Hemoglobin Synthesis in Avian Erythrocytes

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Hemoglobin is present in the nucleus of cells of the mammalian erythropoietic series as well as the nucleated red cells of amphibians and birds. Electron micrographs of frog and chicken erythrocytes demonstrate a direct continuity of hemoglobin between the cytoplasm and the interior of the nucleus through "pores" in the nuclear membrane (1). However, the origin of this nuclear hemoglobin has been a matter of controversy, for protein synthesis is generally considered to be a function of cytoplasmic ribosomes and non-nucleated reticulocyte ribosomes are known to be capable of synthesizing hemoglobin (2). Nevertheless, in the mammalian erythropoietic cell it has been shown that, under normal circumstances, almost the full complement of hemoglobin is synthesized before the nucleus is lost (3, 4). Furthermore, isolated nuclei of calf thymus cells (5) and rat liver cells (6) have been shown to possess a protein-synthesizing system. The possibility, therefore, exists that all or most of the hemoglobin might be synthesized in the nucleus of the erythropoietic cells of all eopies.

Early histologists suggested the nucleus as the site of hemoglobin synthesis because they observed that hemoglobin first appears in the mammalian polychromatophilic normoblast as a few pink spots in, or adjacent to, the nucleus (7). More recently, others have shown that hemoglobin is present not only in human erythroblast nuclei (8) but also in the nuclei of hematopoietic liver cells of the rabbit embryo. This nuclear fraction of rabbit embryo hemoglobin incorporates radioactive iron to about twice the specific activity of the circulating hemoglobin (9, 10). In addition, it has been demonstrated histochemically that early in the prospective blood islands of the chick embryo, there is more hemoglobin in the nucleus than in the cytoplasm, again suggesting a nuclear site of synthesis (11, 12).

In a previous communication (13) we reported preliminary evidence for protein synthesis in the nucleus of the pigeon red cell. The present report gives evidence that this protein is hemoglobin which is formed solely or preponderantly in the nucleus of both the mature red cell and reticulocyte.

EXPERIMENTAL PROCEDURE

Preparation of Cells—Heparinized (1 mg/10 ml) whole blood from adult pigeons was centrifuged at 2500 r.p.m. (1500 × g) at 0-4° for 10 minutes and the plasma anduffy coat were removed. The red cells were washed in 3 to 5 volumes of cold 0.9% sodium chloride solution three times. The third time the top 5% of the red cell layer was discarded to insure complete removal of white cells. The washed erythrocytes were finally suspended in an equal volume of Krebs-Ringer-bicarbonate buffer, pH 7.4, and aliquots of this suspension were used for the various whole cell incubations.

For some experiments, reticulocytosis was induced by daily withdrawal of approximately 10 ml of blood from the axillary veins of each pigeon. After 4 to 7 days a reticulocyte count of 50 to 70% was usually produced, as determined by counts of blood smears stained with brilliant cresyl blue.

Isolation of Nuclei—Washed, packed red cells were hemolyzed with a solution of 1% saponin in 0.25 M sucrose-0.005 M CaCl₂ (1 ml of solution per ml of packed cells) and allowed to stand for 5 minutes. The hemolysate was then diluted with 5 to 10 volumes of sucrose-CaCl₂ solution and centrifuged at 1000 × g for 20 minutes. The nuclear pellet was washed four times by recentrifugation and resuspension in 5 volumes of sucrose-CaCl₂. The final supernatant solution and nuclei were pale pink. During the final two washes, the button of unhemolyzed cells below the nuclear sediment was eliminated by resuspending only the nuclear layer and decanting it into fresh tubes.

This procedure was used both for the preparation of isolated nuclei for incubation and for the separation of nuclei and cytoplasm following intact cell incubations. In the latter instance, the hemolysate supernatant and all washings were combined, and an aliquot of this mixture was used for the various cytoplasmonic determinations.

