PART I

PROTEIN SYNTHESIS DURING CHICKEN ERYTHROCYTE DIFFERENTIATION

PART II

STUDIES OF ORDERED DNA· PROTEIN FIBERS

Thesis by

David Kabat

In Partial Fulfillment of the Requirements

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My thesis is dedicated
in fond memory of Lucien,
who held my hand in Redwing
and said
"That is a bird."

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I give my deepest appreciation to my wife, Susan, who has been so encouraging and helpful during these difficult years.

I. PROTEIN SYNTHESIS DURING CHICKEN ERYTHROCYTE DIFFERENTIATION

II. STUDIES OF ORDERED DNA·PROTEIN FIBERS

by David Kabat

Abstract

Part I

It was the major purpose of this research to study changes of protein synthesis during chicken erythrocyte differentiation.

In Chapter 1, erythrocytes from the blood of normal and anemic birds were fractionated by buoyant density centrifugation in bovine serum albumin gradients. It is shown that this procedure fractionates the erythroid cells according to their physiological maturity. Reduction of RNA synthesis, RNA content, and protein synthesis are shown to accompany cell maturation. Inhibition of RNA synthesis with actinomycin D does not affect hemoglobin synthesis in erythroid cells from anemic birds. The two hemoglobins, present within single chicken erythrocytes, appear to be synthesized in constant ratio throughout erythropoiesis, suggesting that the factors involved (at the genetic and translation levels) in the regulation of these syntheses operate in a coordinate manner.

In Chapter 2, it is shown by peptide mapping that the two chicken hemoglobins contain many common amino acid sequences; their genes presumably arose by duplication of common ancestral genes. The site of hemoglobin synthesis is the cytoplasmic polyribosome, as shown in part by the similar appearance of autoradiographic peptide maps of [^{14}C] leucine-labeled purified hemoglobin and of [^{14}C] leucine-labeled nascent polypeptides associated with the ribosomes. Labeled growing polypeptide chains, isolated from ribosomes following pulses with [^{3}H] leucine, are shown to be

heterogeneous in size, ranging from very small peptides up to complete globin chains. The kinetics of labeling the different sized polypeptides is in agreement with the well-established model of protein synthesis in which amino acids are sequentially polymerized from one end of the growing chains. At 37° in vitro, it requires roughly one minute to synthesize a complete globin chain (approximately 150 amino acids). It is also demonstrated that both histone and non-histone chromosomal proteins are synthesized in non-dividing avian erythrocytes. The histones synthesized are, selectively, the "arginine-rich" histones.

In Chapter 3, a study is made of the nuclear hemoglobin of chicken erythrocytes. One percent of the total cell hemoglobin remains associated with nuclei isolated in low ionic strength solutions, but this bound hemoglobin fraction can be extracted with 0.14 M NaCl. A ubiquitous and widely-studied class of nuclear protein molecules from plant and animal cells ("nuclear soluble proteins") has similar nuclei-binding properties. It is demonstrated that the bound hemoglobin molecules consist exclusively of one of the two hemoglobin types, that they are complexed to the erythrocyte chromosomes, and that they co-sediment with the chromatin in sucrose gradients.

Part II

Mixtures of chicken hemoglobin and DNA or horse heart cytochrome c and DNA form highly-ordered and tightly-packed fibrous complexes only in the presence of divalent metal ions. Polarization and electron microscopy are used to demonstrate that the DNA molecules are strongly oriented parallel to the fiber axes. Polarized visible absorption spectra are used to determine the hemoprotein orientation in the fibers; spectra of

several derivatives of horse hemoglobin crystals and also horse heart ferricytochrome c crystals are analyzed as controls. A model for the role of divalent metal ions in the tightly-packed nucleoprotein fibers is deduced from these experiments. The well-known strong stabilizing effects of divalent metal ions on tightly-packed ribosome and chromosome structures are also discussed.

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PART I

PROTEIN SYNTHESIS DURING CHICKEN ERYTHROCYTE DIFFERENTIATION

INTRODUCTION

The avian erythroid system was chosen for biochemical studies of cellular differentiation, mainly for the following reasons:

(a) The protein products of avian erythropoiesis consist of a small number (1-2 main components) of easily separated hemoglobins (1-4).

(b) The erythroblasts and the most immature erythrocytes are localized in the bone marrow, and are therefore separated from the more mature, predominantly non-dividing, erythroid cells found in the blood (5). This is helpful, for example, in studying nuclear metabolism without the complications of cell division.

(c) Erythrocytes can be easily obtained in large amounts in an almost pure form as a suspension of single cells (5, 6). Whereas blood from normal mammals contains very few immature erythroid cells, normal avian blood contains immature erythroid cells in higher proportion (5, 7). Moreover, maturity of the blood cell population can be influenced experimentally; in phenylhydrazine-induced anemia the number of immature erythrocytes increases dramatically and early cell stages become numerically preponderant (8-10). This allows comparison of mature with relatively immature cell populations.

(d) Avian erythrocytes contain a nucleus and a full diploid quantity of DNA (11). This affords the opportunity, unlike with mammalian red blood cells, of studying nuclear and chromosomal

changes associated with cell maturation.

(e) Avian erythrocytes are morphologically simple, and the maturation is accompanied by clear cytological changes (5, 12).

(f) Mammalian erythropoiesis has been widely studied (for example, 12-15). It was hoped that a study of avian erythropoiesis might provide interesting comparisons with the mammalian system.

Previous knowledge concerning the morphological aspects of avian erythrocyte differentiation is discussed more fully by Lucas and Jamroz (5) and the biochemical aspects by Schweiger (12), Sturkie (6), Cameron and Prescott (7) and Hammarsten, Thorell, Aqvist, Eliasson and Akerman (10).

It was the major purpose of this research to study protein synthesis in chicken erythrocytes. Particular emphasis was placed on the relative syntheses of the two chicken hemoglobins (3,4) in different stages of differentiation, the controlling factors involved in the synthesis of the various erythrocyte proteins, and the cellular sites of hemoglobin synthesis at various developmental stages. The chicken was selected for this work because studies had been made concerning the structure, amino acid composition, and immunochemical behavior of its hemoglobins (2-4, 16-19), and concerning the development of erythropoiesis in the embryo (3,4,20,21). A parallel project in this laboratory involved analysis of RNA synthesis in duck erythrocytes (9). The goal of such research is to obtain information useful to understanding the regulation of gene expression which occurs in normal animal cells.

Chapter 1

SYNTHESIS OF CHICKEN HEMOGLOBINS DURING ERYTHROCYTE DIFFERENTIATION

INTRODUCTION

The erythrocytes of individual adult chickens contain two hemoglobins[1] which are present in the approximate ratio 3:1 (1, 3, 4, 17, 19). These two hemoglobins have different amino acid compositions (2), and differ in oxygen-binding ability (4) as well as in electrophoretic and chromatographic properties. Although it has been shown that the two hemoglobins are present within single erythrocytes (23, 24), it is not known whether they are formed at different stages of cellular differentiation.

In the present study we have determined the relative rates of synthesis of the two chicken hemoglobins in red cells fractionated according to physiological maturity. As a prerequisite for this investigation, conditions suitable for separating erythrocytes at different stages of development were investigated. A satisfactory fractionation, as judged by several biochemical parameters of erythrocyte maturation, was achieved by applying, with minor modifications, the density gradient centrifugation procedures developed by Leif and Vinograd (25) for fractionating mammalian red cells.

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[1] Several investigators (17, 19) have found a more acidic hemoglobin in low concentration (about 1% of the total), but the possibility that this may represent an artifact has not been eliminated. An electrophoretic pattern containing 5 hemoglobin bands has been described by one group (22) who used freezing and thawing to lyse the cells. It has recently been found, however, that freezing and thawing of chicken hemoglobin solutions results in the formation of additional electrophoretic components (R. Sanders, personal communication).

MATERIALS AND METHODS

Animals

3-day-old or adult White Leghorn chickens were utilized in this work. Adult chickens were made anemic by daily subcutaneous injection for 5-7 days of 20 mg of neutralized phenylhydrazine hydrochloride and were bled on the day following the last injection. In some experiments adult normal Pekin ducks were also utilized.

Incubation of blood cells

Blood cells, removed from the animals by heart puncture, were washed 3 times with 0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$ (NKM) and resuspended in the medium to be utilized for incubation at a concn. of $1.5\text{-}2.0 \cdot 10^9$ cells/ml, unless otherwise specified. Incorporation of [2-^{14}C] uridine (30 μC/μM, added at a final concn. of 0.30 μC/ml) was carried out in modified Eagle's medium (26) supplemented with 5% dialyzed chicken or duck serum. Incorporation of individual ^{14}C-labeled amino acids (leucine, 131 μC/μM; valine, 200 μC/μM; isoleucine, 110 μC/μM; tyrosine, 226 μC/μM, each added at a concn. of 1.0 μC/ml unless otherwise mentioned) was performed in modified Eagle's medium usually lacking the corresponding amino acid and supplemented with 5% dialyzed serum. All incubations were carried out at 37°. Aliquots of the incubation mixture (containing 0.5-1.0 mg of acid-insoluble material) were precipitated with 5% trichloroacetic acid and filtered through Millipore membranes; these were glued onto aluminum planchets and assayed with a Nuclear-Chicago gas-flow counter. All measurements of radioactivity were corrected for self absorption.

In the experiments in which the amino acid- or uridine-incor-

porating capacity of cells of different buoyant density had to be measured, the incubation with labeled precursors was done either before or after cell fractionation (see below). In the case of blood cells from normal ducks or chickens, which had only low levels of protein- and RNA-synthesizing activity and therefore required a relatively long incubation in vitro (4 h), this was carried out after buoyant-density fractionation in order to avoid the possible effects on cell density of the prolonged incubation. On the contrary, in the case of the much more active blood cell populations from anemic chickens, the relatively short incubation with radioactive precursors (1 h) was carried out before cell fractionation.

Subcellular fractionation and analytical procedures

For the preparation of the cell lysates for paper electrophoresis, the cells were spun down at 500 X g for 10 min at 4°, and were then resuspended in 4 volumes of distilled water. After 30 min at 0-2°, when hemolysis was complete, the suspension was centrifuged at 9000 X g for 10 min to sediment the big cell debris. The supernatant (cell lysate) was concentrated by dialysis against Sephadex G-100 and then throughly dialyzed versus 0.04 M Veronal buffer (pH 8.5) containing 0.005 M KCN. The highly concentrated hemoglobin solutions were finally clarified by centrifugation at 10 000 X g for 10 min in the 0.3-ml microtubes of the SW-39 Spinco rotor. The cell lysates prepared as described above contained about 70% of the total cell hemoglobin. The remainder was ionically bound to the lysis sediments and could be extracted with NKM solution, but not with water. In the experiments involving analysis of hemoglobin synthesis in cell populations

fractionated in bovine serum albumin gradients, the portion of hemoglobin extractable with NKM solution was pooled with the cell lysate before preparing the samples for electrophoresis.

Paper electrophoresis was performed in a Spinco Durrum-type paper electrophoresis cell in 0.04 M Veronal buffer (pH 8.5). Whatman No. 3 paper was cut to fit the carrier of the cell and was immersed in 0.04 M Veronal buffer (pH 8.5) containing 0.1% bovine serum albumin prior to sample application. The bovine serum albumin greatly reduced adsorption of hemoglobin to the paper. The electrophoresis was performed at a constant current of 15 mA for 20 h. Following the electrophoresis, the paper was dried in a stream of air at 45° for 20 min. Fig. 6 shows a typical paper electrophoresis pattern. Hemoglobin bands ('Hb1' and 'Hb2' in Fig. 6) were eluted from the paper by descending chromatography with NKM solution in a water-saturated atmosphere. Eluted hemoglobins were converted into the cyanmethemoglobin derivatives in order to obtain reliable extinction coefficients (27). Aliquots of purified labeled hemoglobin solutions were precipitated with 5% trichloroacetic acid and the precipitates collected on Millipore membranes for radioactivity determinations.

In some experiments the two chicken hemoglobins were separated by chromatography on Amberlite IRC-50 (ref. 16) carried out in small columns (3 cm X 0.8 cm).

Samples of cell suspensions to be utilized for RNA determinations were precipitated and washed with 5% trichloroacetic acid, and were then treated with 0.30 M KOH at 37° for 24 h; after addition of

perchloric acid to 0.5 M, the insoluble residue was separated by centrifugation and washed, and the combined supernatants were analyzed by the orcinol reaction (28) using D-ribose as a standard.

The intracellular amino acid pools in erythrocytes from normal chickens were determined by an adaptation of the method of Warner and Rich (29).

Fractionation of blood cell populations in bovine serum albumin gradients

The deionized concentrated bovine serum albumin solution was prepared as described by Leif and Vinograd (25). pH and ion content were adjusted by adding to 100 g of this solution the following salts: 0.3270 g Na_2CO_3, 0.2302 g NaCl, 0.1124 g $MgCl_2 \cdot 6H_2O$, .0286 g KCl. This concentrated bovine serum albumin solution had the same osmolarity as, and a very similar cation composition to NKM solution, as calculated for 67% water content (25), a density of 1.100 g/cm^3 and a pH of 7.1. Solutions of lower densities, but of the same osmolarity, were prepared by diluting (on the basis of weight determinations) the dense solution with NKM solution. For the purposes of these experiments we define dense and light bovine serum albumin solutions prepared in this way as 'isotonic'. Bovine serum albumin solutions of lower tonicity were made by diluting isotonic bovine serum albumin solutions with deionized preparations having the same densities. For example, a solution of 90% tonicity was prepared by adding 10 g of deionized bovine serum albumin solution to 90 g of isotonic bovine serum albumin of the same density. Densities were evaluated from refractive indices (25).

Formation of linear density gradients in Spinco SW-39 tubes was performed by a peristaltic pump technique (25). The cells to be

fractionated (0.10-0.15 ml of packed volume) were spun down and the pellet was resuspended, with the aid of a Vortex mixer, in 2.5 ml of dense bovine serum albumin solution which was then utilized for the formation of the gradient; this procedure reduces centrifugation of cells outward against the centrifuge tube walls, which occurs if the cells are layered onto the gradients, resulting in contamination of light cells by denser ones during fraction collection (25). Centrifugation was at 17 000 rev./min for 40 min at 4° in the SW-39 rotor in a Model L Spinco ultracentrifuge.

Following gradient collection, carried out as described by Leif and Vinograd (25), each fraction was diluted with 2 ml of NKM solution and the cells were centrifuged down. In the experiments involving incorporation of radioactive RNA or protein precursors following cell fractionation, the cells of the individual fractions were resuspended in 1.3 ml of incubation medium; 0.3 ml aliquots were removed for cell counts and for cytological examination, while the remainder was incubated with radioactive isotopes, as described previously. In the experiments in which incorporation of radioactive isotopes preceded cell fractionation, the cells of the individual fractions were lysed by resuspension in 1.0 ml H_2O; after 30 min at 4°, the cell debris was spun down and extracted with 1.0 ml NKM solution; the H_2O and NKM extracts were combined, and the absorbance of hemoglobin was measured at 542 mμ; in these experiments the absorbance profile was used as a measure of the erythrocyte distribution in the density gradient (control experiments showed that the distribution thus obtained was not substantially different from that determined on

the basis of cell counts). Absorbances of oxyhemoglobin at 542 mμ were multiplied by the factor 1.14 (30) in order to determine concentrations in mg/ml.

Autoradiography

Erythrocytes from 3-day-old chicks or from anemic adult chickens were suspended, at a cell concn. of 10^9 cells/ml, in modified Eagle's medium lacking leucine and supplemented with 5% chicken serum. 20 μC of L-[4,5-^{3}H] leucine (8.2 C/mM) were added to 1.0 ml of each suspension and incorporation was carried out at 37° for different times, as specified below. At the end of the incubation, 0.5 ml of cold serum was added to each cell suspension and smears were made onto slides previously coated with gelatin-chrome alum solution (0.5 g gelatin-0.05 g chrome alum-100 ml H_2O); after 1 min the cells were fixed with cold methanol for 10 min, washed twice in 2% $HClO_4$ for 20 min (40 ml/slide), rinsed in water and dried in air. Coating of slides with Kodak AR.10 fine grain stripping film was performed according to Boyd (31). After 13 days exposure carried out in light tight boxes at 4°, the films were developed with Kodak D-19 for 7 min at 25°, then stained and mounted by the method of Gude, Upton and Odel (32).

RESULTS

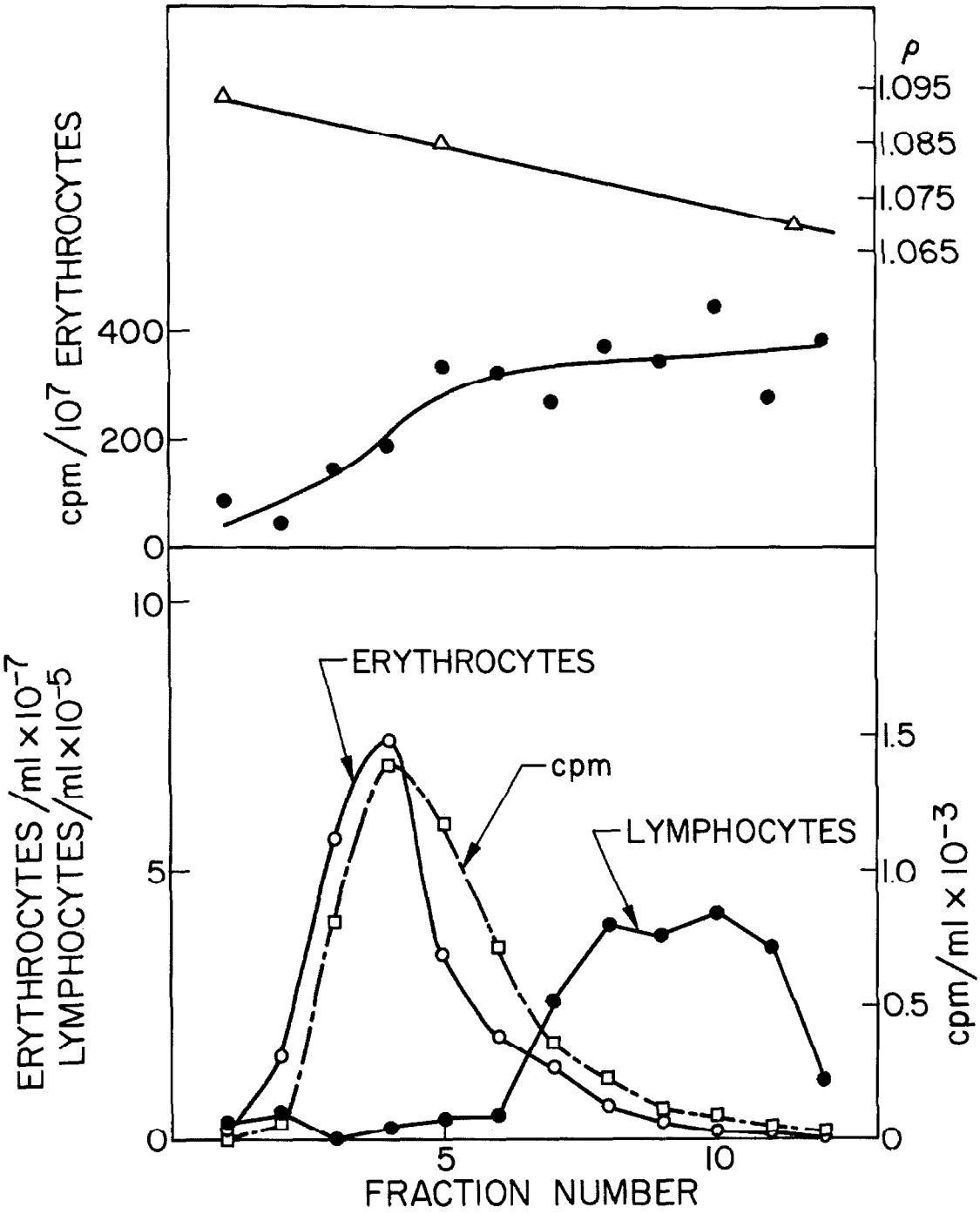
Distribution of RNA- and protein-synthesizing activity and of RNA content among erythrocytes from normal and anemic birds fractionated on the basis of buoyant density in bovine serum albumin gradients

In the blood of the normal adult birds used in this work, there are on the average about $3.5 \cdot 10^9$ erythrocytes, $4 \cdot 10^7$ leucocytes, and $3 \cdot 10^7$ thrombocytes per cm^3. Preliminary experiments showed that

chicken and duck erythrocytes have a similar buoyant density distribution in bovine serum albumin density gradients. Although banding variations occurred with different birds and with different preparations of serum albumin, no systematic species differences were observed. Nucleated red cells from normal birds have a density in isotonic bovine serum albumin which is close to the mazimum density obtainable with bovine serum albumin solutions. Therefore in the experiments involving fractionation of cells from normal animals, it was found to be advantageous to decrease the cell density by using bovine serum albumin solutions with lowered salt concentrations, which caused cell swelling due to the intake of water. This procedure did not alter the RNA- or protein-synthesizing activity of the cells, nor was there any appreciable influence on the distribution of these activities relative to the cell distribution.

Fig. 1 shows the buoyant density fractionation of normal duck erythrocytes in bovine serum albumin of 90% tonicity. It can be seen that there is a shift of [^{14}C] uridine incorporating activity toward the low density side of the red cell distribution. As reported elsewhere (9), about 90% of the [2-^{14}C] uridine incorporation by normal duck blood cells is into RNA, and the bulk of RNA synthesis occurs in erythrocytes. Lymphocyte counts of individual fractions are also plotted in Fig. 1. It appears that the RNA synthesizing activity does not follow the lymphocyte distribution, although a small amount of RNA synthesis probably does occur in lymphocytes (7). That the RNA synthetic activity shift is mainly attributable to separation of inactive or less active dense erythrocytes from more active lighter red

Figure 1. Incorporation of [2-^{14}C] uridine by normal duck blood cells fractionated in a bovine serum albumin gradient of 90% tonicity (see text). Blood cells from a normal adult duck were washed in NKM solution, and 0.10 ml of packed cells was resuspended in dense bovine serum albumin (1.100g/cm^3) of 90% tonicity, which was mixed with light bovine serum albumin (1.064 g/cm^3), also of 90% tonicity, so as to make a linear gradient. Following centrifugation and gradient collection, the cell fractions were washed and used for incorporation of [2-^{14}C] uridine. Erythrocytes and lymphocytes were counted with a hemacytometer.

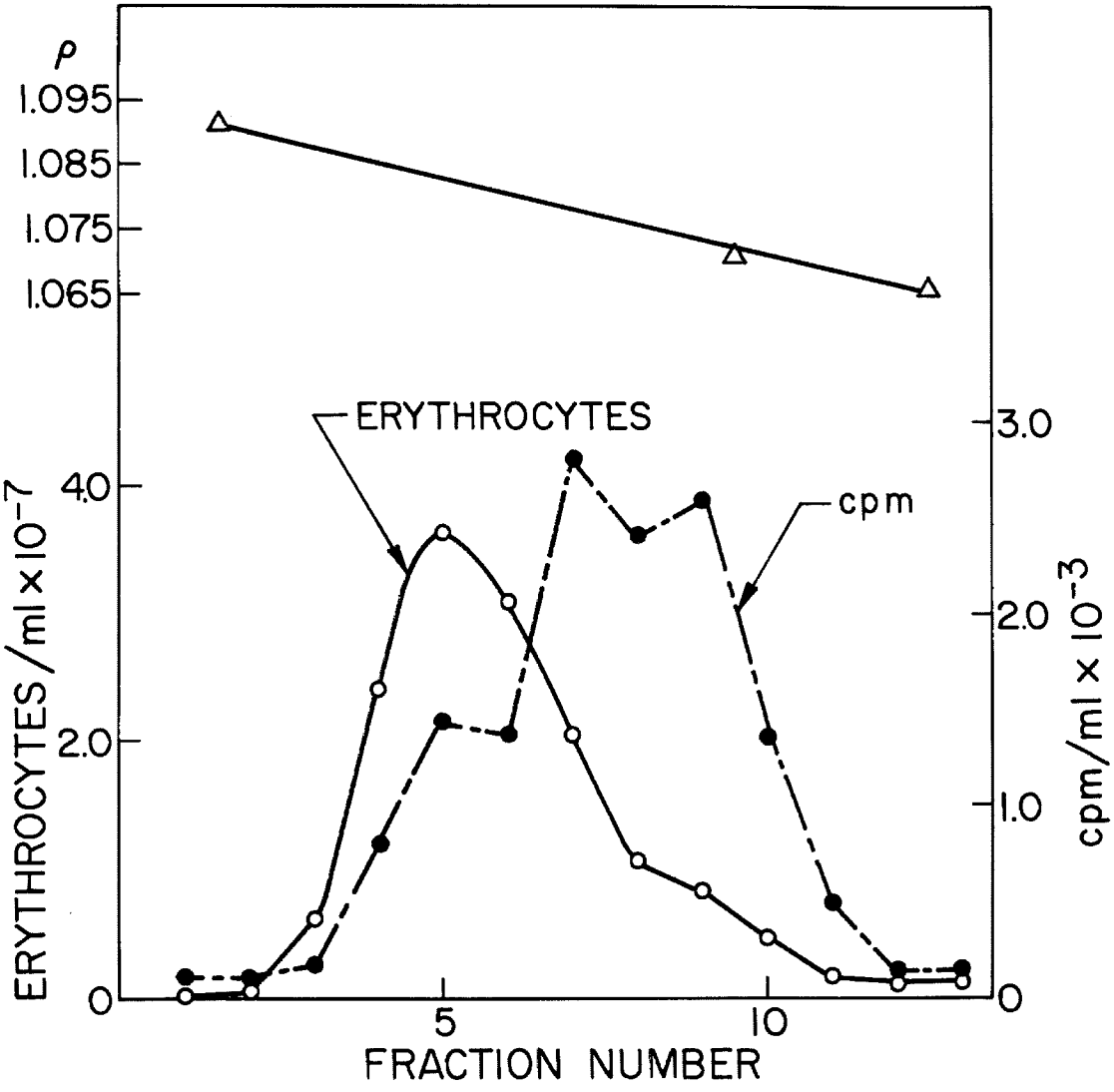

ρ
1.095
1.085
1.075
1.065
cpm/10⁷ ERYTHROCYTES
400
200
0
10
5
0
ERYTHROCYTES
cpm
LYMPHOCYTES
ERYTHROCYTES /ml x10⁻⁷
LYMPHOCYTES/ml x10⁻⁵
1.5
1.0
0.5
0
cpm/ml x 10⁻³
5
10
FRACTION NUMBER

cells is shown by the fact that the uridine incorporated per cell decreases as a function of density in the region of the gradient nearly devoid of lymphocytes (Fractions 2-6).

The separation between active and inactive red cells from normal birds was considerably enhanced by employing linear tonicity gradients superimposed upon the linear density gradients. Among the experimental conditions tested, the best results with cells from normal adult ducks or chickens were consistently obtained by using a dense bovine serum albumin solution (1.100 g/cm^3) of 93% tonicity and a light bovine serum albumin solution (1.065 g/cm^3) of 82% tonicity. This type of gradient caused a relatively greater swelling and decrease in density of the lighter red cells and a consequent broadening of the red cell distribution. Attempts to enhance the cell separation by employing shallower density gradients were less successful.

Fig. 2 shows the incorporation of L-[^{14}C] valine into normal adult chicken erythrocytes which had been fractionated in a tonicity-density gradient. It appears that the amino acid incorporation profile is, like that of uridine, displaced toward the low density side of the red cell distribution. Experiments with unfractionated normal and anemic cell populations showed that at least 90% of the radioactivity incorporated into trichloroacetic acid-precipitable material after 15 min to 5 h incubation in the presence of L-[^{14}C] valine or L-[^{14}C] leucine is alkali stable, as expected for incorporation into polypeptides. Accordingly, the curve representing [^{14}C] valine incorporation in Fig. 2 may be interpreted to reflect the distribution of protein synthesizing activity in the gradient. Under the same conditions of cell fraction-

Figure 2. Incorporation of L-[^{14}C] valine by normal chicken blood cells fractionated in a bovine serum albumin density gradient containing a superimposed tonicity gradient. Normal chicken blood cells were treated as before except that the density gradient was made with dense bovine serum albumin (1.100 g/cm^3) of 93% tonicity and light bovine serum albumin (1.065 g/cm^3) of 82% tonicity. The cell fractions were washed and used for incorporation of L-[^{14}C] valine.

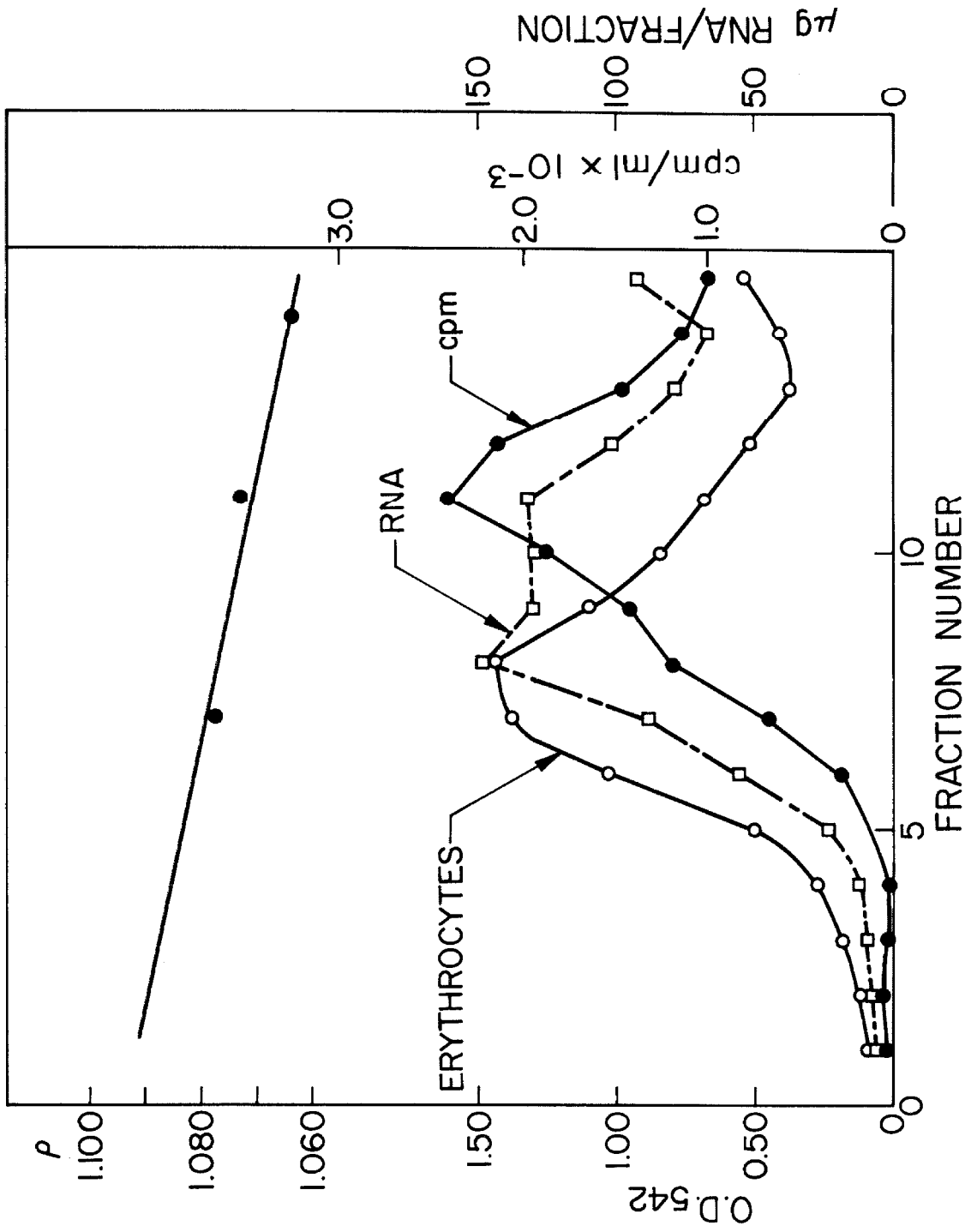

ρ
1.095
1.085
1.075
1.065
ERYTHROCYTES
cpm
4.0
2.0
0
3.0
2.0
1.0
0
ERYTHROCYTES /ml x 10⁻⁷
cpm/ml x 10⁻³
5
10
FRACTION NUMBER

ation, the distribution of RNA-synthesizing activity was close to that of the amino acid incorporation pattern.

Red cell populations from phenylhydrazine-treated chickens or ducks, which contain a large proportion of immature cells (mostly mid- and late-polychromatic erythrocytes and reticulocytes) (5), band in bovine serum albumin gradients at lower densities than do the mature red cells from normal birds (approx. 1.075 g/cm^3 and 1.090 g/cm^3, respectively, under isotonic salt conditions). In view of the lower density of erythrocytes from anemic animals, isotonic bovine serum albumin gradients were routinely used in their fractionation. The density heterogeneity of erythrocytes from anemic animals was found to be greater than that of cells from normal animals, and the curves representing RNA- and protein-synthesizing activity were relatively more displaced towards the light side with respect to the red-cell distribution. In addition, the displacement was significantly more pronounced for the peak of uridine incorporation than for the curve of amino acid incorporation.

Chemical analysis of RNA content of blood cells from anemic chickens fractionated on the basis of density showed that the lightest cells contain on the average 3-5 times more RNA than the heaviest ones (Fig.3). In 3 different experiments the cells containing the least RNA gave respectively values of 0.82, 0.52, 0.33 pg/cell, whereas the cells richest in RNA contained respectively 2.14, 2.10, and 3.40 pg/cell. The bulk of RNA in blood cell populations from anemic chickens is contained in erythrocytes, as shown both by cytochemical evidence and by the large increase in RNA content which occurs in blood cells from

Figure 3. Distribution of RNA content and protein-synthesizing activity among blood cells from an anemic chicken, fractionated in a bovine serum albumin gradient. Blood cells (0.1 ml packed volume) from a severely anemic chicken, which had been labeled in vitro with L-[^{14}C] leucine, were centrifuged in a gradient made with dense (1.102 g/cm^3) and light bovine serum albumin (1.060 g/cm^3), both isotonic. The total hemoglobin was extracted from each fraction as described in MATERIALS AND METHODS, and its absorbance at 542 mμ was used to determine the red cell distribution. The lysate supernatant and the lysis residue from each fraction were remixed and 0.05-ml aliquots were used for radioactivity determination. The remainder was analyzed for RNA content.


ρ
1.100
1.080
1.060
O.D.542
1.50
1.00
0.50
0
0
5
10
FRACTION NUMBER
ERYTHROCYTES
RNA
cpm
cpm/ml x 10⁻³
3.0
2.0
1.0
0
μg RNA/FRACTION
150
100
50
0

anemic animals, as compared to normal animals, in parallel with the increase in proportion of immature erythrocytes and in the absence of any change in the number of leukocytes or thrombocytes; therefore, the curve representing RNA content in Fig. 3 reflects mainly the distribution of RNA among erythrocytes of different density. As appears in Fig. 3, the average protein-synthesizing activity per cell is at least 20-25 times higher in the lighter cells than in those present in the heavier tail of the red cell distribution. At least 75% of the protein synthesis in whole blood cell populations from anemic chickens is hemoglobin, and the distribution of hemoglobin synthesis was found to be equal within experimental error to the distribution of total protein synthesis.

The experiments discussed in this section indicate that in the fractions of the erythrocyte population separated in bovine serum albumin gradients there is an inverse relationship between cell buoyant density on the one hand, and average RNA content, and RNA- and protein-synthesizing activities on the other. In view of the extensive available evidence indicating that both in avian (7) and mammalian erythrocytes (12-14, 25) there is a progressive decrease in RNA content and in RNA- and protein-synthesizing capacity with cell maturation, the results presented here strongly suggest that fractionation on the basis of buoyant density in bovine serum albumin gradients separates cells of increasing degree of maturity from the top to the bottom of the gradient; in this sense the situation appears to be analogous to that observed for mammalian red cells (13, 14, 25).

Characteristics of protein synthetic activity in vitro by blood cell populations from normal and anemic chickens

Fig. 4 shows the kinetics of L-[^{14}C] valine incorporation in vitro into acid-insoluble products by blood cell populations from a newborn chick (3-days-old), a normal adult chicken, and two anemic chickens. It appears that in the four cell populations the amino acid incorporation is approximately linear for the first 4-5 h, and then continues at a decreasing rate up to about 24 h. The initial rates of amino acid incorporation by these cell populations are in the approximate ratios of 1:7:50:100 for the normal adult, the newborn, and the two anemic chickens, respectively. As mentioned above, at least 90% of the radioactive amino acid incorporation is into alkali-stable material, therefore representing presumably protein synthesis.

That the differences between various blood cell populations in amino acid incorporation reflect differences both in the proportion of active cells and in the average incorporating activity per cell was shown by autoradiographic experiments. In the blood cell population from an anemic chicken shown in Fig. 5b more than half of the erythrocytes are labeled, whereas in the blood cell population from a newborn chicken shown in Fig. 5a, even after a pulse 5 times as long as in the previous case, only about 15% of the erythrocytes contain a grain count above background. From the distribution of cell types in the blood cell populations from anemic chickens and from the autoradiographic results, it appears that the bulk of protein synthesis in these populations occurs in the mid- and late-polychromatic erythrocytes. The autoradiographic experiments help also to clarify the bovine serum

Figure 4. L-[^{14}C] valine incorporation by chicken blood cell populations. The cells were incubated at a concn. of $3 \cdot 10^8$ cells/ml in valine-free Eagle's medium supplemented with 5% dialyzed chick serum in the presence of 0.5 µC/ml L-[^{14}C] valine (100 µC/µM). 0.10-ml aliquots were removed at different times for radioactivity determination.

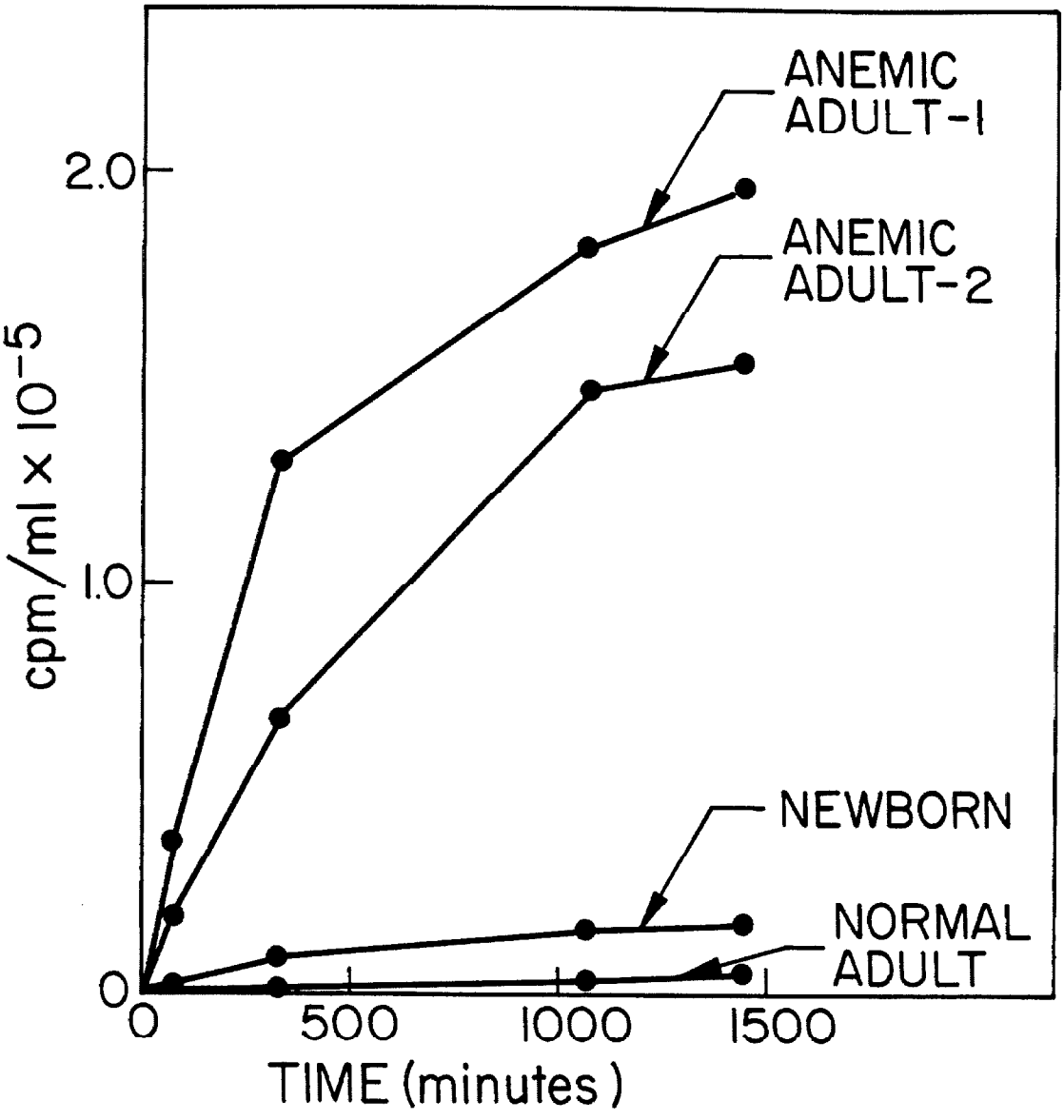
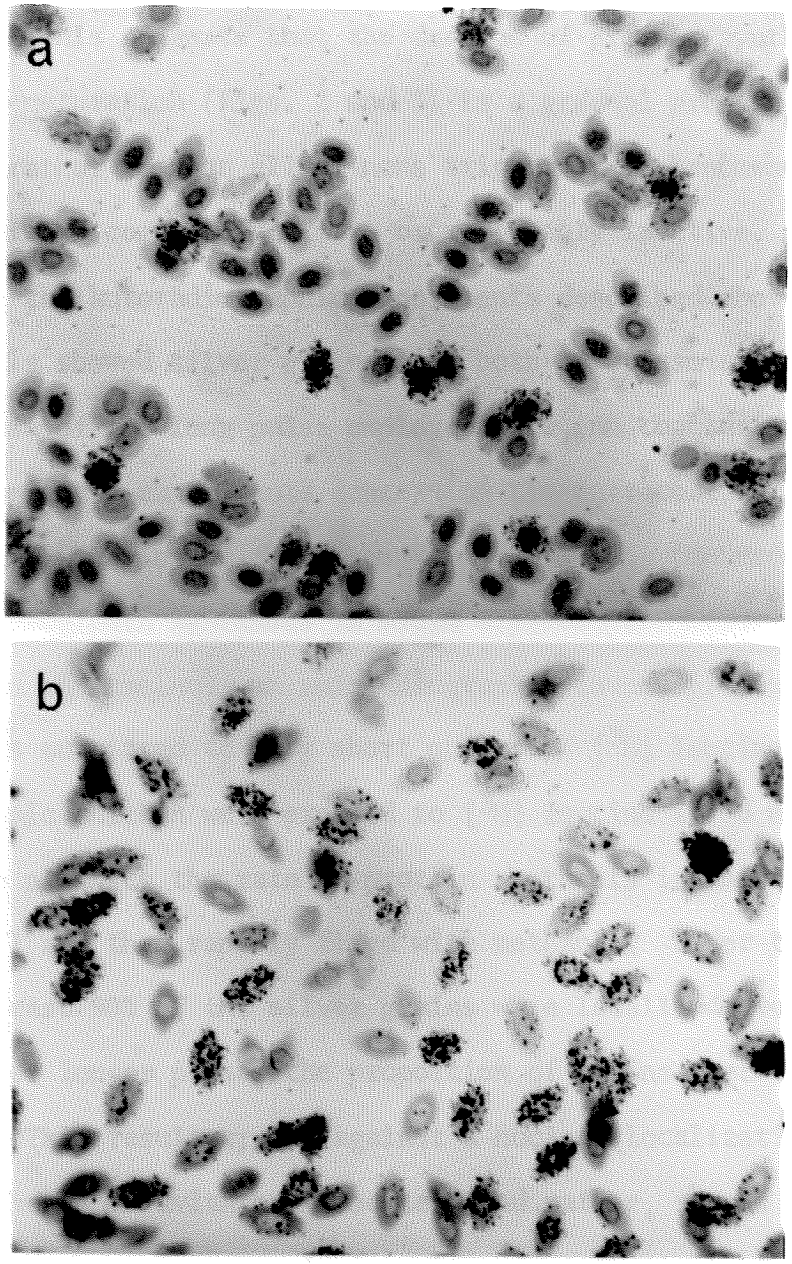

ANEMIC
ADULT-1
ANEMIC
ADULT-2
NEWBORN
NORMAL
ADULT
2.0
1.0
0
cpm/ml x 10⁻⁵
0
500
1000
1500
TIME (minutes)

Figure 5. Autoradiograms showing [^{3}H] leucine incorporation by erythrocytes from a 3-day-old chick and from an anemic adult chicken. See MATERIALS AND METHODS for the experimental conditions. a. 3-day-old chick; 210 min pulse; b. Anemic adult chicken; 40 min pulse. Magnification X 1100.


a
b

albumin density gradient fractionation experiments (Figs. 2 and 3) in that they show that the blood cell populations are heterogeneous in terms of protein synthetic capacity, with a wide spectrum of synthetic activities. This suggests that the decline of protein synthesis occurring during maturation (Figs. 2 and 3) is a gradual process at the single cell level rather than an all-or-none trigger type phenomenon, whereby cells are either completely active or completely inactive.

In the autoradiographic experiments described above, the labeled cells showed silver grains over both cytoplasm and nucleus; however, because of the long pulse employed here, no conclusion can by derived from these experiments concerning the intracellular localization of synthesis. In view of the suggestion which has been made recently (33) that hemoglobin synthesis in avian erythrocytes occurs exclusively or mainly in the nucleus, an autoradiographic investigation was carried out on cells submitted to very short pulses of [^{3}H] leucine. Blood cells from an anemic chicken were exposed to [^{3}H] leucine for 3 min at 25° (a temperature at which the rate of protein synthesis is about 20% of the rate at 37°) and then rapidly fixed with methanol. Under these conditions, at least 90% of the silver grains were localized over the cytoplasm. After longer pulses the proportion of grains over the nucleus increased. This result argues against a nuclear localization of hemoglobin synthesis in avian erythrocytes, and rather, speaks in favor of the idea that the main or exclusive site of synthesis is in the cytoplasm, with the nuclear label resulting both from secondary diffusion into the nucleus of newly formed hemoglobin (in agreement with the electron microscopic evidence of Davies) (34) and from possible

endogenous protein synthesis.

In order to obtain an estimate of the absolute levels of protein synthesis in vitro by chicken blood cell populations, it was necessary to correct the values of incorporation of labeled amino acids for the dilution by the intracellular pools. In the case of newborn and normal adult chickens, a reasonably good estimate of the intracellular pools for several essential amino acids was obtained by an adaptation of a method devised by Warner and Rich (29). This method is based on the assumption, which has been verified in all tested animal cell systems, of a rapid intermixing between intra- and extracellular amino acids (29, 35-37). In the case of chicken blood cells the assumption of rapid equilibration is supported by the observation that addition of a radioactive amino acid to a cell suspension results in linear incorporation of radioactivity into acid-insoluble products without any measurable lag. In the present determination of pool sizes, the initial rate of total incorporation of labeled amino acid into 5% trichloroacetic acid-precipitable material (as measured during the first 2-h incubation) was utilized, rather than the steady-state level of incorporation into polysomes as in the experiments of Warner and Rich (29). As shown in Table I, the close agreement between the experimental incorporation values and those calculated on the basis of the value for the intracellular pool which gave the best fit (see explanation in Table I) supports the model on which the calculations were based.

