered through four layers of cheesecloth and centrifuged at 750 g for 10 min (Sorval SS-34 rotor). The pellet was resuspended in Buffer A and again centrifuged. The resulting pellet was then resuspended in 3 volumes of Buffer B (10 mM Tris-HCl, pH 8.0, 0.6 M NaCl) and centrifuged at 12,000 g for 30 min. The pellet was resuspended in 3 volumes of Buffer B and centrifuged. These steps removed the majority of the nonhistone chromosomal proteins and histone H1. The pellet was extracted with 0.4 N H_2SO_4 (30 min on ice) and insoluble material was removed by centrifugation (12,000 g for 30 min). The acid extract was dialyzed overnight at 4°C against 0.1 N acetic acid, lyophilized and redissolved in distilled water. The extracted proteins consisted of histones, H2A, H2B, H3, H4, and

some histone H1. Calf thymus and chicken erythrocyte histones were prepared as described by Davie et al. (96).

Polyacrylamide Gel Electrophoresis

a) Analytical Gel Electrophoresis

The minislab apparatus was purchased from Idea Scientific, Corvallis, Oregon. The polyacrylamide SDS minislab gels were prepared as described by Davie (58) except that in some cases riboflavin (0.0004%) was used as the catalyst. The "native" or nondenaturing gels were made up using the Davis method (29) except riboflavin (0.0004%) was used as the catalyst. For the SDS gels, either Tris-glycine-SDS (58) or Tris-borate-SDS tray buffers were used. The borate tray buffer was made up as follows: 0.05 M Tris, 0.06 M boric acid, 10^{-4} M EDTA with 0.1% SDS. For the native gels, only the Tris-borate tray buffer was used (without the SDS). The acetic acid-urea minislab gels were prepared as described by Davie (58).

b) Staining

After electrophoresis the gel was stained with 0.25 % Coomassie blue G-250 in 45% methoanol and 9% acetic acid. The gel was destained briefly (30 to 60 min) by diffusion in methanol/acetic acid/water (2:1:5, v/v) and then destained further in 7.5% acetic acid and 5% methanol. Alternatively, the gel was

stained with ANS (8-anilino-1-naphthalene sulfonic acid, magnesium salt, Kodak). SDS gels were prewashed with 20 mM Tris-acetate, pH 8.8, for 30 min. This step removed the unbound SDS which would also stain with the ANS. This step was not required for the native or AU gels. The gel was then stained for 10 to 30 min with 0.003% (w/v) ANS (94,95) in a 0.1 M sodium phosphate buffer, pH 7.0. Destaining was not necessary. The band was visualized with ultraviolet light. A comparison of Coomassie blue-stained duplicate gels with or without previous ANS staining indicated that this procedure did not result in noticeable protein losses.

c) Electroelution

Following staining, the track of interest was cut out from the gel and equilibrated for 20 to 30 min in Buffer O of O'Farrell (63) (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 mM Tris-HCl, pH 6.8). The gel slice was placed into a dialysis bag containing either the Tris-glycine-SDS or the Tris-borate-SDS tray buffer. Electrophoresis was performed at 100 V for 14 to 48 h at 4°C.

d) Removal of Dye and SDS

Following electroelution, the contents of the dialysis bag (except the gel slice) were dialyzed against distilled water, lyophilized and redissolved in 40 µl

distilled H_2O. The sample was treated by ion-pair extraction using solvent system A as described by Henderson et al. (97).

Results and Discussion

I. Boric acid may be substituted for glycine in tray buffers

As the presence of glycine interfered with the determination of a protein's amino acid composition (i.e. a protein isolated from a gel where glycine was present in the tray buffer), we wanted to know if a low concentration of boric acid could be effectively substituted for the glycine (borate buffers were first used in electrophoresis by Consden (138) for carbohydrate separation). The results suggested that indeed, boric acid could be used in both SDS and native gels to resolve the different proteins (Fig. VII.1) and exhibited the same stacking capabilities as glycine. In addition, the resolution of various proteins on the gels containing either boric acid or glycine in the tray buffer was similar [compare trout histone pattern (Fig. VII.1, lane c) to calf thymus histone pattern (Fig. VII.2, lane c)]. However, borate complexes with and changes the charge on certain carbohydrates (138), so the migration of glycoproteins in native gels may be noticably altered.