For certain experiments, nuclei were isolated nonaqueously according to Behrens' procedure as modified by Alfrey and Mirsky (14). Suspensions of red cells (in 0.9% NaCl solution) were quickly frozen and lyophilized, and the dried powder was homogenized in petroleum ether. Instead of using separate centrifugations of increasing specific gravity, gradient centrifugation was done with five layers of benzene-carbon tetrachloride mixtures of progressively increasing specific gravity from top to bottom. After centrifugation at 0° for 80 minutes at 2000 × g, pure nuclei were found to sediment into a single separate layer distinct from the cytoplasm and cytoplasmic-ribbed nuclei, which remained in the layers above (Fig. 1).

Incubations—Incubations of intact cells were carried out at 37° in an atmosphere of 95% O₂-5% CO₂. The medium consisted of the red cell suspension, 0.005 M glucose, and 0.5 μc per ml of a ¹⁴C labeled amino acid. The reaction was stopped by pouring the incubation mixture into 0.001 M 2,4-dinitrophenol in 0.9% NaCl solution at 0°. Preliminary experiments showed complete and instantaneous cessation of incorporation of amino acid into protein by this procedure.

Isolated nuclei suspended in 0.25 M sucrose-0.003 M CaCl₂ solution were incubated aerobically at 37° in 1.0 ml of nuclear suspension, 0.5 ml of 0.1 M sodium phosphate-0.25 M sucrose.
buffer, pH 7.4, 0.4 ml of 0.1 M glucose solution containing 3.75 mg of NaCl per ml, and 0.1 ml of water containing 1 μc of radioactive amino acid.

Preparation of Protein for Counting—The proteins of the separated nuclear and cytoplasmic fractions were precipitated with 10% trichloroacetic acid, washed once with 10 ml of 5% trichloroacetic acid, recentrifuged, and boiled at 91° for 15 minutes after resuspension in 10 ml of 5% trichloroacetic acid. The precipitates were dissolved in 3 ml of 0.4 N NaOH and centrifuged, and the sediments, which contained little radioactivity, were discarded. The protein was reprecipitated from the supernatant solutions with 5 ml of 10% trichloroacetic acid, centrifuged and washed with equal volumes of 95% ethanol and ether, and then suspended in ethanol for plating on 1-inch aluminum planchets. The samples were counted in an end window, gas flow Geiger counter. The counts were corrected for self-absorption.

In some experiments the cells were lysed with 10 volumes of distilled water, and globin was isolated from the supernatant fraction according to the procedure of Anson and Mirsky (15). The globin and the nuclear protein were then purified for counting according to the previously outlined procedure.

Preparation of Crystalline Hemoglobin and Native Globin—

![Fig. 1. Nonaqueous separation of nuclei by gradient centrifugation.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Labeled amino acid</th>
<th>Specific activity of globin</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Intact cell</td>
<td>Hemolysate</td>
</tr>
<tr>
<td>Histidine-14C</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>Histidine-18C</td>
<td>154</td>
<td>2</td>
</tr>
<tr>
<td>Phenylalanine-14C</td>
<td>72</td>
<td>1</td>
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<tr>
<td>Phenylalanine-18C</td>
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**TABLE II**

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<th>Specific activities</th>
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<td>c.p.m./mg.</td>
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<tr>
<td>Whole red blood cells</td>
<td>98</td>
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<td>Hemolysate</td>
<td>22</td>
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<tr>
<td>Isolated nuclei</td>
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**TABLE III**

<table>
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<th>Specific activities</th>
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<tr>
<td></td>
<td>c.p.m./mg.</td>
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<tr>
<td>Nuclear protein</td>
<td>70</td>
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<tr>
<td>Globin</td>
<td>52</td>
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<td>Ratio of nuclear activity to globin activity</td>
<td>1.4</td>
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**TABLE IV**