In the case of blood cell populations from anemic chickens, preliminary experiments utilizing L-[^{14}C] leucine showed that the intra-

TABLE I INTRACELLULAR POOL-SIZE DETERMINATIONS

Source of blood cells	[^{14}C]labeled amino acid	ml packed cells/ml cell suspension	Amount amino acid added (μM/ml·10^{-5})	Incorporated radioactivity per h incubation (disint./min per ml packed cells·10^{-5})	Observed relative incorporation	Expected relative incorporation for P value indicated*	Intracellular pool (μM/ml packed cells)
3-day-old chick	Tyrosine (226 μC/μM)	0.05				P=0.0018	
			2.12	10.8	1.00	1.00	Tyrosine
			1.06	7.40	0.69	0.69	0.036
						P=0.030	
	Leucine (131 μC/μM)	0.05	19.0	28.3	1.00	1.00	Leucine
			3.80	8.35	0.30	0.29	0.60
			1.90	4.34	0.15	0.15	
						P=0.039	
Normal adult chicken	Isoleucine (110 μC/μM)	0.25	95.8	3.52	1.00	1.00	Isoleucine
			47.9	2.71	0.77	0.78	0.156
			12.5	1.20	0.34	0.34	
			6.28	0.55	0.16	0.20	
						P=0.10	
	Valine (200 μC/μM)	0.25	36.0	4.96	1.00	1.00	Valine
			9.00	1.56	0.31	0.31	0.40
			4.50	0.91	0.18	0.16	
						P=0.15	
	Leucine (223 μC/μM)	0.25	39.7	9.77	1.00	1.00	Leucine
			19.9	5.34	0.55	0.56	0.60
			3.97	1.36	0.14	0.12	

*P=μM amino acid in the intracellular pool/ml cell suspension.

Blood cells were suspended, at the concentrations indicated above, in modified Eagle's medium lacking the tested amino acid and supplemented with 5% dialyzed chicken serum. After 15 min incubation at 37°, the tested amino acids were added in the amounts indicated and the rates of radioactivity incorporation were measured. The P values were selected empirically so as to give the best fit of the observed values of incorporation with those expected on the basis of the equation of Warner and Rich (29). The determinations of intracellular pools are most accurate when the value of P falls within the range of the concentrations tested.

cellular pool of this amino acid is low as compared with the rate of leucine utilization, becoming depleted after 1-2 h incubation in a leucine-free medium. This introduced difficulties for obtaining a reasonably accurate measurement of the leucine intracellular pool size by the Warner and Rich method (29). Therefore, in order to have an estimate of the specific activity of leucine incorporated into proteins by these cells it was considered to be more suitable to deplete the intracellular pool (by 2 h preincubation in a medium lacking leucine), and then to add to the medium a large excess of L-[^{14}C] leucine of known specific activity which could be assumed to be incorporated into protein without significant dilution.

The rates of incorporation in vitro of different amino acids by several chicken blood cell populations are shown in Table II. From these incorporation values the rates of protein synthesis were estimated by assuming that the average protein synthesized by these cells has the same amino acid composition as the calculated average for chicken hemoglobins (26 isoleucine, 73 leucine, 17 tyrosine, and 57 valine residues per mol. wt. of $6.8 \cdot 10^4$) (see ref. 2).

As appears in Table II, the over-all rate of protein synthesis in vitro, even in the most active blood cell populations from anemic chickens, is low relative to the cell mass (0.31 g dry wt. per ml of packed cells, of which 0.225 g is hemoglobin in erythrocytes from normal chickens). However, in view of the great heterogeneity of the blood cell populations in terms of protein synthetic capacity, as shown by the autoradiographic experiments, it would appear that the rate of protein systhesis per cell must be high in a fraction of the erythrocytes, presum-

TABLE II RATE OF PROTEIN SYNTHESIS IN CHICKEN BLOOD CELL POPULATIONS

Source of blood cells	Amino acid	μM amino acid incorporated/ml packed cells per $h \cdot 10^3$	μg protein synthesized/ml packed cells per h	% mass increase per day due to protein synthesis
3-day-old chick	Tyrosine	4.0	15.9	0.123
	Leucine	24.9	23.2	0.180
Normal adult chicken	Leucine	9.4	8.7	0.068
	Valine	4.2	5.0	0.039
	Isoleucine	2.0	5.3	0.041
Severely anemic chicken (7 phenyl-hydrazine injections)	Leucine	290	270	2.1
Less anemic chicken (5 phenylhydrazine injections	Leucine	70	65	0.50

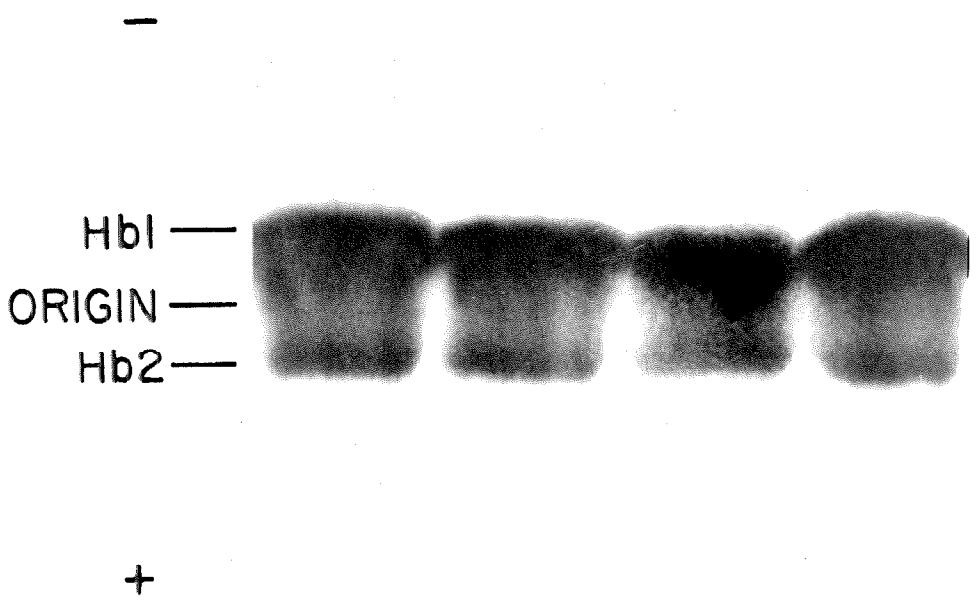
The values for the cells from 3-day old and normal adult chickens were calculated from the data of Table I. The cells from the anemic chickens (1 volume packed cells in 3 volumes of modified Eagle's medium lacking leucine and containing 5 % dialyzed chicken serum) were incubated for 2 h at 37° and then exposed to L-[^{14}C] leucine (1.25 μC/μM; 0.80 μM/ml cell suspension).

ably the youngest ones, from anemic chickens. It appears also from Table II that the great differences in amino acid incorporating capacity detected between erythrocyte populations from newborn, normal adult, and anemic chickens, persist after correction for dilution by the intracellular pools, and reflect, therefore, real differences in protein synthetic capacity.

Distribution and relative rates of synthesis of the two hemoglobins in erythrocytes from anemic chickens

Preparative separation of the two chicken hemoglobins from several cell lysates by paper electrophoresis in 0.04 M Veronal buffer (pH 8.5) is shown in Fig. 6. As already mentioned, such lysates contain approximately 70% of the total hemoglobin, the remainder being ionically bound to the sediment. Analysis of lysates from blood cells of anemic animals, after 1 h labeling with L-[^{14}C] leucine, has shown the following: (a) At least 95% of the incorporated label in the cell lysate is associated with Hb1 and Hb2 isolated by paper electrophoresis or by column chromatography. (b) In the bound hemoglobin, which can be eluted from the sediments with 0.14 M NaCl, the Hb1/Hb2 ratio is higher than in the cell lysate (approx. 11.5 and 2.7, respectively). (c) The bound hemoglobins have, within experimental error, the same specific activities as the lysate hemoglobins. Consequently, reliable specific activity determinations can be made by using the cell lysates alone. (d) In addition to the bound hemoglobins, the lysis sediment contains DNA, nuclear proteins and membranes. These non-hemoglobin sedimentable materials contain, in a trypsin-sensitive form, approximately 25% of the total radioactive leucine incorporated by blood cells.

Figure 6. Preparative separation of the two chicken hemoglobins by paper electrophoresis. Hemoglobin samples were concentrated to at least 200 mg/ml and aliquots containing 10 mg were applied onto Whatman No. 3 paper for the electrophoresis (see MATERIALS AND METHODS).

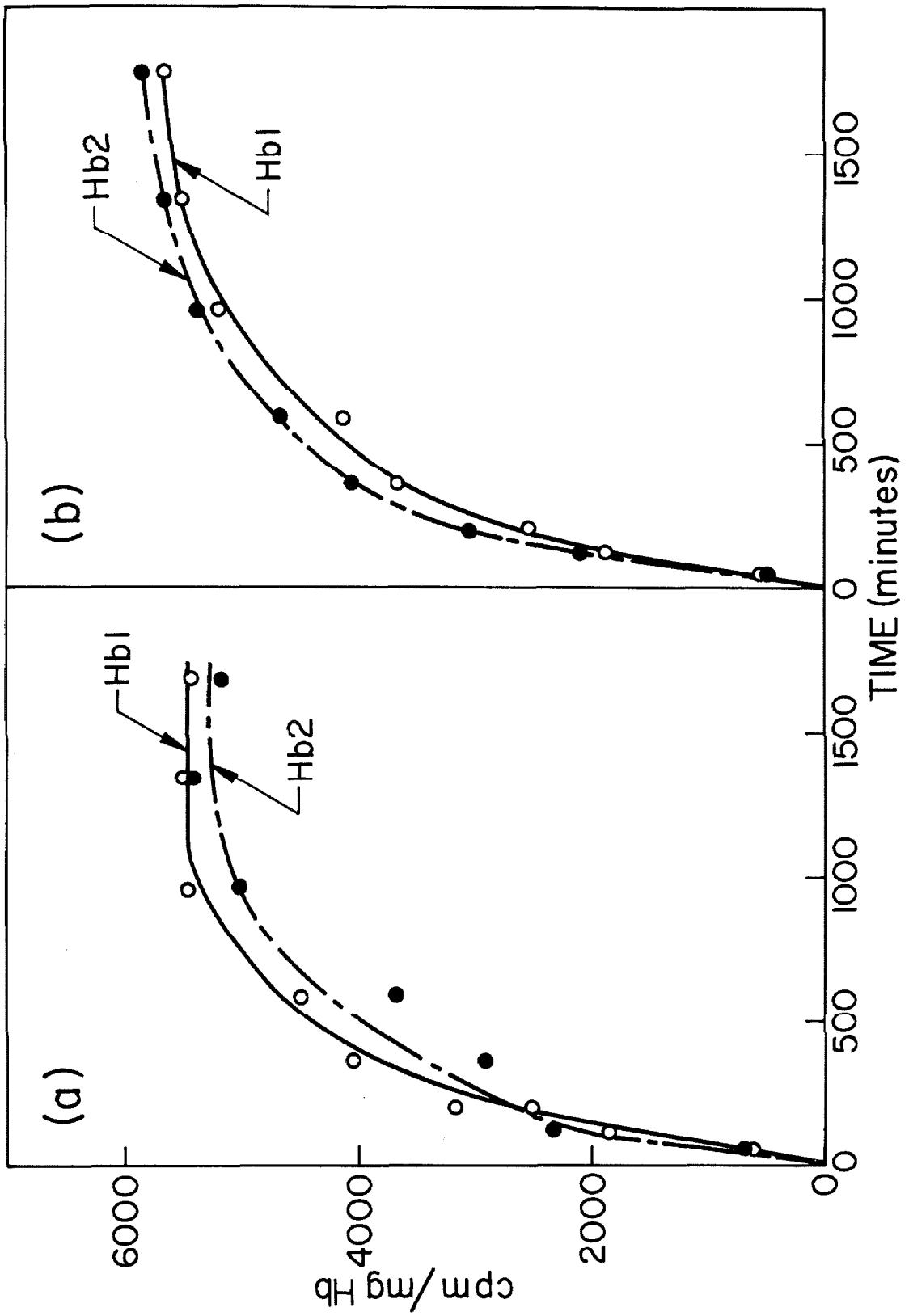

−
Hb1
ORIGIN
Hb2
+

The lysis sediments are complex and were not investigated further.

Fig. 7a shows the kinetics of incorporation of L-[^{14}C] leucine into Hb1 and Hb2 isolated by electrophoresis from the lysates of an unfractionated blood cell population from an anemic chicken. The incorporation data are expressed per unit weight of hemoglobin, so that at any time the slope of each curve shown in Fig. 7a gives a measure of the relative rate of incorporation of the labeled amino acid into the corresponding hemoglobin (that is the rate of incorporation relative to the amount of the hemoglobin already present in the cells). Since Hb1 and Hb2 have been reported to contain a somewhat different number of leucine residues (74 and 68 respectively) (2), the incorporation data have been normalized to the same leucine content by multiplying the determined specific activities of Hb1 by the ratio of the leucine contents (0.92). It is apparent from Fig. 7a that the synthesis of both hemoglobins continues at a decreasing rate for about 24 h. It can also be seen that the relative rates of synthesis of Hb1 and Hb2 are approximately equal. Similar results were obtained by using, instead of electrophoresis, column chromatography for the separation of the two hemoglobins. In red cells from normal chickens the relative rates of Hb1 and Hb2 synthesis were also found to be equal within experimental error. It should be mentioned that a large proportion (about 60%) of the protein synthesized in vitro by whole blood cell populations from normal chickens is non-hemoglobin material, as judged on the basis of precipitability by anti-hemoglobin sera (unpublished results).

The kinetics of incorporation of [^{14}C] leucine into Hb1 and Hb2 in the presence of actinomycin D is presented in Fig. 7b. Compar-

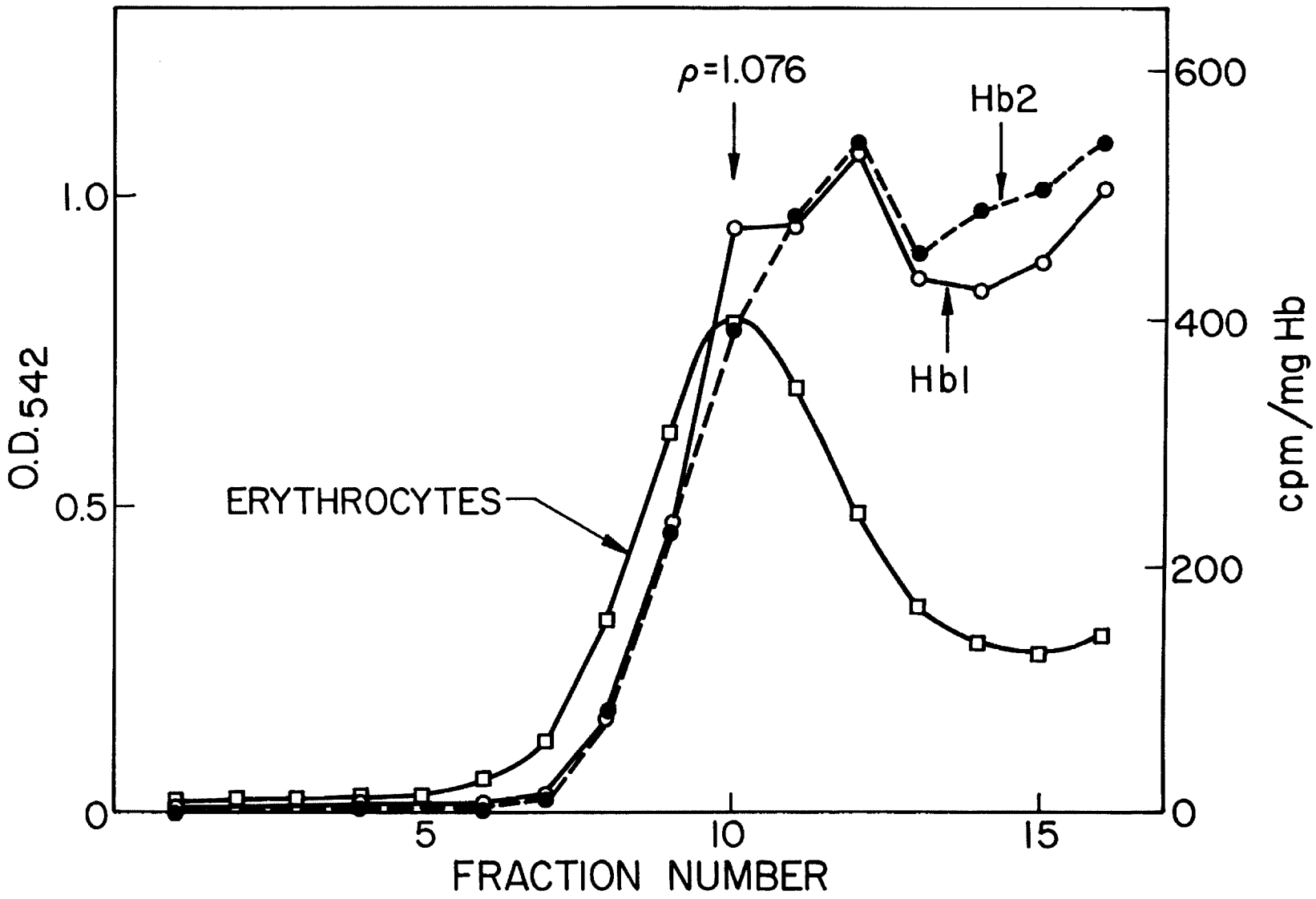
Figure 7. Kinetics of synthesis of Hb1 and Hb2 in the absence (a) or in the presence (b) of actinomycin D by unfractionated erythrocytes from a severely anemic chicken. Blood cells from a severely anemic chicken were incubated at a concn. of $2 \cdot 10^9$ cells/ml in complete Eagle's medium supplemented with 5% dialyzed chicken serum and containing 1 μC/ml L-[^{14}C] leucine (final specific activity 1.25 μC/μM) in the absence (a) or presence (b) of 50 μg/ml actinomycin D. 1.2-ml samples were prepared for electrophoresis of hemoglobin as described in MATERIALS AND METHODS.


(a)
(b)
Hb1
Hb2
Hb1
Hb2
cpm/mg Hb
6000
4000
2000
0
0
500
1000
1500
0
500
1000
1500
TIME (minutes)

ison of Figs. 7a and b shows that a concentration of the drug sufficient to completely block RNA synthesis in chicken erythrocytes (unpublished observation) has no influence on the relative rate of synthesis of either hemoglobin. The close similarity of the amino acid incorporation kinetics in the presence and in the absence of the drug would tend to exclude any appreciable synthesis of 'functional' messenger during the time of incubation in vitro. Unless one is willing to assume a continued mobilization of preformed stored messenger RNA, from the linear portion of the incorporation kinetics in this and other experiments it would appear that the messenger RNA's for the two hemoglobins are stable at least during the first 3—5-h incubation.

The similarity of the relative rates of synthesis of Hb1 and Hb2 in the unfractionated red cell population, as seen in Fig. 7a, b, is in agreement with the idea that in the individual cells both hemoglobins are synthesized at a similar relative rate. More direct evidence on this point was obtained by analyzing the relative synthesis of Hb1 and Hb2 in erythrocytes from anemic chickens fractionated on the basis of buoyant density in a bovine serum albumin gradient. As shown in Fig. 8 the relative rates of synthesis of the two hemoglobins, normalized for the difference in leucine content (2), are equal within experimental error, both in the more active lighter cells and in the less active denser erythrocytes. In the experiment shown in Fig. 8 the total cell hemoglobin (derived from the combined lysis supernatant and salt extracts of the sediment) of each bovine serum albumin gradient fraction was mixed, prior to the electrophoresis, with a constant large amount (50 mg) of unlabeled total cell hemoglobin. This procedure might conceivably have concealed differences

Figure 8. Distribution of the synthesis of Hb1 and Hb2 among erythrocytes from a severely anemic chicken fractionated on the basis of buoyant density in a bovine serum albumin gradient. Blood cells (0.15 ml packed volume) from a severely anemic chicken were incubated for 1 h with [^{14}C] leucine, as described in MATERIALS AND METHODS, prior to centrifugation in a bovine serum albumin gradient made with dense bovine serum albumin (1.100 g/cm^3) and light bovine serum albumin (1.052 g/cm^3), both isotonic. Total hemoglobin was extracted from each fraction and its absorbance used for determination of the erythrocyte distribution. After addition of unlabeled carrier hemoglobin (50 mg per fraction), electrophoresis and determination of specific activities of the two hemoglobins were carried out as described in MATERIALS AND METHODS.


ρ=1.076
Hb2
Hb1
ERYTHROCYTES
O.D.542
cpm/mg Hb
FRACTION NUMBER
0
0.5
1.0
200
400
600
5
10
15

in the relative amounts of Hb1 and Hb2 among cells of different density, thus obscuring the results. However, evidence that the ratio of the two hemoglobins is constant over the whole gradient was obtained in another bovine serum albumin density gradient fractionation experiment run in parallel to that shown in Fig. 8; in this experiment adjacent cell fractions were pooled two by two and their total cell hemoglobin was analyzed directly, without any addition of carrier hemoglobin, by chromatography on Amberlite IRC-50. The red cell banding position in the density gradient was nearly identical to that of Fig. 8 and the Hb1/Hb2 specific activity ratios (normalized to the same leucine content) were 0.97, 0.81, 0.95, and 1.04 for pooled Fractions 7-8, 9-10, 11-12, 13-14, respectively. These ratios can be considered to be within experimental error equal to unity as are the ratios in Fig. 8. Clearly, the presence of carrier hemoglobin in the electrophoresis experiment had no influence on the specific activity ratios, a result which implies an invariance in the relative amounts of the two hemoglobins in erythrocytes of different density.

It appears from Fig. 8 that there is a decrease in the rates of synthesis of the two hemoglobins going from the light portion of the gradient towards the heavier fractions. Inasmuch as fractionation on the basis of buoyant density separates cells of increasing degree of maturity from the top to the bottom of the gradient (see RESULTS, first section), these results have to be interpreted to indicate that the syntheses of the two hemoglobins are equally susceptible to the decline process accompanying cell maturation.

DISCUSSION

The main purpose of this paper has been to provide evidence pertinent to the question of whether in single chicken erythrocytes, which contain two distinct hemoglobins, there is temporal segregation, related to degree of maturity, in the synthesis of the two proteins. A prerequisite for this type of investigation was the availability of a method for separating cells of different degree of maturity. The method of buoyant-density centrifugation in bovine serum albumin gradients developed by Leif and Vinograd (25) was utilized for this purpose.

The interpretation of the results obtained by such a technique depends obviously on the demonstration that this type of fractionation separates cells at different stages of the maturation process. Two complementary types of evidence indicate that this is indeed the case. In the first place, an inverse relationship has been demonstrated in the present work between erythrocyte buoyant density, on the one hand, and average RNA content, RNA- and protein-synthesizing activity on the other. Second, autoradiographic studies of Cameron and Prescott (7) have shown that chicken erythrocytes undergo a progressive loss of RNA- and protein-synthesizing capacity during maturation. Also in non-nucleated mammalian erythrocytes, considerable evidence indicates that RNA content and protein-synthesizing activities decrease with cell maturation (12-14, 25).

In the present work the autoradiographic study of protein synthesis in erythrocytes from anemic chickens (Fig.5b) has helped to clarify the bovine serum albumin gradient centrifugation results by showing that over 50% of the cells in such a population are engaged in protein synthesis and that there is a wide spectrum of degrees of synthetic activity.

These findings, on the one hand, indicate that the decline of protein synthesis during cell maturation is a gradual process at the single cell level rather than an all-or-none phenomenon; on the other hand, they support the idea that the distribution of protein-synthesizing activity among erythrocytes fractionated on the basis of density in a bovine serum albumin gradient does not reflect the density distribution of a minority cell type with uniform protein-synthesizing capacity, but rather the different synthetic capacity of cells of different density. From all these lines of evidence it seems justifiable to conclude that the buoyant density of bird erythrocytes is inversely related to their degree of maturity. In this context, the finding that erythrocyte populations from anemic chickens band in bovine serum albumin gradients in regions of lower density than red cell populations from normal adult chickens, may be interpreted to be a reflection of the fact that they consist in great proportion of 'younger' cells.

A similar relationship between buoyant density in bovine serum albumin gradients and degree of cellular maturity has been demonstrated in mammalian reticulocytes and erythrocytes (13, 14, 25). The basis for this relationship is not known. An increase in the intracellular concentration of protein and other cell constituents due to loss of water (obviously resulting in a decline of cell volume) may contribute to this relationship (25, 38). Such loss of water might possibly reflect changes in the cell membrane; in this connection it should be mentioned that membrane changes have been reported to occur during chicken erythrocyte maturation (39, 40).

Fractions of erythrocyte populations from anemic chickens

separated in bovine serum albumin gradients were analyzed for the relative rates of synthesis of the two hemoglobin components. The ratio of the rates of synthesis of Hb1 and Hb2 was found to be invariant and equal to their mass ratio in cells of different degrees of maturity. It should be pointed out that this invariance was not determined at the level of individual cells, but only as the average behavior of groups of cell of different density; therefore, fluctuations of these ratios within each group of cells are not excluded by the results obtained here. Since the immature cells present in the blood from the anemic chickens utilized here were represented mostly by mid- and late-polychromatic erythrocytes and reticulocytes, the data obtained in this study concern mainly the synthetic capacity of cells relatively advanced in their maturation. It is however likely that cells at earlier stages of differentiation (erythroblasts and early-polychromatic erythrocytes, which remain in their great majority in the bone marrow) also synthesize the two hemoglobins in the same ratio as the cells at the developmental stages examined here. This possibility is supported by the finding that the ratio of rates of synthesis of Hb1 and Hb2 in erythrocytes in the blood equals their mass ratio, a fact which implies that the mean ratio of rates of synthesis of the two proteins in the early stages of differentiation in the bone marrow is equivalent to the invariant ratio observed during maturation in the blood. This apparent constancy throughout erythrocyte differentiation in the ratio of the rates of synthesis of the two hemoglobins suggests that the factors involved (at the genetic or translation level) in the regulation of these syntheses operate in

a coordinate manner.

The fact that the Hb1/Hb2 synthesis ratio remains invariant throughout the phase of decline of protein-synthesizing capacity in the erythrocyte maturation process represents a different situation from that described in human individuals heterozygous for the β-chain gene, where the available evidence suggests a faster decline of the capacity to form the variant β chains (β^S and β^O_{Arabia}) relative to the decline in production of the normal β^A chains (41).

Chapter 2

PATTERN OF PROTEIN SYNTHESIS IN CHICKEN ERYTHROCYTES

INTRODUCTION

In several respects the chicken erythropoietic system is ideally suited for biochemical analysis of cellular structure and differentiation. The erythroblasts and the most immature erythrocytes are localized in the bone marrow and are, therefore, physically separated from the more mature erythroid cells in the blood. Furthermore, the maturity of the blood cell population can be influenced experimentally; in phenylhydrazine-induced anemia the proportion of immature erythrocytes found in the blood increases greatly (9, 10). Additional advantages are that the cells can be obtained in high purity and that the protein product of differentiation consists mainly of two easily separable hemoglobins (1-4). Chicken erythrocytes are morphologically simple; for example nucleoli and mitochondria are absent from the cell stages found in the circulation (5). However, unlike mammalian red blood cells, avian erythrocytes contain a nucleus and the normal diploid content of DNA (11), affording the opportunity of studying chromosomal functions accompanying cell maturation.

In the present work, the pattern of protein synthesis in erythrocytes from the blood of anemic chickens has been analyzed by various methods. The evidence presented shows that hemoglobin synthesis occurs on polyribosomes in the cytoplasm, but that labeled hemoglobin molecules subsequently become distributed between the nucleus and the cytoplasm. Labeled polypeptide chains were isolated from ribosomes following pulses with radioactive amino acids and shown to be heterog-

eneous in size, ranging from very small peptides up to complete globin chains (roughly 150 amino acids). The kinetics of labeling the different sized polypeptides is in agreement with the well-established mechanism of polypeptide chain growth, in which amino acids are sequentially added to one end of the "growing polypeptide chains" (42). It is further demonstrated that there is synthesis of chromosomal proteins, both histone and non-histone, in non-dividing chicken erythrocytes.

MATERIALS AND METHODS

Animals

Adult White Leghorn chickens were made severely anemic by daily subcutaneous injection for 7 days of 20 mg of neutralized phenylhydrazine hydrochloride and were bled, by heart puncture, on the day following the last injection. The cells were washed thoroughly 3 times with 0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$ (NKM). The buffy coat of leucocytes was removed.

Incubation of blood cells

Incorporation of radioactive leucine by erythrocytes from severely anemic chickens was carried out in modified Eagle's medium (26), prepared without leucine and supplemented with 5% dialyzed chicken serum. Erythrocytes were suspended in the latter medium at a concentration of $1.5 \times 10^9 \frac{\text{cells}}{\text{ml}}$ and incubated with swirling for 1 h at 37°; this incubation suffices to deplete the intracellular leucine pool from the cells (see Chapter 1). The incorporation of radioisotope was then carried out either at 37° or at 25° as described in the text; at 25° the rate of protein synthesis by chicken erythrocytes is only 21% as high as at 37°.

For the preparation of labeled proteins for autoradiographic peptide maps, erythrocytes from severely anemic chickens were pre-incubated as described above. To 33 ml of cell suspension, 100 μC of L-[^{14}C] leucine (U.L.) [273 μC/μM] were added and incorporation was continued for 100 min at 25° before cell harvesting, subcellular fractionation, and hemoglobin purification as described below. The final specific activity of the hemoglobins was 410,000 dpm/mg.

Subcellular fractionation and isotope counting procedures

All operations were carried out in the cold at 0-4°. Following incubation of erythrocytes, 12 ml of cell suspension were diluted with 20 ml of cold NKM in a 40 ml plastic Servall centrifuge tube (SS-34 rotor) and centrifuged at 700 X g for 2 min. The resulting pellet of erythrocytes (3 ml packed cells) was resuspended in 4 volumes of 0.01 M tris buffer, 0.01 M KCl, 0.0025 M $MgCl_2$, pH 7.0, containing 1 mg/ml digitonin (Calbiochem). After 1 min, the cells were homogenized for 2-5 min with a motor driven teflon pestle which fit loosely into the tube. Speed of motor and time of homogenization were adjusted to obtain maximum breakage of cytoplasm without any visible rupture of nuclei. The supernatant, containing most of the erythrocyte hemoglobin, was then centrifuged at 10,000 X g for 10 min. If the hemoglobin was to be fractionated by chromatography, the 10,000 X g supernatant was centrifuged at 105,000 X g for 3 h and the final supernatant was dialyzed versus starting buffer (see Chromatography). On the other hand, if ribosomes were to be purified from the 10,000 X g supernatant, the pH was adjusted to 5.0 by addition of 0.1 M acetic acid; under these conditions, ribosomes and polysomes are precipitated (29); they were collected

by centrifugation at 5000 X g for 5 min, and then resuspended in 0.05 M tris buffer, 0.025 M KCl, 0.0025 M $MgCl_2$, pH 7.4 (TKM). Sodium deoxycholate was added to the suspension at a concentration of 0.5%. After standing for 20 min at 2° and a low speed centrifugation (10,000 X g for 10 min), 7 ml of suspension was layered over 2 ml of 30% sucrose in TKM and centrifuged at 105,000 X g for 3 h in the 40 rotor of a Model L Spinco ultracentrifuge. The supernatant was discarded and the ribosomal pellet ("ribosome fraction") was resuspended in TKM. The hemoglobin solution obtained following pH 5.0 precipitation of the ribosomes was adjusted to pH 7.0 with NaOH ("soluble fraction").

The pellet containing the crude nuclear preparation was generally resuspended in 0.25 M sucrose containing 0.0045 M $CaCl_2$ and centrifuged at 500 X g for 4 min. The procedure was repeated (4-5 times) until the supernatant above the nuclei was visually estimated to be free of hemoglobin. In some cases the crude nuclear preparation was alternatively resuspended in 5 ml of 2.0 M sucrose containing 0.0033 M $CaCl_2$ and centrifuged through 20 ml of the same sucrose solution at 22,000 rev./min for 30 min in an SW 25 Spinco rotor. The nuclear preparations, red colored due to complexed hemoglobin, were washed twice by resuspension in 25 ml of 0.0033 M $CaCl_2$, followed each time by centrifugation at 700 X g for 10 min.

Hemoglobin was removed from the nuclear preparations (isolated from 3 ml of packed erythrocytes) by two extractions with 4 ml of 0.14 M NaCl, 0.0024 M magnesium acetate, 0.005 M tris buffer, pH 7.2, followed each time by sedimentation at 7000 X g for 10 min to separate the nuclei. Centrifugation of the combined extracts in the Spinco 40 rotor at 105,000 Xg

for 4 h yielded a small pellet of non-hemoglobin material which contained some ferritin and no detectable ribosomes, as determined with the electron microscope; this material was only weakly labeled upon incubation of the cells with radioactive amino acids. The 105,000 X g supernatant ("nuclear soluble fraction") was further fractionated by anion exchange chromatography as described below.

The nuclei from which the nuclear soluble fraction had been extracted were washed 3 times, by centrifugation, with 25 ml of 0.0037 M NaCl, 0.00037 M sodium citrate and were then sheared in the same solution either in a Virtis homogenizer at 25 volts for 90 sec or by sonication at 7.5 amps for 1 min with a Branson sonifier [Model S (125)] fitted with a solid horn. The preparation was then centrifuged at 10,000 X g for 15 min to pellet the nuclear residue ("residual fraction"). The supernatant, which contained at least 95% of the DNA, was adjusted to 0.0024 M magnesium acetate to precipitate the chromatin; this was then sedimented by centrifugation at 10,000 X g for 10 min. No detectable protein remained in the resulting supernatants which were therefore discarded. Histones were obtained from the chromatin by two extractions (each for 30 min at 0°) with 4 ml of 0.2 M HCl, each extraction being followed by centrifugation at 10,000 X g for 10 min to separate the insoluble residue. The material insoluble in 0.2 M HCl ("non-histone fraction") contained DNA in addition to the chromosomal non-histone proteins.

The protein fractions rich in hemoglobin [soluble fraction and nuclear Hb1 fraction (see Chromatography)] were precipitated with 5% trichloroacetic acid (TCA) at 2° for 1 h, collected by centrifugation at

5000 X g for 10 min, and dissolved in a minimal volume of 0.1 M NaOH. Heme groups were then removed from the globin by adding the protein to a large excess of 1% HCl in acetone at -15°, followed after 1 h by sedimentation of the protein at 7000 X g for 10 min. The other subcellular fractions were precipitated with 5% TCA at 2° for 1 h, except that 15% TCA was used throughout these procedures for the histone fraction, followed by centrifugation at 5000 X g for 10 min.

Subcellular fractions were resuspended in 5% TCA, heated at 90° for 15 min, and then cooled in ice. Following centrifugation at 5000 X g for 10 min, the pellets were once again heated in 5% TCA at 90° for 15 min, cooled in ice, and pelleted at 5000 X g for 10 min. The pellets were then washed first with 100% ethanol and then with ethanol:ether (3:1) at 4°. The pellets resulting from the final washing were then dissolved in 1 ml of 1 M NaOH, by heating if necessary. The samples dissolved in 1 M NaOH were analyzed for protein concentration by the method of Lowry et al (43) using bovine serum albumin as a standard. Then 0.10 ml aliquots were mixed with 15 ml Bray's scintillation fluid (44) and assayed for radioactivity in a Packard Tri Carb liquid scintillation spectrometer. Self absorption corrections were made whenever necessary.

Peptide maps, globin electrophoresis, and protein reduction and alkylation

Globin was prepared by precipitation of hemoglobin in 0.5% HCl in acetone at -15°. Reduction of protein disulfide bonds with 2-mercaptoethanol, sulfhydryl alkylation with iodoacetic acid, and staining of peptide maps with ninhydrin were carried out as described by Bennet, Hood, Dreyer and Potter (45). Trypsin (Worthington) digestion and peptide mapping were performed by the method of Katz, Dreyer and Anfinsen (46).

[^{14}C] leucine-labeled proteins to be utilized for peptide mapping were all isolated from the same batch of cells after labeling at 25° as described above. The peptide maps obtained from these reduced and alkylated proteins were placed in X-ray exposure holders (Kodak) together with Kodak Royal Blue X-ray film and exposed at 2° for 60 or 90 days. Films were developed with Kodak DK-50 developer for 7 min at 25°, rinsed with H_2O, and fixed. Preparation of horizontal acrylamide gels for electrophoresis was carried out as described by Bennet et al (45).

Chemical analyses

DNA and RNA were fractionated by the Schmidt-Thannhauser procedure, as modified by Ts'o and Sato (47). DNA was determined by the diphenylamine method using D-deoxyribose as standard, and RNA by the orcinol reaction (28) using D-ribose as standard. Following hydrolysis of nucleic acids, carried out by heating the samples in 5% TCA at 90° for 30 min, the insoluble residues were washed with 5% TCA, 100% ethanol, and finally with ethanol: ether (3:1). The final precipitates were dissolved in 1M NaOH and assayed for protein by the method of Lowry et al (43), using bovine serum albumin as a standard.

Chromatography of chicken hemoglobins

Chromatographic fractionation of chicken hemoglobins on Amberlite IRC-50 columns (Mallinckrodt CG-50, 200-400 mesh, 10 meq/g) was done by an adaptation of the method of Van der Helm and Huisman (16). Citrate buffer of ionic strength equal to 0.15 M and pH 6.0 (49) was adjusted to lower ionic strength by dilution, and to higher ionic strength by addition of solid NaCl. A column 11 cm X 5 cm diameter was utilized to fractionate about 400 mg hemoglobin. Elution was made by stepwise increments of ionic

strength as described in Fig. 1; in this case, 5 ml fractions were collected. A microcolumn adaptation of this procedure, using columns 4 cm X 1 cm diameter, is described in the legend to Fig. 10. All solutions were saturated with CO. Fractionated hemoglobins were precipitated with 80% $(NH_4)_2\ SO_4$ and dialyzed versus 0.0037 M NaCl, 0.00037 M sodium citrate, pH 7.0.

Immunochemical analyses

Hb1 and Hb2 peaks from chromatographically fractionated erythrocyte lysates were pooled and then fractionally precipitated with $(NH_4)_2\ SO_4$: a 45% cut was discarded and an 80% cut was collected. The precipitated hemoglobins were dialyzed against water prior to injection into rabbits. Five rabbits were injected subcutaneously with 40 mg hemoglobin in Freund's adjuvant. After 15 days the rabbits were injected 5 times with 20 mg hemoglobin on alternate days. Five days after the last injection, each rabbit was injected in the foot pad with 40 mg hemoglobin. Antisera were collected 15 days after the final injection. Precipitin tests, performed according to Heidelberger and Landsteiner (50) showed presence of antibody against hemoglobin in the sera of all 5 rabbits. Immunodiffusion tests with Ouchterlony plates confirmed this result.

Size fractionation of ribosome-associated growing polypeptide chains

Ribosomes purified from [^{3}H] leucine-labeled chicken erythrocytes were resuspended in 0.01 M tris buffer, 0.03 M EDTA, pH 7.3. Carrier chicken globin was then added (1 mg of globin per 0.15 mg of ribosomes, as determined using the extinction coefficients, $E^{1\%}_{1cm}$, 26 for chicken globin at 280 mμ and 112 for erythrocyte ribosomes at 260 mμ [51]). Then, 500 μg ribosome suspension was treated with 100 μg pancreatic ribo-

nuclease (5 times crystallized) (Sigma Chemical Company) for 2 h at 37°; the digestion was stopped by adding one volume of 10 M urea and adjusting the suspension to 0.05 M 2-mercaptoethanol. The digests were incubated for 15 h at room temperature and were then adjusted to pH 2.4 with formic acid. After centrifugation at 5000 X g for 5 min to remove insoluble material which was only approximately 5% of the radioactivity, 0.7 ml portions were placed onto 38 cm X 1 cm diameter columns of Sephadex G-100 previously equilibrated with 8 M urea, pH 2.4. The flow rate was very slow and was increased slightly by use of a Technicon proportioning pump. Fractions were collected in graduated tubes and the volumes and optical densities were measured. Then, 0.2 ml samples were mixed with 15 ml Bray's scintillation fluid for counting.

Determining the kinetics of labeling the various sized polypeptides involved measuring the distribution of radioactive material eluted from the columns after differing pulse times with [^{3}H] leucine. Different columns were normalized to the same ribosome contents in order to quantitatively compare the radioactivity distributions. This was done by correcting the relative radioactivities in the column effluents so that they equaled the relative specific radioactivities (cpm/O.D.$_{260}$) of the purified ribosome fractions. Approximately 0.30 mg of ribosomes were applied to the columns, and the recovery of radioactivity was quantitative.

Cell autoradiography

Procedures employed in the [^{3}H] leucine cell autoradiography have been described previously (see Chapter 1). Chicken erythrocytes are flat disks which lie on their faces when smeared onto microscope slides; furthermore, the nuclear diameter is nearly equal to the cell thickness (3).

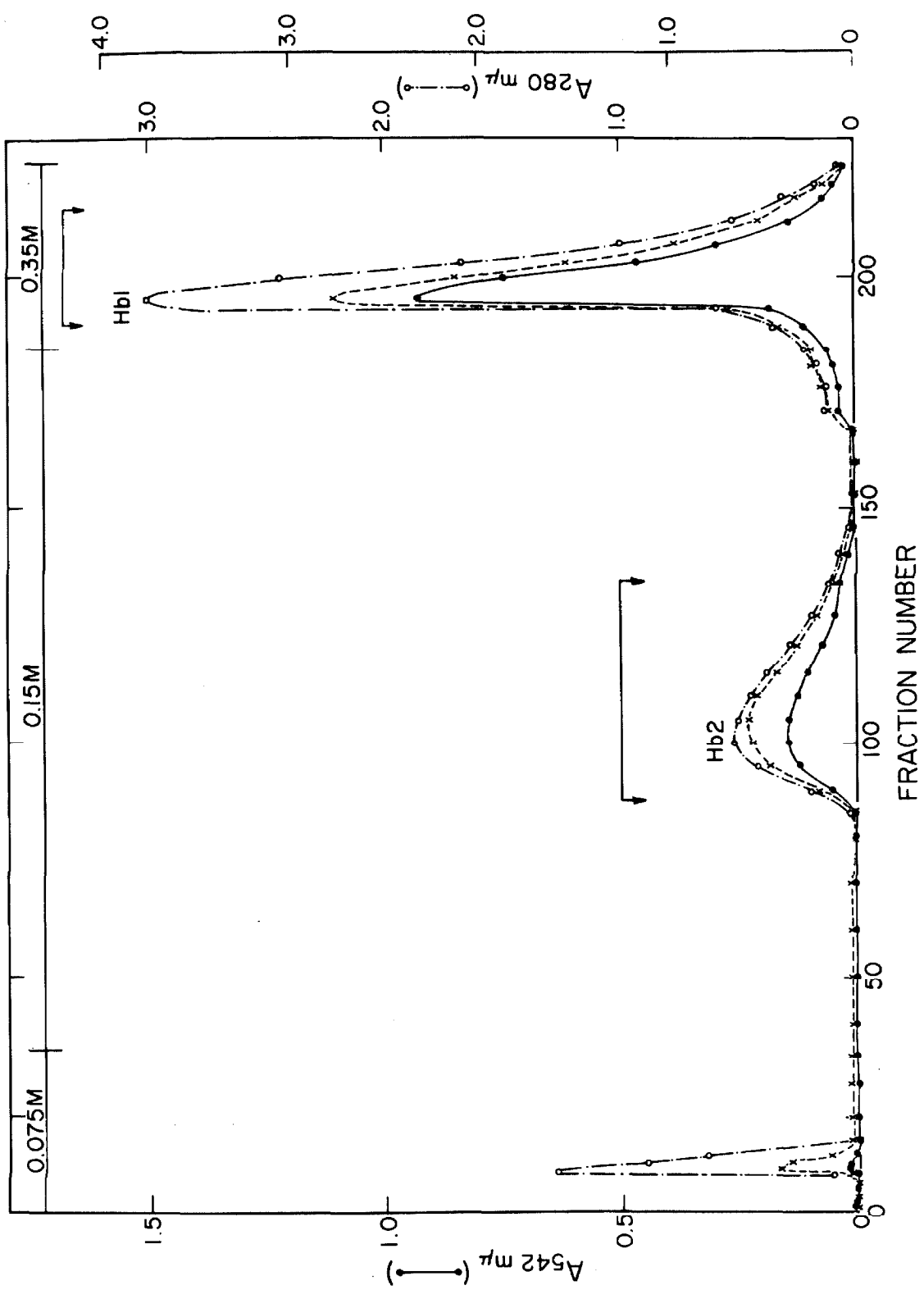
It was therefore assumed that silver grains localized over the nuclei were the result of β-particles emitted from the nucleus rather than from the overlying cytoplasm. Nuclear and cytoplasmic silver grains were counted at 2000 X magnification. Grains located at the border between the nucleus and the cytoplasm were counted for determination of the total grains per cell, but were ignored in calculating the percentage of grains localized over the nucleus. Corrections were always made for background grains. The percentage of nuclear grains did not depend significantly on the total number of cell grains for erythrocytes from the blood of anemic chickens. In calculating the percentage of nuclear grains, only cells having at least 5 times the background grain level were considered, because of the larger errors associated with grain counts of the least active cells. Standard deviations of these percentages were also calculated. At least 75 erythrocytes were counted on each slide.

RESULTS

The hemoglobins present in chicken erythrocytes

Chromatography of the soluble fraction from [^{14}C] leucine-labeled chicken erythrocytes is shown in Fig. 1. The 280 mμ absorbance is predominantly associated (approximately 93%) with the two hemoglobin peaks, labeled "Hb1" and "Hb2"; and at least 96% of the incorporated radioactivity appears also to be associated with the hemoglobins. The radioactivity distribution shown in Fig. 1 was not corrected for the self-absorption of the samples; however when this correction is applied, the cpm/mg protein of Hb1 and Hb2 are equal within experimental error as previously described (Chapter 1). In other experiments, it has been

Figure 1. Anion exchange chromatography of chicken erythrocyte soluble fraction labeled with [^{14}C] leucine. Erythrocytes from a severely anemic chicken were suspended in modified Eagle's medium with 5% chicken serum at 1.5 X 10^9 cells/ ml, and incubated with 2 μC/ml L-[^{14}C] leucine (13.2 μC/μM) for 15 h at 37° (see MATERIALS AND METHODS for preparation of erythrocyte soluble fraction for chromatography). The elution employed stepwise increase of ionic strength as indicated in the figure. Fractions were plated directly on planchets (0.2 ml aliquots) and were assayed for radioactivity in a Nuclear-Chicago gas flow counter. The horizontal lines bounded by arrows show the regions of the eluate pooled for further analysis.


A542 mμ (●——●)
A280 mμ (○——○)
CPM/1.0 ml × 10⁻⁴ (*- - -*)
0.075M
0.15M
0.35M
Hb1
Hb2
FRACTION NUMBER
0
50
100
150
200
0.5
1.0
1.5
2.0
3.0
4.0

shown that even after very short pulses with [^{14}C] leucine (4 or 15 min labeling of an erythrocyte suspension at 25°), the specific radioactivities of Hb1 and Hb2 are equal. Similar chromatographic patterns have been obtained by other investigators (2, 4, 17). Electrophoretic fractionation of freshly prepared chicken erythrocyte lysates likewise resolves two hemoglobins in approximate ratio 3:1 (2-4, 24).