There are several benefits in using boric acid instead of glycine in PAGE. First, when keeping the Tris component (50 mM) of the tray buffer the same as with the conventional glycine buffer, far less boric acid was needed to obtain the same pH as with glycine (e.g. 0.38 M glycine versus 0.06 M borate). This is beneficial, as the gel runs with less current at the same voltage and the gel temperature stays lower. This allows the same system to be run at a high voltage (e.g. 200 V), thus resulting in faster protein separation. Second, the most conspicuous advantage in using boric acid is the absence of the anomalous glycine background in the amino acid analysis data of an excised protein. This advantage is especially significant when attempting quantitation of very low levels of amino acids (e.g. 10^{-12} M).

II. 8-Anilino-1-naphthalene sulfonic acid (ANS) may be used to rapidly stain a variety of gels

ANS has been for the visualization of proteins in nondenaturing polyacrylamide (94) or agar gels (95). ANS will fluoresce when attached to proteins, presumably by the binding of the fluorophore to the hydrophobic portions of the molecule. However, it will not fluoresce alone in water (94,95). We wanted to determine if ANS could be used for staining SDS or acid-urea polyacrylamide gels. The results indicated that

for both of these gel systems, ANS could be used to visualize the proteins (Fig. VII.2). In addition, ANS would stain proteins resolved on native or SDS gels that contained boric acid instead of glycine in the tray buffer (Fig. VII.2, lanes g and i). In the case of the SDS gels, the unbound SDS (that is, SDS not associated with the proteins) had to be removed from the gel as ANS will also fluoresce in the presence of SDS. This was done by soaking the gel in 20 mM Tris-acetate, pH 8.8, which allowed the unbound detergent to diffuse out of the gel. As was stated in the "EXPERIMENTAL" section, this prestain wash did not cause any detectable losses of any of the proteins included in this study. For native gels, the gel can be stained directly with ANS as has been previously described (94,95).

For polyacrylamide SDS gels, a band containing 0.5 μg of protein could be visualized. This sensitivity of staining with ANS was about 2-3 fold less than that of Coomassie blue. For native or acid-urea gels, the amount of protein required for visualization with ANS was greater than that for the SDS gels. As the ANS fluorescence is dependent on the presence of hydrophobic sites to which it can bind, proteins resolved on native or acid-urea gels will vary in their degree of fluorescence and, in some cases, proteins will not

fluoresce at all. For example, the staining of the histones H2A, H2B, H3, H4 and H1, resolved on acid-urea gels, varied for each histone (Fig. VII.2, lanes e and f). Histone H1 and H3 stained poorly compared to histones H2A, H2B and H4. However, high mobility group proteins (HMG) 14 and 17 which contain little in the way of hydrophobic sites (98) to which ANS can bind, cannot be visualized in ANS-stained acid-urea gels (not shown). In addition, heme-containing proteins, such as hemoglobin, resolved on native gels quench the fluorescence of ANS and do not stain. However, most proteins we have examined (histones, HMG 14 and 17, albumins, hemoglobin and transferrin) will stain with ANS when resolved on SDS gels. This is due to 1) the protein is denatured and the hydrophobic sites are available to the ANS and 2) the protein-bound SDS will also bind ANS and will fluoresce.

The utilization of ANS for gel staining has several advantages. Protein patterns can be visualized rapidly, usually within 10-40 minutes. The ANS (and SDS) may be easily removed from the electroeluted protein by ion-pair extraction whereas Coomassie blue cannot (see below). In addition ANS staining does not interfere with electrophoretic transfer of proteins in two-dimensional gel electrophoresis [SDS -> AU (see

Davie (58)]. In the SDS -> AU system (not shown), Coomassie blue staining and destaining significantly lowered the efficiency of protein transfer to the second dimension (58). Furthermore, ANS visualization for subsequent first dimension gel lane excision is preferred to blind excisions as we have found wider gel slices tranfer their contents less efficiently.