<table>
<thead>
<tr>
<th>Total counts per μg of DNA-phosphorus</th>
<th>Intact cell</th>
<th>Isolated nucleus</th>
<th>Efficiency of nucleus</th>
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<tr>
<td></td>
<td>116</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>2</td>
<td>6</td>
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<td></td>
<td>60</td>
<td>0.5</td>
<td>0.8</td>
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<tr>
<td></td>
<td>113</td>
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<td></td>
<td>72</td>
<td>1</td>
<td>1</td>
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<tr>
<td></td>
<td>106</td>
<td>1</td>
<td>0.9</td>
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<tr>
<td></td>
<td>51</td>
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<td></td>
<td>45</td>
<td>2</td>
<td>5</td>
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**Fig. 1.** Nonaqueous separation of nuclei by gradient centrifugation. A homogenate of lyophilized red cells in 1 ml of cyclohexane-carbon tetrachloride mixture of specific gravity 1.299 was layered over four 1-ml layers of benzene-carbon tetrachloride mixtures of specific gravities 1.327, 1.333, 1.338, and 1.392, respectively, from top to bottom for gradient centrifugation. Nuclei free of cytoplasmic tabs sedimented separately into the 1.338 layer. Cytoplasm and nuclei contaminated with cytoplasmic tabs remained in the upper layers.
Fig. 2. Incorporation of $^{34}$C-algal protein hydrolysate into nuclear and supernatant proteins with time. Upper, specific activity of the trichloroacetic acid-precipitable protein; lower, total protein activity. The supernatant protein represents the combined nuclear washings. See "Experimental Procedure" for details.

Fig. 3. Incorporation of leucine-$^1$-$^{34}$C into nuclear and cytoplasmic protein with time. The total protein and its specific activity were determined on the whole hemolysate and the 1000 $\times$ g supernatant. The nuclear protein activity was calculated from the difference between the total hemolysate and its supernatant.

Fig. 4. Incorporation of leucine-$^1$-$^{34}$C into nuclear and supernatant proteins during incubation of isolated nuclei. Upper, specific activity of the trichloroacetic acid-precipitable proteins; lower, total protein activity.
Crystalline pigeon hemoglobin was prepared according to the method of Drabkin (16). The crystals were washed several times with ammonium sulfate and dissolved in water to make a standard solution of pigeon oxyhemoglobin.

Native globin was prepared according to the method of Rossi-Fanelli, Antonini, and Caputo (17).

**Chemical Determinations**—Total proteins were determined by the method of Lowry et al. (18), DNA phosphorus and RNA phosphorus according to the method of Schneider (19), and iron according to Ramsay's method (20).

**Electrophoresis and Chromatography**—The various protein solutions were prepared by dialysis against distilled water for 24 hours. When necessary, the solutions were concentrated after dialysis by vacuum evaporation at 0-4°C through the dialysis sac. All solutions were equilibrated with the appropriate buffer before application of the sample to either the electrophoretic strip or the column.

Electrophoresis was performed with cellulose acetate strips and Veronal buffer, pH 8.6, at 0°C for 4 hours, with constant current at 0.5 mA per cm of width. The strips were counted in an end window, gas flow, strip counter arranged for 4r geometry (Actigraph, Nuclear-Chicago Corporation).

Ion exchange chromatography utilized carboxymethyl cellulose resin obtained from the California Corporation for Biochemical Research. The columns were developed with a linear buffer gradient from 0.02 M pyridine-0.2 M formic acid to 0.2 M pyridine-2 M formic acid, or the first buffer was used throughout with a linear gradient from 0 to 0.3 M NaCl. The effluent was collected in 4.5-ml fractions for which optical densities at 280 nm were measured in a Beckman DU spectrophotometer. The

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**Table V**

**Average rate of loss of nuclear protein counts compared to average rate of gain of cytoplasmic protein counts after pulse**

The experimental details are described in Fig. 5.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>total c.p.m./min.</strong></td>
<td></td>
</tr>
<tr>
<td>Nuclear loss</td>
<td>51.3</td>
</tr>
<tr>
<td>Supernatant gain</td>
<td>53.4</td>
</tr>
</tbody>
</table>

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**Table VI**

**Incorporation of 14C leucine into protein of mature cells and reticulocytes in relation to RNA and DNA content**

Intact cells were incubated for 1 hour, then transferred to ice-cold 0.9% NaCl solution containing 0.001 M dinitrophenol, and washed three times with 10 ml of sucrose-CaCl₂, followed by three washes with 5 ml of NaCl.
RESULTS

Comparison of Intact Cells and Hemolysates—Intact cells actively incorporated ¹⁴C-labeled amino acids into their total protein or into globin, whereas hemolysates of the same cells were relatively inactive (Table I). The nuclear protein fractions of both the whole cell and the hemolysate had consistently higher specific activity than the corresponding supernatants (Table II). The ratio of the specific activities of nuclear protein and globin from the cytoplasm of whole cell incubations was constant regardless of the labeled amino acid used, suggesting some relationship between nuclear protein synthesis and globin synthesis (Table III).