Immunodiffusion analysis of these hemoglobin fractions is shown in Fig. 2. Antiserum made against the purified pooled hemoglobins was placed in the center well, and the different hemoglobin fractions were placed in the peripheral wells. The continuity of the precipitin bands indicates that there are molecular similarities (i.e., cross-reactivity) between the two hemoglobins. However "spurs" occur at the crossover points between Hb1 and Hb2, indicating that the hemoglobins are not identical. The pattern of spurs suggests that the antiserum utilized contains antibodies which react with Hb2, but not with Hb1.

Tryptic peptide maps of the reduced and carboxymethylated hemoglobins likewise suggest that the proteins differ in molecular structure, but that extensive amino acid sequence similarities exist. Figs. 3a, b, c and d show a comparison between the ninhydrin-stained and autoradiographic peptide maps of L-[^{14}C] leucine-labeled Hb1 and Hb2; some peptides lack leucine and can be seen only in the stained maps. Figs. 3e and f are both ninhydrin-stained tryptic peptide maps of unfractionated erythrocyte soluble fraction. Fig. 3f is marked to indicate peptides characteristically associated only with one of the two hemoglobins; also indicated are peptides which appear to be present in both hemoglobins. The similarity between the two proteins is a striking feature of these maps.

Figure 2. Ouchterlony plate. Reaction of Hb1, Hb2, and a mixture (1:1) of Hb1 and Hb2 against anti-hemoglobin antiserum.

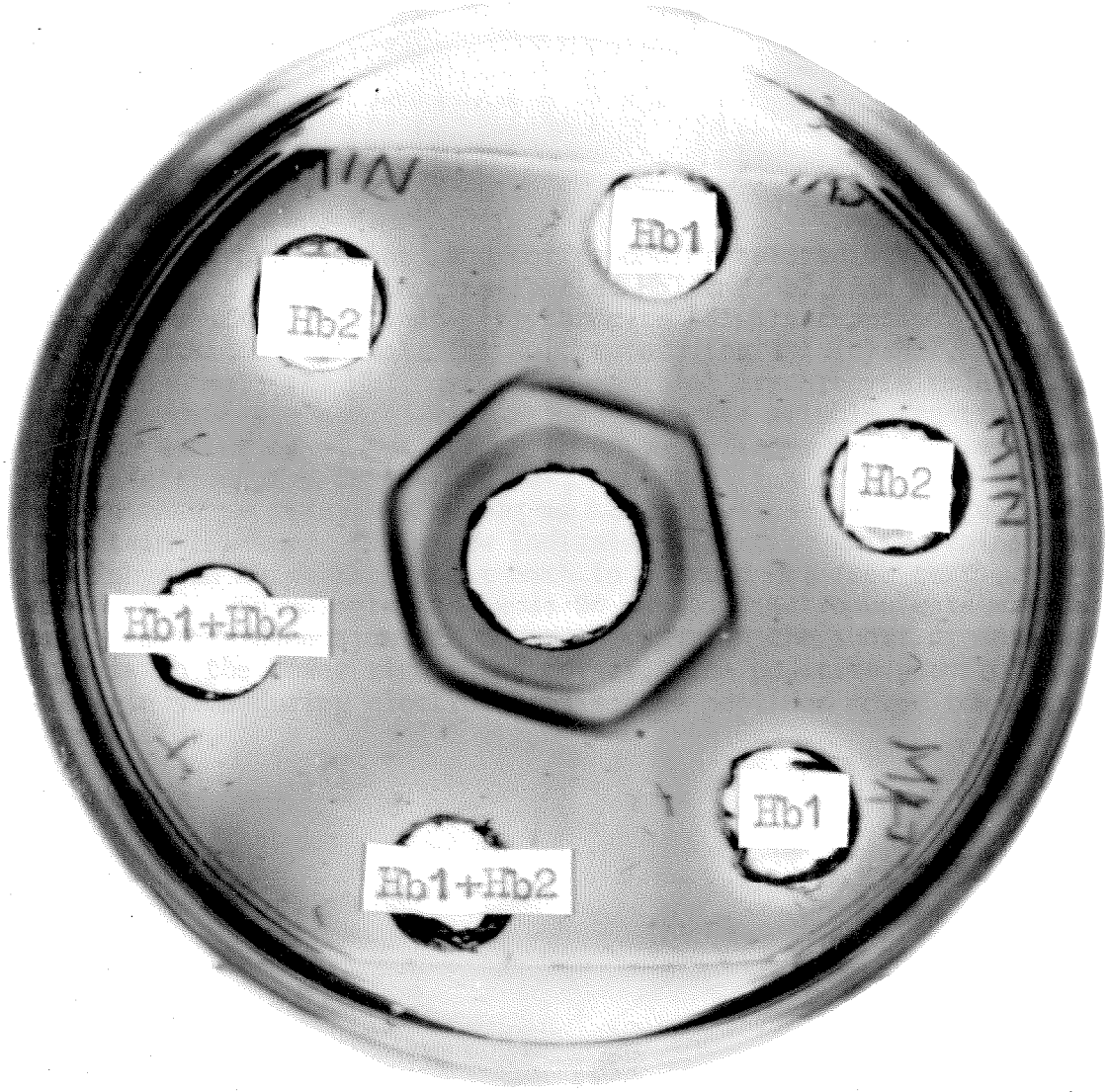
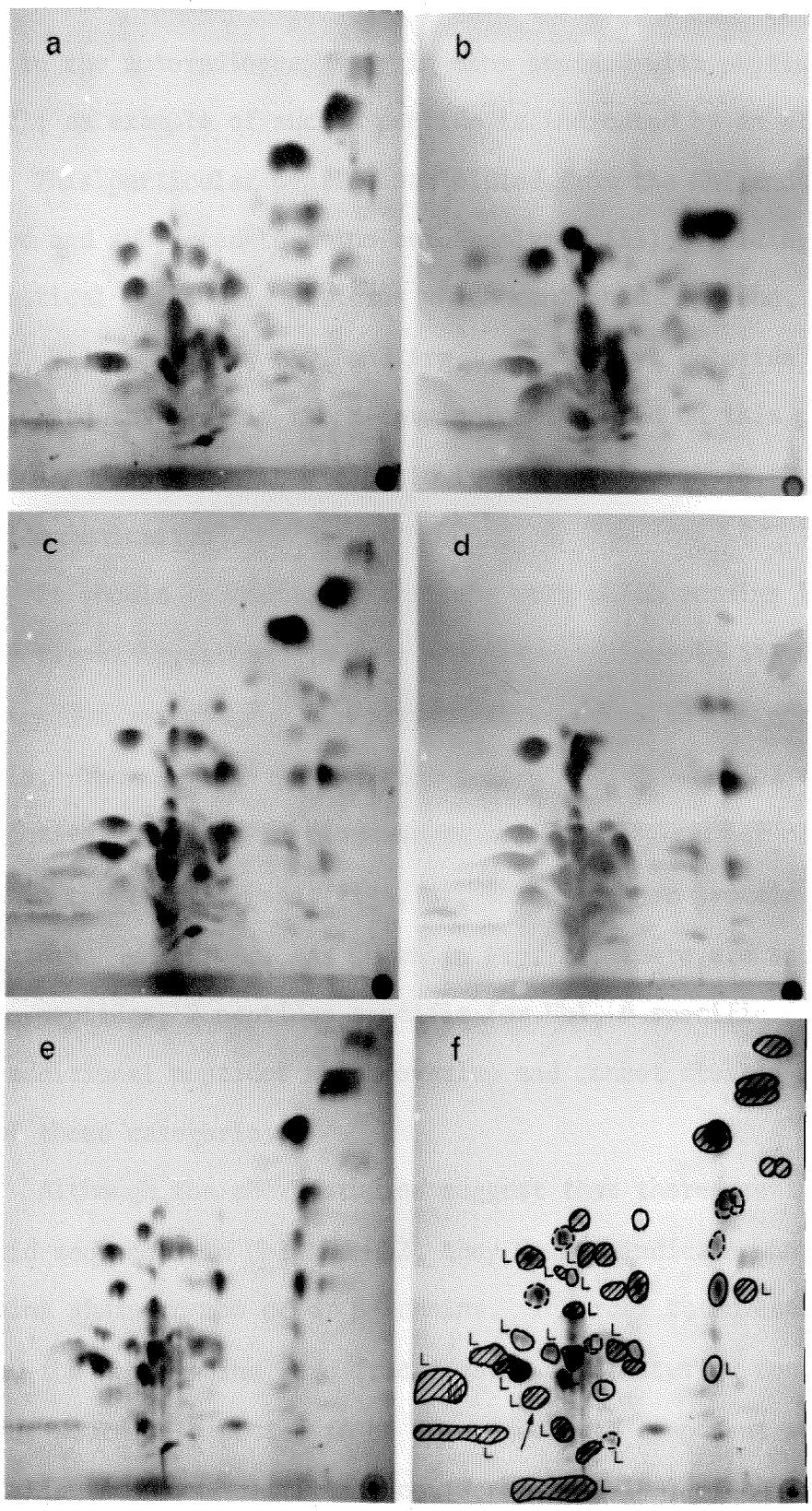

Hb1
Hb2
Hb2
Hb1+Hb2
Hb1
Hb1+Hb2

Figure 3. Ninhydrin-stained and corresponding autoradiographic tryptic peptide maps of L-[^{14}C] leucine-labeled Hb1 and Hb2, and ninhydrin-stained maps of unfractionated erythrocyte soluble fraction. (a) Hb1, ninhydrin-stained (4-5 mg); (b) Hb1, autoradiogram; (c) Hb2, ninhydrin-stained (4-5 mg); (d) Hb2, autoradiogram; (e) erythrocyte soluble fraction, ninhydrin-stained (3 mg); (f) erythrocyte soluble fraction, same map as in latter case, but marked so as to indicate peptides associated with the different hemoglobins. ---, Hb1-specific; ——, Hb2-specific; cross-hatched peptides are common to both proteins; peptides marked with "L" contain leucine. The arrow indicates a peptide which stains slowly with ninhydrin which is present both in Hb1 and Hb2 (see text). Exposure time for the autoradiographic maps: 90 days. Electrophoresis was carried out in the long dimension and chromatography in the short dimension. The origin is in the lower right-hand corner. The positive electrode was on the origin side during electrophoresis; the peptides moved toward the negative side.


a
b
c
d
e
f
O
L

A few peptides stain slowly with ninhydrin, and appear more strongly in the autoradiographs or in maps stained with peptide bond spray (52); an example of such a peptide is indicated by an arrow in Fig. 3f. This particular peptide was eluted from the Hb1 and Hb2 maps, hydrolyzed and subjected to amino acid analysis (53), yielding in both cases identical amino acid patterns which contained Asp, Phe, Glu, Thr, Leu, Ileu, Val, Ser, Ala, Gly, and Arg. The dansyl chloride procedure (54) indicated that Val is the N-terminal amino acid on this peptide; this amino acid is known to stain slowly with ninhydrin [W. R. Gray, personal communication].

It should be mentioned that the core which remains insoluble following trypsin treatment is very small (approximately 5%) when the hemoglobins are reduced and carboxymethylated prior to the enzymic hydrolysis. The arginine plus lysine content of Hb1 and Hb2 (66 and 63, respectively) (2) is in close agreement with the number of peptides observed in the tryptic maps (Figs. 3), assuming each protein to have the structure $\alpha_2\beta_2$. As shown in Fig. 3f, there are approximately 25 common peptides, 7 peptides specific for Hb1, 6 specific for Hb2, and several additional peptides which overlap and cannot clearly be assigned to any of these categories.

Although the above studies suggest that there are identical amino acid sequences in Hb1 and Hb2, they do not indicate whether the hemoglobins share common α- or β- chains. In order to investigate this question, the hemoglobins were fractionated in denaturing conditions in which they would be expected to be disrupted into component polypeptide chains. Fig. 4 shows gel filtration (Sephadex G-100) of chicken globin

under such conditions (8 M urea, titrated to pH 3.5 with formic acid). The globin separates into two main peaks labeled "I" and "II"; however following reduction of the protein with 2-mercaptoethanol, only a single major peak, labeled "III" is obtained. One possible explanation of this result and of the peak positions is that I, II, and III represent, respectively, inter-chain disulfide bonded polypeptides, intra-chain disulfide bonded polypeptides, and open single chain polypeptides. In accord with this explanation is the finding that reduced and carboxymethylated globin migrates in position III. The finding that Hb1 and Hb2 both contain 8 residues of cysteine (2) is consistent with the hypothesis that the globin chains might form intra-chain disulfide bonds, possibly during the experimental manipulations. In order to eliminate complications arising from disulfide bonding, attempts to fractionate chicken globin into constituent polypeptide chains were carried out with protein which had been previously reduced with 2-mercaptoethanol and then carboxymethylated by reaction with iodoacetic acid.

Fig. 5 shows acrylamide gel electrophoresis of reduced and alkylated chicken globins in 8 M urea, 5% formic acid, pH 1.7. The Hb1 globin migrates slightly faster than the Hb2 globin, although a complete separation is not obtained. Furthermore, no resolution of α- and β-chains is observed with either globin. Although able to demonstrate that at pH 2.0 or below chicken hemoglobin is dissociated into quarter-molecules of molecular weight roughly 17,000, Sasakawa et al (18) were also unable to resolve the globin into two different polypeptide chains by electrophoresis, column chromatography, or countercurrent distribution.

Figure 4. Gel filtration of chicken globin through Sephadex G-100 columns in 8 M urea, titrated to pH 3.5 with formic acid. Globin was dissolved in 8 M urea, 0.03 M tris buffer, pH 9.0. One portion was immediately adjusted to pH 3.5 with formic acid. A second portion was adjusted to 0.02 M 2-mercaptoethanol and left for 10 h at room temperature. Both portions were then thoroughly dialyzed against 8 M urea, pH 3.5 prior to placing them on 30 cm columns of Sephadex G-100 previously equilibrated with 8 M urea, pH 3.5. Positions of elution of cytochrome c (molecular weight, 12,300) and tyrosine (molecular weight, 181) are shown.

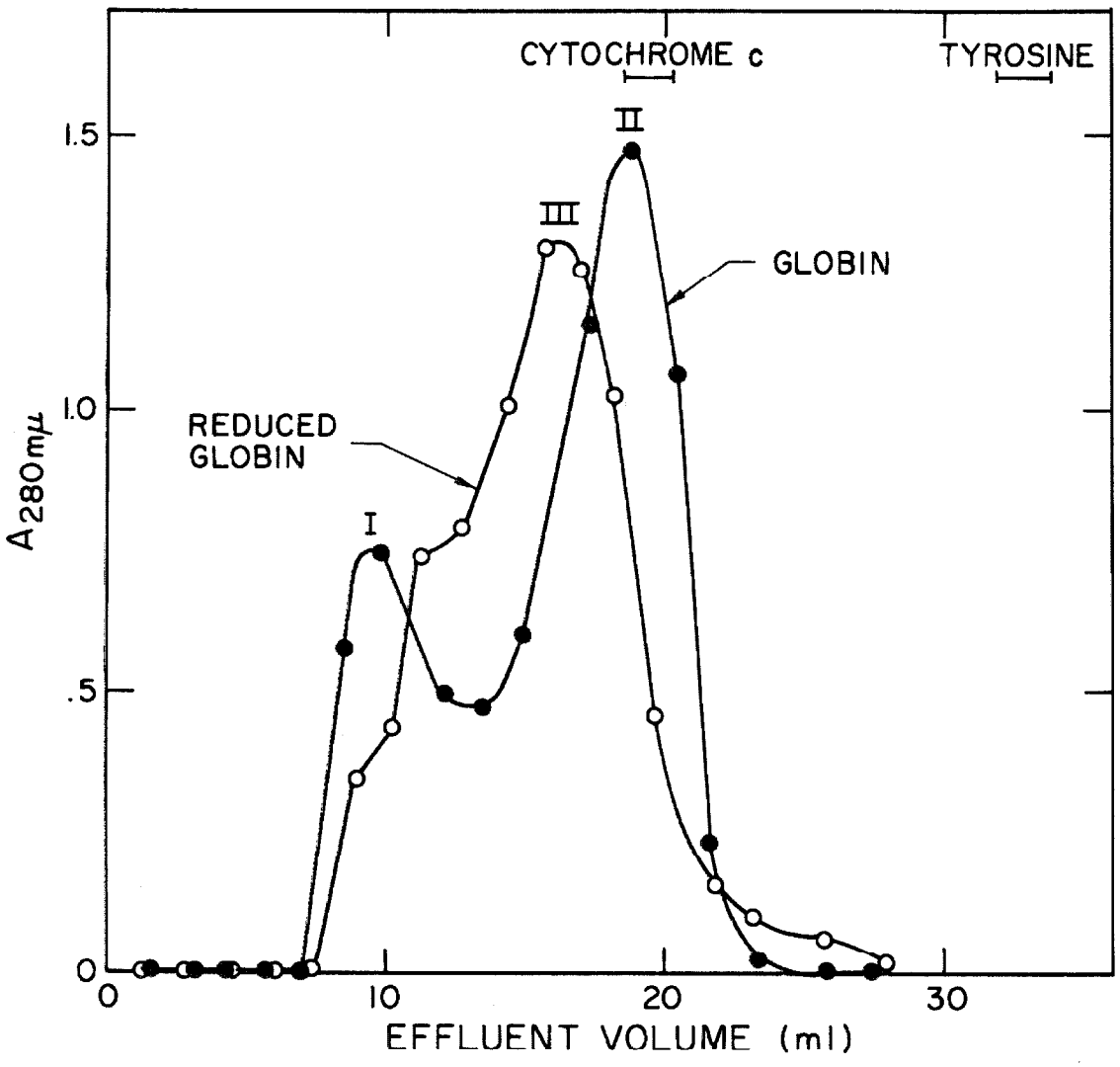
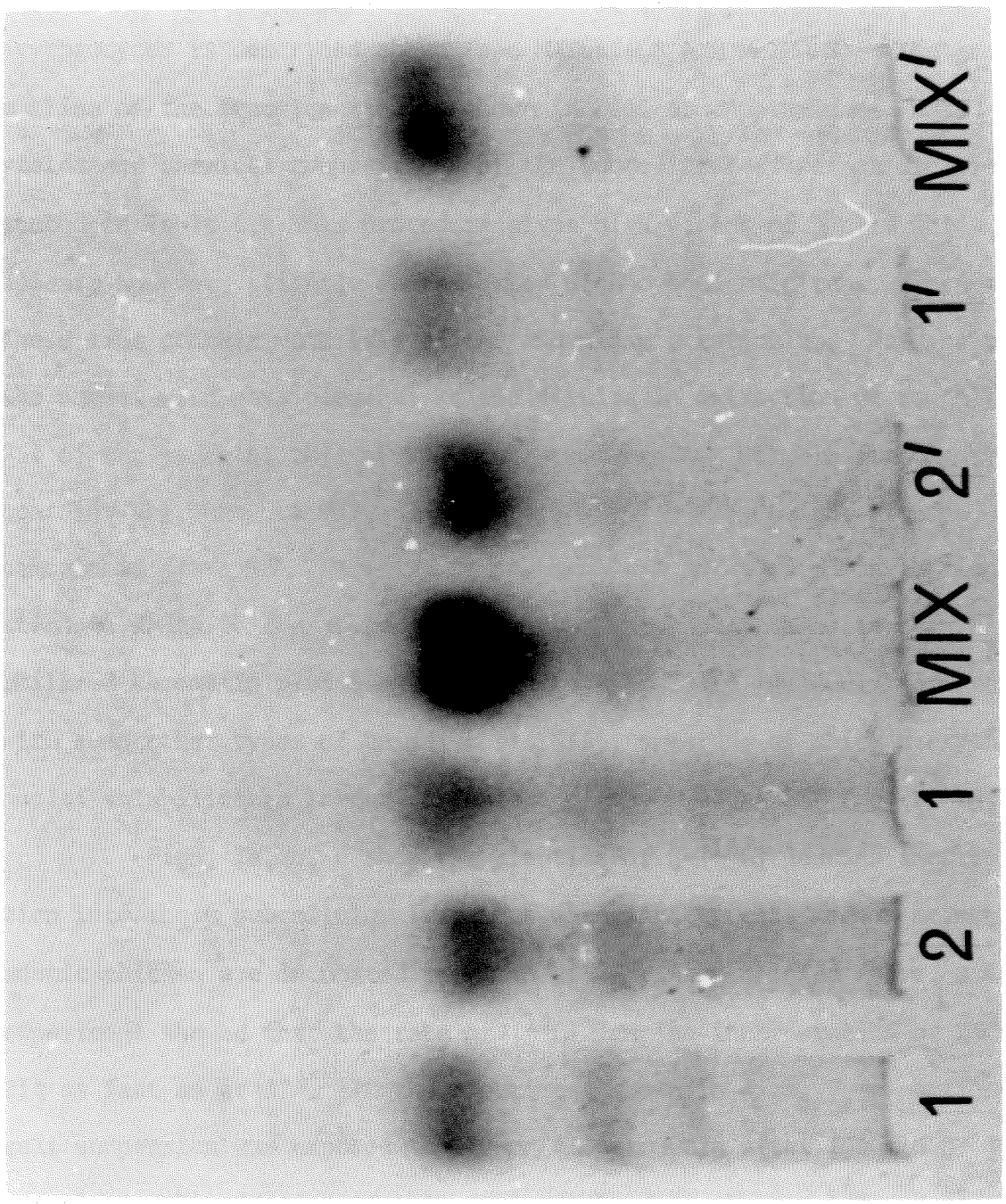

CYTOCHROME c
TYROSINE
1.5
1.0
.5
0
$A_{280m\mu}$
I
II
III
GLOBIN
REDUCED
GLOBIN
0
10
20
30
EFFLUENT VOLUME (ml)

Figure 5. Acrylamide gel electrophoresis of reduced and carboxymethylated chicken globins in 8 M urea, 5% formic acid, pH 1.7. The four samples on the left were reduced and alkylated independently of the three samples on the right (indicated by primed numbers). It is not known whether the faint bands following the main bands toward the cathode represent excessively alkylated polypeptides (e.g., alkylation of lysine side chains) or real microheterogeneity of the polypeptides. However, the difference between the left and right sets of globin samples in the proportion of these minor bands favors the first interpretation.


1
2
1
MIX
2'
1'
MIX'

Kinetics of amino acid incorporation into chicken erythrocyte subcellular fractions

The method employed for subcellular fractionation of chicken erythrocytes is described above (see MATERIALS AND METHODS). The general outline of the fractionation is shown in Fig. 6. Approximate mass yields and chemical compositions of the subcellular fractions are presented in Table I. This procedure gives high yields of nuclei which appear, however, slightly contaminated with cytoplasmic tabs; presumably these tabs contain materials of the endoplasmic reticulum. Washing of the nuclei with physiological saline results in extraction of roughly 25% of the protein; this protein extract ("nuclear soluble fraction") contains Hb1, but not Hb2, plus approximately 50% by weight of non-hemoglobin protein. The extracted nuclei are then sheared in low ionic strength media in the absence of divalent metal ions so as to obtain solubilized chromatin plus a small amount of insoluble residue. Compared with some other types of animal cells (55), shearing of chicken erythrocyte nuclei solubilizes a larger percentage of the nuclear DNA (nearly 100%).

Figs. 7a, b, c show the kinetics of L-[^{14}C] leucine incorporation into these subcellular fractions when intact cells from a severely anemic chicken are incubated in vitro at 25°. As mentioned above, a control experiment showed that the rate of [^{14}C] leucine incorporation at 25° is 21% as fast as at 37°. In the experiment shown in Figs. 7, a portion of the cell suspension was exposed to 100 µg/ml puromycin after 100 min of incorporation; the addition of this drug resulted in the complete cessation of net hemoglobin synthesis and a rapid loss of radioactivity from

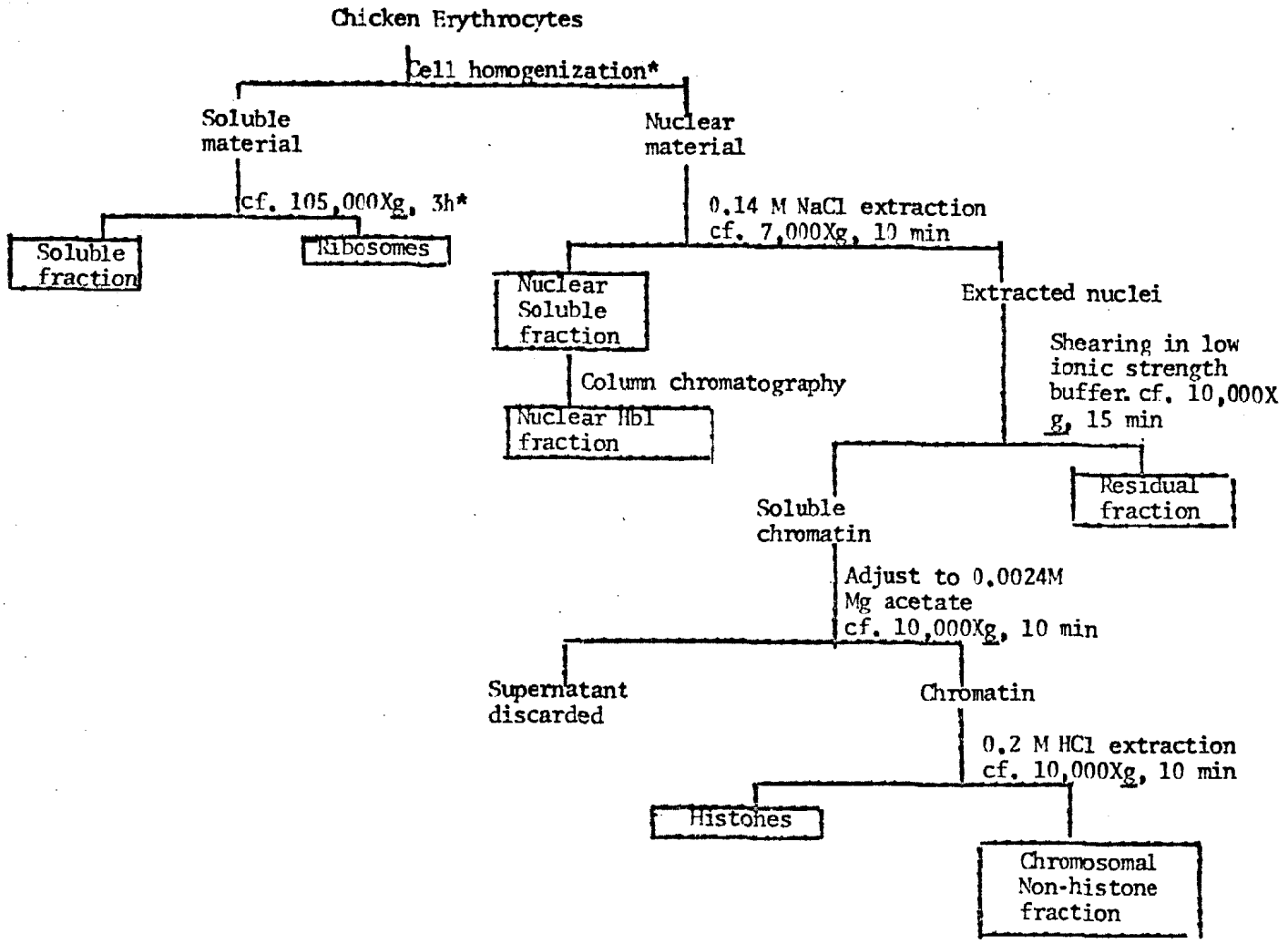


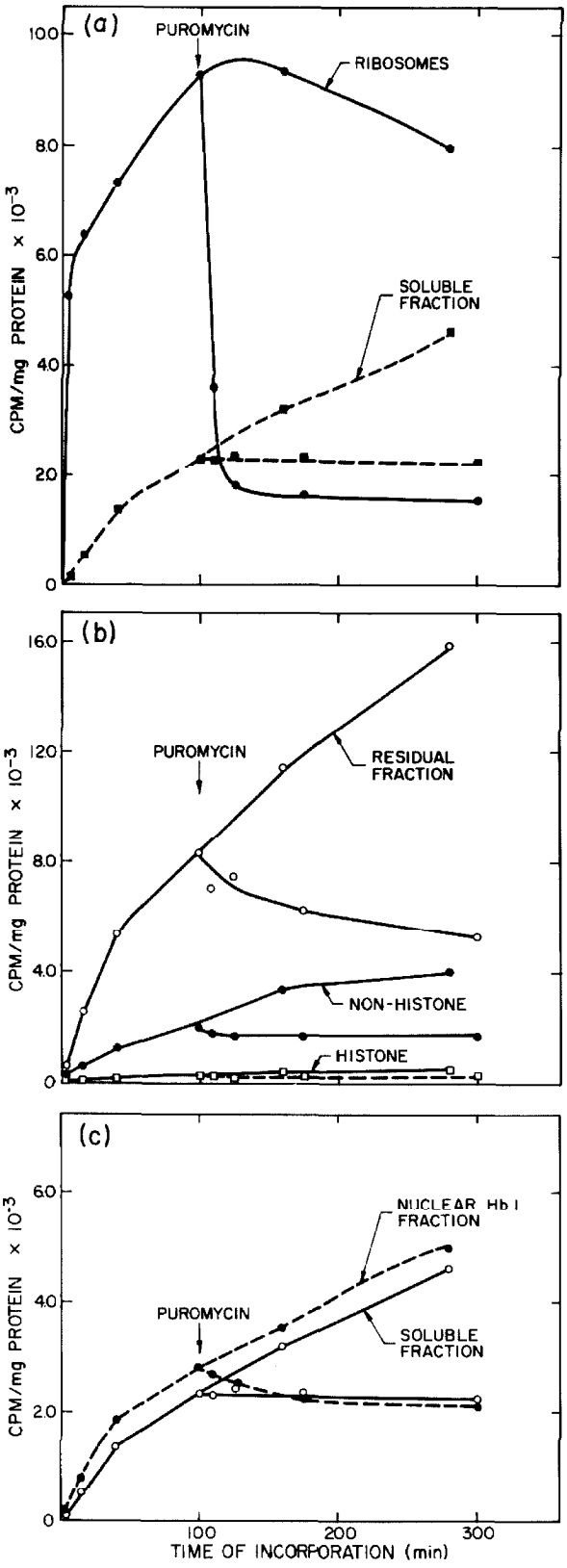
Figure 6. General outline of the subcellular fractionation of chicken erythrocytes. Details of the procedures are described in MATERIALS AND METHODS (subcellular fractionation). The materials enclosed in boxes are the final cell fractions. *Steps indicated with asterisks involve several procedures.

TABLE I. Chemical composition of erythrocyte subcellular fractions

Cell fraction	Approximate mg dry weight of fraction isolated per ml of packed cells	mg DNA/mg fraction	mg RNA/mg fraction	mg Protein/mg fraction
Soluble fraction	190	0.028	0.007	0.94
Ribosomes	4.2	0.021	0.52	0.61
Nuclear soluble fraction	7.6	0.014	0.010	0.87
Nuclear Hb1	5.1	—	—	—
Histones	17	0.009	0.006	0.77
Chromosomal Non-histone fraction	22	0.63	0.06	0.29
Residual fraction	1.5	0.14	0.093	0.53
Whole cells	310	0.071	0.035	0.81

The chemical analyses are average values of several determinations. Because of the differing color values given by various proteins in the assay of Lowry et al (43), the values of mg Protein/mg fraction, as based on the bovine serum albumin standard, are only approximate. The residual fraction is only partially soluble in 1M NaOH following hydrolysis of nucleic acids, and the chemical nature of the insoluble portion was not investigated.

Figure 7. Kinetics of L-[^{14}C] leucine incorporation into chicken erythrocyte proteins. Erythrocytes from severely anemic chickens were preincubated *in vitro* at 25° as described previously (MATERIALS AND METHODS). To the cell suspension, 0.7 μC/ml (2.62 μC/μM) L-[^{14}C] leucine was added and incorporation was continued at 25°. After 100 min of incorporation, 100 μg/ml puromycin were added to a portion of the cell suspension. Samples were removed for subcellular fractionation from the cell suspension incubated in the absence of puromycin after 4, 15, 40, 100, 160 and 280 min, and from the cell suspension incubated in the presence of the drug after 110, 125, 175, and 300 min. (a) Ribosomes and supernatant fraction; (b) chromosomal histone and non-histone proteins, and nuclear residual protein; (c) Nuclear Hb1 and soluble protein fractions.


(a)
PUROMYCIN
RIBOSOMES
SOLUBLE FRACTION
CPM/mg PROTEIN x 10⁻³
100
80
60
40
20
0
(b)
PUROMYCIN
RESIDUAL FRACTION
NON-HISTONE
HISTONE
CPM/mg PROTEIN x 10⁻³
160
120
80
40
0
(c)
NUCLEAR Hb I FRACTION
PUROMYCIN
SOLUBLE FRACTION
CPM/mg PROTEIN x 10⁻³
60
40
20
0
100
200
300
TIME OF INCORPORATION (min)

the ribosomes (Fig. 7a). It should be mentioned that with erythrocytes from severely anemic chickens, at least 90% of the total radioactive amino acid incorporated into TCA-precipitable material does not become TCA-soluble following puromycin addition, implying incorporation into stable products. Furthermore, at least 90% of the incorporated leucine is stable to NaOH treatment, as expected for incorporation into polypeptides. The non-histone chromosomal protein becomes labeled approximately to the same specific activity as does the soluble fraction, whereas the residual protein appears to be labeled several times higher than this (Fig. 7b). Fig. 7c shows a comparison between the labeling of the nuclear Mbl fraction and the protein of the soluble fraction.

As the absolute rate of protein formation is low in chicken erythrocytes, only a small percentage of the protein molecules are synthesized during the incorporation in vitro, even in the fractions with the highest specific activities (see Chapter 1). More detailed analysis of the [^{14}C] leucine incorporation into these subcellular fractions is described below.

Synthesis of chromosomal histone and non-histone proteins

Fig. 8 shows the chromatographic fractionation of [^{14}C] leucine-labeled chicken erythrocyte histones on an Amberlite IRC-50 column (kindly performed by Mr. A. Sadgopal). The radioactivity appears associated primarily with the "run-off" peak and with peak "III+IV" which contain, respectively, non-histone protein and the "arginine-rich" histones (56). Although the unfractionated histones are only weakly labeled as compared with the other cell fractions (Fig.7b), the arginine-rich histones are approximately as highly labeled as the soluble fraction hemoglobins. It seems unlikely that

this histone synthesis occurred in contaminating leucocytes or thrombocytes (which together account for no more than 0.4% of the total protein synthesis, as shown by cell autoradiography). Furthermore, the specific incorporation of label into only some of the histones strongly argues in favor of the idea that the synthesis occurred in non-dividing cells.

Fig. 9a shows a ninhydrin-stained peptide map of unfractionated [^{14}C] leucine-labeled erythrocyte histones. The peptides observed could not derive from the run-off protein because the quantity of the latter protein is too small (Fig. 8). Fig. 9b shows the corresponding autoradiographic peptide map, which appears very weak due to the low degree of labeling. There is a correspondence of the autoradiographic with the ninhydrin spots, consistent with the conclusion that the [^{14}C] leucine is incorporated into histone polypeptides.

Figs. 9c, d show, respectively, the ninhydrin-stained and corresponding autoradiographic peptide maps of [^{14}C] leucine-labeled chromosomal non-histone protein. Characteristic of the peptide map of this fraction is a leucine-containing streak which migrates away from the origin in the electrophoretic dimension. Prior DNase-treatment of the non-histone protein fraction did not affect these patterns. Comparison of the two maps (Figs. 9c, d) strongly suggests that the [^{14}C] leucine is incorporated into the non-histone protein rather than into an unrelated, contaminating material. The cause of the streaked appearance of some of the peptide maps is unclear, but is reproducible and characteristic of certain of the protein fractions. Possibly, the streaked peptides are nearly insoluble in the electrophoretic buffer, and slowly become solubilized during the electrophoresis.

Figure 8. Chromatographic fractionation of chicken erythrocyte histones on Amberlite IRC-50 columns (84), kindly performed by Mr. A. Sadgopal. The histones employed here were also used in the peptide mapping (see Fig 9).

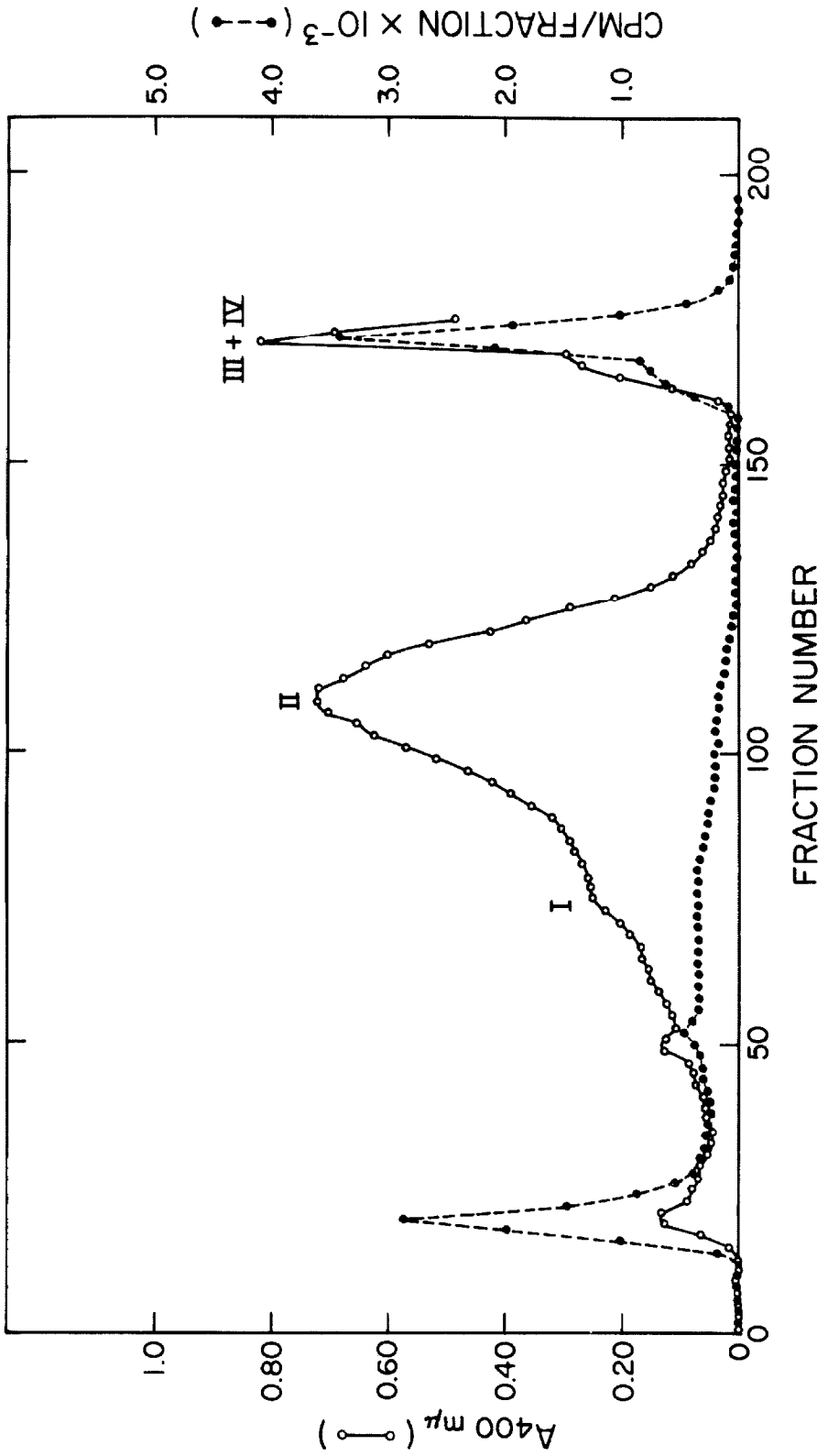
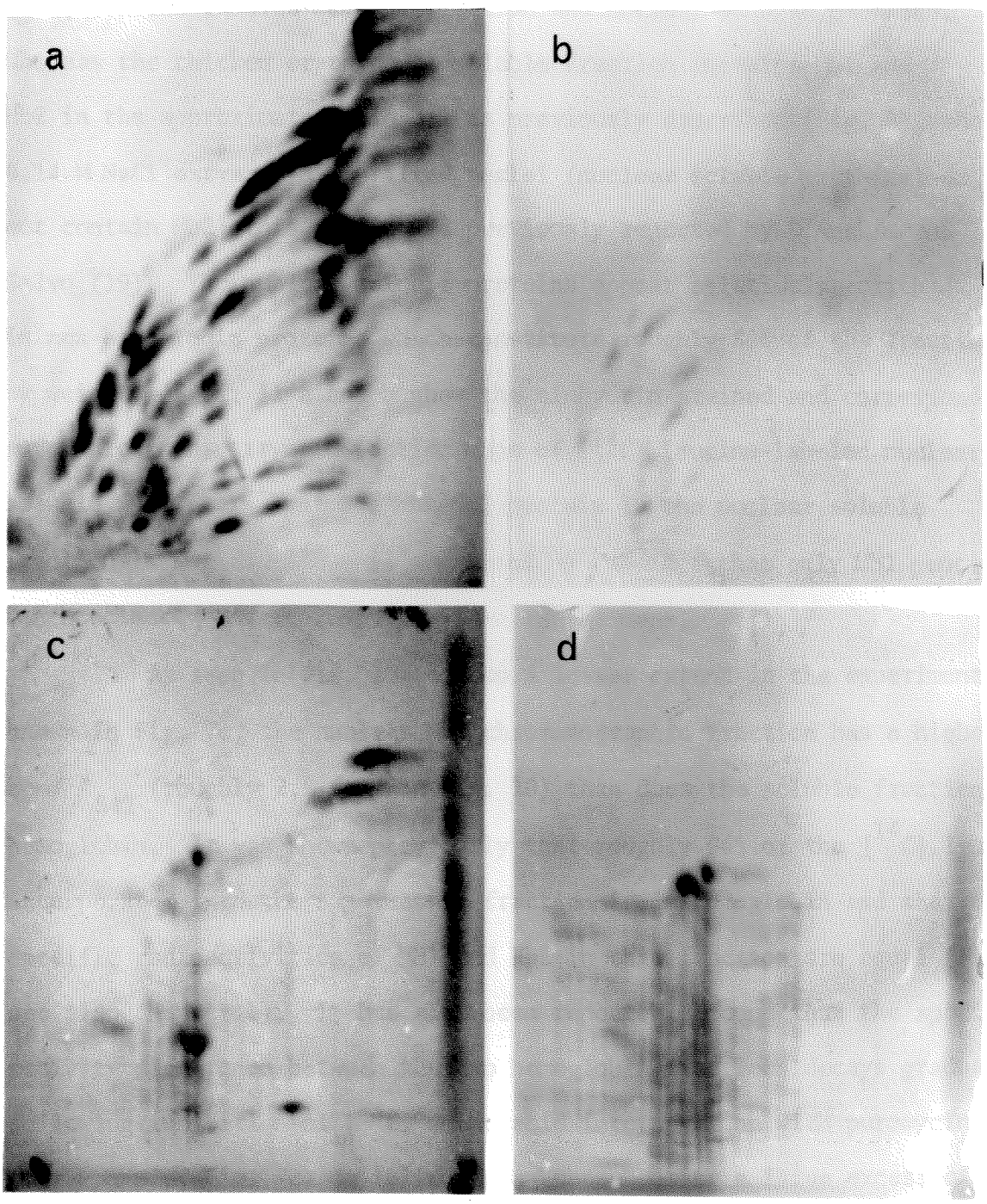

A400 mμ (o—o)
CPM/FRACTION × 10⁻³ (•---•)
FRACTION NUMBER
I
II
III + IV
0
0.20
0.40
0.60
0.80
1.0
1.0
2.0
3.0
4.0
5.0
50
100
150
200

Figure 9. Comparison of ninhydrin-stained with autoradiographic peptide maps of [^{14}C] leucine-labeled chicken erythrocyte histones and of chromosomal non-histone proteins. (a) histone, ninhydrin-stained; (b) histone, autoradiogram; (c) non-histone fraction, ninhydrin-stained; (d) non-histone fraction, autoradiogram. 3 mg of each trypsinized fraction were applied to the origin. Exposure time for autoradiographic maps, 60 days.


a
b
c
d

Synthesis of nuclear soluble proteins

Figs. 10 a and b show a comparison of the chromatography of [^{14}C] leucine-labeled soluble fraction and nuclear soluble proteins. Whereas the chicken erythrocyte soluble fraction contains Hb1 and Hb2 in the approximate ratio 3:1 as previously described (Fig. 1), the 0.14 M NaCl extracts of purified nuclei (nuclear soluble proteins) do not contain Hb2; this result was previously reported by D'Amelio and Salvo (19). The nuclear soluble proteins appear relatively enriched in non-hemoglobin proteins which constitute roughly 50% of the fraction by weight. Figs. 11 a and b show the ninhydrin-stained and corresponding autoradiographic tryptic peptide maps of [^{14}C] leucine-labeled nuclear soluble fraction. That the non-Hb1 protein in the nuclear soluble fraction is heterogeneous is suggested by the fact that only Hb1 peptides can be clearly and reproducibly seen in the maps.

As seen in Fig. 10, and to a lesser extent in the experiment shown in Fig. 7c, the nuclear Hb1 chromatographic fraction has a higher cpm/ A_{542} (roughly 2.5-fold in Fig. 10) than does the soluble fraction hemoglobin. It was shown previously that roughly 96% of the [^{14}C] leucine incorporated into the supernatant fraction is in hemoglobin and that the specific radioactivities of Hb1 and Hb2 in this fraction are equal (Fig. 1; see also chapter 1). It has also been repeatedly found that the specific activity difference between the two hemoglobin fractions decays gradually following inhibition of [^{14}C] amino acid incorporation with puromycin (for example, Fig. 7c) or following a "chase" using a large excess of [^{12}C] amino acid.

One would, in fact, expect that the specific activities of Hb1

Figure 10. Microcolumn chromatography of [^{14}C] leucine-labeled soluble fraction and of nuclear soluble fraction. Erythrocytes from a severely anemic chicken were incubated at 37° as described previously (MATERIALS AND METHODS). The cells were exposed to 2 μC/ml L-[^{14}C] leucine (13.1 μC/μM) for 15 h at 37° prior to cell harvesting and preparation of fractions for chromatography. One ml fractions were collected. The elution employed stepwise increase of ionic strength as follows (fraction numbers indicated in parentheses): 0.075 M (1), 0.15 M (2), 0.20 M (3,4), 0.25 M (5-11), 0.40 M (12-18). (a) Soluble fraction, (b) Nuclear soluble fraction. -●-●- , A_{542}; --■--■-, A_{280}; -○-○- , radioactivity.