III. Recovery of proteins

Following excision and electroelution of a stained band(s) (see "Materials and Methods") which recovered no less than 80% of the protein band, we wanted to know if, first, would the ion-pair extraction technique (solvent system A) described by Henderson et al. (97) work satisfactorily with small amounts of protein (e.g. 10 µg)? Second, would the presence of ANS interfere with the ion-pair extraction technique? This technique is used to remove SDS from the protein. When we addressed the first question, we found that this procedure did not result in losses of protein. We have subjected various amounts of a histone sample (10, 20, 100 and 200 µg) to the procedure and did not observe detectable losses of the histones or their associated modified forms when resolved on acid-urea gels (not shown). When we addressed the second question, we found that ANS did not interfere with the

recovery of the protein when using the ion-pair technique. Furthermore, we have found it to be advantageous to use ANS rather than Coomassie blue to detect the protein bands (see above) as ANS, but not all of the Coomassie blue was removed by ion-pair extraction. The presence of Coomassie blue in the sample led to the formation of a water-insoluble precipitate which contained both dye and protein.

Through the use of the above procedures (SDS or acid-urea polyacrylamide gel electrophoresis, ANS staining, electroelution, and ion-pair extraction), we have been able to purify a variety of proteins including histone H4 and the unacetylated and monoacetylated species of histone H4 (not shown).

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Figure VII.1 a) Human plasma proteins resolved on a 7% polyacrylamide native minislab gel with Tris-borate for the tray buffer, b) human plasma proteins on a 8% polyacrylamide SDS minislab gel with Tris-borate-SDS for the tray buffer, and c) trout testis histones resolved on a 15% polyacrylamide SDS minislab gel with Tris-borate-SDS for the tray buffer. All gels were stained with Coomassie blue.

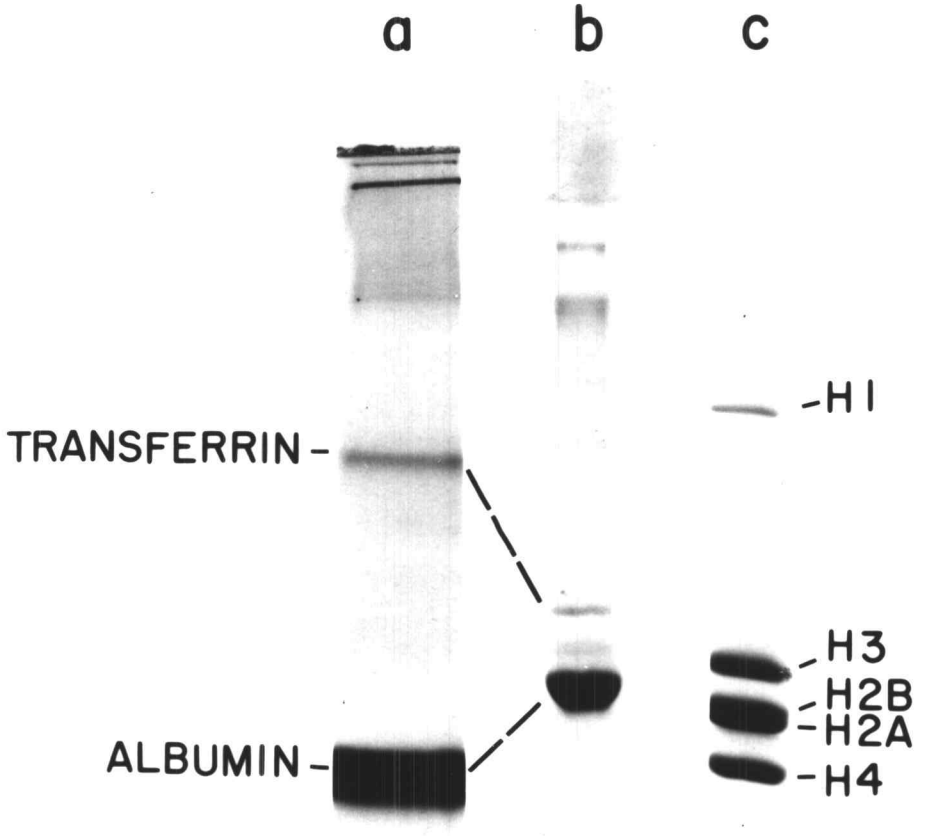

Fig.VII.1

Figure VII.2 a) 15% polyacrylamide SDS minislab gel containing calf thymus histones (lanes a and c) and chicken erythrocyte histones (lanes b and d). The tray buffer was Tris-glycine-SDS. b) 15% polyacrylamide acid-urea minislab gel containing calf thymus histones (lanes e and f). c) 7% polyacrylamide native minislab gel containing human plasma proteins (lanes g and h). The tray buffer was Tris-borate. d) 7% polyacrylamide native minislab gel containing 12 µg of BSA (oxidized) (lanes i and j). Tray buffer again was Tris-borate. a, b, e, g and i were stained with ANS and c, d, f, h and j were stained with Coomassie blue.