Isolated nuclei varied in total protein synthetic capacity from 1 to 10% of the efficiency of intact cells (Table IV). Washed nuclei alone showed about the same protein-synthetic capacity as nuclei incubated in a whole hemolysate.

Kinetic Experiments—In early experiments, at intervals an aliquot of cells from the incubation mixture was hemolysed with saponin and the nuclei were washed several times with sucrose-CaCl₂ solution. The washings were combined with the hemolysate supernatant, and an aliquot of this mixture was precipitated for determination of the supernatant protein specific activity. The washed nuclei were precipitated with trichloroacetic acid separately. A typical experiment is shown in Fig. 2. The nuclear protein showed a rapid rise in the activity during the first few minutes of incubation. Its total activity exceeded that of the supernatant protein during this time. At 5 minutes, there was a change in slope of the nuclear curve to a much slower rate of incorporation. The total activity of the cytoplasmic protein increased at a relatively constant rate during the experiment and surpassed that of the nucleus at about 5 minutes, at the time when nuclear incorporation decelerated. Thus it appeared that labeled protein was being transferred from nucleus to cytoplasm.

A similar relationship of nuclear to cytoplasmic incorporation was seen in later experiments in which the nuclei were not washed, but in which an aliquot of the total hemolysate and an aliquot of the supernatant were taken for total protein and specific activity determinations. The nuclear values were calculated by difference (Fig. 3). Although the total incorporation in the first 15 minutes was comparable to Fig. 2, the time required for the
FIG. 8. Electron micrograph of a nonaqueous preparation of nuclei (45,000 ×)

Incorporation of labeled amino acids into protein in isolated nuclei, although less efficient, followed the same pattern with time as the nuclear fraction of the intact cell, and radioactive protein similarly accumulated in the supernatant fluid (Fig. 4).

"Washout" Experiments—Experiments with intact cells were
done in which, at 5 minutes, a 100-fold excess of unlabeled amino acid was added to the medium. It can be seen in Fig. 5 that after the addition of carrier, both the specific activity and total activity of the nucleus fell concurrently with a rise in supernatant activity, which is consistent with a movement of recently labeled protein from nucleus to cytoplasm. The nuclear incorporation leveled off to a very slow rate of increase as the activity of the supernatant protein continued to increase at a lesser rate, which is consistent with the dilution by carrier. In several experiments, the total number of counts lost by the nucleus was approximately equal to the total counts gained by the cytoplasm in the same period of time, as is shown in Table V.

**TABLE VII**

Incorporation of $^{14}$C-amino acids with time into protein of nuclear fraction nonaqueously isolated from intact cell incubations

The nuclear and whole cell fractions, separated as described in Fig. 1, were extracted with ether and then precipitated with 10% trichloroacetic acid. The protein precipitates were purified as usual before plating and counting.

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonaqueous nucleus</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>160</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>120</td>
<td>87</td>
</tr>
<tr>
<td>240</td>
<td>63</td>
</tr>
</tbody>
</table>

*Comparison of Mature Cells and Reticulocytes*—Protein synthesis in mature cells was compared to that in nucleated reticulocytes. Table VI shows that although the reticulocytes were more active in terms of the total number of counts incorporated into protein per cell based on counts per unit of DNA, their efficiency in terms of RNA content was only 50% of the mature cells. This suggests that the quantity of RNA may not be rate-limiting for protein synthesis in the reticulocyte.