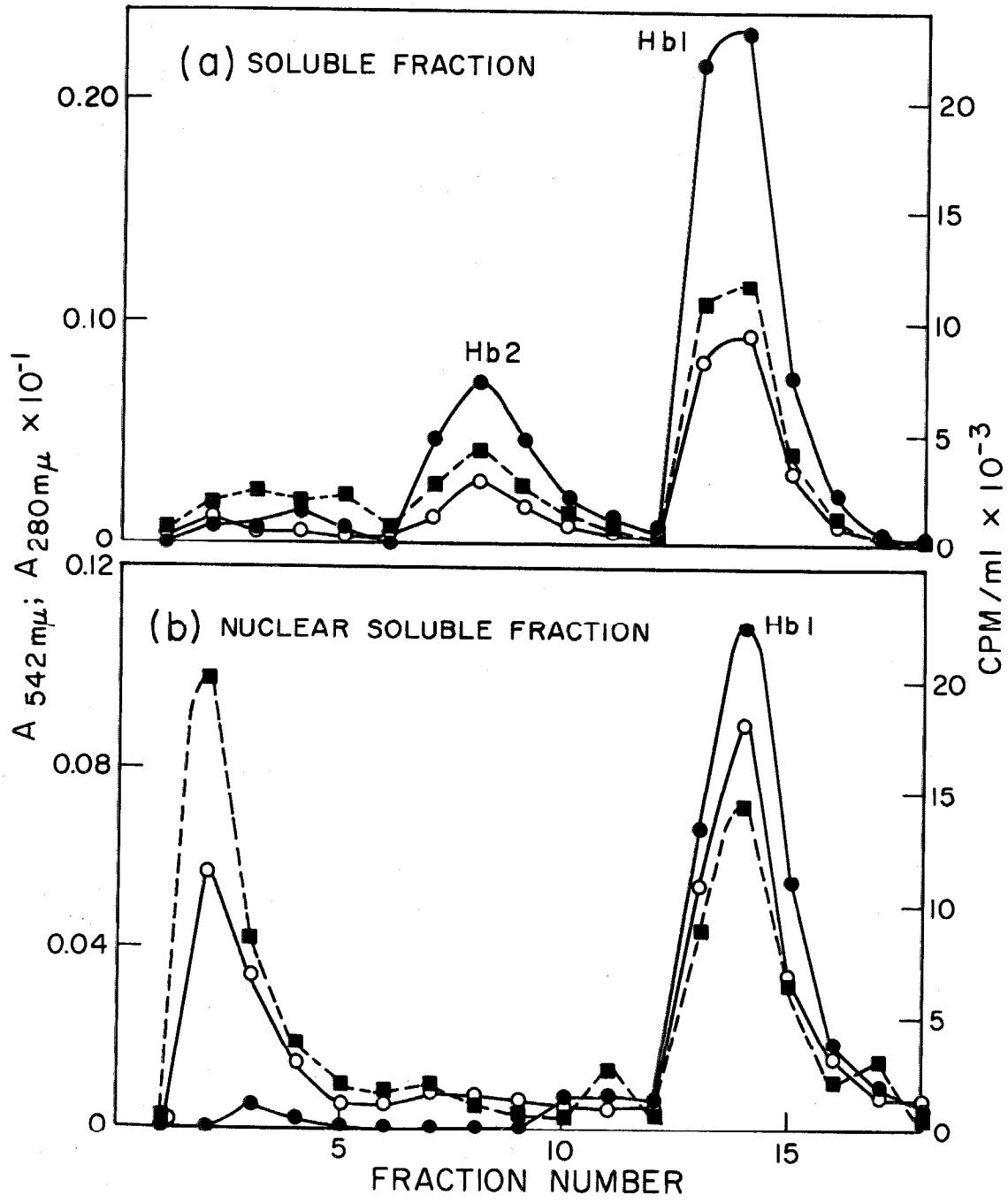
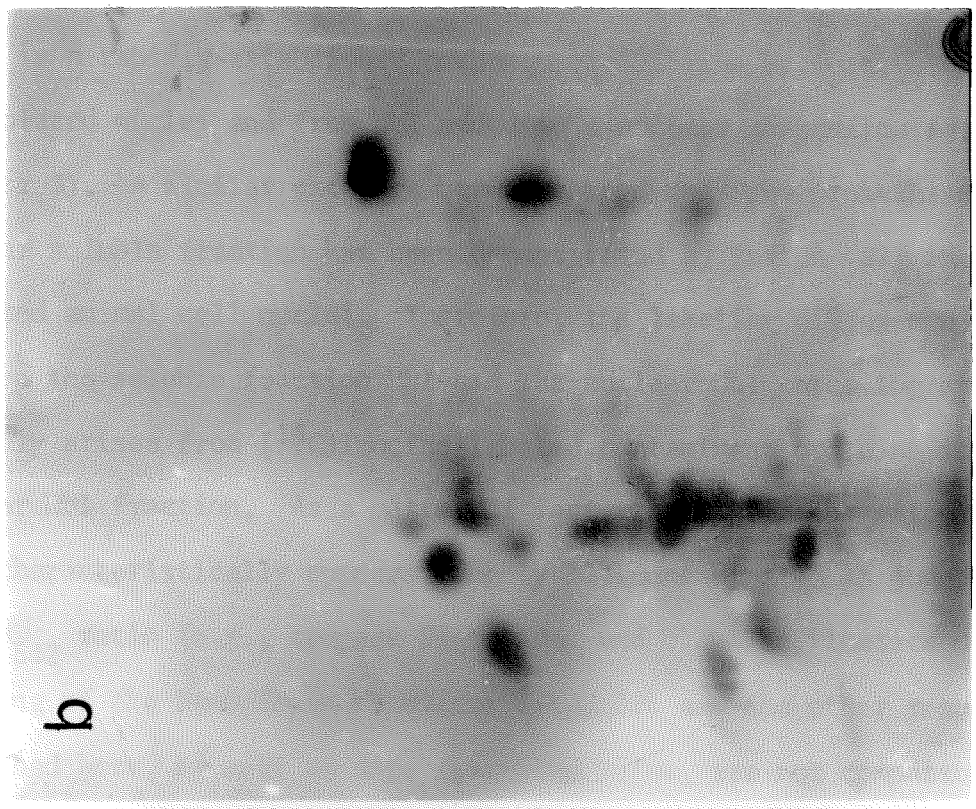
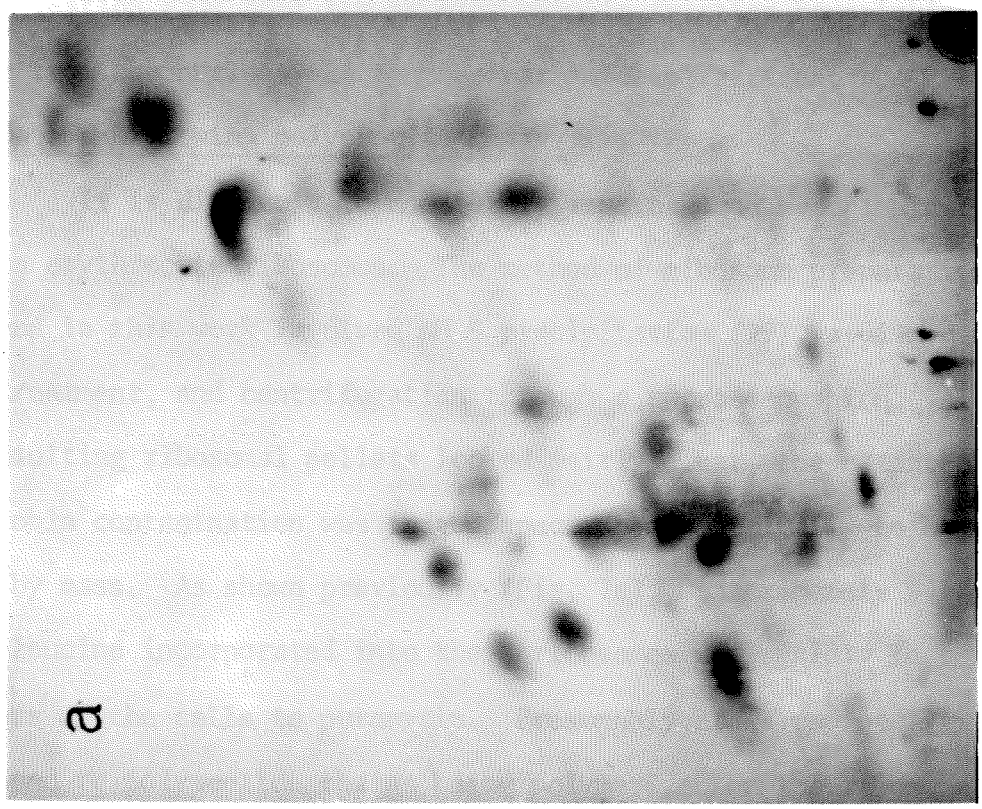

(a) SOLUBLE FRACTION
Hb1
Hb2
0.20
0.10
0
20
15
10
5
0
(b) NUCLEAR SOLUBLE FRACTION
Hb1
0.12
0.08
0.04
0
20
15
10
5
0
5
10
15
FRACTION NUMBER
A 542 mμ; A 280 mμ × 10⁻¹
CPM/ml × 10⁻³

Figure 11. Comparison of ninhydrin-stained with autoradiographic peptide maps of [^{14}C] leucine-labeled nuclear soluble fraction. (a) ninhydrin-stained, (b) autoradiogram. 3 mg of the trypsinized fraction was applied at the origin. Exposure time for the autoradiogram, 60 days.


b


a

in the two erythrocyte subcellular fractions would be equal because there is a rapidly-established exchange equilibrium between Hbl bound to isolated nuclei and free Hbl added to a nuclear suspension (e.g., see Chapter 3). A similar exchange between bound and free proteins of the nuclear soluble fraction has been demonstrated by Barton (57) in other isolated animal cell nuclei. Therefore, the labeling difference observed between the soluble fraction Hbl and the nuclear Hbl chromatographic peaks probably arises from [^{14}C]leucine-labeled contaminant proteins in the nuclear Hbl fraction. As a matter of fact, the latter chromatographic peak characteristically appears roughly 15% contaminated as judged by A_{280}/A_{542} ratio (e.g., compare Figs. 10 a and b). The kinetics of labeling of the hemoglobin fractions (Fig. 7c) and the other results described here, as well as data described below, are not compatible with the suggestion (33) that the labeled soluble fraction hemoglobin molecules derive from the nucleus via the nuclear soluble fraction.

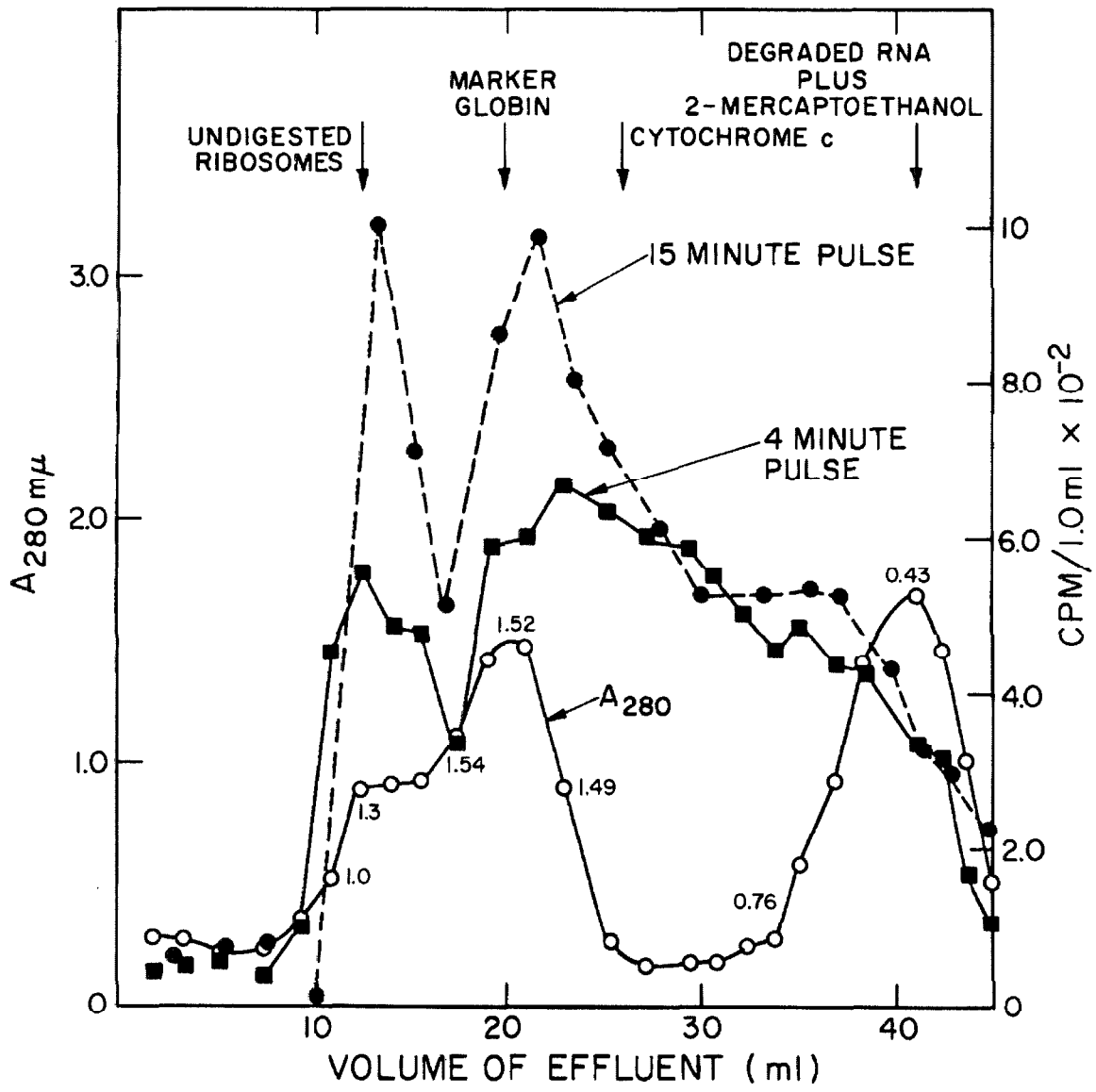
Ribosomes and growing polypeptide chains

It is difficult to completely remove bound hemoglobin from chicken erythrocyte ribosomes. The method of ribosome preparation employed in this work involved pH 5 precipitation (29), sodium deoxycholate treatment, and centrifugation through a sucrose cushion. Although the resulting ribosomal pellets looked fairly clear, the extent of hemoglobin contamination was judged spectrophotometrically to be roughly 5-10% by mass. As shown previously (Fig. 7a), approximately 84% of the [^{14}C] leucine incorporated into these ribosomes was rapidly lost following exposure of the cells to puromycin. Presumably, this radioactivity was bound to polypeptide chains being polymerized on the ribosomes;

such chains are known to be released by puromycin (58). The nature of the remaining 16% radioactivity is unclear; but probably it could not be completely accounted for by the contaminating hemoglobin, as judged on the basis of the known specific activity of hemoglobin in the soluble fraction (Fig. 7a). Conceivably, some of this residual label is incorporated into ribosomal structural proteins. Although there is a net destruction of ribosomes during chicken erythrocyte maturation (9; see also Chapter 1), a small proportion of very immature cells might be engaged in ribosome formation.

A method was developed for stripping the labeled polypeptide chains from chicken erythrocyte ribosomes and fractionating them according to size by gel filtration on Sephadex G-100 columns under denaturing conditions. Fig. 12 shows the size fractionation of the growing chains following [^{3}H] leucine pulses of 4 min or 15 min at 25°. Chicken globin (reduced with 2-mercaptoethanol) was added as an absorbance marker. The A_{280}/A_{260} ratio is written for several points along the A_{280} curve. The radioactivity falls into two main peaks. The first peak off the column contains a variable proportion of the incorporated [H^3] leucine and consists of large-sized ribonucleoprotein material, resistant to RNase. The second [^{3}H]-peak appears to consist of an heterogeneous distribution of labeled polypeptides ranging in size between complete globin chains (roughly 150 amino acids) and very small peptides; this peak presumably contains nascent polypeptide chains freed from the ribosomes. After a very short pulse (e.g., 4 min at 25°) small peptides are, within experimental error, as highly labeled as after longer pulses; however large peptides the size of completed globin chains are more highly labeled after a 15 min

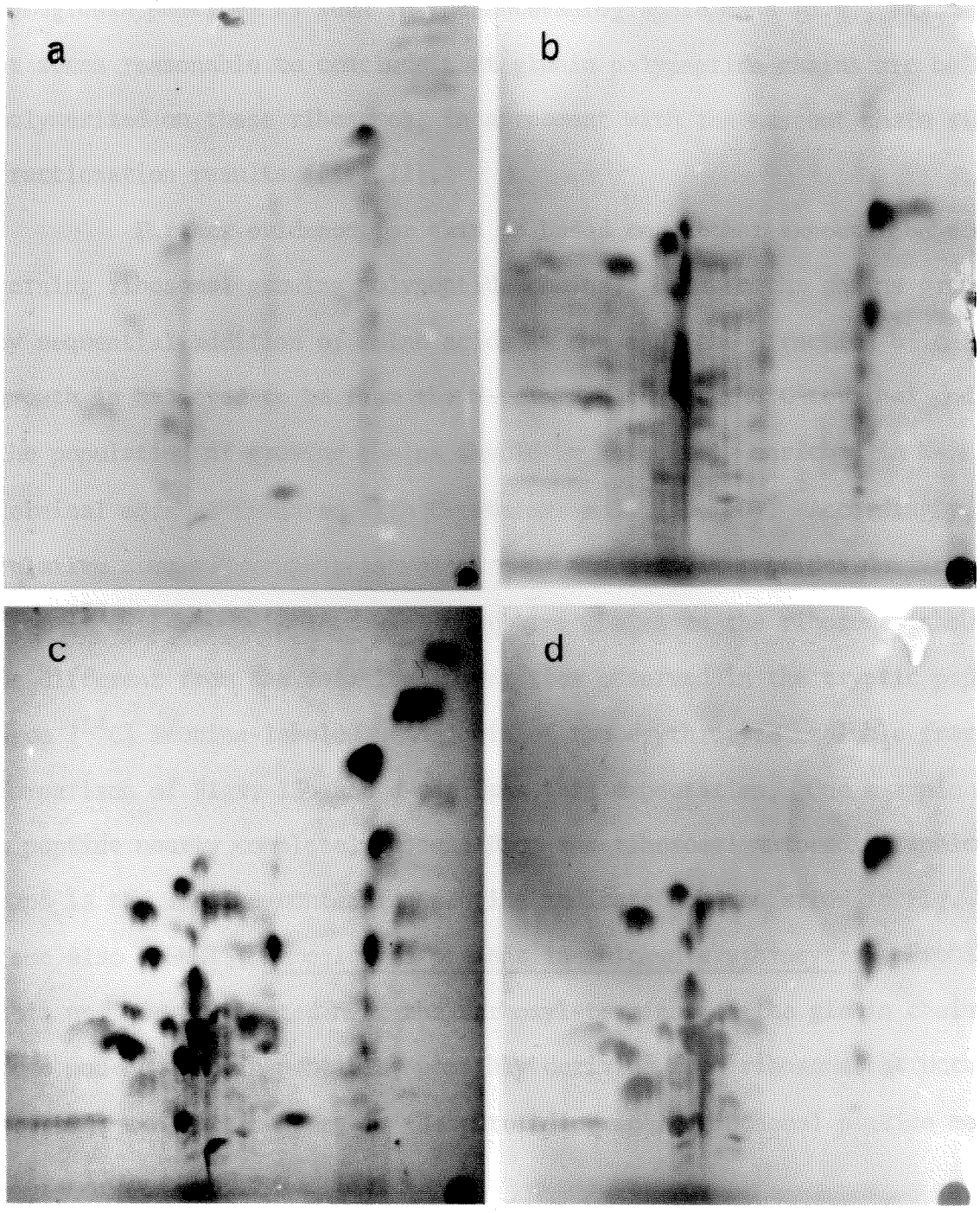
Figure 12. Size fractionation of [^{3}H] leucine-labeled ribosomal growing polypeptide chains. Erythrocytes from a severely anemic chicken were incubated in vitro at 25° at 2 X 10^9 cells/ml as described (MATERIALS AND METHODS). The suspension was then exposed to 20 μC/ml L-[4,5-^{3}H] leucine (8.2 C/mM) for 4 min or 15 min at 25°; the cells were then rapidly cooled, and used for preparation of ribosomes. Growing polypeptide chains were stripped from the ribosomes in the presence of unlabeled carrier globin as described in MATERIALS AND METHODS. Elution of the samples from the 38 cm X 1 cm diameter columns of Sephadex G-100 was performed with 8 M urea, titrated to pH 2.4 with formic acid. Positions of elution of undigested ribosomes, marker globin, cytochrome c and degraded RNA plus 2-mercaptoethanol are indicated. An impurity in the 2-mercaptoethanol contributes to the A_{260} between 37-45 ml of eluate. Numbers written beside the A_{280} profile indicate A_{280}/A_{260} ratios for several fractions. The absorbancy profile was from the 4 min pulse column, but an almost equal profile was obtained for column containing the 15 min sample.


UNDIGESTED RIBOSOMES
MARKER GLOBIN
CYTOCHROME c
DEGRADED RNA PLUS 2-MERCAPTOETHANOL
15 MINUTE PULSE
4 MINUTE PULSE
A280
1.3
1.0
1.54
1.52
1.49
0.76
0.43
A280 mμ
CPM/1.0ml x 10-2
VOLUME OF EFFLUENT (ml)
0
1.0
2.0
3.0
2.0
4.0
6.0
8.0
10
10
20
30
40

pulse (Fig. 12). This result is reproducible and agrees with a model of protein synthesis in which amino acids are added sequentially at one end of the "growing polypeptide chains" (42). For pulse times longer than 15 min (25 min) the distribution of the labeled growing chains was found to be the same as for the 15 min pulses. Assuming that the [^{3}H] leucine added to the suspension of chicken erythrocytes immediately enters the cells (which had previously been starved for leucine so as to deplete the internal pool), the results shown in Fig. 12 also suggest that it takes roughly 5-6 min at 25°, or probably 1 min at 37° (as based on the 5-fold greater extent of protein synthesis at 37°), to polymerize a globin chain. Relatively rapid entry of leucine into the cells is likely, because there is no detectable lag period between leucine addition to a cell suspension and the beginning of the linear rate of incorporation into polypeptides. The possible significance of the [^{3}H] leucine-containing peak which is resistant to RNase is unknown, but it has been shown with polyuridylic acid-directed polyphenylalanine synthesis that the nascent chains are rather resistant to RNase-induced release (59); this peak might therefore contain some nascent chains which the treatment has failed to liberate.

Figs. 13 a and b show ninhydrin-stained and corresponding autoradiographic tryptic peptide maps of [^{14}C] leucine-labeled chicken erythrocyte ribosomes; the analogous peptide maps of the erythrocyte soluble fraction are shown in Figs. 13 c, d for comparison. The faintly-staining hemoglobin spots in the ribosomal ninhydrin map are expected, in view of the above-mentioned contamination of the ribosomes with hemoglobin; in addition to the hemoglobin spots in this map, other peptides may be

Figure 13. Ninhydrin-stained and autoradiographic peptide maps of [^{14}C] leucine-labeled ribosomes and erythrocyte soluble fractions. (a) ribosomes, ninhydrin-stained; (b) ribosomes, autoradiogram; (c) soluble fraction, ninhydrin-stained; (d) soluble fraction, autoradiogram. 2 mg of trypsinized ribosome fraction and 3 mg of trypsinized soluble fractions were applied to the origins. Exposure time for autoradiograms, 60 days.


a
b
c
d

visualized. However the low level of contaminating hemoglobin in the purified ribosomes could not account for the strong intensities of the hemoglobin peptides as seen in the autoradiographic maps (e.g., Fig. 13b). It seems reasonable to conclude that globin polypeptide chains are being polymerized on these ribosomes, in agreement with the nascent chain size fractionation results (Fig. 12).

Further evidence for this is based on another expectation concerning ribosomal growing polypeptide chains. Because the chains grow by sequential addition of amino acids at one end (the direction of chain growth is believed to be from the N-terminal toward the C-terminal end), the population of growing chains should be relatively enriched in N-terminal ends as compared with C-terminal ends (42, 60). Accordingly, relative autoradiographic intensities of the tryptic peptides derived from [^{14}C] leucine-labeled globin growing chains on the ribosomes should be different from the relative intensities observed in the tryptic peptides from [^{14}C] leucine-labeled hemoglobin of the erythrocyte soluble fraction. Comparison of Figs. 13b and d confirms this expectation. One example of a peptide nearly completely absent from the ribosomal autoradiographic maps is the leucine-containing peptide indicated by the arrow in Fig. 3f (see also RESULTS, Hemoglobins present in chicken erythrocytes). Probably this peptide is located near the carboxyl-terminus of the globin chains. Some non-hemoglobin peptides, possibly deriving from ribosomal structural protein, can also be seen in the autoradiographic ribosomal peptide map (Fig. 13b).

Following pulse-labeling with radioactive amino acids, and lysis of the chicken erythrocytes, sucrose gradients of the lysates were run by

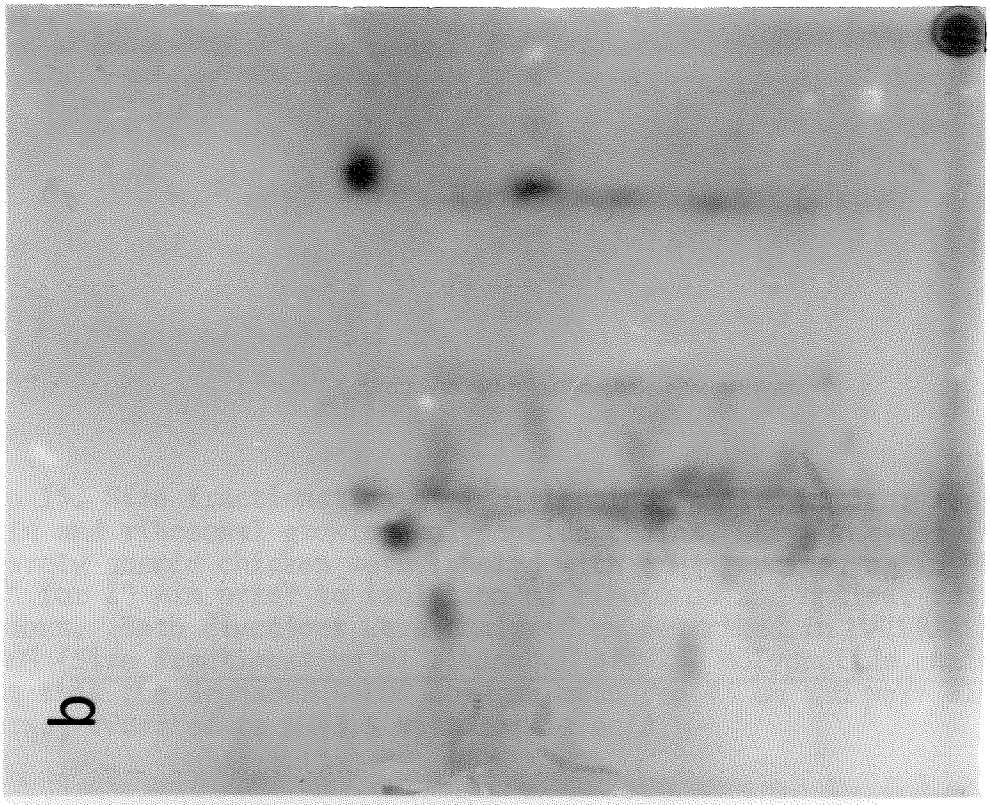
methods previously described (9). Only a minority of the radioactivity was normally associated with 74 s ribosomal particles (monomers), the majority being in polysomal aggregates of various size, with sedimentation coefficients from 110 to 200 s or more. However, as compared with polyribosomes from rabbit erythrocytes (61), the polysomal distribution from chicken erythrocytes was shifted toward smaller aggregates. This can probably be attributed to high levels of ribonuclease known to be present in chicken erythrocytes (62). The results suggest that in the conditions of in vitro incubation used in this work, approximately 50% of the ribosomes are engaged in protein synthesis and that the polyribosomal aggregate is the site of this synthesis.

Residual fraction

Of all chicken erythrocyte subcellular fractions, the nuclear residue fraction is the least understood. Conceivably, this material might derive from structural elements (e.g., membranes) of the nuclei or even from endoplasmic reticulum which contaminates the nuclear preparations. In addition, the residue is slightly red-brown in color, presumably due to low levels of contaminating hemoglobin. Figs. 14 a and b show ninhydrin-stained and corresponding [^{14}C] leucine-labeled autoradiographic peptide maps of the erythrocyte residue fraction. Only relatively weak spots developed in these maps, suggesting that much of the residue protein may be heterogeneous. The peptide patterns visible in these maps, however, appear generally similar to those obtained from the ribosomes (Figs. 13a and b).

In order to determine more directly whether the leucine-labeled polypeptides in the residual fraction might be similar to ribosomal growing

Figure 14. Peptide maps of [^{14}C] leucine-labeled nuclear residual fraction. (a) Ninhydrin-stained, (b) autoradiogram. 2 mg of trypsinized residual fraction were applied to the origin. Exposure time for autoradiogram, 60 days.


b

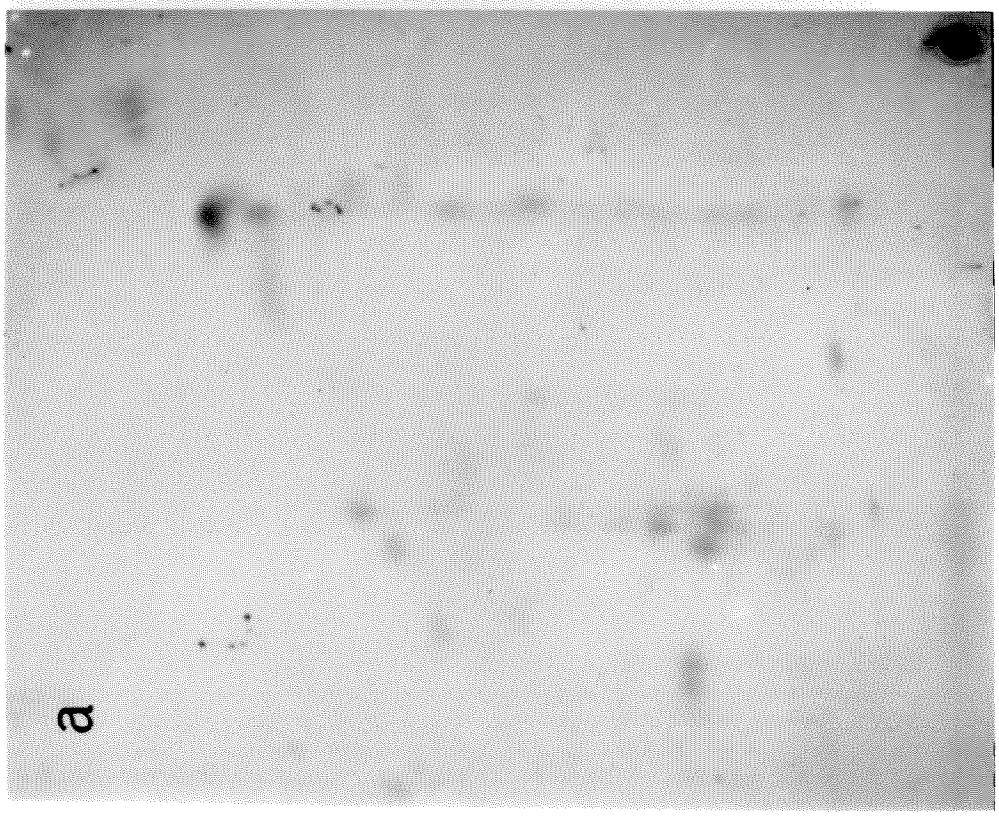
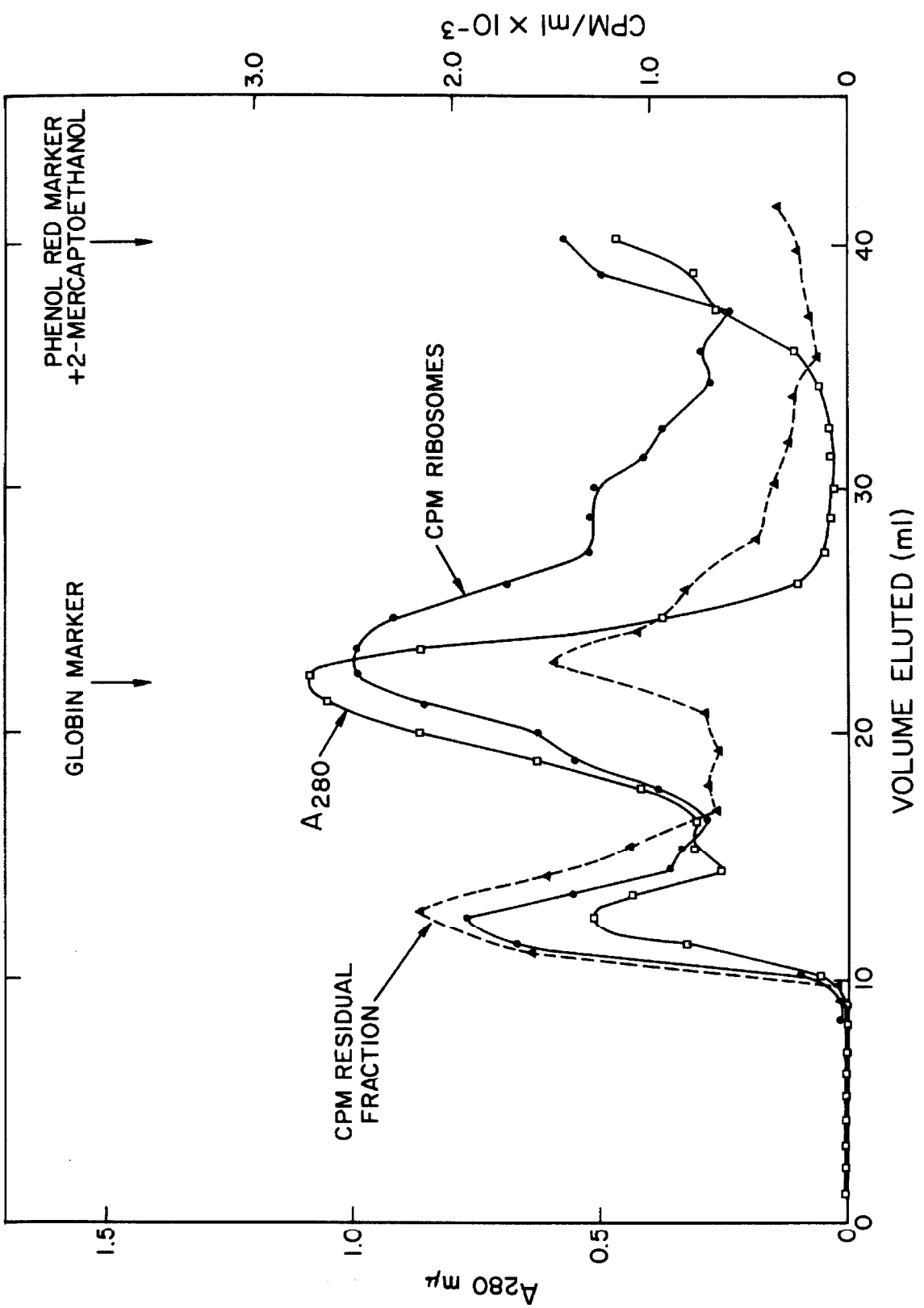

a

Figure 15. Size fractionation of [^{3}H] leucine-labeled nuclear residual protein and ribosomal growing polypeptide chains. Erythrocytes from a severely anemic chicken were labeled with [^{3}H] leucine, as in Fig. 13, at 25° for 20 min prior to isolation of ribosomes and nuclear residue fractions. Both fractions were analyzed as described in MATERIALS AND METHODS (Size fractionation of ribosomal growing polypeptide chains) except that the Sephadex G-100 columns measured 43 cm and were eluted with 8 M urea, titrated to pH 3.5 with formic acid.


A280 mμ
1.5
1.0
0.5
0
CPM/ml x 10-3
3.0
2.0
1.0
0
0
10
20
30
40
VOLUME ELUTED (ml)
PHENOL RED MARKER
+2-MERCAPTOETHANOL
GLOBIN MARKER
CPM RIBOSOMES
A280
CPM RESIDUAL
FRACTION

chains, the residue was treated by methods previously shown to liberate growing chains from ribosomes, followed by size fractionation of the resulting polypeptides on Sephadex G-100 columns. Results of this experiment are shown in Fig. 15 and are compared with the size fractionation of growing chains from ribosomes isolated from the same batch of labeled erythrocytes. Like the ribosomal label, the [^{3}H] leucine-labeled residual protein appears in two peaks, the first containing large-sized materials and the second having an heterogeneous distribution of peptides ranging from the size of full globin chains down to very small peptides. However, whereas approximately 95% of the ribosomal [^{3}H] leucine was soluble in the 8M urea, pH 3.5, following RNase digestion, only roughly 45% of the [^{3}H] leucine bound to the residue was soluble and could be applied to the column.

It is known that large-scale destruction of ribosomes and presumably of messenger RNA accompanies avian erythrocyte maturation (9; also Chapter 1). Furthermore, a rapid loss of ribosomes during rabbit erythrocyte incubation in vitro (50% loss in 24 h) has been demonstrated by Marks, Rifkind and Danon (15). The possibility that the residual fraction studied here contains inactivated ribosomes in the process of degradation (presumably some retaining their nascent globin chains) is not inconsistent with the kinetics of labeling (Fig.7), chemical composition (Table 1), peptide maps (Fig. 14), or gel filtration of the labeled polypeptides (Fig. 15). Obviously, however, further analysis of this fraction is needed before these data can be unequivocally interpreted.

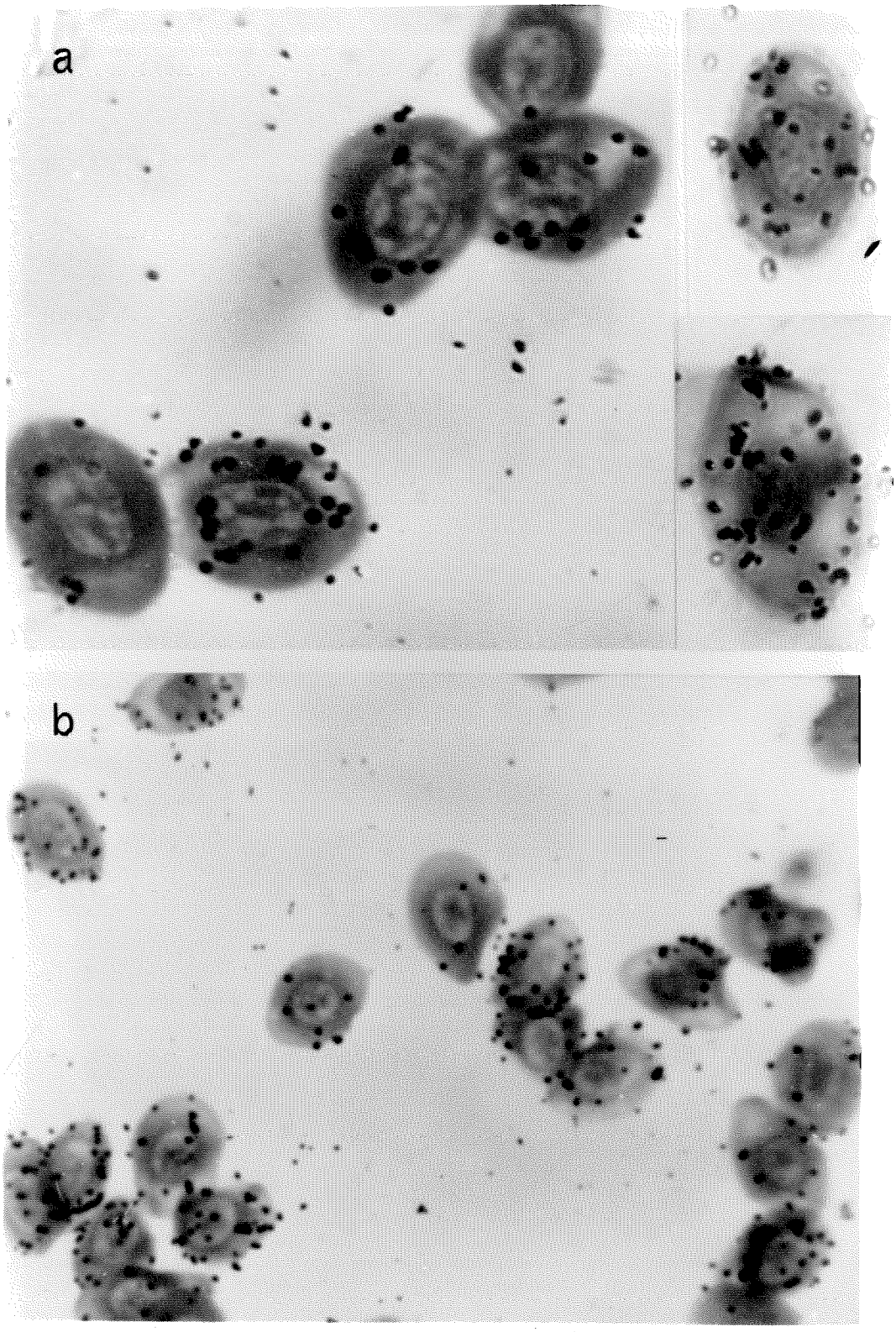
Cytoplasmic localization of protein synthesis

To determine the intracellular localization of protein systhesis in erythrocytes from anemic chickens, an autoradiographic study was carried out on cells submitted to short pulses of [^{3}H] leucine. Figs. 16 and 17 show representative autoradiograms of erythrocytes pulsed at 25° for varying periods prior to rapid fixation with methanol. For the shortest pulse times used (3 min), at least 90% of the silver grains appear over the cytoplasm; however, for longer pulses, or following a chase with [^{12}C] leucine, the proportion of grains over the nucleus increased. Figs. 18 present the data in quantitative form. Very similar autoradiographic data were recently described by Scherrer et al (8).

In erythrocytes from anemic chickens, at least 90% of the leucine incorporated into TCA-precipitable material remains precipitable following puromycin addition to the cells, or administration of a cold [^{12}C] leucine chase. Consequently, the increasing proportion of silver grains located over the nucleus must reflect a slow migration of labeled proteins from the cytoplasm into the nucleus. An autoradiographic experiment was also performed with mature erythrocytes from normal chickens, with nearly identical results to those described here. However, roughly 50-60% of the radioactive leucine incorporated into mature chicken erythrocytes is degraded into TCA-soluble material following a cold chase or puromycin treatment; and this complicates the interpretation of the increasing proportion of nuclear grains.

In the experiments shown here, roughly 93% of silver grains are localized over the cytoplasm after a 3 min pulse at 25°. Since it takes

Figures 16 and 17. Erythrocyte autoradiography, following [^{3}H] leucine incorporation at 25°. Erythrocytes from a severely anemic chicken were incubated at 2 X 10^9 cells/ml at 37° so as to deplete the endogenous leucine pool (MATERIALS AND METHODS), and were then pulsed at 25° with 100 µC/ml L-[4, 5- ^{3}H] leucine (8.2 C/mM). After 12 min incorporation, a portion of cell suspension was given a cold chase with a 200-fold excess of unlabeled leucine. Fig. 16, (a) 3 min pulse, (b) 5.5 min pulse. Fig. 17, (a) 75 min pulse, (b) 185 min pulse.


a
b

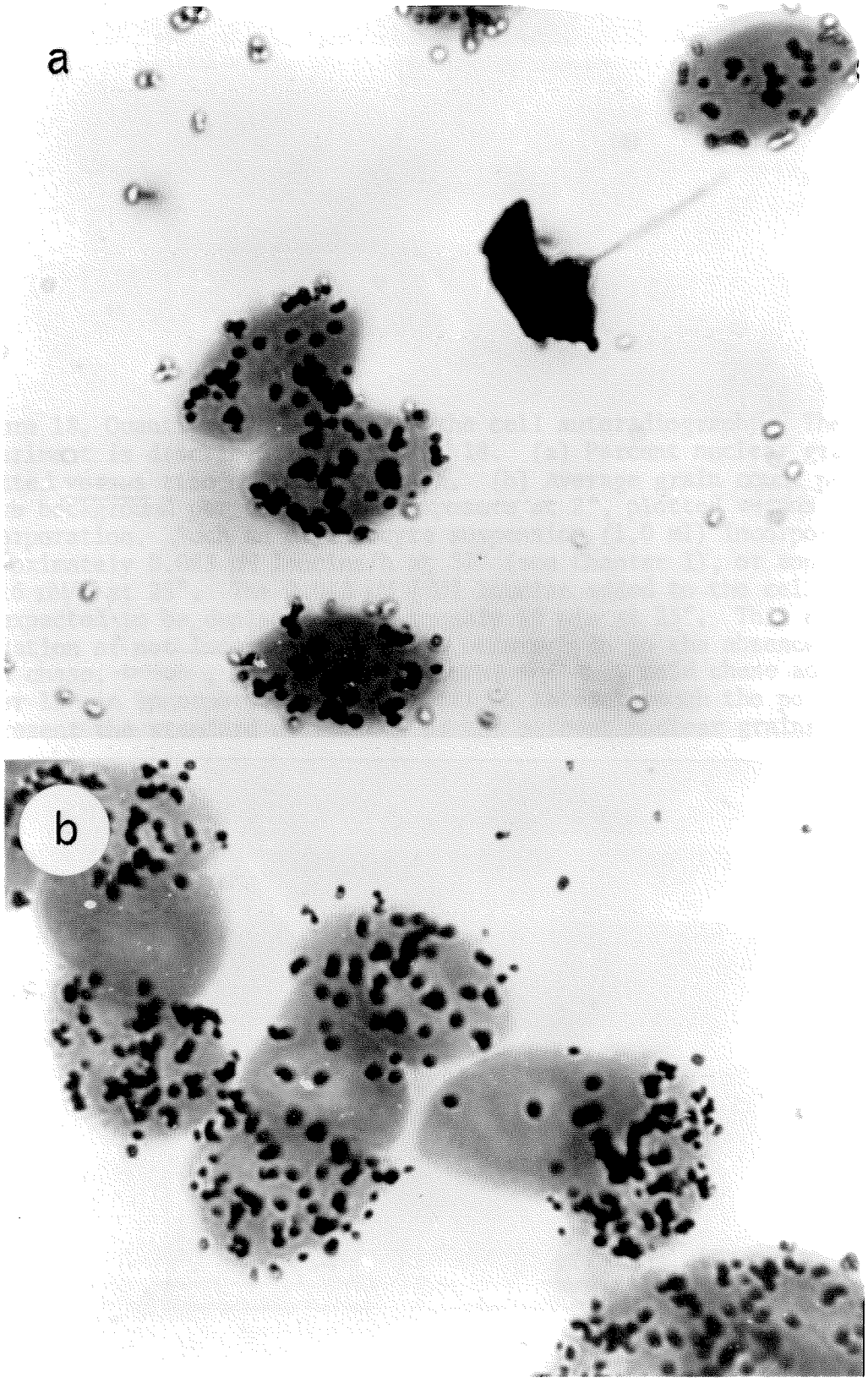
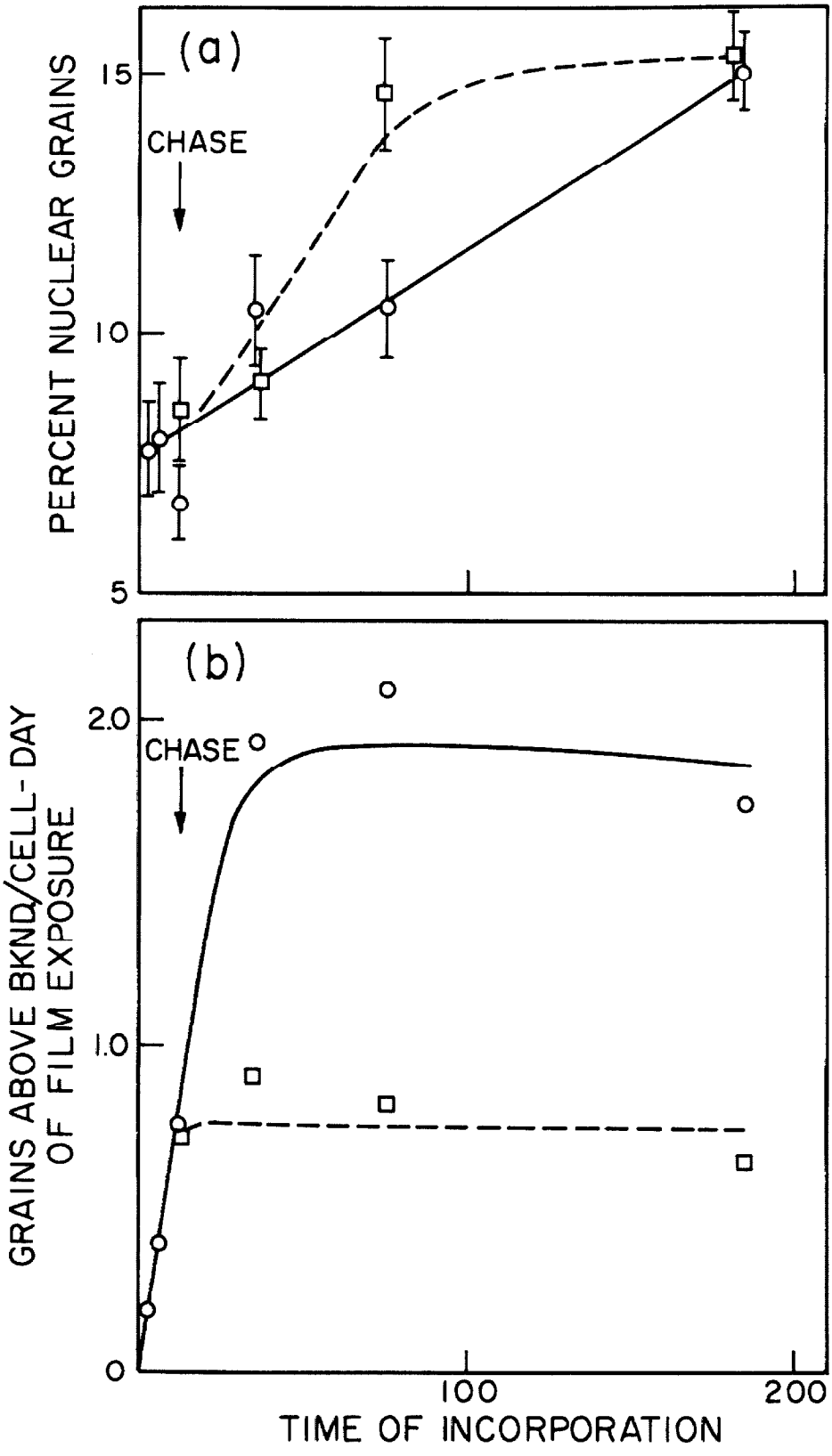

a
b

Figure 18. Quantitative aspects of the cell autoradiography. The same experiment is described in Figs. 17, 18. (a) Percent nuclear grains plotted versus time of incorporation. (b) Average grain count per cell above background per day of film exposure at 2°, plotted versus time of incorporation. Such an erythrocyte suspension (1.0 ml) incorporates approximately 0.083 μM leucine/h at 37° (see Chapter 1), or approximately 0.016 μM/h at 25°. The 0.013 μM [^{3}H] leucine added to the cells would be expected to be depleted after roughly 50 min at 25°. This explains the cessation of net incorporation which occurs even in the absence of the cold chase. -o—o- , no cold chase given; -□---□-, cold chase administered after 12 min incorporation. The vertical lines through the points in (a) represent the standard deviations of the percent nuclear grains.