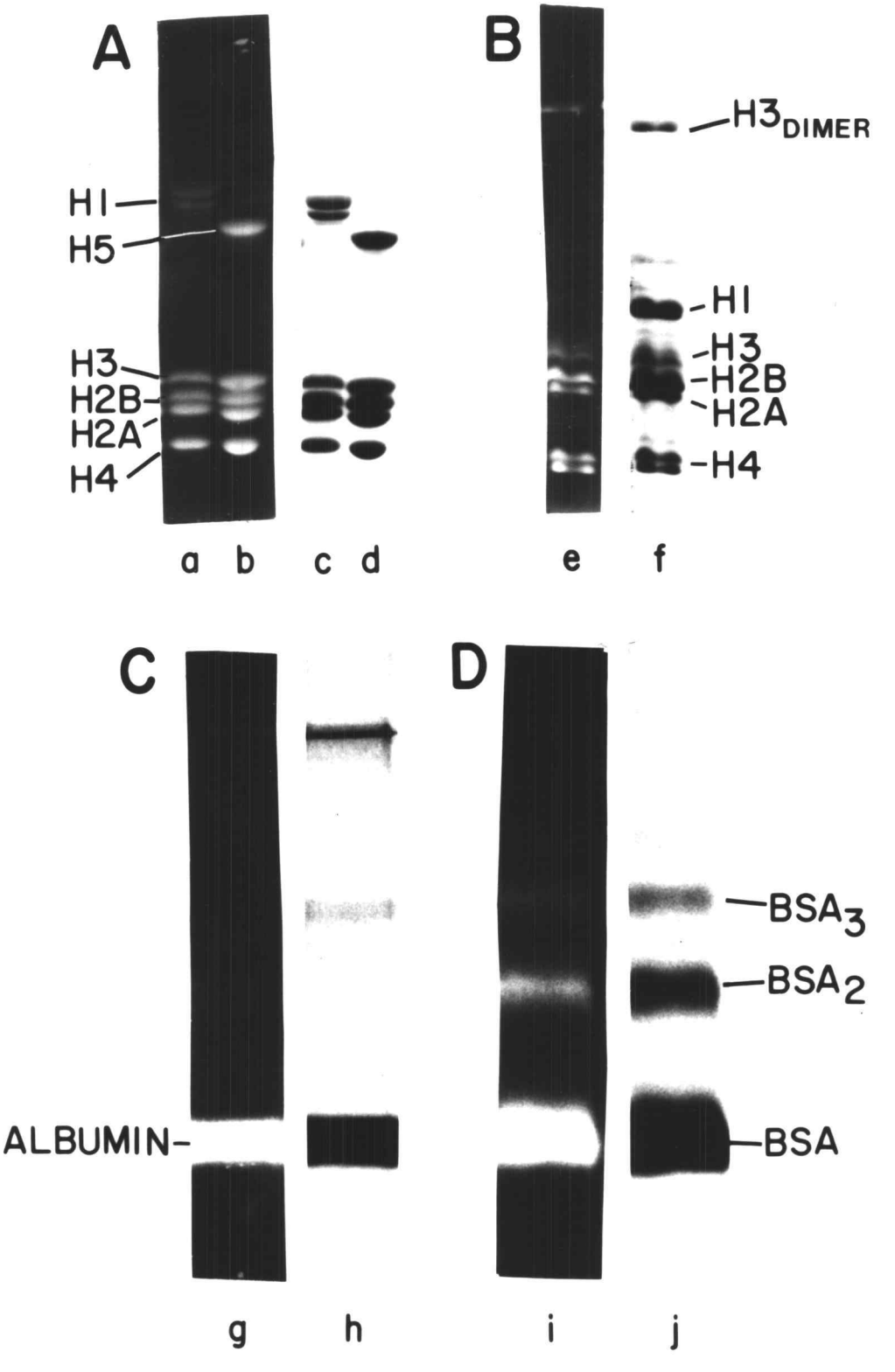
A
H1
H5
H3
H2B
H2A
H4
a b c d
B
H3 DIMER
H1
H3
H2B
H2A
H4
e f
C
ALBUMIN
g h
D
BSA3
BSA2
BSA
i j

Fig. VII.2

SECTION VIII

SUMMARY

The rough-skinned newt Taricha granulosa proved to be an excellent choice for the study of hemoglobin-binding and posthemolytic iron conservation in an amphibian. It is proliferate in western Oregon and especially easy to capture in ponds during Spring. The animal does not require elaborate life-support facilities and is stalwart enough to endure captivity for many weeks.