*Aqueous Extractions of Labeled Nuclei*—In order to answer the question of whether a more highly labeled soluble protein was made in the nucleus than in the cytoplasm, mature erythrocytes previously incubated with $^{14}$C-amino acids were hemolyzed with saponin, and the nuclei were washed several times with sucrose and then with NaCl solution. The quantity of protein and its specific activity and the amount of RNA and DNA in each wash were determined. Fig. 6 shows that each sucrose wash extracted from the nuclear fraction protein of successively greater specific activity. At the same time the total protein and RNA content of the washes decreased, consistent with a washing away of the cytoplasm adjacent to, or still attached to, the nucleus.

There appeared in the NaCl washes, on the other hand, an increased quantity of protein and RNA, and this protein had a still higher specific activity than that of the protein found in the sucrose washes. In addition, the ratio of counts to RNA in the NaCl washes was relatively constant, suggesting a RNA-protein complex of the most recently synthesized protein. There is no evidence of gross nuclear breakdown because DNA never appeared in the washes.

*Kinetic Experiments with Nonaqueous Isolation of Nuclei*—Nuclei isolated in aqueous media are usually “contaminated” with endoplasmic reticulum, for these membranes are adherent...
to or continuous with the nuclear membrane. Fig. 7 is an electron micrograph of an aqueous preparation and shows the reticulum to be present around the nuclei of mature cells as well as reticulocytes, although in the latter it is more abundant, more clearly double layered, and contains more ribosomes. The question therefore arises as to whether the loci for protein synthesis are entirely perinuclear, on this reticulum, or truly intranuclear. An answer to this was sought by isolation of nuclei in nonaqueous media after varying periods of incubation with labeled amino acids. This method of separation has been reported to yield pure nuclei free of all cytoplasmic contamination, but without loss of water-soluble components from the nucleus. Fig. 8 is an electron micrograph of such a preparation, showing that the outer reticulum has been lost but that hemoglobin has been retained in the nucleus.

The data from several experiments are given in Table VII. At 1 minute, the specific activity of the nuclear protein was 5 times greater than that of the whole cell, at 5 minutes the activities were approximately equal, and at later times the whole cell protein had a specific activity greater than that found in the nuclei. This is consistent with the data from aqueous isolation of nuclei, and again shows the nucleus to be far more active than the cytoplasm in protein synthesis.

Identification of Protein Synthesized—Fig. 9 shows an electrophoretic strip and radiometric scanogram of the hemolysate supernatant after incubation of intact cells with 14C-amino acids. There was a single band of hemoglobin corresponding to the single peak of radioactivity and identical with a simultaneously run standard of crystalline pigeon hemoglobin. Similar results were obtained with the sucrose washes of 14C-labeled nuclei from whole cell incubations.

Fig. 10 compares the patterns obtained by elution of several different proteins from carboxymethyl cellulose with pyridine-formate buffers. Crystalline pigeon hemoglobin and native pigeon globin gave identical patterns which were different from all of the other proteins tested. The first peak is probably unchanged hemoglobin, since it invariably contains both iron and heme as determined chemically and by spectral analysis. The second peak also contains traces of iron and heme when hemoglobin solutions are eluted, but not when native globin is chromatographed. Therefore, this peak probably represents globin either alone or associated with one or more heme moieties.

Fig. 11 shows the elution patterns obtained with the sucrose and NaCl washes of the nuclei of 14C-labeled cells. The pattern of the sucrose washes was identical with that of crystalline hemoglobin or globin. The NaCl wash protein produced a smaller initial peak of a different shape, but also gave a second peak which was identical with globin. A similar pattern was seen with the supernatant protein of isolated nuclear incubations.

An examination of the specific activities of the protein in these peaks gave further evidence for hemoglobin or globin as the major protein synthesized. In the case of the sucrose wash, the specific activities of the two peaks were equal with the exception of a small quantity of protein in the earliest portion of the second peak which consistently had an activity 2 or 3 times greater than that of the remaining protein. This segment has not been identified. It is not contamination by free label, nor is it a precursor of globin, as shown by preliminary kinetic studies.
The initial peak of the NaCl wash had a very low specific activity compared to the second peak in addition to being different in quantity and shape from the sucrose wash initial peak. The globin peak, like the sucrose wash, contained an initial more highly active component.