(a)
CHASE
PERCENT NUCLEAR GRAINS
15
10
5
(b)
CHASE
GRAINS ABOVE BKND/CELL-DAY
OF FILM EXPOSURE
2.0
1.0
0
100
200
TIME OF INCORPORATION

approximately 6 min at 25° to synthesize complete globin chains on the ribosomes (Fig. 12), after a 3 min pulse roughly 75% of the incorporated amino acid should still be localized on the ribosomes at the site of synthesis. Therefore, protein formation occurs primarily in the cytoplasm.

DISCUSSION

Heterogeneity of chicken hemoglobin

Many previous investigators have found adult chicken erythrocytes to contain two main hemoglobins, Hb1 and Hb2, in the approximate ratio 3:1 (1-4, 17, 19, 24, 63), in agreement with the results described here. In addition to these hemoglobins, which have different amino acid compositions (2, 16), several of the above workers have also reported a more acidic Hb in relatively very low concentration (approximately 1% of the total) (17, 19); however it is not known whether this latter component contains polypeptides of distinct amino acid sequence, or whether it represents a secondarily altered protein such as is responsible for some of the heterogeneity of human adult and fetal hemoglobins (64, 65) or of cytochrome c from various species (66). Slight oxidation of the hemoglobin iron can also cause marked chromatographic changes (17, 67).

An electrophoretic pattern containing 5 hemoglobin bands was described by one group (22) that lysed the chicken erythrocytes by freeze-thawing. It has recently been shown by Sanders (63), however, that freeze-thawing of chicken hemoglobin solutions results in formation of several electrophoretic components in addition to the Hb1 and Hb2 characteristic of the fresh, untreated solutions. Similarly, aged chicken oxyhemoglobin solutions exhibit an increased heterogeneity (3).

Hashimoto and Wilt (68) have recently resolved chicken hemoglobin into 5 electrophoretic components after precipitating the protein with $(NH_4)_2SO_4$ and passing it through anion exchange columns using phosphate buffers. Chicken hemoglobin is known to react with inorganic phosphate in a complex manner, giving rise to additional electrophoretic bands (4). It should be mentioned that freeze-thawing (69) and aging of protein solutions can stimulate disulfide bonding, which is known to be responsible for artificial heterogeneity of mouse hemoglobins (70) and turtle and frog hemoglobins (71). In the present work, it has been shown by gel filtration of chicken globin on Sephadex G-100 columns under denaturing conditions, that disulfide bonding is probably responsible for the size heterogeneity observed (Fig. 4); therefore, reduced and carboxymethylated globin was used in all experiments designed to determine heterogeneity of the globin polypeptide chains. As discussed below, there was no clear resolution of α- and β- chains upon electrophoresis of reduced and carboxymethylated globin in denaturing conditions (8M urea, 5% formic acid, pH 1.7); however several bands were often obtained using non-reduced chicken globin, as would be expected from the size heterogeneity of this material (Fig. 4). Using similar conditions of electrophoresis in starch gels, Hashimoto and Wilt (68) also observed several bands with non-reduced chicken globin. Although it cannot be concluded definitely that HbI and HbII are homogeneous proteins, the apparent extreme heterogeneity found by some workers (22, 68) using conventional separation methods appears inconclusive in view of the wide variety of conditions known to produce artificial chicken hemoglobin components (3, 4, 17, 63).

In the present work, Hb1 and Hb2 were compared by immunochemical methods, and the reduced and carboxymethylated proteins by peptide mapping techniques. The results suggest that the two proteins differ in structure, but that many common amino acid sequences exist (Figs. 2, 3). At least 95% by mass of the reduced and carboxymethylated hemoglobins were soluble in the tryptic digests, and the number of peptides obtained agrees closely with the arginine plus lysine content of the proteins (2), under the assumption that the hemoglobins have the structure $\alpha_2\beta_2$. These data do not agree with the structure β_4 or γ_4 which occur in certain human anemias [β_4^A (Hb-H) or γ_4^F (Hb-Bart's)] (72). The reduced and carboxymethylated Hb1 and Hb2 proteins were also subjected to acrylamide gel electrophoresis (Fig. 5) under conditions in which chicken hemoglobin is known to be dissociated into quarter-molecules of molecular weight approximately 17,000 (18). However with neither protein was there any clear resolution of α- and β- chains; Sasakawa et al (18) were also unable to resolve chicken hemoglobin into two different polypeptide chains by electrophoresis, column chromatography or countercurrent distribution. The results suggest that there is no gross heterogeneity of the reduced and carboxymethylated hemoglobin polypeptide chains, in agreement with the interpretation that Hb1 and Hb2 may be single proteins. However the results do not show whether Hb1 and Hb2 contain a common subunit polypeptide, or whether the amino acid sequence similarities occur within related, but non-identical, polypeptide chains.

Synthesis of chicken erythrocyte proteins

The blood of anemic chickens contains mainly erythrocytes late in the process of cellular differentiation. Not only are these cells non-dividing, but their capacity for RNA and protein synthesis progressively declines during maturation, and their RNA content decreases (7; also Chapter 1). Furthermore, chromosomal condensation appears to accompany the maturation process (5, 8, 73). The loss of capacity for macromolecular syntheses and the cessation of cell division are typical also of other cell differentiation processes, including those of mammalian erythrocytes (14), muscle cells (74), exocrine pancreas cells (75), and chick embryo lens cells (76, 77).

The pattern of protein synthesis in erythrocytes from the blood of anemic chickens has been analyzed at the molecular level not only by measuring total incorporation of radioactive amino acids into various fractions, but by other methods including autoradiographic peptide mapping. All of the protein synthesis is sensitive to puromycin inhibition; puromycin is known to cause release of incomplete peptide chains from ribosomes by substituting for aminoacyl-transfer RNA at the growing end of the peptide chains (58, 78, 79). Localization of protein synthesis predominantly in the cytoplasm is implied by the cell autoradiography (Figs. 16, 17, 18), in complete agreement with the data of Scherrer et al (8), and by the known localization of avian erythrocyte RNA in the cytoplasm (12, 80). The results obtained here are incompatible with the view (33) that the hemoglobin molecules are synthesized in the nucleus, or that the labeled cytoplasmic hemoglobin derives from the nucleus via the nuclear soluble fraction. As discussed above

(RESULTS, Synthesis of nuclear soluble proteins), this latter protein fraction does not appear to be a metabolic precursor of the cytoplasmic soluble protein. At most 5-10% of the protein synthesis appears to occur in the nucleus in agreement with the experiments of Scherrer et al (8).

Growing polypeptide chains were stripped from the ribosomes and fractionated according to size by gel filtration on Sephadex G-100 columns under denaturing conditions (Fig. 12). After a 4 min pulse with [^{3}H] leucine at 25°, the short chains are as highly labeled as after 15 min or 25 min pulses. However, long chains the size of completed globin polypeptides (approximately 150 amino acids) are only roughly 70% labeled, as compared with the longer pulses. On the one hand, this result is consistent with the well-established model of protein synthesis in which the nascent chains grow by sequential addition of amino acids to one growing end (42) which is known to be the carboxyl-terminal end (42, 60). On the other hand, these data suggest that it requires roughly 6 min at 25°, or probably 1.1 min at 37° (based on the 5-fold greater extent of protein synthesis at 37° than at 25°), to polymerize a complete globin chain. Implicit in these latter estimates is the assumption that entry of [^{3}H] leucine into the erythrocytes (previously the cells had been depleted of endogenous leucine) is very rapid relative to the globin chain synthesis. This is probably a valid assumption and is discussed above (see RESULTS, Ribosomes and growing polypeptide chains). It has been estimated previously by other methods that it requires approximately 1 min at 37° to synthesize rabbit globin chains (81, 82). Globin-specific tryptic peptides can be identified in the autoradiographic

tryptic peptide maps of [^{14}C] leucine-labeled ribosomes (Fig. 13b), further demonstrating that the ribosomes are the site of chicken hemoglobin synthesis.

In view of the decline of RNA synthesis (7) and the chromosomal condensation (5, 73) which accompany chicken erythrocyte maturation, it is of interest that chromosomal proteins, both histone and non-histone, are being synthesized in these non-dividing cells. The low level of histone synthesis consists selectively of the arginine-rich histones (Fig. 8). Selective formation of arginine-rich histones was previously observed by Chalkley and Maurer (56) in several other tissues consisting mainly of non-dividing, differentiated cells. The incorporation of [^{14}C] leucine into the erythrocyte histones is inhibited by puromycin (Fig. 7b), suggesting incorporation into newly-formed polypeptide chains. Previous work with chicken erythrocytes had shown that this cell population synthesizes roughly 0.1% of its protein content per h incubation *in vitro* at 37° (Chapter 1). The arginine-rich histones are synthesized at roughly the same rate as are the hemoglobins, and therefore also at approximately 0.1% per h incubation at 37°. Such a low absolute rate of synthesis need not signify a *turnover* of DNA-bound histones as was suggested by Chalkley and Maurer (56). At the present time there does not appear to exist any unequivocal evidence for a degradation of histones bound to DNA. Conceivably, the synthesis of the erythrocyte chromosomal proteins is causally related to the chromosomal condensation and concomitant decline of RNA synthesis. Experiments by Harris (73) using hybridization of chicken erythrocytes with He La cells suggest that inhibition of RNA synthesis in the erythrocyte nuclei appears to parallel the chromosomal

condensation.

A slow migration of [^{3}H] leucine-labeled proteins from the cytoplasm into the nuclei is suggested by the results observed by cell autoradiography (Fig. 18). Incorporation of labeled leucine into the chromosomal proteins or into the nuclear residual fraction is not preceded by a lag period (Fig. 7b), suggesting that the label transferred into the nuclei, as seen in the cell autoradiography, is not transferred into these protein fractions. More probably, there occurs a slow migration of labeled hemoglobin molecules from the cytoplasm into the erythrocyte nuclei. Approximately 20% of the dry weight of chicken erythrocyte nuclei is hemoglobin (83). Cytological data suggest that the nuclear hemoglobin solution lies in channels between the condensed chromosome masses and is continuous with cytoplasmic hemoglobin solution through pores in the nuclear membrane (34). Since cytoplasmic membrane breakage results in the loss of hemoglobin from the intranuclear channels (33, 34), it has been proposed that the nuclear pores are open, allowing passage of hemoglobin molecules. The cell autoradiography experiments reported here support this model of the nuclear membrane. It has also been found (unpublished results) that the Hb1:Hb2 ratios are identical in the nuclear and cytoplasmic solutions, suggesting that passage across the nuclear membrane is not selective for either hemoglobin.

Chapter 3

THE NUCLEAR HEMOGLOBIN OF CHICKEN ERYTHROCYTES

INTRODUCTION

Evidence based on a variety of techniques has shown that hemoglobin is present within avian, amphibian and reptilian erythrocyte nuclei, as well as within mammalian erythrocyte nuclei prior to nuclear loss during cellular differentiation (19, 21, 33, 34, 85-88). Davies (34) has presented evidence that the concentration of hemoglobin within chicken erythrocyte nuclei is the same as that in the cytoplasm (approximately 340 mg/ml , or 5.0 mM) and that the nuclear hemoglobin solution bathes the chromosome masses. Single red cells from adult chickens contain two different kinds of hemoglobin in the ratio Hb1:Hb2 equal to 3:1 (4, 23); this ratio is maintained both in the nuclear and in the cytoplasmic solutions (Kabat & Attardi, unpublished observation).

The extremely high concentration of hemoglobin surrounding the chromosomal masses raises the possibility that hemoglobin influences the structure and function of the erythrocyte chromosomes. In this paper, some chemical properties of chicken erythrocyte chromosomes are described. It is shown that one of the chicken hemoglobins, Hb1, binds *in vitro* and possibly *in vivo* to these chromosomes.

MATERIALS AND METHODS

Cytological staining

Cytochemical staining specific for hemoglobin was performed using o-dianisidine (21). Stages of erythrocyte differentiation were identified by the criteria of Lucas and Jamroz (5).

Peptide maps

Reduction of protein disulfide bonds with 2-mercaptoethanol, sulfhydryl alkylation with iodoacetic acid, and tryptic peptide mapping procedures have been described previously (46; see also Chapter 2).

Isolation of chicken erythrocyte nuclei

Methods used for homogenization of erythrocytes from normal adult chickens and isolation of chicken erythrocyte nuclei in low ionic strength media have been described (see Chapter 2). Such nuclei contain 25% by weight "nuclear soluble proteins", proteins extractable with 0.14 M NaCl but not with low ionic strength buffers. Approximately 50% by weight of these proteins consist of Hbl (see Chapter 2). Chromatin was purified from these nuclei by the two following methods.

(1) Magnesium precipitation method

Nuclei from 3 ml packed erythrocytes were washed 3 times, by centrifugation, in 25 ml of SSC/40 (a forty-fold dilution of 0.15M NaCl, 0.015M sodium citrate) and were then sheared in 25 ml of SSC/40 either in a Virtis homogenizer at 25 volts for 90 sec or by sonication at 7.5 amps for one min with a Branson sonifier [Model (S125) fitted with a solid horn]. The preparation was then centrifuged at 10,000 g for 15 min. The supernatant, which contains at least 95% both of the nuclear DNA and of the hemoglobin, was adjusted to 0.0024M magnesium acetate to precipitate the chromatin-nuclear soluble protein complex; the latter was sedimented by centrifugation at 10,000 g for 10 min. Purified chromatin was obtained by extracting the complex with 20 ml of 0.14M NaCl, 0.0024M magnesium acetate, 0.005M Tris buffer, pH 7.2, followed by sedimentation of the chromatin at 10,000 g for 10 min.

(2) Sucrose sedimentation method

An alternative method of chromatin purification employed nuclei from which the nuclear soluble proteins were removed by extraction with 0.14M NaCl. In this case, the extracted nuclei from 3 ml of packed erythrocytes were washed twice with 25 ml of saline-EDTA (0.075M NaCl, 0.024M sodium EDTA, pH 8), followed each time by sedimentation at 1500 g for 10 min. The chromatin was then purified by the sucrose centrifugation method of Marushige and Bonner (55).

Preparation of proteins

Nuclear soluble proteins were obtained from erythrocyte nuclei (isolated from 3 ml of packed erythrocytes) by two extractions with 4 ml of 0.14 M NaCl, 0.0024M magnesium acetate, 0.005 M tris buffer, pH 7.2, each extraction followed by sedimentation at 7,000 g for 10 min. Centrifugation of the extracts in the Spinco 40 rotor at 105,000 g for 3 h yields a small pellet which contains some ferritin but negligible ribosomes as seen in the electron microscope; this pellet was discarded. In a few cases, the nuclei were alternatively suspended in SSC/40 containing 0.0012 M magnesium acetate (to give approximately 2 mg DNA/ml) and the nuclear soluble proteins were extracted by treatment for 30 min at 37° with 10 mg/ml pancreatic DNase (Worthington) followed by centrifugation at 10,000 g for 10 min. This DNase treatment released approximately 60% of the deoxyribose from the nuclei and 80% of the hemoglobin.

Approximately 90% of the erythrocyte hemoglobin was present in the first supernatant following cell homogenization. Ribosomes were sedimented from these supernatants by centrifugation at 105,000 g for 3 h; the resulting hemoglobin solutions will be referred to as "total

cell hemoglobin".

Preparation of chicken hemoglobin labeled with L-[^{14}C] leucine has been described (Chapter 1). Radioactive samples were precipitated with 5% trichloroacetic acid, filtered onto millipore membranes, and counted in a Nuclear-Chicago gas flow counter. Corrections were made for efficiency of counting.

Chromatographic purification of chicken hemoglobins on Amberlite IRC-50 columns (Mallinckrodt CG-50, 200-400 mesh, 10 meq./g) has been described (Chapter 1).

Protein preparations were dialyzed versus 0.005M tris buffer, pH 7.0, containing 0.005M KCN, and were stored in a nitrogen atmosphere in Thunberg tubes.

Spectrophotometric measurements

Absorbance readings were made with a Zeiss PMQII spectrophotometer. Concentrations of oxyhemoglobin solutions in mg/ml were obtained using the factors 1.14 x O.D. at 542 mμ (30) or 0.108 x O.D. at the Soret band at 414 mμ. Molar extinction coefficients were calculated from these experimentally determined constants by using the value 6.8 x 10^4 for the molecular weight of avian hemoglobin (2, 89).

Melting profiles of DNA and chromatin were determined using a Gilford model 2000 multiple sample absorbance recorder equipped with a temperature controlled cuvette holder having a constant heating rate of 0.67° per min. Determinations were made in 0.015M NaCl, 0.0015M sodium citrate. DNA for melting controls was purified from chicken erythrocyte nuclei by the Marmur procedure (90).

Chemical analyses

DNA and RNA were fractionated by the Schmidt-Thannhauser procedure as modified by Ts'o and Sato (47). DNA was determined by the diphenylamine method (48) using D-deoxyribose as standard, and RNA by the orcinol reaction (28) using D-ribose as standard.

Histone was extracted with 0.2M HCl (4°, 30 min), precipitated with 20% trichloroacetic acid and assayed by the method of Lowry, Rosebrough, Farr and Randall (43) using purified chicken erythrocyte histone as standard. After removal of nucleic acids by heating in 5% trichloroacetic acid at 90° for 15 min, non-histone protein was determined on the 0.2M HCl-precipitable material by the method of Lowry et al. (43) using bovine serum albumin (Sigma) as standard.

Measurement of hemoglobin-chromatin binding

Chicken erythrocyte chromatin was resuspended 3 times in binding buffer (to give approximately 1 mg DNA/ml), followed each time by centrifugation at 10,000 g for 10 min. The binding buffer depended on the particular experiment, but always contained at least 0.0020M magnesium acetate. Cacodylic acid buffers were prepared from sodium cacodylate as described by Gomori (91). The pH of cacodylate buffers increases slightly upon temperature increase; consequently, pH was adjusted to the desired value at the temperature at which binding was studied. One-half ml hemoglobin samples at various concentrations in binding buffer were mixed with 1.5 ml suspensions of chromatin (containing approximately 1.2 mg DNA/ml) in the same buffer. After one h equilibration at 4°, the chromatin was firmly sedimented at 10,000 g for 10 min, and the supernatant hemoglobin was removed. Complexed

hemoglobin was extracted from the chromatin with 2.0 ml. of 0.25M NaCl, 0.0024M magnesium acetate for 15 min at 4°, followed by centrifugation at 10,000 g for 10 min. Complexed and free hemoglobin concentrations were determined spectrophotometrically at the Soret bands. DNA remained quantitatively associated with the pellets throughout these procedures and was analyzed by the method of Burton (48).

RESULTS

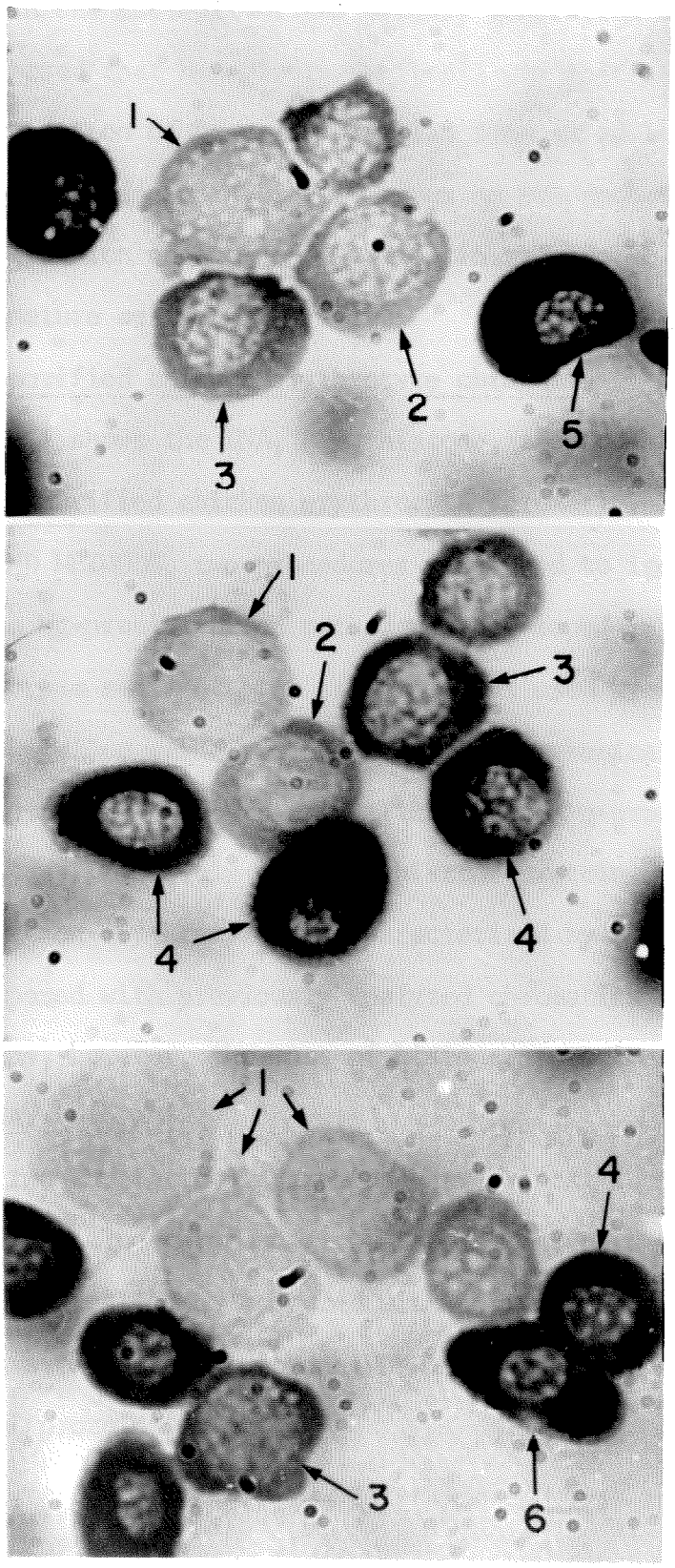
Cytological staining for hemoglobin

Cytological staining for hemoglobin in erythroid cells from the bone marrow of an adult chicken is shown in Fig. 1. The hemoglobin increase during erythrocyte differentiation is accompanied by nuclear condensation and by other morphological changes, such as transition from round to oval shape. Regions containing hemoglobin are visible in the nuclei and surround the relatively less stained chromosomal masses. Since there are no membranes or other diffusion barriers within the nuclei (34), it may be concluded that nuclear hemoglobin solution is in direct contact with the chicken erythrocyte chromosomes.

According to Davies (34), pores in the nuclear membrane connect the nuclear and cytoplasmic hemoglobin solutions. These pores apparently allow diffusion of hemoglobin molecules since cytoplasmic membrane breakage is accompanied by loss of nuclear hemoglobin from the interchromosomal spaces (33, 34). Furthermore, autoradiographic studies suggest that hemoglobin molecules synthesized on polyribosomes in the cytoplasm can migrate into the nucleus, implying that there occurs, in vivo, a mixing of cytoplasmic and nuclear hemoglobins (see Chapter 2).

O'Brien (21) reported that hemoglobin first appears during chick

Figure 1. Cytochemical staining of hemoglobin in bone marrow cells from an adult chicken. Erythrocyte differentiation occurs in the sequence (1) blast cell, (2) late erythroblast, (3) early polychromatic erythrocyte, (4) mid-polychromatic erythrocyte, (5) late polychromatic erythrocyte, (6) reticulocyte or mature erythrocyte (not distinguishable by this staining procedure). Mature chicken erythrocytes contain the normal diploid content of DNA (11). Mag'n. X3200.


1
2
3
5
1
2
3
4
4
1
4
3
6

embryogenesis in the nucleoli of the primary generation of erythroblasts; he proposed that hemoglobin synthesis was initiated in the nucleoli. Examination of bone marrow cells from an adult chicken by the same methods, as shown in Fig. 1, gives no evidence for a preferential localization of stained material in the nucleoli or nuclei of the most immature cells.

Properties of purified chicken erythrocyte chromatin

Table I shows the DNA, RNA, histone, and non-histone protein compositions of purified chicken erythrocyte chromatin. As described in MATERIALS AND METHODS, two procedures were used to isolate the chromatin. The Mg-precipitation method was developed in this work, whereas the sucrose sedimentation procedure was used by Marushige and Bonner (55) to isolate rat liver chromatin. The chemical compositions of the two preparations are similar, although the Mg-precipitated chromatin contains relatively more non-histone protein. Chromatin from mature chicken erythrocytes is characterized by a very low RNA content as compared with previously analyzed chromatins (55, 92). Like other chromatins, however, the mass of histone approximately equals the mass of DNA.

The two chromatin preparations have almost identical melting profiles (Fig. 2). The chromosomal DNA's are considerably stabilized against heat denaturation as compared with purified chicken erythrocyte DNA. It appears that the chromosomal DNA is directly associated with the chromosomal proteins.

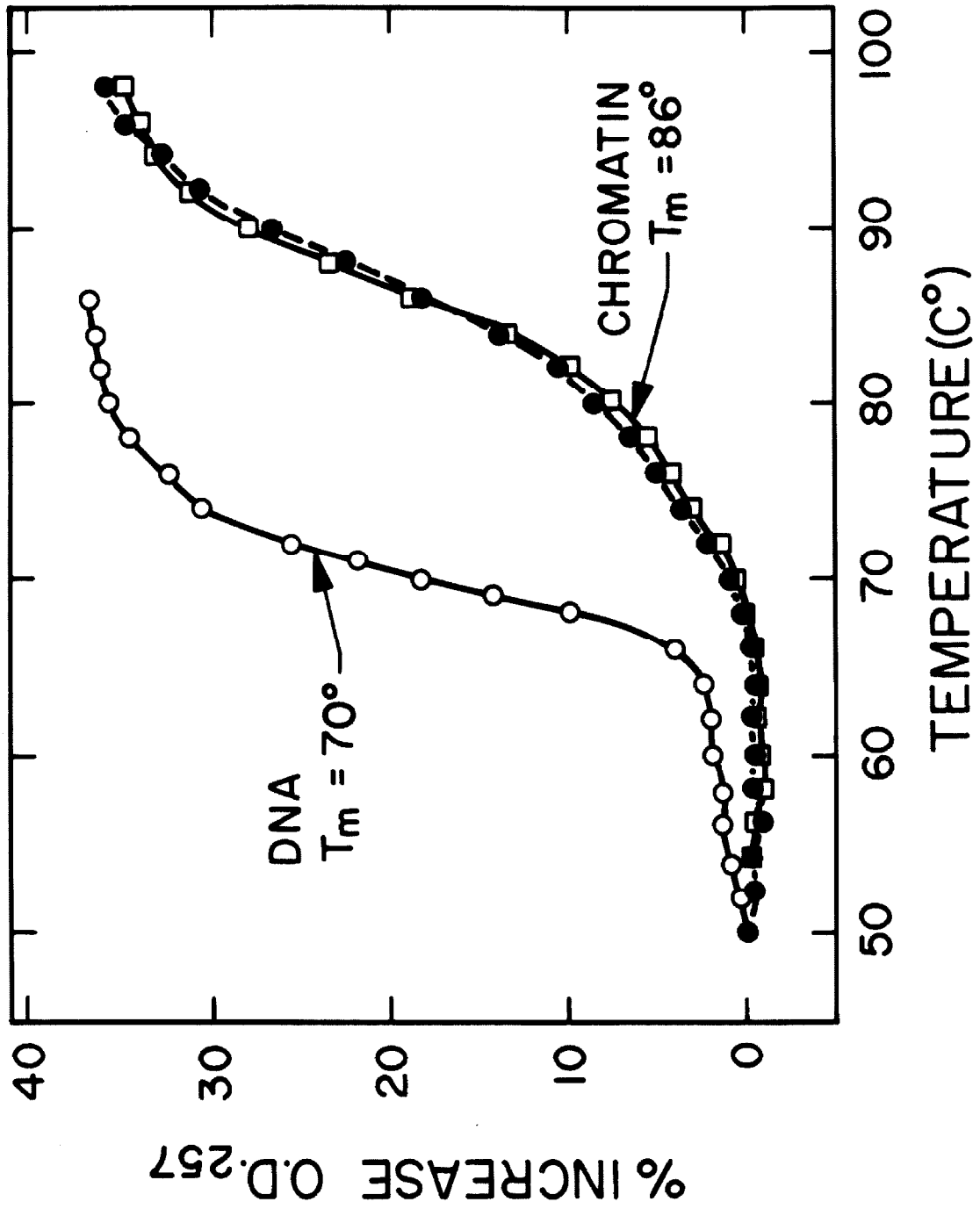
TABLE I

CHEMICAL COMPOSITION OF CHICKEN ERYTHROCYTE CHROMATIN

Component	Mass ratio *1 Mg-precipitated chromatin	Mass ratio *1,2 sucrose-sedimented chromatin
DNA	1.0	1.0
RNA	0.014	0.004
Histone	1.06	0.99
Non-histone protein	0.38	0.29

*1. Average values of two preparations.
2. Prepared by the method of Marushige and Bonner (55).

Figure 2. Melting profiles of chicken erythrocyte chromatin and DNA. —○—○— purified DNA, -●--●- chromatin prepared by the magnesium ion precipitation method, —□—□— chromatin prepared by the sucrose centrifugation method (55).


% INCREASE O.D.257
40
30
20
10
0
50
60
70
80
90
100
TEMPERATURE (C°)
CHROMATIN
Tm =86°
DNA
Tm = 70°

Isolation and properties of a native hemoglobin·chromosome complex

(a) Isolation

Chicken erythrocyte nuclei purified in low ionic strength media are red, due to the presence of hemoglobin in the nuclear soluble fraction (19). The nuclear soluble fraction is that class of nuclear proteins, present in all cells examined, which can be extracted with 0.14 M NaCl, but not with low ionic strength solutions (57, 93, 94). Although chicken erythrocytes contain two kinds of hemoglobin in the relative proportions Hb1:Hb2 equal to 3:1 (see Chapter 2), the nuclear soluble fraction contains only the most basic hemoglobin (Hb1) as shown by D'Amelio and Salvo (19) who used electrophoretic and immunochemical criteria to identify the proteins. This conclusion has been confirmed in this study using column chromatography and peptide map analysis to identify the hemoglobins (see Chapter 2). Tryptic peptide maps of chicken erythrocyte Hb1, Hb2, and nuclear soluble fraction are compared in Fig. 3. The Hb1 in the nuclear soluble fraction constitutes approximately 1% of the total cell hemoglobin.

Soluble chromatin has frequently been obtained by shearing nuclei or chromosomes in low ionic strength solutions in the absence of divalent cations (95, 96). This treatment results in the solubilization of at least 95% both of the bound hemoglobin and of the DNA in the case of chicken erythrocyte nuclei, whereas both hemoglobin and DNA remain insoluble following shearing in the presence of 0.0020M magnesium acetate. In addition, the solubilized chromatin and the hemoglobin are both precipitated by addition of 0.0020M magnesium acetate (see MATERIALS AND METHODS). Fig. 4a shows sucrose gradient sedimentation of chicken

Figure 3. Peptide mapping of chicken hemoglobins. In (a) the protein is Hb1, purified by chromatography. In (b) the protein is the 0.14 M NaCl extract of purified nuclei. In (c) the protein is Hb2, purified by chromatography. Electrophoresis was carried out in the long (vertical) dimension and chromatography in the short (horizontal) dimension. The origin is in the lower right-hand corner. The positive electrode was on the origin side during electrophoresis; the peptides moved toward the negative side.

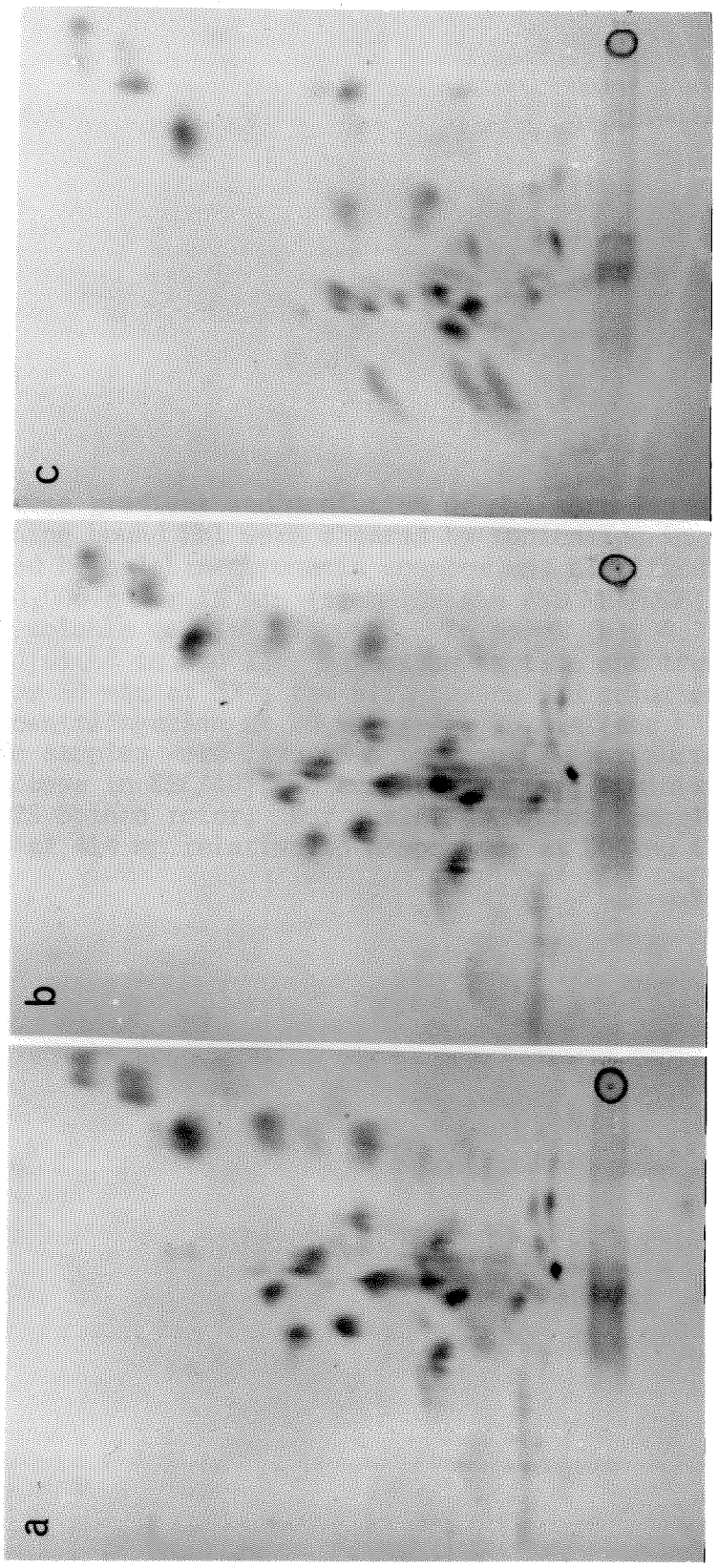
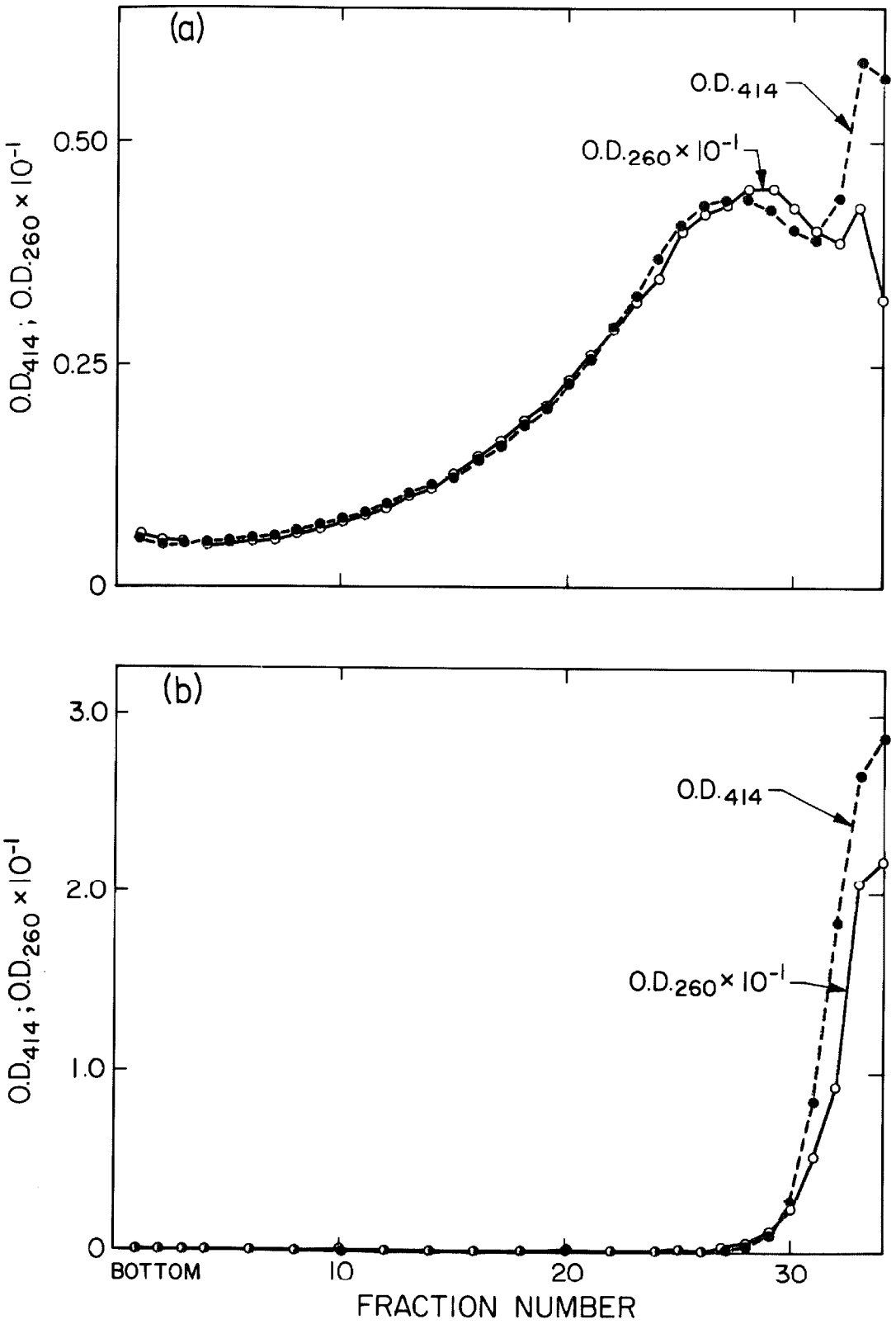

c
b
a

Figure 4. Sucrose gradient sedimentation of Hbl·chromosome complex. Nuclei containing bound Hbl were sheared by sonication in SSC/40 and the solubilized hemoglobin-chromatin complex was clarified by centrifugation at 10,000 g for 15 min (see MATERIALS AND METHODS). In panel (a) 1.0 ml of soluble complex was used. In panel (b), 0.2 ml of soluble complex was adjusted to 0.0012 M magnesium acetate and treated with 10 ug/ml DNase for 30 min at 37°; the slightly turbid solution was then clarified by centrifugation at 10,000 g for 10 min (see MATERIALS AND METHODS). The samples were layered onto 25 ml linear sucrose gradients (10 to 20% sucrose in SSC/40) and centrifuged for 70 min at 25,000 rev./min in the SW25 Spinco rotor. For purified chicken hemoglobin, the ratio of absorbance at 414 mμ relative to absorbance at 260 mμ is 3.9.


(a)
O.D.414
O.D.260 × 10⁻¹
0.50
0.25
0
O.D414 ; O.D260 × 10⁻¹
(b)
O.D.414
O.D.260 × 10⁻¹
3.0
2.0
1.0
0
O.D414 ; O.D260 × 10⁻¹
BOTTOM
10
20
30
FRACTION NUMBER

erythrocyte chromatin (solubilized by sonication). Hemoglobin sediments with the DNA; the ratio of Hbl/DNA in this case was 0.2 mass ratio, which is approximately one hemoglobin molecule per 1,100 deoxyribonucleotides. The ratio of hemoglobin to DNA is nearly constant throughout the gradient. Pancreatic DNase treatment reduces the sedimentation rate of both the hemoglobin and the chromatin (Fig. 4b).

(b) Influence of salts

The comparative influence of salts of monovalent and of divalent metal ions on the Hbl-chromatin binding is shown in Fig. 5. The binding is inhibited approximately 10 times more efficiently by divalent than by monovalent cations on a molar basis.

(c) Influence of pH

The effect of pH on Hbl·chromatin binding is shown in Fig. 6. Increase of pH results in strong inhibition of the chromosome-hemoglobin interaction.

(d) Reversibility

Evidence that Hbl molecules complexed to chicken erythrocyte chromatin are in equilibrium with free molecules was obtained by adding [^{14}C] leucine-labeled Hbl to unlabeled Hbl·chromatin complex and by then determining the specific radioactivities of complexed and of free hemoglobin at intervals following the addition. Table II shows the data from this experiment. By 2-3 min following the [^{14}C] hemoglobin addition, specific activities of bound and free Hbl molecules were equivalent, indicating that equilibrium had been achieved.

Figure 5. Inhibition of Hb1·chromosome binding by divalent and by monovalent metal ions. Magnesium-precipitated Hb1·chromatin complex was suspended at 2.3 mg DNA/ml in 0.005M sodium cacodylate, 0.0024M magnesium acetate, pH 6.3. Aliquots of the suspension were adjusted to the salt concentrations indicated by addition of 2.0M $MgCl_2$, 2.0M $CaCl_2$, 2.0M magnesium acetate, 2.0M NaCl, or 2.0M KCl. After incubation at 4° for 2 h, tubes were centrifuged at 10,000 g for 10 min and absorbance in the supernatants was measured at the Soret band (414 mμ). The Hb1:DNA mass ratio in the complex used in this study was 0.2. —o—o— NaCl, --●---o-- KCl, --o---o-- $MgCl_2$, —□—□— $CaCl_2$, —△—△— magnesium acetate.

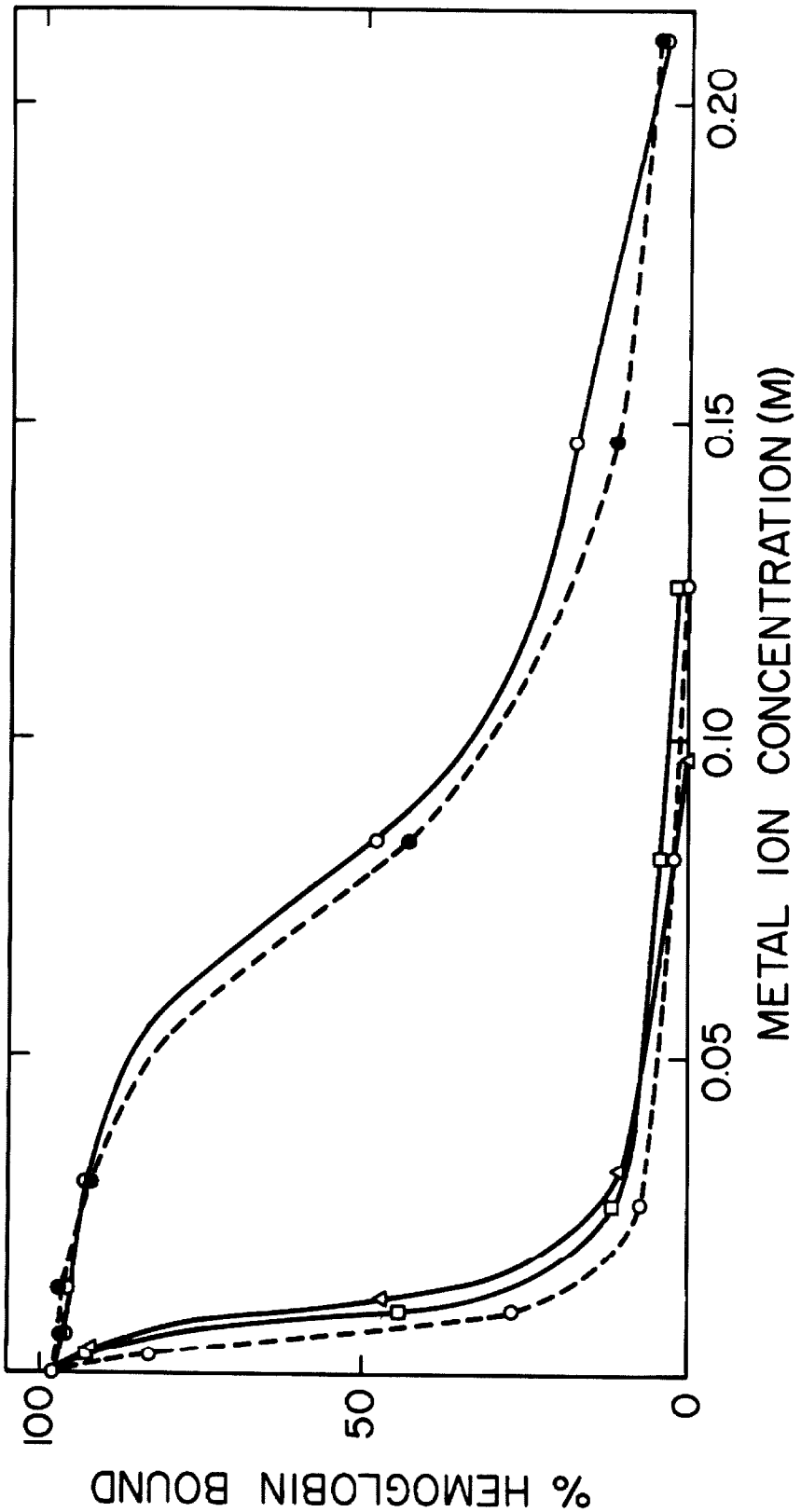

% HEMOGLOBIN BOUND
100
50
0
0.05
0.10
0.15
0.20
METAL ION CONCENTRATION (M)

Figure 6. pH influence on hemoglobin·chromatin binding. The binding buffers contained 0.01M NaCl, 0.01M sodium cacodylate, 0.0020 M magnesium acetate. Chromatin concentration in the binding mixture was 2.1 mg DNA/ml. Portions of chromatin were suspended in 1.5 ml of the buffers, prepared as described by Gomori (91). Samples were analyzed as described in the legend to Fig. 5.