The studies contained in this dissertation have centered on assessing the newt's ability to bind erythrocyte-free hemoglobin and prevent significant hemoglobin iron loss through excretion, along with comparing its hemoglobin-binding proteins to mammalian haptoglobin and serum albumin. Also included are studies that improved methodology regarding protein purification and identification which enhanced the quality and accuracy of data produced in the above-mentioned sections.

This representative of the most primitive class of land vertebrates was shown to lack haptoglobin, reported to exist in snakes, birds and mammals. The "acute-phase" response to inflammation that greatly increases haptoglobin levels in birds and mammals was not observed in the amphibian. Taricha hemoglobin

demonstrated an inability to bind to human haptoglobin, and instead of splitting into αβ dimers (as is required for hemoglobin-haptoglobin complexation) it was shown to octamerize.

Although appearing to lack haptoglobin, Taricha was observed to possess three hemoglobin-binding proteins. One is a glycoprotein of about 75,000 daltons in molecular weight. The remaining two, after a comparison with known serum albumins, were shown to be albumins. The presence of two albumins, a condition called "bisalbuminemia" is rare in vertebrates. Even rarer is the hemoglobin-binding ability of a serum albumin, which was observed in Taricha. Only one other vertebrate has been suggested to possess such a mechanism, and it was also an amphibian (frog).

The excretion of iron was followed after induced hemolysis in the newt. The amphibian was observed to exert a proficient degree of iron conservation even at life-threatening hemolytic levels.

Since haptoglobin has been reported to exhibit bacteriostatic influence when complexed with hemoglobin, Taricha hemoglobin-plasma protein complexes were introduced to Staphylococcus aureus and Streptococcus pyogenes cultures. The uptake of iron-59 from mouse and Taricha hemoglobins by the bacteria was

compared to that of the mouse hemoglobin-human haptoglobin complex and Taricha complexes. Neither haptoglobin nor the hemoglobin-binding proteins of the amphibian exhibited bacteriostatic influence.

It appears that although an amphibian is devoid of the model hemoglobin-binding protein haptoglobin, it does possess other plasma proteins that bind its hemoglobin. The amphibian also exhibited the ability to conserve hemoglobin upon hemolysis, a function attributed to haptoglobin in mammals.

The results suggest that through evolutionary time, the responsibility of hemoglobin binding has traversed from a multipurpose protein such as serum albumin to a more complicated, specialized protein such as haptoglobin.

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APPENDIX

APPENDIX

This manuscript is currently in press with the Journal of Chromatography to be published as a "Note." The method of Dubray and Bezard (127) which was claimed to specifically reveal glycoproteins in gels, was used for a short time in this thesis research when attempting to identify glycoproteins in Taricha plasma. When Taricha hemolyzed plasma was resolved in SDS gels and stained with this method, the Hb stained intensely. Due to the specificity claimed by Dubray and Bezard, this result suggested Taricha Hb to be substantially glycosylated (which would be a rare situation for that class of protein). This paper clearly describes the reason for conducting the research and for the submission of the results.

Cautionary Note: A Silver Stain Devised to Indicate Glycoproteins in Polyacrylamide Gels Also Reveals Hemoproteins and Hemin

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Introduction

The identification of glycoproteins electrophoretically separated in polyacrylamide gels has been a focus of increasing attention, with various staining procedures employed (93,111,126). However, the relatively low sensitivities of those methods have limited their usefulness.