The globin thus isolated from each successive sucrose and NaCl washes of nuclei from intact cell incubations with 14C-leucine. The protein solutions, after dialysis against water and equilibration with the buffer, were eluted from CM-cellulose columns (135 X 9 mm) with 300 ml of pyridine-formic acid buffer gradient from 0.02 M pyridine-0.2 M formate to 0.2 M pyridine-2 M formate.

The question of whether the sites for synthesis are intranuclear or on perinuclear ribosomes of the endoplasmic reticulum has been convincingly answered by the kinetic experiments with nonaqueous preparations. It is in this preparation that the most rapid rise in specific activity of nuclear protein is seen initially. The labeled protein appears subsequently in the cytoplasm, but at a more rapid rate than in any of the aqueous preparations. One explanation for this phenomenon might be that the small percentage of nuclei with attached cytoplasmic "tabs" contaminating the nonaqueous preparation of pure nuclei could be enough to account for all of the radioactivity, i.e. that all of the nuclear activity is in reality always perinuclear on the ribosomes of the endoplasmic reticulum rather than intranuclear. According to this interpretation, the nuclei should become less radioactive as the tabbed nuclei are removed, and a preparation of close to 100% tabbed nuclei should have a higher specific activity than either more purified nuclei or pure cytoplasm from the same cells.

However, neither was found to be the case since a further purification of 1-minute nuclei resulted in an increase in specific activity, However, neither was found to be the case since a further purification of 1-minute nuclei resulted in an increase in specific activity, and fractions rich in "tabbed" nuclei did not have a higher specific activity. Furthermore, isolated ribosomes have been consistently shown to be, at best, only 1% as efficient as the intact cell. Since the isolated nuclei from these cells without added cofactors are 1% to 10% as efficient as the whole cell, the observed protein synthesis must take place in the nucleus itself.

Ribosomes have been demonstrated in the nucleus together with the known components of the protein-synthetic system (25). The finding that a single protein, which represents essentially all of the protein of a special cell, is synthesized in the nucleus, even in the ribosome-rich reticulocyte, makes the efficiency lent to the protein-synthesizing system by proximity of DNA shown by Tissières and Hopkins (26) in bacteria a possibly.
Fig. 12. Comparison of fingerprints of sucrose and NaCl washes of nuclei with crystalline pigeon hemoglobin. A, sucrose wash; B, NaCl wash; C and D, crystalline pigeon hemoglobin. Horizontal axis, electrophoresis; vertical axis, chromatography. Developed with ninhydrin.

significant factor in protein synthesis in an animal cell. This is underlined by the finding by Allfrey, Mirsky, and Osawa that the most highly labeled protein synthesized by thymus nuclei was associated with DNA.

SUMMARY AND CONCLUSIONS

The presence of hemoglobin in the nuclei of erythropoietic cells of all species has led to the hypothesis that it might be synthesized there. The following evidence obtained in the pigeon red cell lends support to this hypothesis.

1. When intact cells or hemolysates are incubated with $^{14}C$-labeled amino acids, the nuclear fraction is found to contain protein of the highest specific activity.

2. Labeled protein is transferred from the nuclear fraction to the supernatant with time, both in intact cell and in isolated nuclei incubations, and the most highly labeled protein can be extracted from the nuclear fraction with isotonic sucrose or NaCl.

3. Although reticulocytes are more active than mature cells in synthesizing protein, their efficiency in terms of ribonucleic acid content is only 50% of that of mature cells.

4. The nonaqueous preparation of nuclei from intact cell incubations, shown by electron microscopy to be free of endoplasmic reticulum and to retain intranuclear hemoglobin, becomes labeled more rapidly than the cytoplasm.

5. By its behavior on electrophoresis and ion exchange chromatography, and by peptide analysis, the major protein synthesized is hemoglobin.

6. It is therefore concluded that, in the avian erythrocyte, hemoglobin is synthesized in the nucleus and transferred to the cytoplasm. In the light of findings by other investigators, it is possible that the major site of synthesis of other proteins in other types of cells may be the nucleus.

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