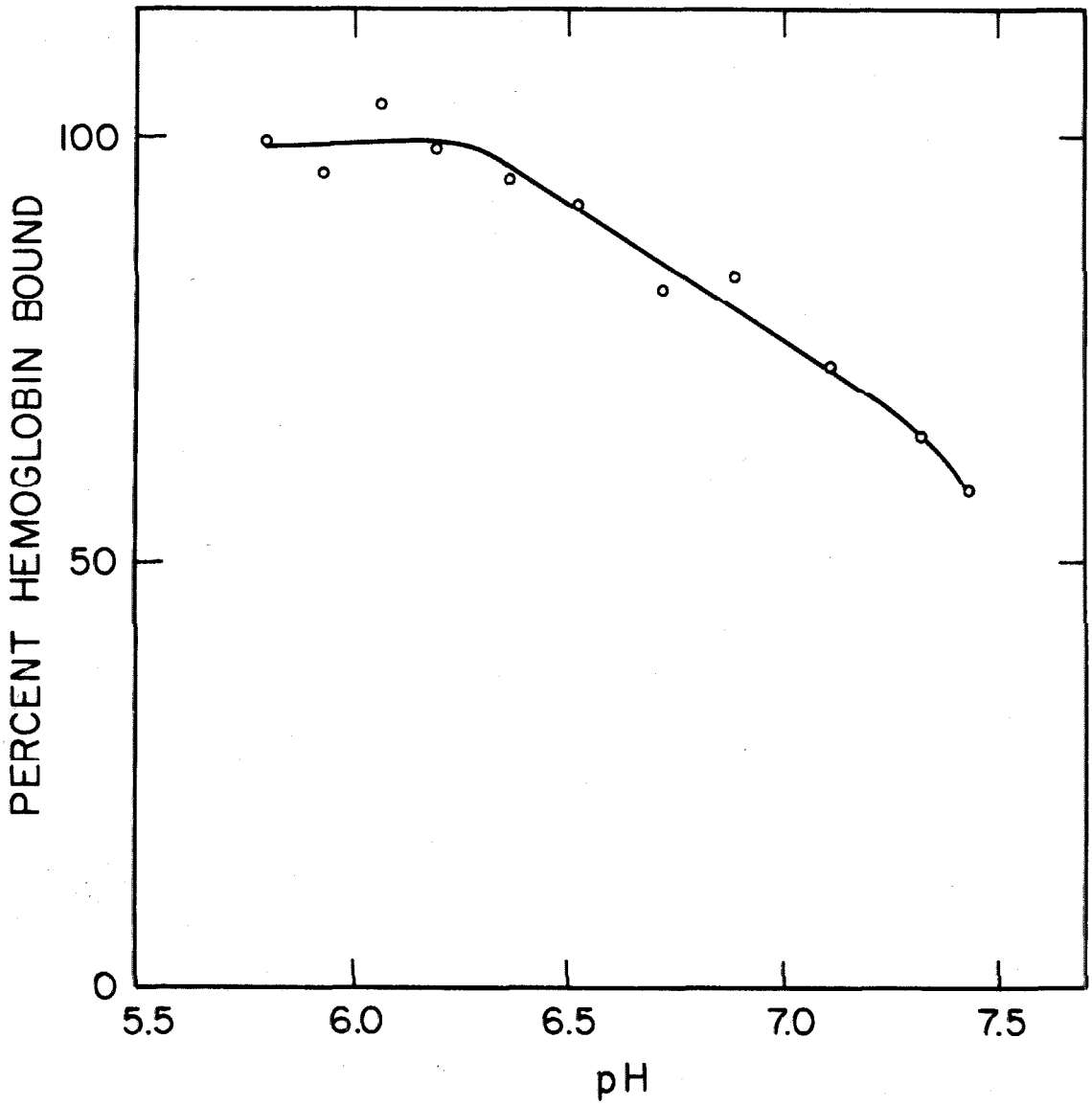

PERCENT HEMOGLOBIN BOUND
100
50
0
5.5
6.0
6.5
7.0
7.5
pH

TABLE II

REVERSIBILITY OF HEMOGLOBIN-CHROMATIN BINDING

Minutes after addition of C^{14}-leucine labeled Hbl	Hemoglobin analyzed	Specific radioactivity (cpm/O.D.414 mμ)
2-3'	Free	76
	Bound	71
8'	Free	72
	Bound	79
20'	Free	73
	Bound	69
40'	Free	77
	Bound	72
130'	Free	77
	Bound	70

Magnesium-precipitated Hbl.chromatin complex (see MATERIALS AND METHODS) was resuspended in 0.03M NaCl, 0.005M sodium cacodylate, 0.0024M magnesium acetate, pH 6.6 at 4°C. The Hbl:DNA mass ratio in the complex used was 0.24. L-[^{14}C] leucine-labeled Hbl (2030 cpm/O.D.414 mμ) was added and at intervals following this addition, 2.0 ml. aliquots of chromatin solution (containing 1.8 mg DNA/ml.) were centrifuged at 10,000 g for 3 min. Bound hemoglobin was extracted from the chromatin pellets with 2.0 ml. of 0.25M NaCl, 0.0024M magnesium acetate. Absorbance of the hemoglobin fractions at 414 mμ was determined and aliquots were assayed for radioactivity as described in MATERIALS AND METHODS.

In vitro formation of complexes with purified erythrocyte chromatin and added hemoglobins

Binding of isolated Hb1 and Hb2 to purified erythrocyte chromatin was measured as described previously (see MATERIALS AND METHODS). In Fig. 7a, the number of hemoglobin molecules bound per nucleotide in the reaction mixture is plotted versus the concentration of unbound hemoglobin for Hb1 and Hb2 (the binding buffer was 0.03M NaCl, 0.005M sodium cacodylate, 0.0024M magnesium acetate, pH 6.3). Chromosomal binding of Hb1 is clearly stronger than binding of Hb2.

An attempt can be made to quantitatively analyze such binding curves according to the simple mass-action expression

$$k_d = \frac{c(n-r)}{r} \qquad (1)$$

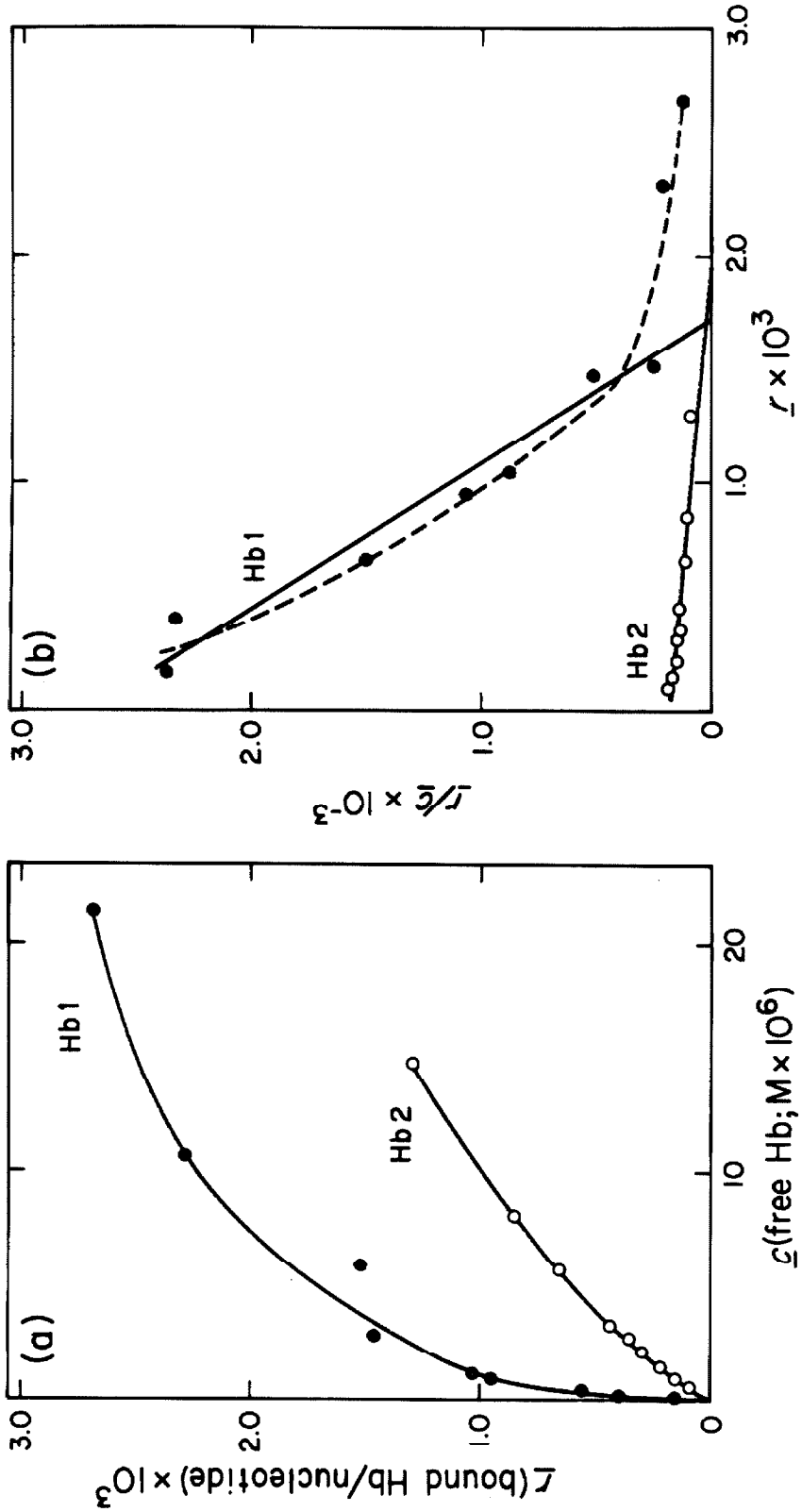
or

$$\frac{r}{c} = \frac{n}{k_d} - \frac{r}{k_d} \qquad (2)$$

where k_d is the dissociation constant of the complex, c is the molar concentration of free hemoglobin, n is the number of chromosomal binding sides per deoxyribonucleotide, and r is the number of hemoglobin molecules bound per deoxyribonucleotide. According to this treatment, a plot of r/c versus r would yield a straight line of slope $-\frac{1}{k_d}$, with the intercept on the r-axis equal to n (97). If, however, the chromosomal sites are heterogeneous in terms of hemoglobin affinity, then a more detailed treatment may be considered (97); such heterogeneity is indicated by curvature in plots of r/c versus r.

Fig. 7b shows the same data as Fig. 7a plotted as r/c versus r. The binding curves are not straight lines. However straight lines can

Figure 7. Binding of Hb1 and Hb2 to purified chicken erythrocyte chromatin. 1.5 ml aliquots of chromatin (purified by magnesium acetate precipitation), in 0.005 M sodium cacodylate, 0.03 M NaCl, 0.0024 M magnesium acetate, pH 6.3, were mixed with 0.5 ml dilutions of the isolated hemoglobins dissolved in the same buffer. After 2 h at 4°, samples were centrifuged and bound and free hemoglobin levels were determined (see MATERIALS AND METHODS, section h). Chromatin concentration in the binding mixture was 0.77 mg DNA/ml. In panel (a) the hemoglobin bound per nucleotide is plotted versus unbound hemoglobin concentration. In panel (b), r/c is plotted versus r (see text). —●—●— Hb1, —○—○— Hb2.


(a)
Hb1
Hb2
3.0
2.0
1.0
0
10
20
r̄ (bound Hb/nucleotide) × 10³
c̄(free Hb; M × 10⁶)
(b)
Hb1
Hb2
3.0
2.0
1.0
0
1.0
2.0
3.0
r̄/c̄ × 10⁻³
r̄ × 10³

be drawn which are reasonably close to most of the experimental points and these are drawn in the graph. The number of primary binding sites for Hb1 and Hb2 is the same within experimental error (one site per 600 nucleotides). The difference between the binding of the two molecules appears to result from the great difference in their binding constants (Hb1 association constant is approximately 15 times larger than that of Hb2). The values of k_d estimated from Fig. 7b are 6.0×10^{-7}M and 8.8×10^{-6}M for Hb1 and Hb2 respectively.

DISCUSSION

Because of the complex macromolecular nature of the reactants, it is not possible to understand the hemoglobin-chromosome association in detail. That the chromosomal sites which bind Hb1 molecules (one such site per approximately 600 deoxyribonucleotides as suggested by Fig. 7) may be regions of DNA not covered with histones is consistent with the following evidence: (1) Chicken hemoglobin binds more extensively to purified DNA than to erythrocyte chromatin (complexes have been made containing one Hb molecule per 50 deoxyribonucleotides, or having a Hb: DNA mass ratio equal to 4:1); like the chromosomal binding, the interaction with pure DNA is much stronger for Hb1 than for Hb2 (see this thesis, Part II). Hb1 is more basic than Hb2, its isoelectric point being above pH 8.6 (2); this is consistent with the hypothesis that Hb1 forms electrostatic bonds with negatively charged chromosomal regions. (2) Divalent cations are known to strongly interact with nucleic acid phosphate groups (97, 98); this latter interaction often severely inhibits the binding of other molecules to nucleic acids, and the inhibition has been used as a probe for detecting ionic interactions involving nucleic acid phosphates (99). Divalent cations very strongly inhibit both histone-DNA

interaction (100) and Hbl-chromatin interaction (Fig. 5). (3) The complexing of protein to a negatively charged region would be expected to increase the pK_a values of titratable amino acids on the protein surface, thereby leading to a net proton uptake (101). The effect of pH on Hbl·chromatin binding is consistent with this expectation since pH increase weakens the interaction (Fig. 6). In histone binding to DNA, there is an increase in the pK_a of carboxyl, histidyl and tyrosyl residues upon combination of the protein with DNA (102). According to the available evidence, therefore, the binding of Hbl to the chromosomes is similar to the binding of histones; it is likely that there occurs an ionic interaction between positive charges on the protein and negatively charged DNA phosphate groups.

The following conditions are believed to approximately represent the intracellular environment of mature avian erythrocytes from the blood: 0.105 M KCl, 0.025 M NaCl, 0.002 M $MgCl_2$, pH 7.2 and 42° (6). Examination of Figs. 5 and 6 indicate that the Hbl·chromosome binding is greatly weakened under these conditions as compared with lower ionic strength or lower pH values. In this context it should also be mentioned that temperature increase between 4° (the temperature used in the binding studies reported) and 42° also weakens the binding; however this effect is much smaller than the effects of pH or salt and was not studied in detail. However, it is important to recognize, as discussed previously, that the <u>in vivo</u> chicken erythrocyte chromosomes are bathed in extremely concentrated hemoglobin solutions (5.0 mM, which is 3.8 mM in Hbl in the most mature erythrocytes). Consequently, although the binding <u>in vivo</u> may be very <u>weak</u>, the complexing might still be <u>extensive</u> due to the mass action principle. The data available do not indicate, with any accuracy, the

possible level of intracellular Hbl·chromosome binding.

The binding experiments reported here were done in the presence of at least 0.0020M magnesium acetate, that is under conditions in which chromatin is insoluble. Non-aggregated chromatin can only be obtained by shearing nuclei or chromosomes in low ionic strength in the absence of divalent cations (95, 96); moreover, the soluble product is insoluble in physiological salt conditions (103). X-ray diffraction studies of nuclei from chicken erythrocytes and from calf thymocytes in 0.14M NaCl led Wilkins to propose a model of *in vivo* chromosomes in which histone bridges link DNA molecules into a large three-dimensional aggregate (103, 130). The mechanisms of the NaCl-induced and of the magnesium acetate-induced nucleohistone precipitations are both likely to be due to action of these salts in masking interchain electrostatic repulsion, thereby allowing formation of the labile histone bridges (103). Since salt-aggregated nucleo-histone gives the same X-ray diffraction pattern as isolated nuclei (103), it seems reasonable to infer that the chromatin used in the present study may be structurally similar to the *in vivo* chromosomes. Consistent with this idea is the fact that the morphology of isolated nuclei at the light microscope level is well preserved in the binding media employed. It should be mentioned that salt-aggregated chromatin has a highly hydrated and open structure and there is sufficient separation of DNA molecules for quite large molecules to diffuse into the chromosomal network (103, 104).

A common method of fractionating nuclear proteins employs 0.14 M NaCl extraction of nuclei previously isolated in low ionic strength media, and the class of nuclear proteins so obtained have been termed the "nuclear

soluble proteins" (57, 93) or the "nuclear globulins"(94). This class of proteins differs in various animal cells (93, 94); moreover, addition of these proteins back to nuclei in low ionic strength results in rebinding to the nuclei (57). Other common features of this class of proteins are their presence also in the cell cytoplasm (57, 93), and their generally more-basic character as compared with most of the cytoplasmic soluble proteins (57, 93, 105). These similar properties of this class of nuclear proteins suggests that many of these proteins may bind to the nuclei in a similar fashion. By all of the above criteria, chicken erythrocyte Hbl is a typical nuclear soluble protein. The evidence described here shows that the Hbl molecules form ionic bonds to the erythrocyte chromosomes; the electrostatic interaction is weakened when the ionic strength increases (Fig. 5), resulting in extraction of the protein from the chromosomes. Possibly, the nuclear soluble proteins constitute a class of weakly-bound chromosomal proteins. Further studies of the interactions between the nuclear soluble proteins and the chromosomes might lead to broadened understanding of the possible involvement of these proteins in chromosomal structure and function.

It should be emphasized, however, that the binding of nuclear soluble proteins to the cell nuclei may be in part a consequence of the conditions of nuclear isolation. For example when the intact cells are suspended in low ionic strength media for homogenization, there might occur a chromosomal binding by the proteins due to the cell swelling and consequent decrease in ionic strength. Whatever the factors involved, an understanding of this widely studied group of nuclear protein molecules will contribute to our knowledge of nuclear function and of the relation-ship between the intracellular and the isolated organelle.

GENERAL DISCUSSION

The results have been discussed in each chapter. It is the purpose of this general discussion to consider some of the findings in a larger perspective, rather than to review all of the data or to further discuss their validity.

The constant ratio of rates of synthesis of Hb1 and Hb2 (3:1, respectively) during erythropoiesis in the chicken suggests that the factors regulating these syntheses (at the genetic and translation level) operate in a coordinate fashion throughout cell development. The two proteins are metabolically stable and are therefore present in mature erythrocytes also in the ratio 3:1. From these results, and from the known localization of the hemoglobins within single cells (23, 24), it may be concluded that the Hb1:Hb2 ratio in mature cells does not result from unequal rates of destruction during cell development of messenger RNA's (mRNA's) initially transcribed in equal numbers. In other experiments it has been shown that even after very short pulses with [^{14}C] leucine (4 and 15 min labeling of an erythrocyte suspension at 25°), the radioactivity associated with Hb1 and Hb2 in the soluble fraction are in the 3:1 ratio (Chapter 2). This eliminates the possibility that the mRNA molecules are present in equal numbers but are translated into protein at different rates as a result of a "modulating triplet" or other slow step involved in the translation of Hb2 mRNA; if such modulation occurred, radioactivity associated with Hb1 would initially be released into the soluble fraction relatively faster than that associated with Hb2 (41). It seems likely, therefore, that the mRNA molecules specific for the two hemoglobins are initially synthesized in the unequal ratio

of roughly 3:1 for Hb1 and Hb2, respectively.

The suggestion that there occurs a coordinate regulation of the syntheses of Hb1 and Hb2 is consistent also with the bulk of evidence concerning the appearance of these hemoglobins in the chick embryo. Hb1 and Hb2 appear to be formed simultaneously and in approximately the 3:1 ratio during chick embryogenesis (3, 106, 107). It is of interest that the first appearance of Hb1 and Hb2 in the 6-7 day chick embryo occurs in coincidence with a decline of formation of the embryonic hemoglobins (3, 106, 107). It was shown by peptide mapping that the chicken embryonic hemoglobins differ considerably from Hb1 and Hb2 in amino acid sequence (3). Fraser (108) was unable to resolve the early embryonic hemoglobins from the adult Hb1 and Hb2, and concluded that the proportions of Hb1 and Hb2 changed gradually throughout embryonic life. An early conclusion of Wilt (109) that Hb2 is already present in newly-laid eggs is apparently believed incorrect in later publications (20, 68). The earlier work was based on immunochemical criteria; however the rabbit antiserum used clearly contained antibodies directed against non-hemoglobin materials. It is well known that many egg proteins cross-react immunologically with serum proteins (110), and it is likely that enough highly-antigenic serum proteins were injected into the rabbits to stimulate antibody production. In summary it seems to be well established that the adult hemoglobins, Hb1 and Hb2, appear simultaneously and in the normal adult proportions during chick embryogenesis.

In view of the apparent coordinate regulation of Hb1 and Hb2 syntheses, it is of interest that the two hemoglobins have extensive amino

acid sequence homology as suggested by analysis of peptide maps of the reduced and carboxymethylated proteins (Chapter 2, Fig. 3). Each of the hemoglobins appears to yield, upon tryptic hydrolysis, only approximately 7 peptides not present in the other protein. This indicates that there may be only approximately 7 positions altered between Hb1 and Hb2 per symmetrical half-molecule (approximately 300 amino acids consisting of one α plus one β polypeptide chain). This is undoubtedly a minimum estimate of the differences between the two proteins, but it seems reasonable to conclude that the number of positions altered per symmetrical half-molecule may be only few. It is not known whether these amino acid changes are all localized on one of two polypeptide chains or whether both the α and β subunits of these hemoglobins differ. Presumably, the genes specifying the two chicken hemoglobins arose from common ancestral genes recently, as compared with genes specifying the human α^A and β^A chains (87 position changes) (72,111,112) or the human β^A and γ^F chains (42 position changes) (72, 112-114). The structural similarity of the two chicken hemoglobins suggests that their mRNA molecules and their genes are also structurally similar.

In adult humans, Hb-A ($\alpha_2^A \beta_2^A$) occurs within single erythrocytes containing also 2.5% of Hb-A_2 ($\alpha_2^A \delta_2^{A2}$) (72). The β^A and δ^{A2} genes are closely linked on the genetic map (115), and preliminary evidence suggests that these genes are under a common genetic control (72, 116). For example, Hb-A and Hb-A_2 appear in fetuses at the same age and increase in concentration in parallel (117). It is of great interest that the increase of Hb-A and Hb-A_2 during embryogenesis appears to coincide with a decline of fetal hemoglobin (72), and that both Hb-A and Hb-A_2 are absent from adult

individuals homozygous for the "high-F" gene (118). Such individuals synthesize high levels of fetal-type hemoglobin in adulthood. It may be mentioned that β^A and $\delta^A 2$ polypeptide chains have very similar amino acid sequences (only approximately 8 position changes) (72, 119); and it has been suggested that the δ gene arose from a duplication of the β gene, followed by independent mutations (120). These matters of human genetics are discussed here because of their apparent similarity, and possible relatedness, to the phenomena described above concerning Hb1 and Hb2 synthesis in chickens. The mechanisms involved in the control of protein synthesis at the genetic level in higher organisms are not understood and are of profound importance to modern biology. No evidence is available concerning the location on the genetic map of the genes specifying Hb1 and Hb2; it would be very interesting to know whether linked genes are involved in this case.

In the developing chick embryo at 38°, hemoglobin synthesis begins at 34 hours (7-somite stage). However inhibition of RNA synthesis with actinomycin blocks the hemoglobin formation only if added prior to 22 hours of development (head-fold stage) (20, 121). In erythrocytes from the blood of anemic chickens, hemoglobin synthesis is also insensitive to actinomycin (Chapter 1, Fig. 7), implying that globin mRNA synthesis is completed fairly early in erythropoiesis, probably during the late erythroblast or early polychromatic erythrocyte stages (cells at these stages are very rare in peripheral blood from anemic chickens) (5; see also Chapter 3, Fig. 1). Studies of RNA synthesis in avian erythrocytes support the concept that ribosomes and mRNA are formed very early in differentiation; although metabolically-labile nuclear RNA is formed and degraded

in erythrocytes from the blood of anemic ducks, entry of newly-formed RNA into the cytoplasm is negligible (9). By all available criteria, therefore, the synthesis of mRNA molecules, and also of functional ribosomes, appears to be an early event in erythropoiesis. Subsequent control of protein synthesis during cell differentiation may occur solely at the translation level; one obvious example of such regulation is the destruction of ribosomes which occurs during the erythroid cell maturation (9; see also Chapter 1). The results are consistent with the hypothesis that the bulk of nucleic acids needed for the initiation of specialized protein synthesis and cytodifferentiation are available by the time that overt differentiation begins.

An analogous change from sensitivity to insensitivity to actinomycin occurs in other developing animal cell systems including muscle cells (122), metanephros (123), lens cells (124), and exocrine pancreas cells (125). Messenger RNA's related to cleavage or differentiation are transcribed in advance of their use also in other systems including sea urchin cleavage (126-128), feather synthesis (129) and Acetabularia cap synthesis (128).

During the differentiation of chicken erythrocytes, the chromatin becomes progressively condensed (5; also, Chapter 3, Fig. 1). Experiments by Harris (73) suggest that the decline of RNA synthesis which occurs during chicken erythrocyte development may be functionally related to the chromosomal condensation. At least in the later stages of differentiation, the chromosomal masses are bathed in concentrated hemoglobin solution (34, 88), and the hemoglobin molecules migrate between the nucleus and cytoplasm, probably passing through pores in the nuclear

membrane (34, 88; also Chapter 2, Fig. 17). The chromosomal condensation in avian erythrocytes appears analogous to the nuclear loss which occurs during mammalian red cell maturation.

While considering the role of the chicken erythrocyte nucleus in hemoglobin synthesis, it should be mentioned that O'Brien (21) reported, using a cytochemical stain specific for hemoglobin, that hemoglobin first appears during embryonic life in the nucleoli of the primary generation of erythroblasts. Analysis of bone marrow cells from adult chickens by the same procedure shows no preferential staining of the erythroblast nuclei or nucleoli (Chapter 3, Fig. 1). However it should be recognized that early embryonic chicken hemoglobins differ from those of the adult (3, 106, 107) and other differences between the primary and adult generations of erythroblasts are known (5). It should also be mentioned in this connection that Hammel and Bessman (33) reported that the nucleus is the major or sole site of protein synthesis in pigeon erythrocytes. This conclusion was based on the relative kinetics of labeling different erythrocyte subcellular fractions; however the ribosome fraction was not examined. As described in Chapter 2, the results obtained in the present study using a variety of methods of analysis are not in agreement with the Hammel and Bessman conclusion. On the contrary, it appears to have been well established here that the cytoplasmic polyribosome is the major site of hemoglobin synthesis in avian erythrocytes, in agreement with the results of Scherrer et al (8).

REFERENCES

1. W. B. Gratzer and A. C. Allison, Biol. Rev. Cambridge Phil. Soc. 35, 459 (1960).

2. A. Saha, Biochim. Biophys. Acta 93, 573 (1964).

3. C. Manwell, C. M. A. Baker, J. D. Roslansky, and M. Foght, Proc. Natl. Acad. Sci. U.S. 49, 496 (1963).

4. T. H. J. Huisman, J. M. Schillhorn Van Veen, A. M. Dozy, and C. M. Nechtman, Biochim. Biophys. Acta 88, 352 (1964).

5. A. M. Lucas and C. Jamroz, Atlas of Avian Hematology, U.S. Dept. of Agr., Washington, D.C. (1961).

6. P. D. Sturkie, Avian Physiology, Cornell University Press, Ithaca, N.Y. (1965).

7. I. L. Cameron and D. M. Prescott, Exptl. Cell Res. 30, 609 (1963).

8. K. Scherrer, L. Marcaud, F. Zajdela, B. Breckinridge, and F. Gros, Bull. Soc. Chim. Biol. 48, 1037 (1966).

9. G. Attardi, H. Parnas, M. I. H. Hwang, and B. Attardi, J. Mol. Biol. 20, 145 (1966).

10. E. Hammarsten, B. Thorell, S. Aqvist, N. Eliasson, and L. Akerman, Exptl. Cell Res. 5, 404 (1953).

11. A. E. Mirsky and H. Ris, Nature 163, 666 (1949).

12. H. G. Schweiger, Intern. Rev. Cytol. 13, 135 (1962).

13. H. Borsook, J. B. Lingrel, J. L. Scaro, and R. L. Millette, Nature 196, 347 (1962).

14. E. R. Glowacki and R. L. Millette, J. Mol. Biol. 11, 116 (1965).

15. P. A. Marks, R. A. Rifkind, and D. Danon, Proc. Natl. Acad. Sci. U.S. 50, 336 (1963).

16. H. J. Van der Helm and T. H. J. Huisman, Science 127, 762 (1958).

17. G. Matsuda and H. Takei, J. Biochem. 54, 156 (1963).

18. S. Sasakawa, K. Satake, T. Isemura, T. Yamashita and K. Kakiuchi, J. Biochem. 53, 513 (1963).

19. V. D'Amelio and A. M. Salvo, Z. Naturforsch 14B, 455 (1959).

20. F. H. Wilt, J. Mol. Biol. 12, 331 (1965).

21. B. R. A. O'Brien, Exptl. Cell Res. 21, 226 (1960).

22. H. Schall and F. Turba, Biochem. Z. 339, 219 (1963).

23. G. Matioli and B. Theorell, Blood 21, 1 (1963).

24. R. C. Fraser, Exptl. Cell Res. 36, 429 (1964).

25. R. C. Leif and J. Vinograd, Proc. Natl. Acad. Sci. U.S. 51, 521 (1964).

26. L. Levintow and J. E. Darnell, J. Biol. Chem. 235, 70 (1960).

27. Natl. Acad. Sci. U.S.-Natl. Res. Council, Science 127, 1376 (1958).

28. W. C. Schneider, in Methods in Enzymology, Vol. III, eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York (1957) p. 680.

29. J. R. Warner, and A. Rich, J. Mol. Biol. 10, 202 (1964).

30. R. T. Jones and W. A. Schroeder, J. Chromatog. 10, 421 (1963).

31. G. A. Boyd, in Autoradiography in Biology and Medicine, Academic Press, New York (1955) p. 210.

32. W. D. Gude, A. C. Upton, and T. T. Odel, Jr. Stain Technol. 30, 161 (1955).

33. C. L. Hammel and S. P. Bessman, J. Biol. Chem. 239, 2228 (1964).

34. H. G. Davies, J. Biophys. Biochem. Cytol. 9, 671 (1961).

35. J. E. Darnell and L. Levintow, J. Biol. Chem. 235, 74 (1960).

36. H. Eagle, K. A. Piez, and M. Levy, J. Biol. Chem. 236, 2039 (1962).

37. E. Heinz and H. A. Mariani, J. Biol. Chem. 228, 97 (1957).

38. G. Brecher and F. Stohlman, Proc. Soc. Exptl. Med. 107, 887 (1961).

39. H. Walter, F. W. Selby, and R. Winge, Federation Proc. 24, 420 (1965).

40. H. Walter, J. M. Brake, and F. W. Selby, Exptl. Cell Res. 37, 420 (1965).

41. S. H. Boyer, P. Hathaway, and M. D. Garrick, Cold Spring Harbor Symp. Quant. Biol. 29, 333 (1964).

42. H. M. Dintzis, Proc. Natl. Acad. Sci. U.S. 47, 247 (1961).

43. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. J. Biol. Chem. 193, 265 (1951).

44. G. W. Bray. Analyt. Biochem. 1, 279 (1960).

45. J. C. Bennett, L. E. Hood, W. J. Dreyer, and M. Potter. J. Mol. Biol. 12, 81 (1965).

46. A. M. Katz, W. J. Dreyer, and C. B. Anfinsen. J. Biol. Chem. 234, 2897 (1959).

47. P.O.P. Ts'o and C. Sato. Exptl. Cell Res. 17, 227 (1959).

48. K. Burton. Biochem. J. 62, 315 (1956).

49. T. H. J. Huisman and H. K. Prins. J. Lab. Clin. Med. 46, 225 (1955).

50. M. Heidelberger and K. Landsteiner. J. Exptl. Med. 38, 561 (1923).

51. P. O. P. Ts'o and J. Vinograd. Biochim. Biophys. Acta 49, 113 (1961).

52. H. N. Rydon and P. W. G. Smith. Nature 169, 922 (1952).

53. W. J. Dreyer and E. Bynum. in Methods in Enzymology, Academic Press, New York, in the press.

54. W. R. Gray. in Methods in Enzymology, Academic Press, New York, in the press.

55. K. Marushige and J. Bonner. J. Mol. Biol. 15, 160 (1966).

56. G. R. Chalkley and H. R. Maurer. Proc. Natl. Acad. Sci. U.S. 54, 498 (1965).

57. D. Barton. in The Cell Nucleus, Academic Press, New York (1960) p. 142.

58. D. W. Allen and P. C. Zamecnik. Biochim. Biophys. Acta 55, 865 (1962).

59. M. Cannon, R. Krug, and W. Gilbert. J. Mol. Biol. 7, 360 (1963).

60. R. E. Canfield and C. B. Anfinsen. Biochem. 2, 1073 (1963).

61. A. Rich, J. R. Warner, and H. M. Goodman. Cold Spring Harbor Symp. Quant. Biol. 28, 269 (1963).

62. Z. B. Miller and L. M. Kozloff. J. Biol. Chem. 170, 105 (1947).

63. R. Sanders. personal communicagion.

64. W. A. Schroeder, J. T. Cua, G. Matsuda, and W. D. Fenninger. Biochim. Biophys. Acta 63, 532 (1962).

65. W. R. Holmquist and W. A. Schroeder. Biochim. Biophys. Acta 82, 639 (1964).

66. E. Margoliash and A. Schejter. Advanc. in Protein Chemistry 21, 113 (1966).

67. D. W. Allen, W. A. Schroeder, and J. Balog. J. Amer. Chem. Soc. 80, 1628 (1958).

68. K. Hashimoto and F. H. Wilt. Proc. Natl. Acad. Sci. U.S. 55, 1477 (1966).

69. O. P. Chilson, L. A. Costello, and N. O. Kaplan. Fed. Proc. 24 part I, 5 (1965).

70. A. Riggs. Science 147, 621 (1965).

71. A. Riggs, B. Sullivan, and J. R. Agee. Proc. Natl. Acad. Sci. U.S. 51, 1127 (1964).

72. C. Baglioni. in Molecular Genetics part I., ed. J. H. Taylor, Academic Press, New York (1963) p. 405.

73. H. Harris. Proc. Roy. Soc. Brit. 166, 358 (1966).

74. D. Yaffe and S. Fuchs. Devel. Biol. 15, 33 (1967).

75. N. K. Wessells. Devel. Biol. 9, 92 (1964).

76. R. B. Scott and E. Bell. Science 147, 405 (1965).

77. R. Peeder and E. Bell. Science 150, 71 (1965).

78. M. B. Yarmolinsky and G. L. De LaHaba. Proc. Natl. Acad. Sci. U.S. 45, 1721 (1959).

79. A. J. Morris, R. Arlinghaus, S. Favelukes, and R. Schweet. Biochem. 2, 1084 (1963).

80. V. G. Allfrey, A. E. Mirsky, and H. Stern. Advances in Enzymology Vol. 16, Academic Press, New York (1955) p. 411.

81. H. M. Dintzis, H. Borsook, and J. Vinograd. in Microsomal Particles and Protein Synthesis. ed. R. B. Roberts, Pergamon Press, New York (1958) p. 95.

82. R. S. Schweet, J. Bishop, and A. Morris. Lab. Invest. 10, 992 (1961).

83. H. Stern, V. Allfrey, A. E. Mirsky, and H. Saetren. J. Gen. Phys. 35, 559 (1952).

84. P. S. Rasmussen, K. Murray, and J. M. Luck. Biochemistry 1, 79 (1962).

85. V. Allfrey, H. Stern, A. E. Mirsky, and H. Saetren. J. Gen Physiol. 35, 529 (1952).

86. S. Granick and R. Levere. Fed. Proc. 23, 222 (1964).

87. K. W. Taylor, H. M. Kaplan, and T. Hirano. Cytologia 28, 248 (1963).

88. H. G. Davies. J. Cell Biol. 16, 501 (1963).

89. A. Rossi Fanelli, E. Antonini, and A. Caputo. Advanc. Protein Chem. 19, 73 (1964).

90. J. Marmur. J. Mol. Biol. 3, 208 (1961).

91. G. Gomori. in Methods in Enzymology Vol.I, eds. S. P. Colowick and N. O. Kaplan. Academic Press, New York (1955) p. 142.

92. J. Bonner and R. C. Huang. J. Mol. Biol. 6, 169 (1963).

93. G. Patel and T. Y. Wang. Exptl. Cell Res. 34, 120 (1964).

94. H. Busch. Histones and other Nuclear Proteins. Academic Press, New York (1965) p. 197.

95. J. Bonner, R. G. Chalkley, M. Dahmus, D. Fambrough, F. Fujimura, R. C. Huang, J. Huberman, R. Jensen, K. Marushige, H. Ohlenbusch, B. Olivera, and J. Widholm. in Methods in Enzymology. eds. S. P. Colowick and N. O. Kaplan. Academic Press, New York, in press.

96. G. Zubay and P. Doty. J. Mol. Biol. 1, 1 (1959).

97. R. F. Steiner and R. F. Beers, Jr. in Polynucleotides. Elsevier Publishing Co., Amsterdam, (1961) p. 290.

98. J. Shack and B. S. Bynum. Nature 184, 635 (1959).

99. M. J. Waring. J. Mol. Biol. 13, 269 (1965).

100. E. O. Akinrimisi, J. Bonner, and P. O. P. Ts'o. J. Mol. Biol. 11, 128 (1965).

101. C. Tanford. J. Amer. Chem. Soc. 79, 5340 (1957).

102. I. O. Walker. J. Mol. Biol. 14 381 (1965).

103. M. H. F. Wilkins. in Nucleoproteins. Interscience Publishers, New York (1959) p. 73.

104. M. H. F. Wilkins. Cold Spring Harb. Symp. Quant. Biol. 21, 75 (1956).

105. C. Poort, Biochim. Biophys. Acta 46, 373 (1961).

106. T. H. J. Huisman and J. M. Schillhorn Van Veen, Biochim Biophys. Acta 88, 367 (1964).

107. V. D'Amelio and A. M. Salvo, Acta Embryol. Morphol. Exper. 4, 250 (1961).

108. R. C. Fraser, Exptl. Cell Res. 25, 418 (1961).

109. F. H. Wilt, Proc. Natl. Acad. Sci. U.S. 48, 1582 (1962).

110. A. M. Schechtman, in Biological Specificity and Growth, ed. E. G. Butler, Princeton Univ. Press, Princeton, New Jersey (1955) p.3.

111. W. Konigsberg, G. Guidotti and R. J. Hill, J. Biol. Chem. 236, PC55 (1961).

112. J. Goldstein, G. Guidotti, W. Konigsberg, and R. J. Hill, J. Biol. Chem. 236, PC77 (1961).

113. W. A. Schroeder, R. T. Jones, J. R. Shelton, J. B. Shelton, J. Cormick, and K. McCalla, Proc. Natl. Acad. Sci. U.S. 47, 811 (1961).

114. W. A. Schroeder, J. R. Shelton, J. B. Shelton, and J. Cormick, Proc. Natl. Acad. Sci. U.S. 48, 284 (1962).

115. R. Ceppelini, in Ciba Foundation Symposium, Biochemistry of Human Genetics, (1959) p. 133.

116. E. Zuckerkandl, J. Mol. Biol. 8, 128 (1964).

117. H. G. Kunkel, R. Ceppelini, U. Müller-Eberhard, and J. Wolf, J. Clin. Invest. 36, 1615 (1957).

118. J. T. Wheeler and J. R. Krevans, Clin. Res. 9, 168 (1961).

119. V. M. Ingram and A. O. W. Stretton, Nature 190, 1079 (1961).

120. V. M. Ingram, Nature 189, 704 (1961).

121. B. R. A. O'Brien, Nature 184, 376 (1959).

122. D. Yaffe and M. Feldman, Dev. Biol. 9, 347 (1964).

123. J. Jainchill, L. Saxén and T. Vainio, J. Embryol. Exp. Morph. 12, 597 (1964).

124. T. Yamada and M. Roesel, J. Embryol. Exp. Morph. 12, 713 (1964).

125. N. K. Wessells and F. H. Wilt, J. Mol. Biol. 13, 767 (1965).

126. P. C. Denny and A. Tyler, Biochem. Biophys. Res. Comm. 14, 245 (1964).

127. P. R. Gross, J. Exp. Zool. 157, 21 (1964).

128. J. Brachet, Nature 213, 650 (1967).

129. T. Humphreys, S. Penman, and E. Bell. Biochem. Biophys. Res. Comm. 17, 618 (1964).

130. M. H. F. Wilkins, G. Zubay and H. R. Wilson, J. Mol. Biol. 1, 179 (1959).

PART II

STUDIES OF ORDERED DNA-PROTEIN FIBERS

INTRODUCTION

The packing of nucleic acid molecules in viruses, ribosomes, chromosomes, and sperms appears to involve associations of the nucleic acid with protein molecules. Although little is known concerning the organization of nucleic acids in such structures, this subject is of obvious importance to understanding the biological functioning of these molecules. Interest in this area is heightened by recent studies of hetero- and euchromatin (1) and of the phenomenon of "puffing" in dipteran polytene chromosomes (2) which suggest that the tightness of packing of chromosomal DNA chains might be an important factor in the regulation of genetic activity.

There are many instances in which divalent cations exert a profound influence on nucleoprotein structure; this has been most thoroughly studied in the cases of ribosomes (3,4), chromosomes (5,6), and sea urchin sperms (5, 7). In all cases the effect appears similar in that tight-packing of the nucleoprotein occurs in the presence of divalent cations, whereas swelling of the nucleoprotein occurs upon divalent cation removal; this raises the possibility that a common mechanism might be involved. Unfortunately, the naturally occurring nucleoproteins generally contain heterogeneous protein components of unknown structure which tend to aggregate in solution; this has greatly complicated analysis of the protein-nucleic acid interactions.

It is the purpose of this paper to describe the divalent cation-dependent formation of the tightly-packed fibrous complexes

chicken Hb·DNA[1] and horse heart Cy c·DNA. A role for divalent cations in these fibers, possibly relevant to the general involvement of divalent cations in nucleoprotein structures, is suggested to account for the observations. It is further shown that the DNA double helices are oriented parallel to the nucleoprotein fiber axes, and that the cytochrome c molecules (possibly also the hemoglobin molecules) are strongly oriented with respect to the fiber direction.

MATERIALS AND METHODS

(a) Protein preparation

Blood of normal adult White Leghorn chickens was obtained by heart puncture with heparin as anticoagulant, and the cells were washed thoroughly with 0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$. The buffy coat of leukocytes was removed. Hemolysis was accomplished by suspending 1 volume of packed cells in 2 volumes of distilled water at 2° for 30 min. The hemolysates were centrifuged at 105,000 g for 3 h to sediment the ribosomes. The resulting Hb solutions were dialyzed exhaustively versus SSC/100 (0.0015 M NaCl, 0.00015 M sodium citrate, pH 6.9), and were then stored in a N_2 atmosphere in Thunberg tubes. Fresh heparinized horse blood was obtained from Dr. W. D. Ommert (Monrovia, California). The washing of horse erythrocytes and preparation of Hb was the same as for the chicken erythrocytes.

Chromatographic purification of chicken hemoglobins on Amberlite IRC-50 columns (Mallinckrodt CG-50, 200-400 mesh, 10 meq/g)

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[1] Abbreviations used: Hb, hemoglobin

Cy c, cytochrome c

was done as previously described (Part I, Chapter 2). Fractionated hemoglobins were precipitated with 80% $(NH_4)_2SO_4$, resuspended and dialyzed versus SSC/100, and then stored under N_2 in Thunberg tubes. Oxidation to give methemoglobin was done by dialysis of the hemoglobin against 0.001 M K_3 $Fe(CN)_6$, followed by dialysis versus SSC/100. Methemoglobin azide was prepared by adding a few grains of NaN_3 to solutions of methemoglobin.

Horse heart ferricytochrome c (type III) was purchased from Sigma Chemical Co. Ferrocytochrome c was prepared by adding a few grains of $Na_2S_2O_4$ to solutions of the oxidized protein.

(b) Preparation and composition analysis of nucleoprotein fibers

Highly polymerized calf thymus DNA (type I) was purchased from Sigma Chemical Co. DNA was dissolved and then throughly dialyzed with SSC/100. DNA was denatured by heating such dialyzed solutions (100 μg/ml) at 100° for 10 min followed by rapid cooling in ice. DNA was sheared by sonication at 8 mA. for 2 min with a Branson sonifier (Model S125). Human liver 28S ribosomal RNA was a gift of Drs. C. Attardi and F. Amaldi.

A soluble complex between Cy c and DNA was prepared similarly to Olivera (8). Solutions of Cy c and DNA in 1M NaCl were mixed in different proportions and were then throughly dialyzed (good agitation is necessary) versus 2.5×10^{-4} M EDTA, pH 6.8. Some factors involved in the preparation of chicken Hb·DNA and horse heart Cy c·DNA fibers are described below (RESULTS, sections a and d). Unless otherwise mentioned, nucleoprotein fibers for structural studies were formed by adding 0.05 ml of protein solution (25 mg/ml in SSC/100) to 1.5 ml solutions of DNA

(0.10 mg/ml in 0.002 M NaCl, 0.002 M sodium cacodylate, 0.0012 M $MgCl_2$, pH 6.6). Such fibers reproducibly had the mass ratios Hb:DNA equal 3.4 (1 Hb/64 P), or Cy c:DNA equal 3.8 (1 Cy c/20 P).

Occasionally, nucleoprotein fibers were disrupted by treatment with DNase. Fiber suspensions in SSC/100 at the concentration of 100 μg/ml were digested with 10 μg/ml DNase (Worthington) for 20 min at 37° after addition of 0.0024 M $MgCl_2$.