The periodic acid-silver (PA-AG)[1] staining method of Dubray and Bezard (127) exhibited superior sensitivity for the staining of human serum glycoproteins, as observed in this laboratory. Glycoproteins such as haptoglobin (128), α^2 macroglobulin (129) and transferrin (130) stained while serum albumin did not. However, when serum was hemolyzed (containing hemoglobin), the hemoglobin (Hb) subunit bands separated in sodium dodecyl sulfate (SDS) polyacrylamide gels also stained intensely for glycosylation. Since in some cases of diabetes a portion of human Hb has been seen to be glycosylated (131,132), this result was thought to indicate a diabetic condition in the human donor. However, when commercially available human

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[1]Abbreviations: PA-AG, periodic acid-silver; Hb, hemoglobin; Mb, myoglobin; SDS, sodium dodecyl sulfate; cyto c, cytochrome c.

met-Hb and other nonglycosylated hemoproteins were resolved in SDS gels, all stained intensely. The staining reaction with hemoproteins and hemin was observed to be independent of periodic acid oxidation.

Experimental

Materials

Human serum was supplied by the Red Cross of Portland, Oregon. The low molecular weight marker kit was purchased from Pharmacia, while human met-hemoglobin, sperm whale myoglobin and cytochrome c were obtained from Sigma. Electrophoresis was performed on a mini-slab (8 x 10 cm) apparatus made by the Idea Scientific of Corvallis, Oregon.

Polyacrylamide Gel Electrophoresis

The electrophoresis involved the use of sodium dodecyl sulfate (SDS) according to Laemmli (57) with 15% acrylamide slab gels 0.8 mm thick. Samples were dissolved in 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue and were heated at 100°C for 5 minutes.

Staining of Gels

The gels were stained according to the PA-AG procedure of Dubray and Bezard (127) and for peroxidase activity according to Francis and Becker (118).

Results and Discussion

When human serum was electrophoresed in SDS gels and stained using the PA-AG method, proteins such as α^2 macroglobulin, haptoglobin and transferrin were indicated while serum albumin was not. Since the first three proteins are glycosylated (93,111,126) while albumin is not (133), the staining results supported the contention that this silver-stain method was specific for glycoproteins. However, when human hemolyzed plasma samples were electrophoresed and stained, the Hb subunit bands also gave a positive reaction. When commercially supplied human Hb was resolved in SDS gels and PA-AG treated, the subunit bands again reacted positively. Since normal human Hb is not glycosylated (131,132), it was apparent that another component of the Hb was reacting with the ammoniacal silver under these conditions. In order to demonstrate the possible role of the heme moiety in the reaction, other hemoproteins such as sperm whale myoglobin (Mb) and cytochrome c (cyto c) (neither one of which is glycosylated) were also run in SDS gels. The PA-AG staining of these samples resulted in a positive reaction of each (Fig. A.1). The evidence strongly suggested that the heme component was reacting with the silver. Myoglobin had been reported to

react with PA-AG stain when used as a molecular weight marker in another study (134).

In order to support the contention of a heme-silver interaction, two experiments were conducted. The first involved the resolution of the hemoproteins along with the molecular weight standard in SDS gels. The samples were applied so that the gel could be bisected (after electrophoresis) resulting in duplicate samples in each half. One-half was stained for peroxidase activity (118) and the other using the PA-AG procedure (127). The result is shown in Figure A.1. The hemoprotein bands along with the diffuse band previously identified as hemin (118) stained for peroxidase activity. The silver-stained gel also revealed the hemoprotein and hemin bands. Of the proteins in the molecular weight standard sample, ovalbumin stained intensely with silver while phosphorylase b and carbonic anhydrase faintly stained. This is the same result as observed in the original PA-AG paper (127).

The second experiment involved removing the heme group from Hb and Mb using the method of Teale (135). The dehemed and hemed globins were electrophoresed in SDS gels and compared regarding peroxidase activity and silver staining. The hemed proteins reacted with both stains while their dehemed counterparts failed to

react (data not shown). The staining of hemoproteins and hemin using the PA-AG method was observed to be independent of the periodic acid oxidative step.

The purpose of this report is to present evidence that a sensitive silver stain devised to indicate glycoproteins specifically also reveals hemoproteins and hemin in SDS gels. Since hemoproteins are present in both plant and animal tissues, the results of this study should be taken into account when assaying a sample for glycoproteins using the periodic acid-silver staining procedure of Dubray and Bezard.