Generally, nucleoprotein fibers were suspended in 1.0 M NaCl for 15 h at 4°; this treatment caused slow solubilization of the fibers. In the case of Cy c, absorbance of the solutions was measured following reduction of the iron with $Na_2S_2O_4$. Absorbance of solutions containing methemoglobin was measured at 260 mμ before conversion to cyanmethemoglobin [by the addition of 10 μl/ml solution of 0.08 M KCN, 0.06 M K_3 $Fe(CN)_6$]. Concentrations of hemoprotein and of DNA were determined spectrophotometrically using the following molar absorptivities: Calf thymus DNA at 260 mμ (6.40×10^3 l/mole DNA P). Hemoglobin at 260 mμ (4.03×10^4 l/mole Fe). Oxyhemoglobin at 414 mμ (15.7×10^4 l/mole Fe), or at 542 mμ (1.49×10^4 l/mole Fe). Cyanmethemoglobin at 540 mμ (1.15×10^4 l/mole Fe). Ferrocytochrome c at 521 mμ (1.59×10^4 l/mole Fe) (9) and at 260 mμ (3.62×10^4 l/mole Fe). In all cases, absorbance at 260 mμ due to DNA was taken to be the measured absorbance minus the absorbance due to protein. The above molar absorptivities were calculated from measured values of absorbance per mg using the molecular weights 6.8×10^4 for chicken Hb (4 Fe/molecule) (10), and 1.23×10^4 for horse heart Cy c (1 Fe/molecule) (9). The average molecular weight of a DNA nucleotide was taken to be 320.

Water content of the nucleoprotein fibers was measured as the weight change when the fibers were dried in an oven at 100° for 45 min.

(c) Polarization microscopy

Nucleoprotein fibers were withdrawn from the mother liquor with forceps and pressed gently against glass to blot away adhering solution. After allowing the fibers to dry slightly in air (5 min at 25° at about 35% relative humidity), the fibers were placed onto clean glass slides, covered with immersion oil (n_D = 1.515; Cargille Laboratories, Inc.), and observed in a Carl Zeiss (Jena) polarizing microscope with a tungsten arc light source and transformer (Model 393) purchased from Spencer Lens Co. (Buffalo, N.Y.). Nucleoprotein fibers handled in this manner contained reproducibly 35-50% water by weight. The birefringence of colored materials is complicated due to light absorption by the sample (11); consequently, regions of the fibers so thin as to appear colorless were selected for analysis. Birefringence was quantitatively measured by compensator methods (12) using both a quarter-wave mica plate (125 mμ shift) and a selenite plate (550 mμ shift).

(d) Electron microscopy

In order to prepare nucleoprotein fibers for electron mircroscopic observation, it was necessary to break the fibers into small pieces. However, in solutions containing divalent metal ions, the fibers associate and dispersion is not possible. Consequently, fibers were dispersed by vigorous agitation in low ionic strength solution lacking divalent cation (SSC/100). Dispersed nucleoprotein was placed onto 300 mesh copper grids previously covered with parlodion film and excess liquid was drawn off.

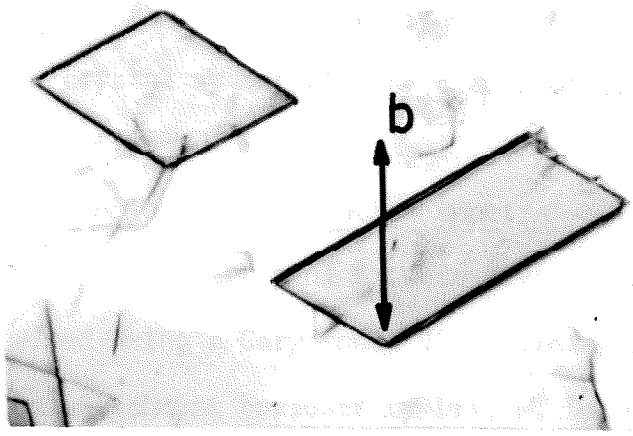
Staining of grids was accomplished by floating them face-down for 15 min on aqueous solutions either of 4% phosphotungstic acid, 0.4% sucrose, pH 6.5, or else on 2% uranyl acetate. Excess stain was drawn off by touching the edge of the grids with filter paper. Some grids were shadowed with Pt-Pd metal at an angle of 30°. Preparations were viewed with a Phillips 200 electron microscope.

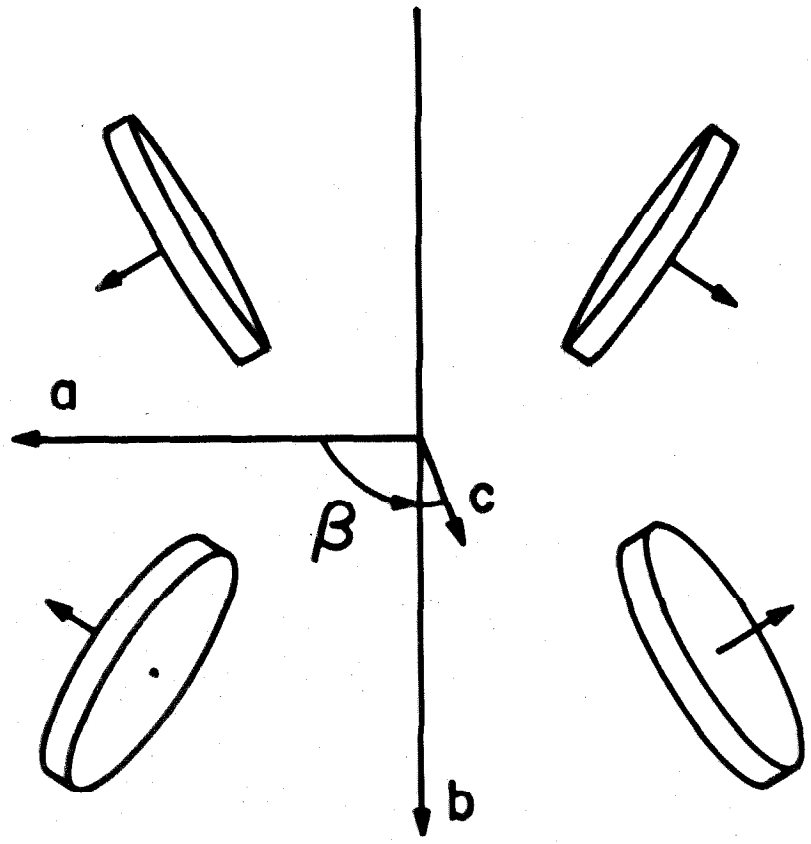
(e) Protein crystals

There has been confusion in the literature concerning the appearance and absorption of light by horse hemoglobin crystals (13). Fig. 1 shows a photograph taken of monoclinic (space group C2) horse methemoglobin crystals. Crystals were grown by dialysis against 50% $(NH_4)_2SO_4$ or 2.4 M K_2HPO_4, pH 7.0 (14). Such crystals are flat plates in the [001] plane, the only other faces being commonly well-developed are the [110] and [1$\bar{1}$0] faces. Unit cell dimensions are $\underline{a}$ = 109 Å, $\underline{b}$ = 63.2 Å, $\underline{c}$ = 54.4 Å, and β = 111°. The accompanying diagram shows the orientation of the unit cell $\underline{b}$ axis relative to the crystal faces and also the orientation of the heme groups in the crystal as given by Ingram, Gibson & Perutz (18). The two hemoglobin molecules in the unit cell have identical orientations relative to the crystal axes. A principal direction of refraction of these crystals is the $\underline{b}$ axis, as is generally true for monoclinic crystals (16).

Tetragonal crystals (space group $P4_1$) of horse heart ferricytochrome c were a gift of Dr. R. E. Dickerson. These crystals have been described (17). The unit cell dimensions are $\underline{a}$ = 58.45 Å, $\underline{b}$ = 58.45 Å, $\underline{c}$ = 42.34 Å and β= 90°; there are 4 molecules in the unit cell related by a 4-fold screw axis parallel to the $\underline{c}$ direction which is also the

Figure 1. Photograph of horse hemoglobin crystals and arrangement of the heme groups. Positions of the heme groups were taken from Ingram et al (18). The upper and lower discs represent respectively α- and β-chain hemes. The angles between the heme normals and b (the 2-fold rotation axis) are 58° and 57° for the α- and β-chain hemes respectively. The b axis bisects the obtuse, 120°, angle of the crystals.


b


a
β
c
b

major axis of the needle-like crystals. The predominant crystal faces parallel to c are the [100] and [010] planes, which are optically equivalent due to crystal symmetry. The c axis of tetragonal crystals is the optic axis (16).

(f) Measurement of polarized absorption spectra

The absorption spectra of protein crystals and nucleoprotein fibers were obtained using a Cary Model 15 recording spectrophotometer with a microscope attachment borrowed by Drs. M. Delbrück and K. Zankel from Applied Physics Corp. The microscope attachment replaced the usual sample compartment of the spectrophotometer. The sample beam of the spectrophotometer was focused with a 20X, 0.5 N.A. microscope objective to form a 10μ x 10μ image at the microscope stage. Specimens analyzed in these experiments were always at least several times larger than this image. The light was collected by a 90X, 1.30 N.A., oil immersion objective. Viewing was accomplished by means of a removable mirror and auxiliary ocular which contained reference lines drawn parallel and perpendicular to the polarizer directions. Metal discs with holes were used in the reference beam as neutral density filters to balance losses in the microscope optics. A base line was obtained for each spectral curve by moving the specimen out of the beam and sending the beam through a section of the slide adjacent to the specimen; the absorbance was taken as the difference between these curves. The monochromator slits were held fixed at 0.3 mm, resulting in a spectral band width of 2.5 to 8.5 mμ in the wavelength range from 400 to 600 mμ. The apparatus was capable of measuring absorbance to 0.003. Calibration and adjustment of the instrument were performed by K. Zankel and P. Burke.

The protein crystals and nucleoprotein fibers, suspended in mother liquor, were placed on microscope slides and covered with coverslips which were spaced from the slides with dabs of stopcock grease. This served to immobilize the crystals, without crushing them. Immersion oil was placed between the coverslip and the collecting objective. The specimens could be positioned by the usual methods on a rotating microscope stage. The specimen selected for observation was then rotated so that its principal direction of refraction was accurately aligned parallel to one of the reference lines marked on the viewing ocular.

The polarizer used was a piece of polaroid mounted in clear glass and marked so as to indicate the directions parallel and perpendicular to the electric vector of the transmitted light. It is important that the eccentricity of the polarizer be known if large dichroic ratios are to be measured (15). For the polarizer used, the out-of-plane component was a maximum of 1/160 of the in-plane intensity (measured using two equivalent polaroids on top of each other and assuming that the intensity transmitted when the polarizers are crossed at 90° is at least twice the out-of-plane intensity passed by either filter). It is readily calculated that for a specimen with a dichroic ratio of 2.0 and a measured absorbance of 1.0 in the direction of strong absorption, the error in the measured absorbance will consequently be less than $\pm$ 0.01 absorbance units (15). The polarizer was placed in the sample beam just above the 90X collecting objective and was taped onto a metal platform containing an aperture through which the light passed. By aligning the reference markings on the polarizer with markings on the metal bar, the polarizer could be accurately positioned either parallel or perpendicular to the principal

directions of the specimen. Such polarized absorption spectra were reproducible to within a few percent (ca., ± 4%).

(g) Analysis of porphyrin polarized absorption spectra

Circularly polarized light shone through a birefringent crystal face will be resolved into two mutually perpendicular plane-polarized beams. The polarization directions (x and y), called here the "principal axes of refraction", are generally fixed by crystal symmetry (16). Absorbance of light polarized in each of these directions is measured, and the ratio A_x/A_y is termed the dichroic ratio, or D. Absorbance in either direction is proportional to the square of the direction cosine between the polarization direction and the direction of the transition moment for allowed electronic exitations of the chromophore. As discussed below (DISCUSSION, section a), the heme group can, as a first approximation, be considered an "ideal two dimensional absorber" between 470-600 mμ. According to this approximation, the absorption in this region is interpreted as being due to two degenerate (i.e., equal energy) and perpendicularly polarized $\pi \rightarrow \pi^*$ transitions lying in the heme plane. This approximation is similar to assuming that the heme group has square symmetry, all 4 pyrrole rings being electronically equivalent (19).

The absorption of polarized light by monoclinic horse methemoglobin crystals has previously been shown to be consistent with the idea that the heme groups act as ideal two-dimensional absorbers (18). For such absorption, the dichroic ratio ($A_{\parallel b}/A_{\perp b}$) will agree closely with the equation $D = \tan^2\theta$, where θ is the mean angle between the heme normals and the b axis (in these crystals the α-chain heme normals lie

at 58° and the β-chain heme normals at 57° with respect to b).

If the heme groups in the tetragonal horse ferricytochrome c crystals are simple two-dimensional absorbers as just defined, then the dichroic ratio ($A_{\parallel c}/A_{\perp c}$) will be given by $D = \frac{2 \sin^2\theta}{1 + \cos^2\theta}$, where θ is the angle between any heme normal and c (the 4-fold rotation axis).

In the nucleoprotein fibers, the fiber axis (also a principal axis of refraction) can probably be considered (from the standpoint of the protein molecules) an axis of rotational symmetry; this assumption is based on the helical nature of DNA and is described below (DISCUSSION, section a). The calculation of light absorption by heme groups located on such an axis is very similar to that just described for the c axis of Cy c crystals. That is, for ideal two-dimensional absorbers the dichroic ratio ($A_{\parallel}$ fiber/$A_{\perp}$ fiber) is given by $D = \frac{2 \sin^2\theta}{1 + \cos^2\theta}$ where θ is the angle between the heme normal and the fiber axis. For Cy c·DNA fibers, the dichroism for ideal absorption will be given by the latter equation, assuming that the Cy c molecules are all oriented with respect to the fiber axis as depicted in Fig. 12. On the other hand, the interpretation of absorption by Hb·DNA fibers is further complicated by the fact that the protein contains 4 hemes (Fig. 1).

RESULTS

(a) Formation of chicken hemoglobin·DNA complexes

When chicken Hb is added to solutions of highly polymerized DNA in low ionic strength buffers (pH 7.0) containing divalent cations (Mg^{++}, Ca^{++}, or Mn^{++}), large red fibers rapidly form; these fibers may be sedimented by low speed centrifugation (2000 g for 2 min) or withdrawn on forceps. In the absence of added divalent cations, mixtures

of Hb and DNA become turbid upon prolonged standing, but no fibers appear; non-fibrous nucleoprotein can be sedimented from these turbid solutions (e.g., 10,000 g for 10 min). In all buffer systens examined, the Hb:DNA mass ratio in the non-fibrous nucleoprotein is less than the mass ratio in the fibers formed at low Mg^{++} levels (e.g., 3×10^{-4} M $MgCl_2$). Apparently, the divalent cation-induced change of nucleoprotein structure is associated with an increased level of Hb binding.

Some characteristics of the fiber formation are presented in Fig. 2 which shows the DNA and Hb content of the insoluble fibers at varying levels of added $MgCl_2$. The Hb:DNA mass ratio in the fibers declines as the $MgCl_2$ concentration is increased; at concentrations above approximately 0.01-0.02 M $MgCl_2$, no fibers form. The buffer used in the latter experiment was 0.002 M tris, pH 7.0; similar dependence of fiber composition on $MgCl_2$ concentration was observed also in 0.002 M sodium cacodylate, pH 6.9.

To solutions containing constant $MgCl_2$ and DNA concentrations, various amounts of Hb were added with rapid mixing, and the fibers were analyzed for Hb and DNA composition (Table I). The Hb:DNA ratio in the fibers remains constant over a wide range of input Hb; in the conditions used (0.002 M tris, 0.005 M $MgCl_2$, pH 7.0), the mass ratio is approximately 2.6. As also seen in Table I, when Hb is added in amounts insufficient to fully complex all of the DNA, a portion of the DNA forms fibers fully covered with the Hb, whereas the Hb: DNA mass ratio in the mother liquor is relatively low (it will be shown below that there is a fraction of chicken Hb - ca. 25% - which does not complex with DNA). This suggests that Hb molecules bind much more strongly to the fibers

Figure 2. Composition of Hb·DNA fibers as a function of $MgCl_2$ concentration. To 1.5 ml aliquots of DNA (0.17 mg) in 0.002 M tris buffer, pH 7.0 were added varying amounts of 1.2 M $MgCl_2$ at 2°. Chicken Hb (0.1 ml containing 2.5 mg Hb) was added to each aliquot and the tubes were immediately centrifuged at 2000 *g* for 2 min to sediment the fibers. Fiber composition was analyzed as described in MATERIALS AND METHODS, section b.

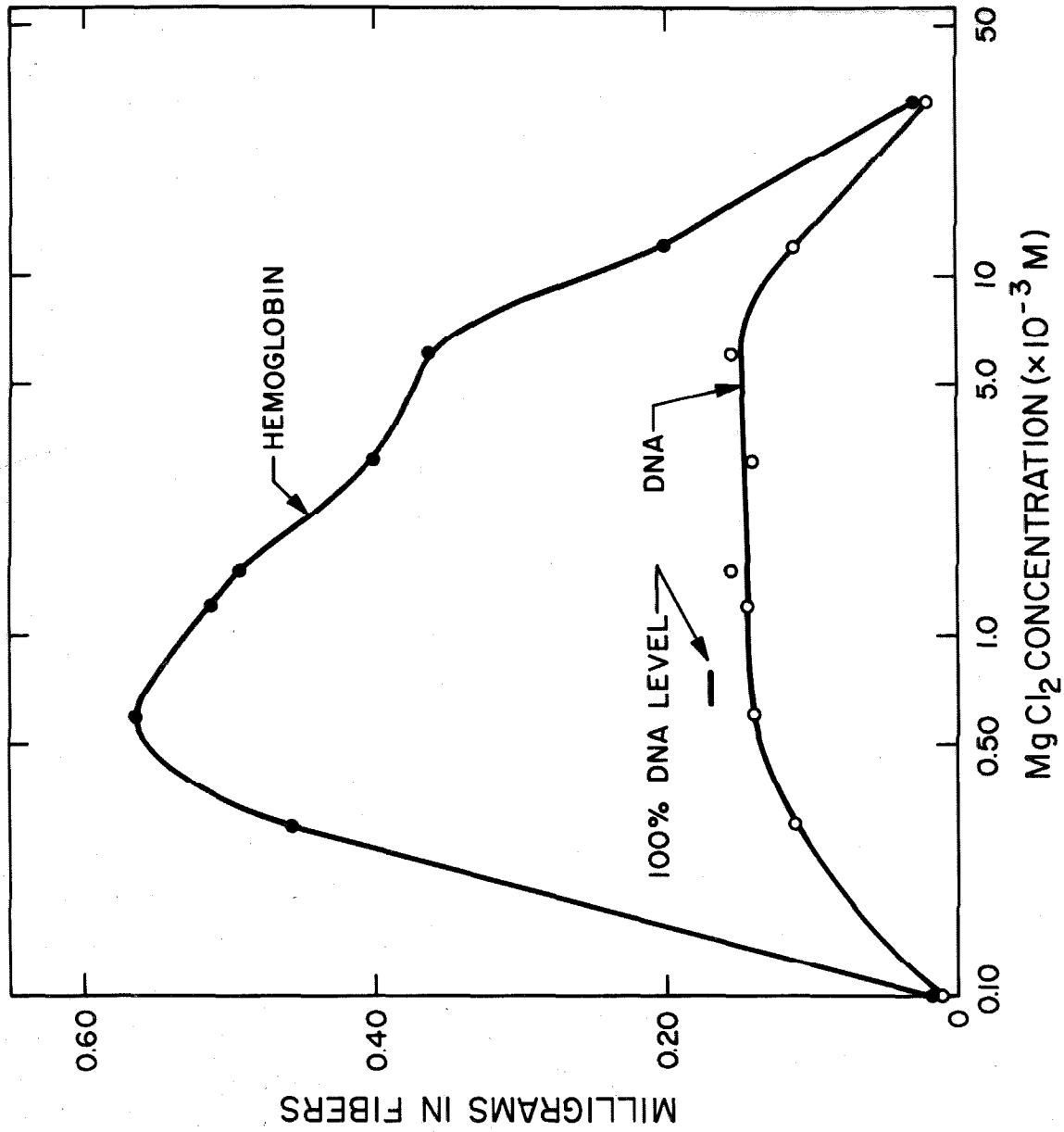

HEMOGLOBIN
DNA
100% DNA LEVEL
MILLIGRAMS IN FIBERS
0.60
0.40
0.20
0
0.10
0.50
1.0
5.0
10
50
Mg Cl2 CONCENTRATION (×10-3 M)

TABLE I

INDEPENDENCE OF FIBER COMPOSITION ON HEMOGLOBIN CONCENTRATION

Sample	mg Hb added	mg Hb in fibers	mg DNA in fibers	Mass ratio in fibers (Hb:DNA)
1	0.13	.097	.041	2.4
2	0.27	.20	.076	2.6
3	0.81	.41	.15	2.65
4	1.20	.42	.16	2.6
5	1.91	.43	.17	2.6
6	2.76	.42	.15	2.7

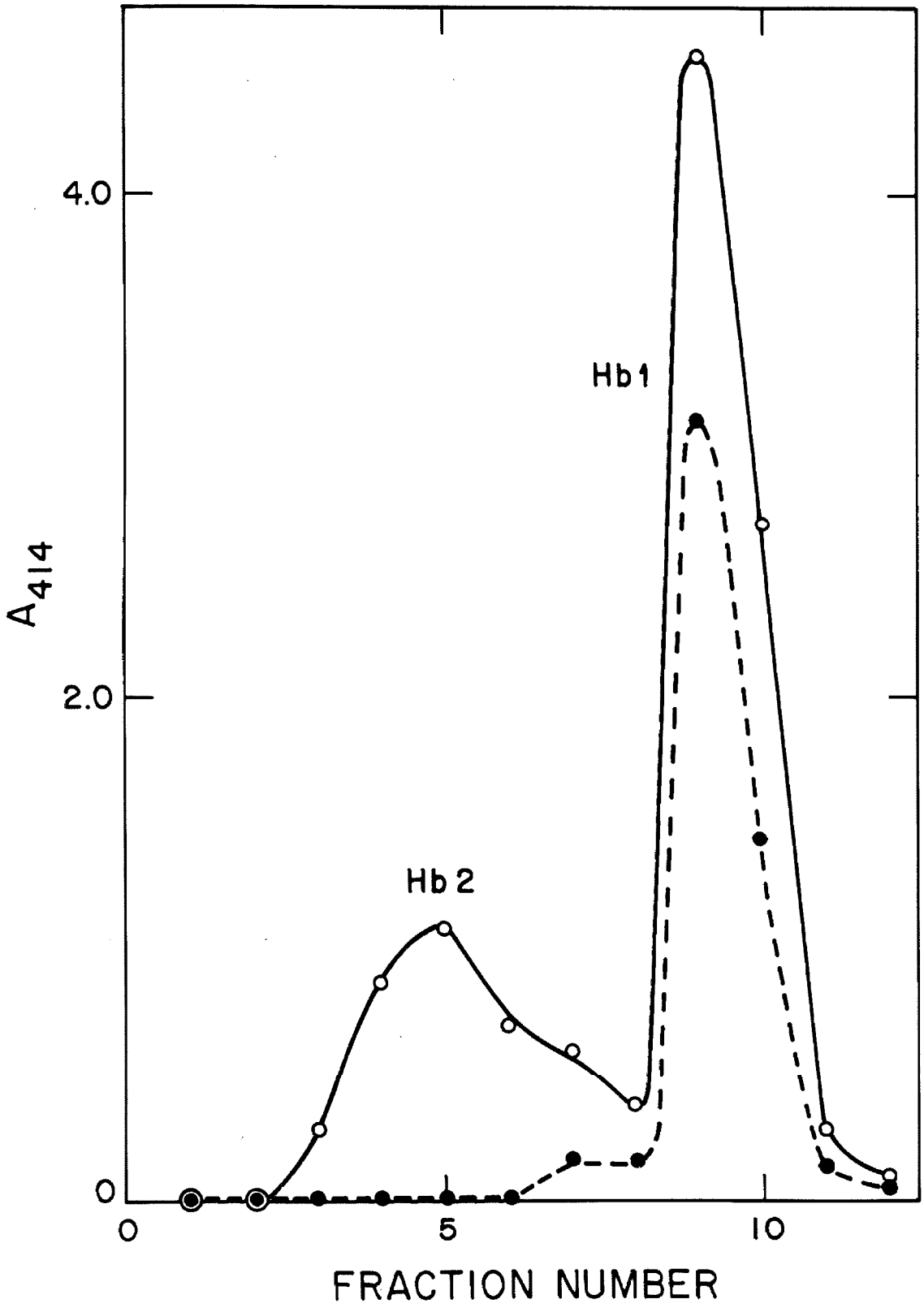
To 1.5 aliquots of DNA (0.18 mg) in 0.002 M tris, pH 7.0 containing 0.0030 M $MgCl_2$ were added 0.1 ml dilutions of Hb in the same buffer at 2°. Fibers were sedimented at 2000 g for 5 min and fiber composition was analyzed as described in MATERIALS AND METHODS, section b.

than to DNA molecules in solution, implying that the binding to the fibers may be highly cooperative. In an experiment run in parallel to that shown in Table I, it was found that the fiber Hb:DNA mass ratio was also not significantly affected by varying the DNA concentration between 35 and 200 μg/ml.

Although two different kinds of Hb are present in chicken erythrocytes in the approximate ratio Hb1:Hb2 equal to 3:1 (10,20,21), the Hb isolated from nucleoprotein fibers consists exclusively of the more basic protein, Hb1 (Fig. 3). In agreement with this result, studies with the pure proteins showed that Hb1, but not Hb2, forms fibers when mixed with DNA solutions containing divalent cation. It should be mentioned that although several investigators have described heterogeneity of the Hb within the peak labeled Hb2 (Fig. 3) (22,23), the available evidence supports the idea that Hb1 is a single protein. Chicken Hb1 is fairly basic, being isoionic at approximately pH 8.7; this is consistent with the possibility that the protein forms electrostatic bonds with the negatively charged DNA.

A test was made to determine the influence of nucleic acid structure on its association with chicken Hb. Mixtures of Hb with 28S ribosomal RNA or with sheared or thermally denatured DNA(in all cases 100 μg/ml in 0.002 M tris, pH 7.0) became immediately turbid when adjusted to 0.001 M $MgCl_2$; however no fibers formed in these solutions. It appears that fiber formation requires native highly-polymerized helical DNA, but that divalent cation-induced aggregation of nucleoprotein occurs also with sheared or denatured DNA and with ribosomal RNA.

Figure 3. Anion exchange chromatography of chicken hemoglobins. Amberlite IRC-50 columns measured 1 X 4 cm, and fractions contained 1.0 ml eluate. The elution employed stepwise increment of ionic strength as follows (fraction numbers indicated in parenthesis): 0.075 M (1), 0.15 M (2), 0.20 M (3-8), 0.40 M (9-13). -o—o- total cell Hb, --o---o-- Hb extracted from Hb·DNA fibers (Hb:DNA mass ratio, 3.4) by DNase treatment (see MATERIALS AND METHODS, section b).


4.0
2.0
0
A_{414}
Hb 1
Hb 2
0
5
10
FRACTION NUMBER

The requirement for divalent cations - Mg^{++}, Ca^{++}, or Mn^{++}- appears stringent in the sense that NaCl, KCl, or NH_4Cl are ineffective at all concentrations in stimulating Hb·DNA fiber formation. The above divalent metal ions are known to interact strongly with nucleic acids, probably binding to the negatively charged phosphate residues of the backbone (24, 25). No study was made of the effects on the nucleoprotein structure of multivalent metal ions or of other ions present in biological materials in only trace amounts; such metal ions frequently interact with nucleic acids or proteins in a relatively complex fashion (26-28).

The results described in this section indicate that divalent metal ions contribute strongly to the stabilization of the Hb·DNA fibers. In agreement with this conclusion, the fibers are unstable and break apart when placed in low ionic strength solutions which lack divalent metal ions (MATERIALS AND METHODS, section d). In view of the complexity of the nucleoprotein aggregation, which presumably involves a variety of forces, no attempt was made to investigate all conceivable environmental parameters. It should be mentioned, however, that at pH values below 6.7 and in the absence of divalent cations, the non-fibrous aggregates appear more rapidly, upon mixing the Hb and DNA, than at the higher pH values described above. Furthermore, fibers formed at the lower pH values in the presence of $MgCl_2$ are larger and are less easily broken than those formed at pH 7.0. Presumably, the protein-DNA binding is stronger at the lower pH values because the protein is more positively charged. Probably, other environmental factors not studied in this work would also influence the nucleoprotein aggregation. A model consistent with the observed stimulation of fiber formation by divalent metal ions is

described below (DISCUSSION, section b).

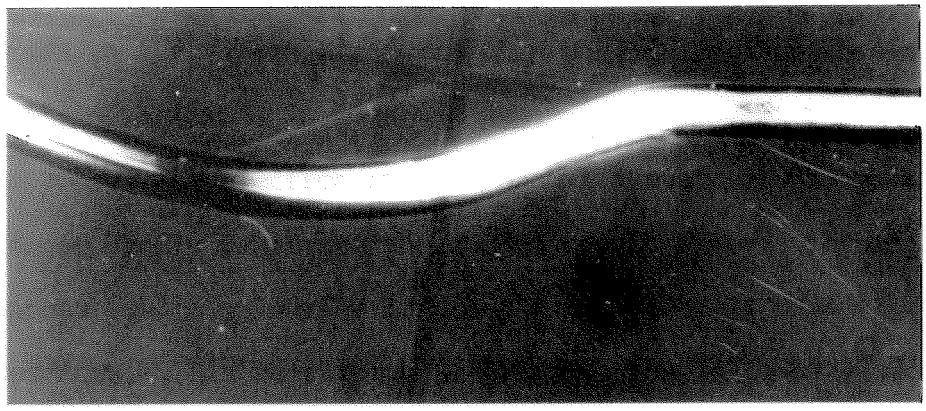
(b) Birefringence of hemoglobin DNA·fibers

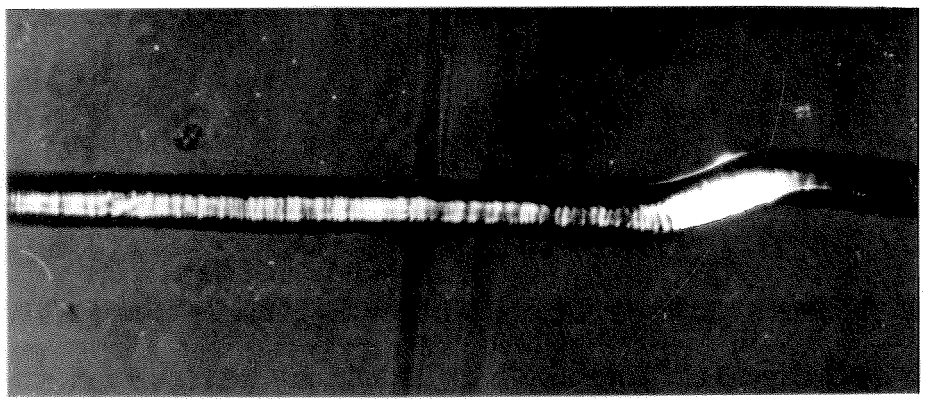
Viewed in a polarization microscope, the Hb·DNA fibers appear highly birefringent (Fig. 4), indicating that the refractive indices differ in the directions parallel and perpendicular to the fiber axis (these refractive indices are designated $n_{\parallel}$ and $n_{\perp}$ respectively, the magnitude of birefringence being $n_{\parallel} - n_{\perp}$). The strong negative birefringence of DNA fibers is intrinsic and due largely to the unsaturated purine and pyrimidine bases which lie perpendicular to the long axis of the molecules; the form birefringence of fibers is positive in sign (29). Protein molecules have negligible intrinsic birefringence as compared with DNA; accordingly, the negative sign of birefringence in a large number of sperm heads is in agreement with the orientation of the DNA molecules (30, 31). In the present case of Hb·DNA fibers, the birefringence is also negative (approximate value, - 0.015 for fibers having Hb:DNA mass ratio 3.4), implying an orientation of DNA molecules parallel to the fiber axis.

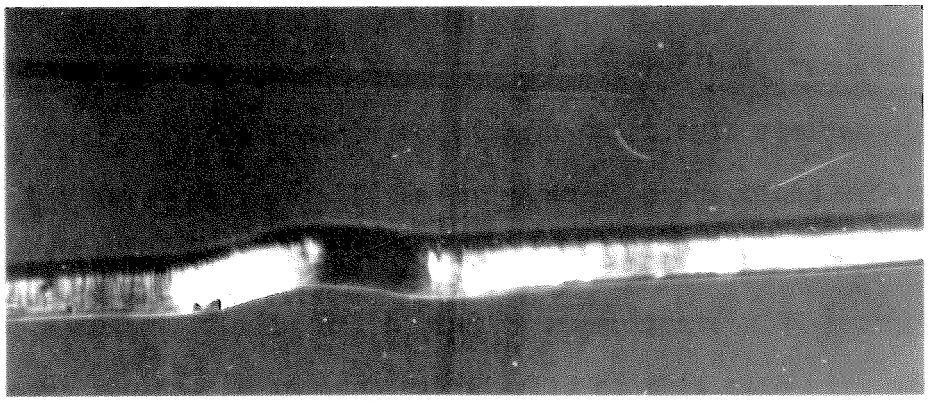
(c) Electron microscopy of hemoglobin·DNA fibers

Figs. 5, 6, 7a show representative features of Hb·DNA fibers as seen in the electron microscope. Large fibers were disrupted (see MATERIALS AND METHODS, section d) in order to obtain material sufficiently small for convenient observation. The large fibers are composed of much smaller fibrillar elements running parallel to the main fiber axis (e.g., Figs. 5, 6). In partially disrupted fibers, branching is observed and the "cleavage" direction runs parallel to the fiber axis (e.g., Fig. 7a). This information is all consistent with the idea that the individual DNA molecules are oriented parallel to the Hb·DNA fiber axis and that the lateral associations between DNA molecules are due to non-covalent linkages,

Figure 4. Birefringence of chicken Hb·DNA fibers. The main fiber axis is a principal direction of refraction. DNA molecules are aligned parallel to the fiber axis (see text). Occasional fibers appear to contain transverse bands which might indicate that ordered regions in a fiber are occasionally interrupted by more disordered regions or that the parallel DNA molecules occasionally bend (as in a crystal dislocation) in a new direction. That the banding is probably not an interference phenomenon is suggested by the finding that the pattern of banding does not change when colored filters are placed over the light source. The Hb:DNA mass ratio of the fibers was 3.4. Magnification (320X).





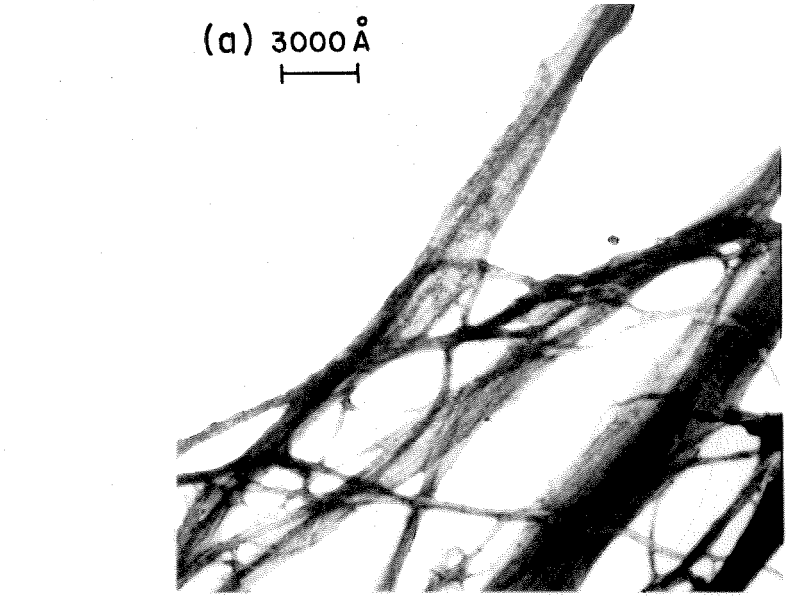


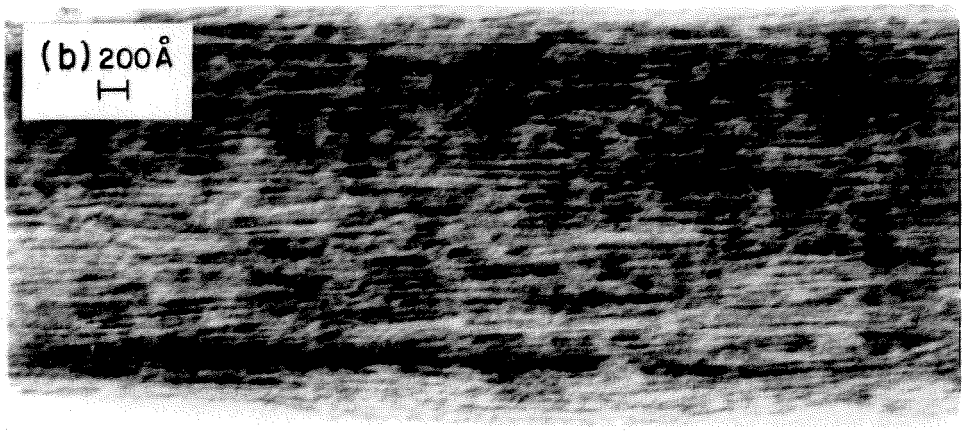
presumably involving protein bridges. In this context it is of interest that fiber formation and growth can be watched in the light microscope; small fibers adhere laterally and the area of association then rapidly grows as the fibers are forcefully pulled together in a zipper-like fashion.

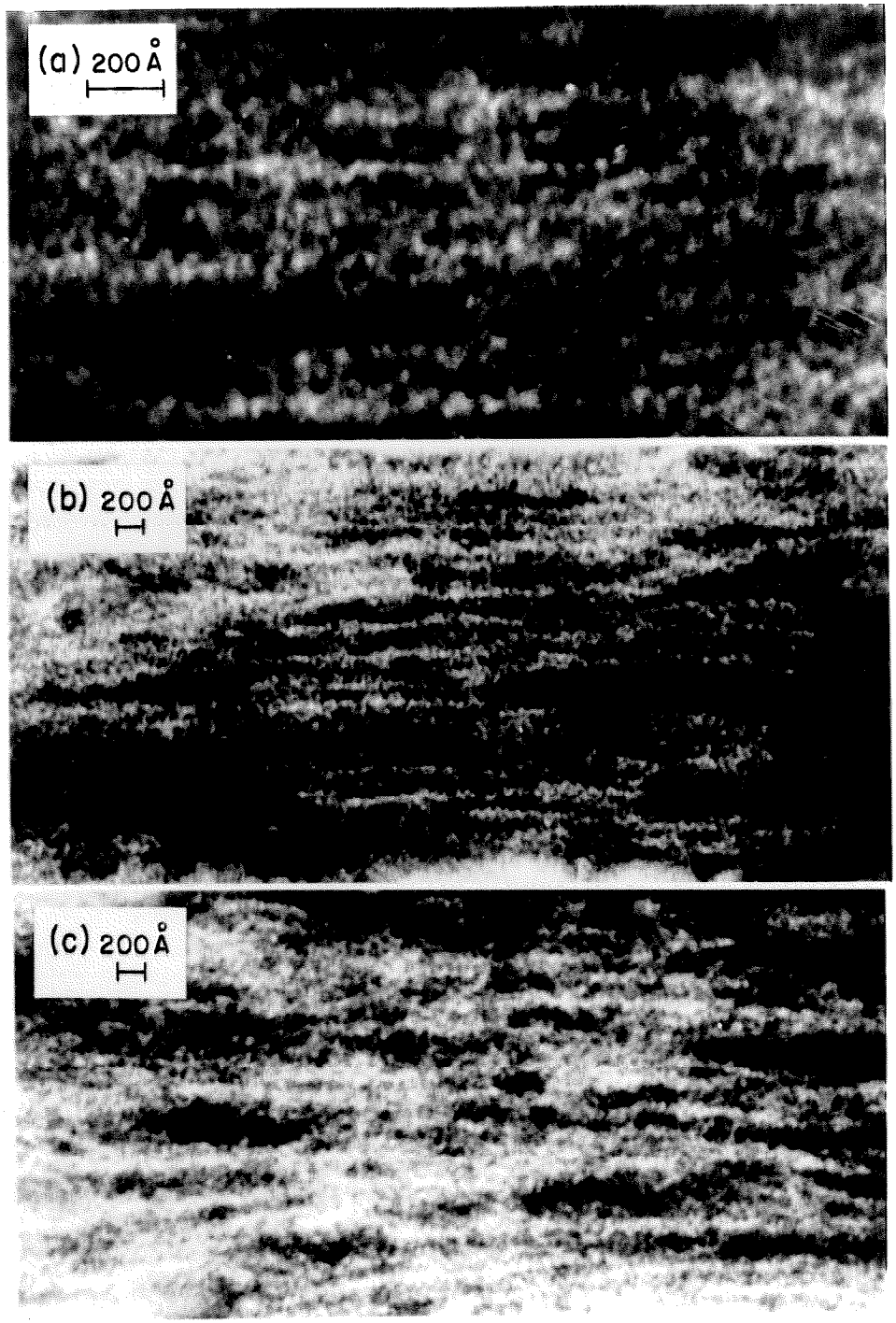
The electron stains (uranyl acetate or phosphotungstic acid) do not deeply penetrate the nucleoprotein fibers; rather, the stains appear to lie primarily in troughs and other irregularities on the fiber surfaces. This conclusion is based on the following information: (i) Although the fibers are rounded as seen by Pt-Pd shadowing (Fig. 7a), the staining is not systematically more intense for the larger fibers (Fig. 5a). (ii) At high magnification one can see fine structure in the fibers (Figs. 5b, 6). If the stain deeply penetrated into the fibers, small objects would probably not be discernible. (iii) When stained grids are briefly washed with water, the stain is mostly removed, as expected if it were merely lying on the fiber surfaces. Consequently, the unstained fibrillar elements seen in the fibers may be identified with nucleoprotein, whereas the dark areas are probably inter-fibril troughs filled with stain.

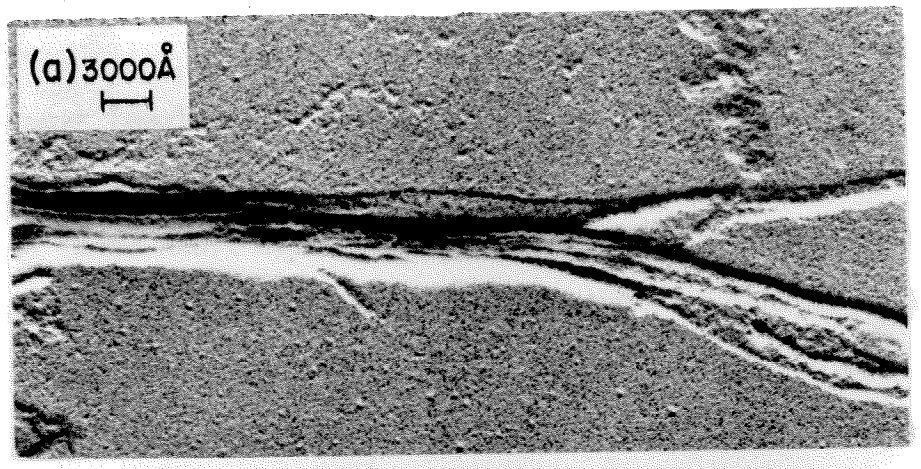
At high magnification, Hb·DNA fibers stained with uranyl acetate (Fig. 5b) appear very different from fibers stained with phosphotungstic acid (Figs. 6). The uranyl acetate stained fibers appear to be composed of parallel fibrils which measure approximately 20 Å thick (Fig. 5b). It seems reasonable to identify fibrils of this size with single DNA molecules. Since no hemoglobin molecules can be seen in fibers stained in this fashion, I infer that uranyl acetate solutions may extract the protein from the surface DNA.

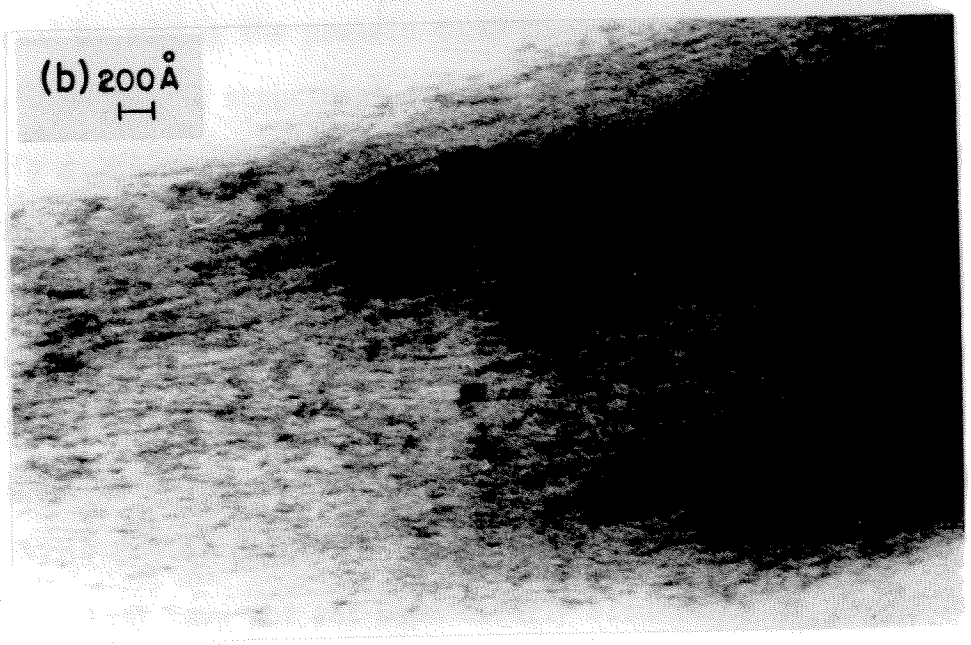
Figures 5, 6 and 7. Electron micrographs of chicken Hb·DNA and horse heart Cy c·DNA fibers. Nucleoprotein fibers were prepared for observation in the electron microscope as described (MATERIALS AND METHODS, section d). Fig. 5: Uranyl acetate-stained Hb·DNA a (X 33,000); b (X 183,000). Fig. 6: Phosphotungstic acid-stained Hb·DNA, a (X 550,000); b and c (X 183,000). Fig. 7: a (Pt-Pd shadowed Hb·DNA, X 20,200); b (uranyl acetate-stained Cy c·DNA, X 194,000).


(a) 3000 Å


(b) 200 Å


(a) 200 Å
(b) 200 Å
(c) 200 Å


(a) 3000Å


(b) 200Å

On the other hand, the surfaces of fibers stained with phosphotungstic acid appear to contain numerous small particles (roughly 35-55 Å) often arranged in rows approximately parallel to the fiber axis (Figs. 6). These particles are the size expected for single hemoglobin molecules (32); I therefore suggest that these globular particles are Hb molecules complexed with the DNA.

(d) Horse heart cytochrome c·DNA fibers

Horse heart cytochrome c forms a soluble complex with DNA in the absence of divalent metal ions (8). In the present study it was found that addition of divalent cation (Mg^{++}, Ca^{++}, or Mn^{++}) to such soluble complexes results in precipitation of insoluble nucleoprotein fibers, strikingly similar in appearance to Hb·DNA fibers. Cy c·DNA fibers can also be formed by adding the protein to mixtures of the metal ion and DNA; fibers used in all structural studies were formed by the latter route and had a Cy c:DNA mass ratio of 3.8 (see MATERIALS AND METHODS, section b for fiber preparation).

Cy c·DNA fibers are negative birefringent ($\Delta n = -0.026$), suggesting an alignment of DNA molecules parallel to the fiber axis. Preliminary studies with the electron microscope are in agreement with this idea, the fibers appearing to consist of laterally associated small fibrils (e.g., Fig. 7b). However particles the size expected for Cy c molecules (roughly 25 Å globules) have not yet been clearly visualized in these fibers.

(e) Polarized visible spectra of hemoglobin·DNA fibers

To determine the orientation of Hb molecules with respect to the nucleoprotein fiber axis, the visible absorption spectrum of the fibers was measured in polarized light. As a control, horse Hb crystals of

known structure (32) were investigated. Horse oxyhemoglobin, methemoglobin, and methemoglobin azide crystals are isomorphous and were all analyzed.

Fig. 8 shows the polarized absorption spectra of horse Hb crystals for light passing through the [001] plane. For methemoglobin, the dichroic ratio (approximately 2.1) is nearly independent of wavelength. Previous polarized absorption studies of horse methemoglobin crystals, viewed also through [001], showed a dichroic ratio independent of wavelength and equal to 2.6 (33). The findings reported here generally substantiate the results of Perutz (33) and are in agreement with the idea that the absorption transition moments lie in the heme planes ($\pi \rightarrow \pi^*$ transitions) and that the heme normals lie roughly in the a direction (see Fig. 1). The fact that the methemoglobin crystal spectra are indistinguishable in shape from the methemoglobin solution spectrum, supports the reliability of the spectroscopic method employed.

Figs. 8b and c show polarized absorption spectra of crystals of oxyhemoglobin and methemoglobin azide, respectively. For these derivatives, the dichroic ratios vary more with wavelength than in the case of methemoglobin crystals; however, also in these derivatives, the dichroic ratio is almost uniformly greater than 1.0, consistent with the idea that the absorption is mainly due to $\pi \rightarrow \pi^*$ excitation. In the methemoglobin azide crystals (Fig. 8c), the 572 mμ shoulder in the absorption spectrum appears relatively more pronounced when the incident light is polarized perpendicular, rather than parallel to b. In the same crystals there also appears to be a weak absorption band (620 mμ) which is polarized perpendicular to b.

Figure 8. Polarized absorption spectra of horse hemoglobin crystals. Spectra were taken with light shone through the [001] plane and polarized parallel and perpendicular to the b axis. (a) Methemoglobin, (b) oxyhemoglobin, (c) methemoglobin azide —o—o—, electric vector parallel to b (" ∥ b"); —o—o—, electric vector perpendicular to b (" ⊥ b"); --□---□-- dichroic ratio.

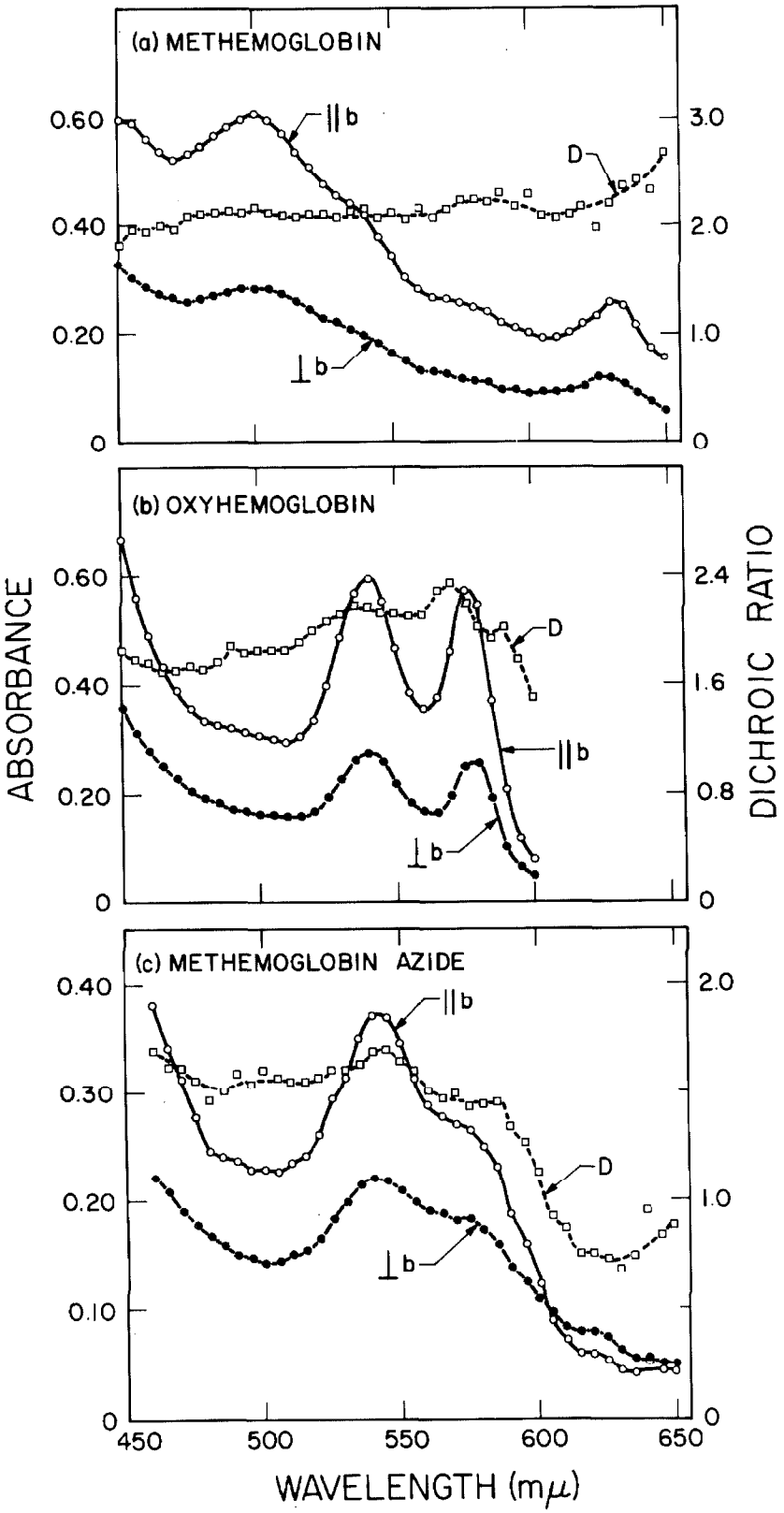
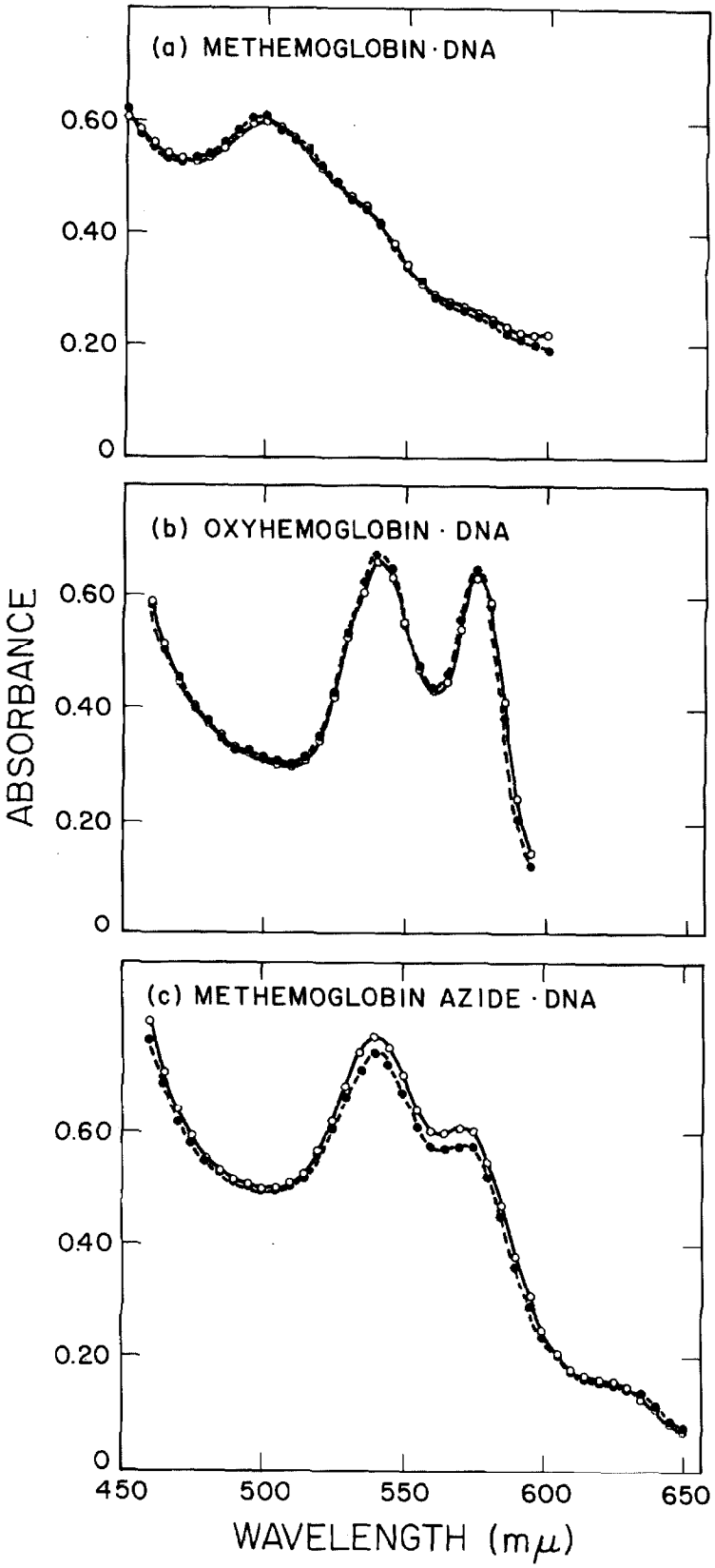

(a) METHEMOGLOBIN
0.60
0.40
0.20
0
3.0
2.0
1.0
0
||b
⊥b
D
(b) OXYHEMOGLOBIN
0.60
0.40
0.20
0
2.4
1.6
0.8
0
D
||b
⊥b
ABSORBANCE
DICHROIC RATIO
(c) METHEMOGLOBIN AZIDE
0.40
0.30
0.20
0.10
0
2.0
1.0
0
||b
D
⊥b
450
500
550
600
650
WAVELENGTH (mμ)

Figure 9. Polarized absorption spectra of chicken Hb·DNA fibers. Spectra were taken with light polarized parallel and perpendicular to the fiber axis (a principal axis of refraction). (a) Methemoglobin·DNA, (b) Oxyhemoglobin·DNA, (c) Methemoglobin azide·DNA. -o-o-, electric vector parallel to fiber axis; --o--o-- , electric vector perpendicular to fiber axis.


(a) METHEMOGLOBIN·DNA
(b) OXYHEMOGLOBIN · DNA
(c) METHEMOGLOBIN AZIDE · DNA
ABSORBANCE
0.60
0.40
0.20
0
450
500
550
600
650
WAVELENGTH (mμ)

Figs. 9 show the polarized absorption spectra of chicken Hb·DNA fibers. Fiber absorbance does not significantly depend on the direction of polarization of the incident light. This result is compatible with the idea that the Hb molecules are randomly oriented with respect to the fiber direction. However, restricted orientation of the Hb molecules in certain positions with respect to the fiber axis is not excluded by the data (DISCUSSION, section a).

The visible spectra of the Hb·DNA fibers are indistinguishable from spectra of the pure Hb derivatives in solution. Since the spectrum of Hb is affected by changes in the folding of the polypeptide chains (34, 35), it may be concluded that there is no gross structural rearrangement of the Hb molecules upon combination with DNA.

(f) Polarized visible spectra of cytochrome c·DNA fibers

Crystals of oxidized and reduced horse heart cytochrome c are not isomorphous (17); accordingly, reduction of the iron causes disruption of the ferricytochrome c crystals (the latter crystals were a gift from Dr. R. E. Dickerson and are described in MATERIALS AND METHODS, section e).

Fig. 10 shows the polarized visible spectrum of the ferricytochrome c crystals for light shone through the [100] or [010] planes. The dichroic ratio varies with the wavelength (2.0 at 570 mμ, and 1.2 at 530 mμ), the shoulder in the absorption spectrum at 555 mμ being most pronounced when the electric vector is polarized parallel to c.

Fig. 11a and b shows the polarized visible spectra of horse heart Cy c·DNA fibers. The absorbance of these fibers depends significantly on the direction of polarization of the incident light. In the case of both the oxidized and reduced Cy c derivatives, the dichroic ratio is

Figure 10. Polarized absorption spectra of horse heart ferricytochrome c crystals. Light was shone through [100] or [010] which are optically equivalent due to crystal symmetry. —o—o—, electric vector parallel to c; —o—o—, electric vector perpendicular to c;--□--□-- , dichroic ratio.

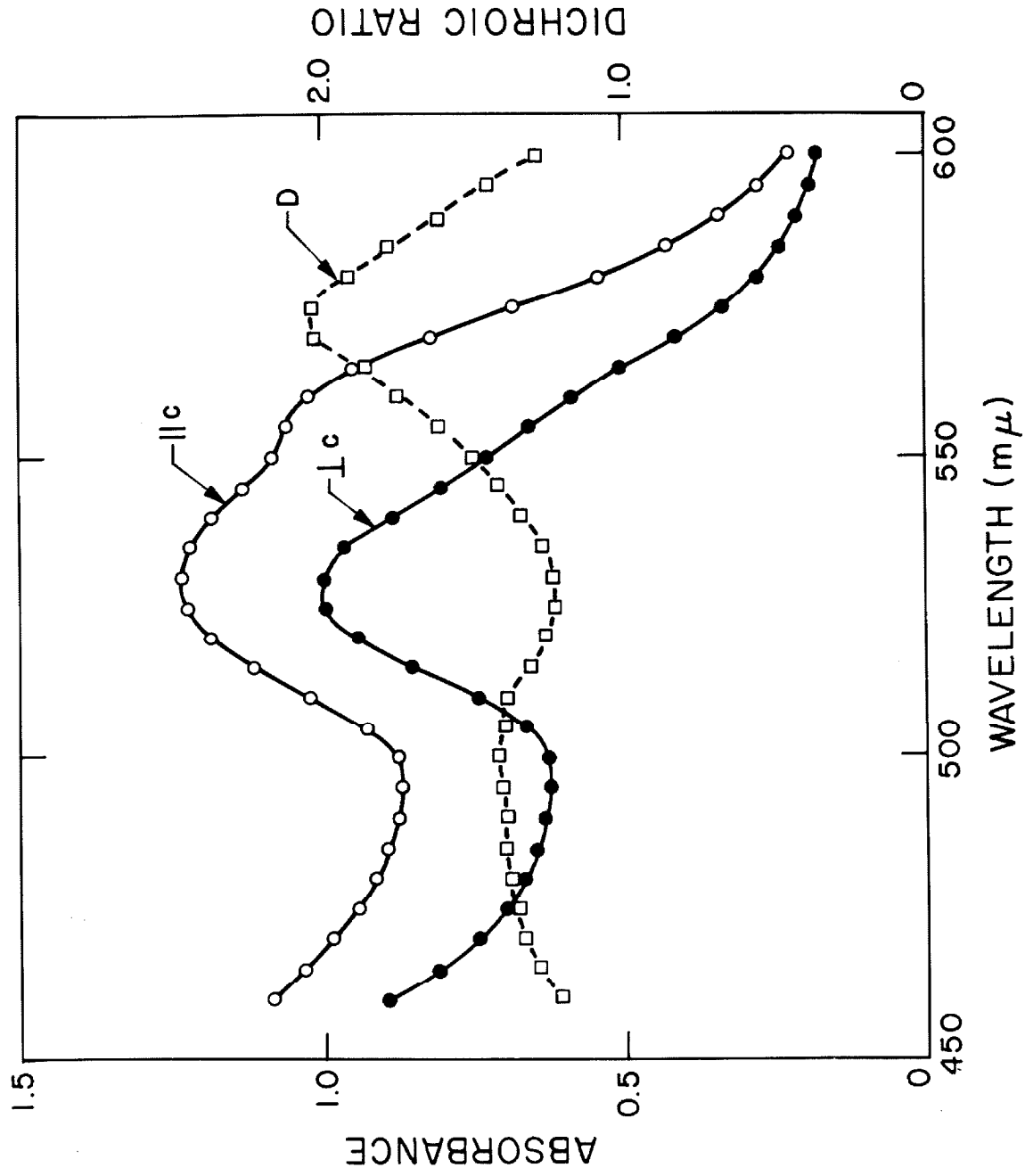
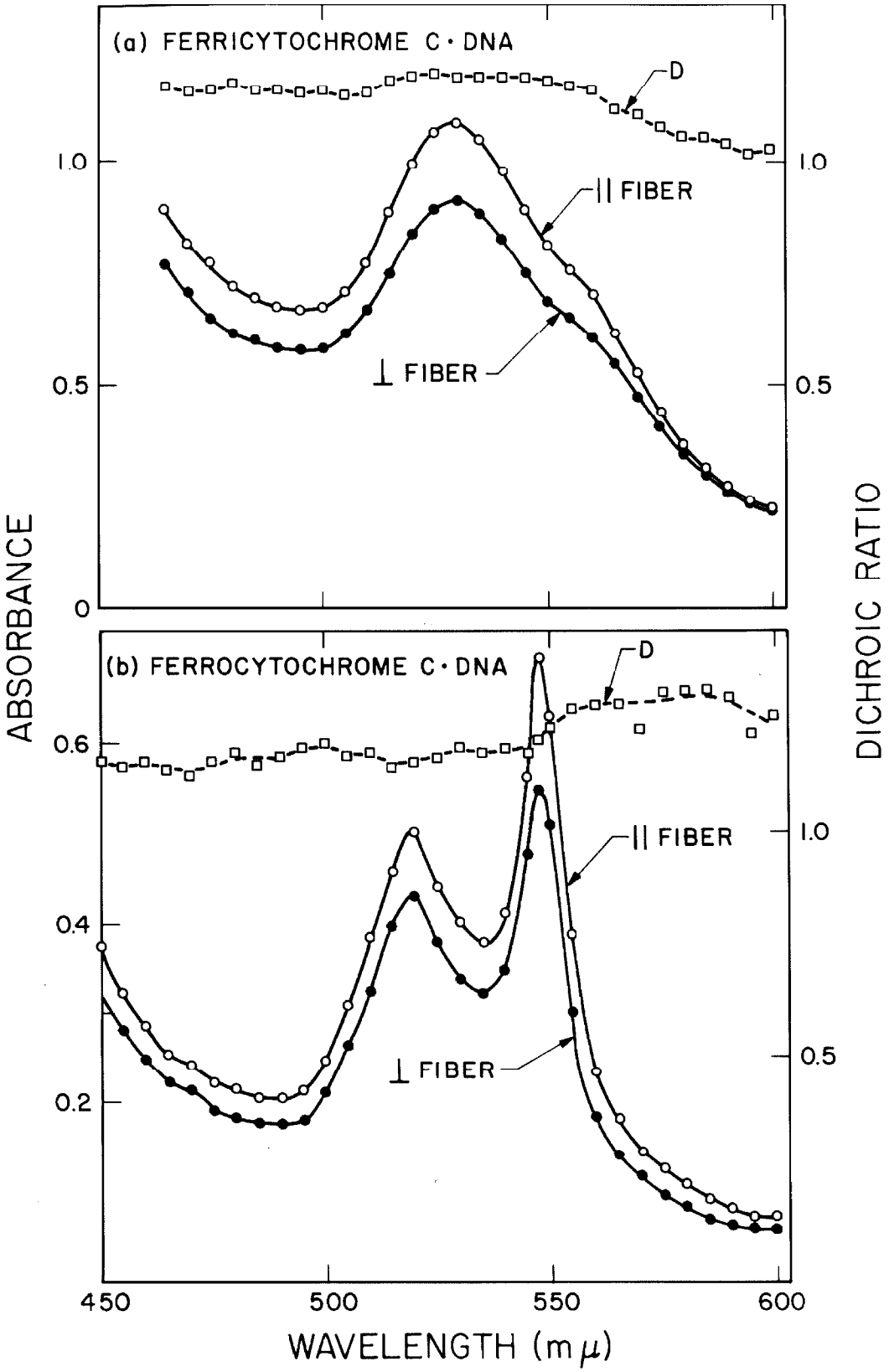

DICHROIC RATIO
2.0
1.0
0
D
∥c
⊥c
1.5
1.0
0.5
0
ABSORBANCE
450
500
550
600
WAVELENGTH (mμ)

Figure 11. Polarized absorption spectra of horse heart Cy c·DNA fibers. The 550 mμ band of the ferrocytochrome c spectrum is extremely narrow (9) and consequently absorbance here is very sensitive to spectral band width. (a) ferricytochrome c·DNA, (b) ferrocytochrome c·DNA. -o—o-, electric vector parallel to fiber axis; -o—o- , electric vector perpendicular to fiber axis; -□--□-, dichroic ratio.


(a) FERRICYTOCHROME C · DNA
D
|| FIBER
⊥ FIBER
1.0
0.5
0
(b) FERROCYTOCHROME C · DNA
D
|| FIBER
⊥ FIBER
0.6
0.4
0.2
1.0
0.5
ABSORBANCE
DICHROIC RATIO
450
500
550
600
WAVELENGTH (mμ)

greater than 1.0, suggesting that the heme group normals tend to lie perpendicular to the nucleoprotein fiber axis.

The shape of the visible spectra of the Cy c·DNA fibers are not significantly different from spectra of the pure Cy c derivatives in solution [e.g., see Margoliash and Frohwirt (9)]. Since the Cy c spectrum is affected by changes of configuration of the polypeptide chain (35), this result suggests that the protein structure is not grossly altered upon binding to DNA. Furthermore, although ferrocytochrome c molecules undergo spontaneous oxidation when slightly denatured (17), it was observed that the ferrocytochrome c molecules bound into the nucleoprotein fibers do not undergo spontaneous oxidation when placed in solutions exposed to air.

DISCUSSION

(a) Polarized absorption spectra of the protein crystals and nucleoprotein fibers

The hemoproteins and other porphyrin compounds all have similar visible spectra, suggesting that the absorption bands result from similar electron excitations (19, 36). It has been clearly shown, in fact, that the absorption spectrum of the porphyrins between 470-600 mμ is due to a pair of approximately degenerate (i.e., equal energy) and perpendicularly polarized electronic transitions of the $\pi \rightarrow \pi^*$ class, each being split due to the several vibrational levels of the excited molecules (these associated vibronic bands are termed the 0-0, 0-1, etc., in order of their increasing energy) (19, 37). The transition moments for such absorption are polarized in the porphyrin plane.

These two electronic transitions will be strictly degenerate

only if the porphyrin has square symmetry, all four pyrrole rings being electronically equivalent (19). Clearly, if these transitions are not degenerate, then the dichroic ratio can be expected to vary with the wavelength, the spectrum being anisotropic in the porphyrin plane. It is known that porphyrin compounds can be rendered asymmetric by substitution of side chains (e.g., vinyl groups) onto the porphyrin ring; asymmetry can also be introduced as a consequence of double bonding between d-orbitals of the metal and π-orbitals of the tetrapyrrole (37, 38). This latter type of interaction is known to depend greatly on the oxidation state of the metal and also on the other ligands (e.g., O_2, H_2O, CN^-, imidazole) attached to the metal (37, 38). Of the different hemoprotein crystals examined, horse methemoglobin crystals (Fig. 8a) are exceptional in that the dichroic ratio between 470-600 mμ is nearly constant, as previously shown by Perutz (33). Presumably, in this protein the electronic distribution in the tetrapyrrole rings is more symmetrical than in the other hemoglobin derivatives, possibly due to the relatively weak double bonding between the iron and the porphyrin as suggested by paramagnetic resonance studies (38). In the other hemoproteins examined, however, the variation of D between 470-600 mμ suggests that the electronic transitions involved are not strictly degenerate.

As mentioned previously (MATERIALS AND METHODS, section g), when these $\pi \rightarrow \pi^*$ transitions are degenerate then the heme group can be expected to act as an "ideal two-dimensional absorber" and the absorption dichroism will closely agree with the equations described. In cases of derivatives for which D varies with wavelength, the equations given can be expected to provide only approximate estimates of the orientations of

TABLE II

Estimates of Angles Between Heme Normals and the Principal Axes of Refraction

Material	Expected dichroism for ideal absorber*[a]	Definition of angle θ	Observed average D 470-600 mμ	Calculated θ	Actual θ
Horse Met Hb crystals	$D=\tan^2\theta$	Between heme normal and b-axis	2.1	56°	58° for α-heme and 57° for β-heme*[b]
Horse Oxy Hb crystals			1.9	54°	
Horse Met Hb azide crystals			1.5	51°	
Chicken Hb·DNA fibers (Met Hb, Oxy Hb, and Met Hb azide)	no simple relationship	——	1.0*[c]	——	——
Horse heart Ferri Cy c crystals	$D=\frac{2\sin^2\theta}{1+\cos^2\theta}$	Between heme normal and c-axis	1.4-1.6	65-70°	70° *[d]
Horse heart Cy c·DNA fibers (Ferri Cy c and Ferro Cyc)	$D=\frac{2\sin^2\theta}{1+\cos^2\theta}$	Between heme normal and fiber axis	1.2	60°	unknown

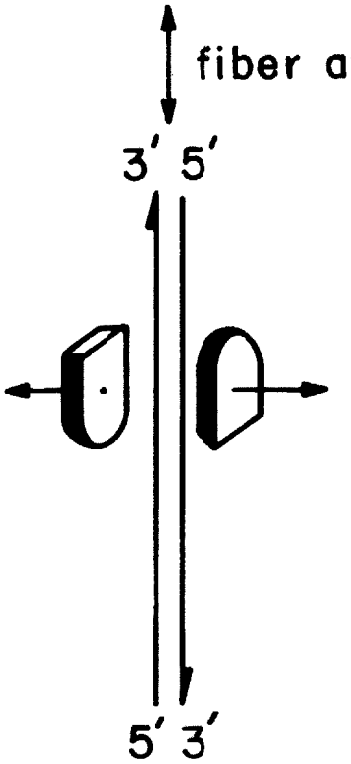
a. The method of obtaining these equations is described in MATERIALS AND METHODS, section g. Assumptions made in the derivations are described in the text.

b. Heme orientation in the horse hemoglobin crystals based on Ingram, Gibson, and Perutz (18).

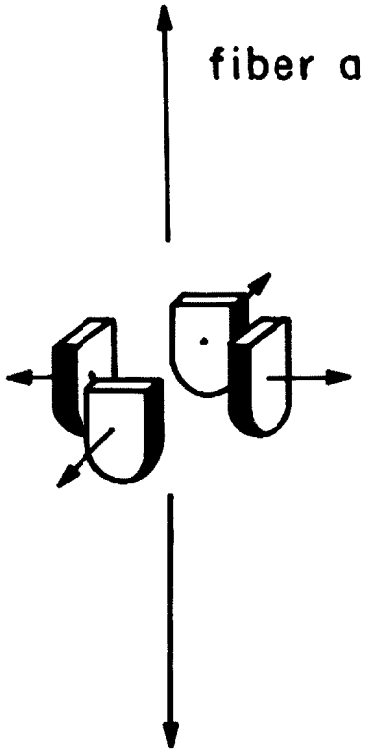
c. The absorbance of Hb·DNA fibers is independent of the polarization directions. No unique interpretation of this is possible, as the result might reflect random orientation of Hb molecules, unique orientation such that the transition moments lie on the average at 45° with respect to the fiber axis, or even several stable orientations with cancelling dichroism.

d. Independently of the work described here, X-ray diffraction studies by R. E. Dickerson (41) gave the angle indicated; and Eaton and Hochstrasser (42) recently obtained polarized absorption spectra of Cy c crystals in close agreement with the data described here and estimated the angle as 72±3°.

Figure 12. Likely degeneracies in the orientations of protein molecules with respect to a DNA fiber axis as based on the known symmetry properties of DNA (60). The structures with arrows represent protein molecules having DNA-binding sites on the face opposite the arrow. A chromophoric prosthetic group might conceivably have any orientation with respect to the DNA-binding site under consideration. In (a), it is assumed that the fibers have no polarity. In (b), it is assumed that the fibers are isotropic in cross-section.



(a) 180° rotation around axis perpendicular to fiber due to antiparallel orientation of the two strands composing the DNA double helix.



(b) Rotational freedom around the fiber axis due to the helical nature of the DNA molecules.

heme groups. Table II presents calculated estimates of the orientations of heme groups in the different materials studied, as based on the observed dichroic ratios and on the dichroism expected for ideal two-dimensional absorbers. It is of interest that the estimated angles agree fairly well with the actual angles for the different derivatives of the horse hemoglobin crystals, and for the ferricytochrome c crystals.

In the chicken Hb·DNA and horse heart Cy c·DNA fibers, the DNA molecules appear to be aligned approximately parallel to the fiber axes (RESULTS, sections b, c, d). Since the protein molecules interact with DNA, their orientation in the fibers is likely to reflect the symmetry properties and orientation of the DNA. In Fig. 12 are depicted some equally favorable protein orientations with respect to the fiber axis as based on the symmetry of DNA. The type of position degeneracy shown in Fig. 12a can have no effect on the light absorption by a chromophoric protein (rotation of such a protein by 180° around the fiber axis will not alter the angle between a transition moment and the fiber axis). However, the type of position degeneracy shown in Fig. 12b (making the fiber axis a rotational axis of symmetry) would strongly influence the light absorbing properties of the fibers. In the calculations presented in Table II, it was assumed that such position degeneracy exists in the nucleoprotein fibers. It should be recognized, however, that protein orientation in the fibers might also be strongly influenced by protein-protein interactions and other factors not considered in Fig. 12.

(b) Function of divalent metal ions in tightly-packed nucleoprotein structures

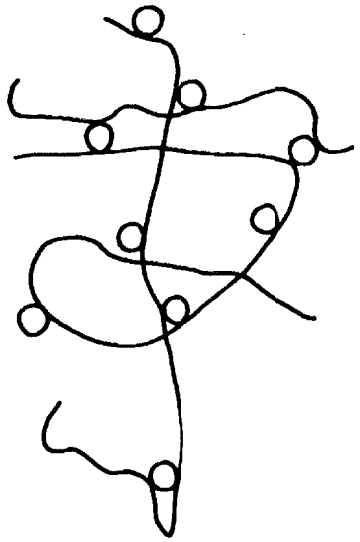
The tightly-packed Hb·DNA fibers studied here form only in the

presence of divalent metal ions (RESULTS, section a); in the absence of these cations, the Hb·DNA mixtures form only non-fibrous aggregates. Since the structurally related Cy c·DNA fibers also form only in the presence of divalent cations (RESULTS, section e), it seems likely that the divalent metal ions play a common role in both of the nucleoprotein fibers. It is well known that the divalent metals used (Mg^{++}, Ca^{++}, Mn^{++}) bind strongly to nucleic acids, probably interacting with the negatively charged phosphate residues of the backbone (24, 25, 39, 40, 43, 44). This suggests that the divalent metal ions might act by virtue of their binding to the nucleic acids rather than to the protein moieties.

Fig. 13 shows a model of the divalent cation-induced formation of Hb·DNA fibers. This model contains the following basic ideas: (1) Electrostatic repulsion in the absence of divalent cations inhibits close association and tight packing of DNA chains (a "DNA chain" is considered to be a double helical segment of a DNA molecule; association between DNA chains might therefore be either inter- or intramolecular). (2) Hb molecules have at least two "sites" able to interact with, and thereby to crosslink, adjacent DNA chains. The noncovalent crosslinking of DNA chains is favored when interchain electrostatic repulsion is reduced by divalent cation addition. (3) Parallel DNA orientation and fiber formation is a natural consequence of the crosslinking of DNA chains, the parallel arrangement allowing more crosslinks per length of DNA than any other simple structure.

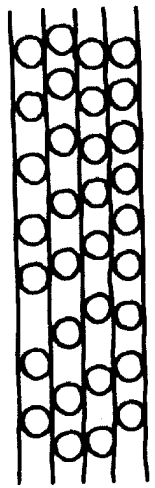
In the model shown in Fig. 13, the protein molecules cooperatively hold adjacent DNA chains in a favorable binding position; this is consistent with the observed cooperative binding of Hb molecules to the

Figure 13. Possible role of divalent metal ions in tightly-packed nucleoprotein structures. The illustrations refer to Hb·DNA fiber formation (see RESULTS, section a). The circles represent Hb molecules and the lines represent double helical DNA molecules. However the basic model may be relevant to other nucleoproteins (see text). The essential changes accompanying the divalent metal ion-dependent structural transition are printed below the illustrations.



(a) Absence of Mg^{++}, Ca^{++}, or Mn^{++}

- Turbidity develops, but no fibers form.
- Probably some DNA chains are crosslinked by protein, but interchain electrostatic repulsion prevents tight-packing of chains.



(b) Presence of Mg^{++}, Ca^{++}, or Mn^{++}

- The ions bind to the nucleic acid phosphates, thereby reducing interchain electrostatic repulsion.
- Maximum crosslinking of DNA results in fiber formation (the fiber structure has more crosslinks per length of DNA than any other simple structure).
- Protein molecules are cooperatively bound into the nucleoprotein structure.

fibers (RESULTS, section a). A bidentate ligand (in this case a ligand having two binding sites for DNA) would be expected to bind many times more strongly to the fibers (where both sites could simultaneously interact with DNA chains) than to DNA molecules in solution (where presumably only one site could interact with DNA). Therefore, the binding of such ligands to DNA should be weakened in conditions where extensive cross-linking is prevented (e.g., in the absence of divalent metal ions); this might explain the increased level of Hb binding which initially accompanies the divalent cation-induced fiber formation (RESULTS, section a). The decline of Hb:DNA ratio as the $MgCl_2$ concentration is further raised (Fig. 2) could be due to various secondary effects such as competition between the Mg^{++} and Hb for sites on the DNA. The dyad symmetry and subunit structure of Hb (10) is consistent with the requirement that this protein must have at least two binding sites. Furthermore, the existence of strong electrostatic repulsion between DNA chains in low ionic strength media in the absence of divalent cations, even when the chains are separated by 50 Å (the approximate diameter of a Hb molecule), is consistent with theoretical studies concerning electrostatic fields surrounding cylindrical polyions (44).

A second model by which the effects of the divalent cations might be explained is that these ions themselves form bridges between adjacent DNA chains, thereby causing nucleoprotein aggregation. This latter explanation is unlikely for several reasons. In the first place, this model explains neither the role of the protein nor the cooperative binding of the protein molecules into the fibers. In the absence of protein, it may be noted, divalent cations (Ca^{++}, Mg^{++}, or Mn^{++}) do not precipitate DNA.

In addition, from the composition of typical hydrated Hb·DNA fibers used in this study (DNA:protein:H_2O present in the mass ratio 1:3.4:2.1), it can be calculated that the average distance between adjacent DNA chains is roughly 46 Å (i.e., approximately 30 Å between the outer surfaces of the two adjacent chains); therefore, metal ion bridges, if they exist, could only occur infrequently between adjacent nucleic acid chains.

One major feature of the model shown in Fig. 13 is the concept that the divalent metal ions influence the Hb·DNA interaction as a result of their binding to the nucleic acid rather than to the protein moieties. This suggests that other proteins having at least two binding sites might also crosslink nucleic acid chains most readily in the presence of at least low levels of divalent cations. That the role of divalent metal ions as elaborated in Fig. 13, is indeed not unique to the Hb·DNA interaction, and may in fact be relevant to many nucleoproteins, is consistent with the information below.

(i) Treatment of animal or plant cell chromosomes in low ionic strength solutions causes nucleoprotein swelling only if the divalent metal ion concentration is very low (e.g., less than about 5×10^{-4} M $MgCl_2$). This has been reported in a wide variety of systems including Drosophila melanogaster salivary glands and mitotic and meiotic cells from grasshopper testis (5), chicken erythrocytes (45), Vicia faba root cells (46), isolated metaphase chromosomes from mouse leukemia cells, Chinese hamster cells, and HeLa cells (6, 47, 48), and sea urchin sperms (5, 7). In all cases, the native condensed morphology of the chromosomes appears to depend critically upon the divalent cation concentration.

Soluble chromatin, or nucleohistone, can only be obtained by shearing chromosomes in the swollen state (i.e., in low ionic strength solutions in the absence of divalent cations) (49, 50); the soluble

chromatin so obtained is precipitated by low levels (c a, 0.001 - 0.002 M $MgCl_2$) of divalent metal ions (51, 52). X-ray diffraction studies of intact nuclei from chicken erythrocytes and calf thymocytes suggested a model of in vivo chromosomes in which histone bridges crosslink parallel oriented DNA chains into a large three-dimensional aggregate (53, 54). That the histone bridges are labile and are broken in low ionic strength media as a consequence of electrostatic repulsion between DNA chains was suggested by Wilkins (53). Histone crosslinking of chromosomal DNA chains was also suggested by Luzzatti and Nicolaieff (55) on the basis of their X-ray diffraction studies.

Studies of heterochromatin and euchromatin (1) and of "puffing" in Dipteran salivary gland chromosomes (2), suggest that the tightness of packing of chromosomal DNA chains might be an important factor in the regulation of genetic activity. If the difference between tightly-packed and swollen chromatin is indeed a matter of histone crosslinking, then the factors regulating such crosslinking are of obvious biological importance. According to the model shown in Fig. 13, one factor of importance in the heterochromatin to euchromatin transition might be the electrostatic repulsion between adjacent DNA chains; such electrostatic repulsion might be increased in a chromosomal region by removal of metal ions from the DNA, by removal of histones from the DNA, or by addition of polyanions to the DNA. The cooperative nature of the protein crosslinking of nucleic acid chains (Fig. 13) leads to the expectation that neighboring histone crosslinks would tend to break synchronously, giving rise to a localized "melting" phenomenon; consequently, small changes in the environment might induce large changes of chromosomal structure.

(ii) It is well known that magnesium ions are necessary for the structural integrity of ribosomal particles (e.g., ref. 3). The association of smaller ribosomal particles into faster-sedimenting components appears to be critically dependent upon magnesium concentration.

Only about 12% of the phosphate groups in E. coli ribosomes are neutralized by the basic groups of the heterogeneous subunit proteins; approximately 0.1 Mg/P are very strongly bound to the ribosomes whereas 0.3 Mg/P are bound more loosely (3). In concentrated CsCl solutions, protein subunits dissociate from ribosomes (56, 57). Furthermore, the protein dissociation from ribosomes is a cooperative stepwise process in which groups of protein molecules are released simultaneously (57), the extent of dissociation increasing as Mg-ions are removed. In the latter studies, the sedimentation coefficients of the partially dissociated ribosomal particles were lowered with respect to the complete particles; however, it was unclear to what extent structural changes of the nucleoprotein "core" contributed to this reduction of sedimentation coefficient. Methods were subsequently described (4) by which Mg-ions could be removed from ribosomal particles without significant release of subunit proteins. In these conditions, removal of Mg clearly caused considerable swelling of the nucleoprotein; it was concluded that "the deciding factor resulting in the conversion of ribosomal particles to less compact structures is the removal of endogenous magnesium" (4).

I suggest that these observations might possibly be understood by assuming that, in the presence of Mg-ions, protein subunits crosslink adjacent regions of the tightly-coiled ribosomal RNA (see Fig. 13). The RNA molecule coils around in such a way that adjacent segments of the nucleic acid tend to lie parallel, thereby allowing maximum crosslinking;

this picture agrees with the model of the 50S E. coli ribosomal particle constructed by Hart (58) on the basis of electron microscope observation. As the Mg^{++} level is reduced, electrostatic repulsion induces separation of adjacent chains due to breaking of the protein crosslinks; the weakest coils break first and the group of protein subunits involved is then only weakly bound to the particles (being bound to only one RNA chain). These weakly-bound proteins dissociate from the nucleoprotein core in concentrated CsCl solutions.

As compared with divalent metal ions, monovalent cations (e.g., Na^+, K^+, NH_4^+) exert only a relatively weak influence not only on the nucleoprotein structures discussed here, but also on many reactions of nucleic acids, including thermal denaturation of double stranded nucleic acids (39, 43), and binding of some catonic dyes to nucleic acid phosphates (25, 59). Such monovalent cations probably don't strongly bind onto the phosphates, but rather form a relatively loose atmosphere of counterions in the surrounding solution (44). Therefore, if two nucleic acid chains were packed together such that very little solution were interposed between them (either by very close association or by having a larger inter-chain space completely occupied by protein), then the concentration of monovalent salt in the surrounding solution would be expected to have little influence on the electrostatic repulsion between the chains. In such a case, divalent metal ions might exert a primary influence on the stability of protein crosslinks (see Fig. 13) even at relatively high concentrations of monovalent salts.

REFERENCES

1. V. C. Littau, V. G. Allfrey, J. H. Frenster, and A. E. Mirsky. Proc. Natl. Acad. Sci. U.S. 52, 93 (1964).

2. H. Kroeger and M. Lezzi. Ann. Rev. Entomology 11, 1 (1966).

3. A. Goldberg. J. Mol. Biol. 15, 663 (1966).

4. L. P. Gavrilova, D. A. Ivanov, and A. S. Spirin. J. Mol. Biol. 16, 473 (1966).

5. D. Mazia. Proc. Natl. Acad. Sci. U.S. 40, 521 (1954).

6. C. E. Somers, A. Cole, and T. C. HSU. Exptl. Cell. Res., Suppl. 9, 220 (1963).

7. A. J. Solari. Proc. Natl. Acad. Sci. U.S. 53, 852 (1965).

8. B. Olivera. Ph.D. Thesis, California Institute of Technology, Pasadena, California (1966).

9. E. Margoliash and N. Frohwirt. Biochem. J. 71, 570 (1959).

10. A. Saha. Biochim. Biophys. Acta 93, 573 (1964).

11. M. F. Perutz and J. M. Mitchison. Nature 166, 677 (1950).

12. J. L. Stoves. in Fibre Microsopy, National Trade Press. London (1957) p. 61.

13. M. Murayama, R. A. Olson, and W. H. Jennings. Biochim. Biophys. Acta 94, 194 (1965).

14. M. F. Perutz. Proc. Roy. Soc. A. 191, 83 (1947).

15. R. F. Stewart and N. Davidson. J. Chem. Phys. 39, 255 (1963).

16. E. A. Wood. in Crystals and Light. D. Van Nostrand Co. Inc., Princeton N.J. (1964)

17. E. Margoliash and A. Schejter. Advanc. Protein Chem. 21, 113 (1966).

18. D. J. E. Ingram, J. F. Gibson, and M. F. Perutz. Nature 178, 906 (1956).

19. J. R. Platt. in Radiation Biology III, ed. A Hollaender. McGraw-Hill Book Co., Inc., New York (1956) p. 71.

20. T. H. J. Huisman, J. M. Schillhorn Van Veen, J. M. Dozy and C. M. Nechtman. Biochim. Biophys. Acta 88, 352 (1964).

21. C. Manwell, C. M. A. Baker, J. D. Roslansky, and F. Foght, Proc. Natl. Acad. Sci. U.S. 49, 496 (1963).

22. G. Matsuda and H. Takei, J. Biochem. 54, 156 (1963).

23. K. Hashimoto and F. H. Wilt, Proc. Natl. Acad. Sci. U.S. 56, 1477 (1966).

24. J. Shack and B. S. Bynum, Nature 184, 635 (1959).

25. R. F. Steiner and R. F. Beers, in Polynucleotides, Elsevier Publishing Co., Amsterdam (1961) p. 290.

26. G. L. Eichorn and P. Clark, Proc. Natl. Acad. Sci. U.S. 53, 586 (1965).

27. R. Jensen and N. Davidson, Biopolymers 4, 17 (1966).

28. B. Singer and H. Fraenkel-Conrat, Biochemistry 1, 852 (1962).

29. M. F. Maestre and R. Kilkson, Biophys. J. 5, 275 (1965).

30. H. O. E. Pattri, Z. Zellforsch. 16, 83 (1932).

31. S. Inoue and H. Sato, Science 136, 1122 (1962).

32. M. F. Perutz, J. Mol. Biol. 13, 646 (1965).

33. M. F. Perutz, Nature 143, 731 (1939).

34. E. M. Jope, in Haemoglobin, eds. F. J. W. Roughton and J. C. Kendrew, Butterworth Scientific Publications, London (1949) p. 205.

35. K. Kaziro and T. Tshushima, in Haematin Enzymes, eds. J. E. Falk, R. Lemberg, and R. K. Morton, Pergamon Press, London (1959) p. 80.

36.

36. J. E. Falk and D. D. Perrin, in Haematin Enzymes, eds. J. E. Falk, R. Lemberg and R. K. Morton, Pergamon Press, London (1959) p. 56.

37. R. J. P. Williams, Chem. Rev. 56, 299 (1956).

38. L. E. Orgel, in Haematin Enzymes, eds. J. E. Falk, R. Lemberg, and R. K. Morton, Pergamon Press, London (1959) p. 1.

39. W. F. Dove and N. Davidson, J. Mol. Biol. 5, 467 (1962).

40. G. Felsenfeld and S. Huang, Biochim, Biophys. Acta 34, 234 (1959).

41. R. E. Dickerson, Manuscript in preparation (1967).

42. W. A. Eaton and R. M. Hochstrasser, J. Chem. Phys., in the press (1967).

43. G. Felsenfeld and A. Rich, Biochim. Biophys. Acta 26, 457 (1957).

44. A. Katchalsky, Biophys. J. 4 (part 2), 9 (1964).

45. H. G. Davies and M. Spencer, J. Cell Biol. 14, 445 (1962).

46. B. B. Hyde, Genetics 41, 648 (1956).

47. M. Chorazy, A. Bendich, E. Borenfreund, and D. J. Hutchinson, J. Cell Biol. 19, 59 (1963).

48. J. Huberman, personal communication.

49. G. Zubay and P. Doty, J. Mol. Biol. 1, 1 (1959).

50. K. Marushige and J. Bonner, J. Mol. Biol. 15 160 (1966).

51. J. Bonner and R. C. Huang, Biochem. Biophys. Res. Comm. 22, 211 (1965).

52. B. P. Sonnenberg and G. Zubay, Proc. Natl. Acad. Sci. U.S. 54, 415 (1965).

53. M. H. F. Wilkins, in Nucleoproteins, ed. R. Stoops, Interscience Publishers, New York (1959) p. 45.

54. M. H. F. Wilkins, G. Zubay, and H. R. Wilson, J. Mol. Biol. 1, 179 (1959).

55. V. Luzatti and A. Nicolaieff, J. Mol. Biol. 1, 127 (1959).

56. M. Meselson, M. Nomura, S. Brenner, C. Davern, and D. Schlessinger, J. Mol. Biol. 9, 696 (1964).

57. M. I. Lerman, A. S. Spirin, L. P. Gavrilova, and V. F. Golov, J. Mol. Biol. 15, 268 (1966).

58. R. G. Hart, Proc. Natl. Acad. Sci. U.S. 53, 1415 (1965).

59. M. J. Waring, J. Mol. Biol. 13, 269 (1965).

60. R. Langridge, D. A. Marvin, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins, and L. D. Hamilton, J. Mol. Biol. 2, 38 (1960).

ABBREVIATIONS

h	hour
min	minute
37°	37° centigrade
µ	micro
C	curie
g	gram
ml	milliliter
mg	milligram
pg	picogram
rev./min	revolutions per minute
meq/g	milliequivalents per gram
mA	milliamps
µl	microliters
N.A.	numerical aperture
cpm	counts per minute
dpm	disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid, disodium salt
Tris	Tris (hydroxymethyl) aminomethane
concn.	concentration
dry wt.	dry weight
mol. wt.	molecular weight
µC/µM	microcuries per micromole
O.D.	optical density
Asp	aspartic acid
Phe	phenylalanine
Glu	glutamic acid
Thr	threonine
Leu	leucine
Ileu	isoleucine
Val	valine
Ser	serine
Ala	alanine
Gly	glycine
Arg	arginine