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Figure A.1

A 15% polyacrylamide-SDS slab gel containing the following: 1) Pharmacia low molecular weight markers; 2) human Hb; 3) sperm whale Mb; 4) cyto C. The gel half left of the arrow was stained for peroxidase activity (note that none of the molecular weight marker proteins were indicated). The gel half on the right reveals the silver staining of duplicate samples. The small arrows indicate hemin. Of the molecular weight marker proteins, ovalbumin (oval) with a molecular weight of 43 Kd stained intensely while phosphorylase b (phos b) at 94 Kd and carbonic anhydrase (ca) at 30 Kd stained faintly.

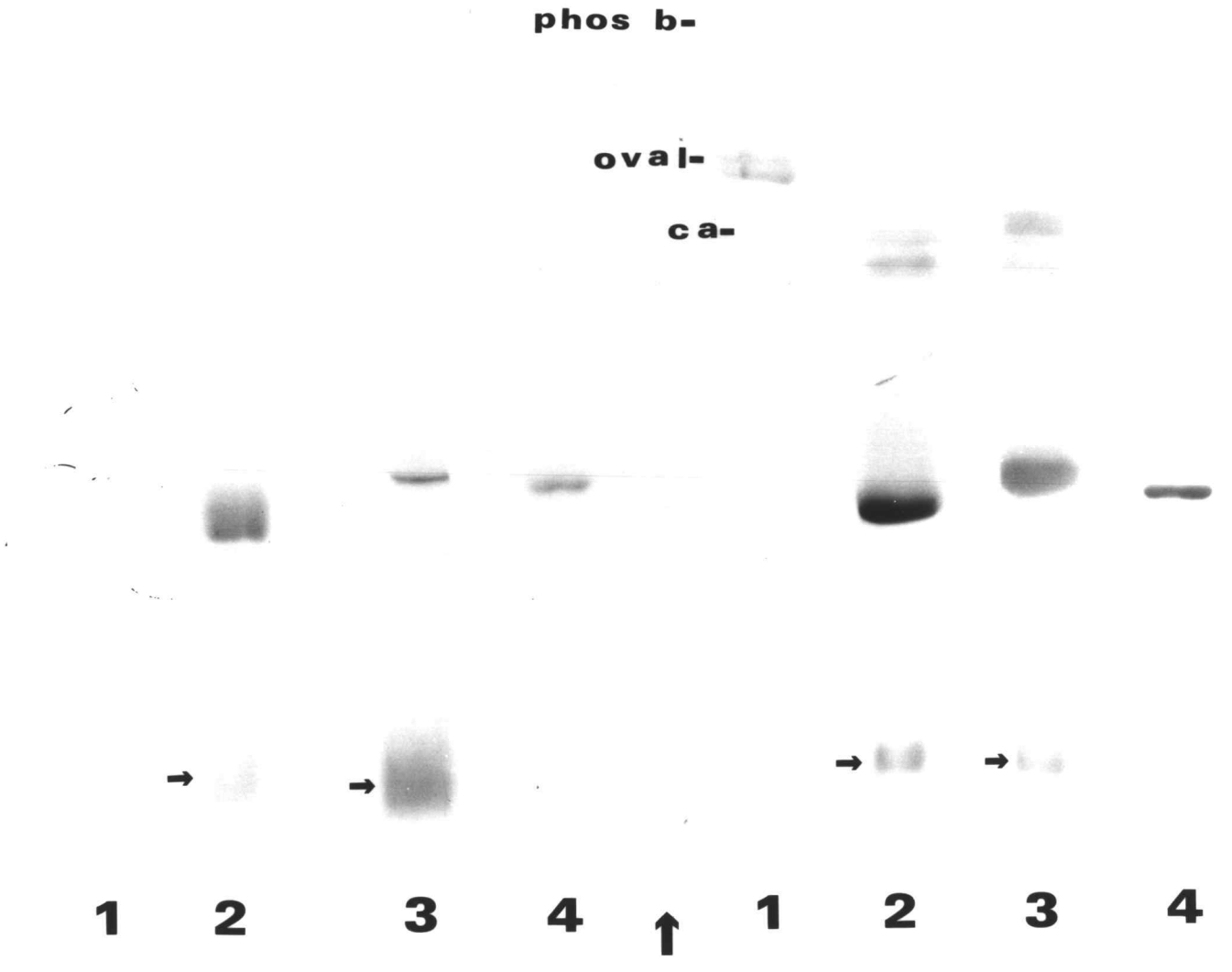

Fig. A.1