THE ROLE OF THE AVIAN ERYTHROCYTE NUCLEUS IN HEME BIOSYNTHESIS

by

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ABSTRACT

Though the enzymes involved in heme biosynthesis have been studied by several groups using in vitro avian erythrocyte systems, no attempt has been made to relate these enzymes to the morphology of the intact cell. Previous work indicates that the chicken erythrocyte nucleus is metabolically active and therefore must contain numerous enzymes. In the present study fractionation of the chicken red cell has been undertaken and the role of the nucleus in heme biosynthesis investigated.

Fractionation into cytoplasmic and nuclear fractions was achieved by hemolysis of the washed red cells with hypotonic sodium chloride solution followed by centrifugation at 9000 g. The isolated nuclei were further fractionated into a soluble fraction and an insoluble residue by Waring Blendor treatment and centrifugation. Microscopic study and remarkably consistent experimental results indicated that the cell fractions thus prepared were relatively homogeneous.

Isolated cell fractions and combinations thereof were incubated with various heme precursor substrates. Heme formation was evaluated by tracer methods using C^{14}-labelled glycine or Fe^{59}, and in certain experiments the uro-, copro-, and protoporphyrin formed were also quantitated.

The heme biosynthetic pathway was divided into four segments for study: (1) the condensation of glycine and succinate (with decarboxylation of the α-amino-β-keto adipic acid formed) to yield δ-aminolevulinic acid (ALA); (2) the conversion of ALA through porphobilinogen (PBG) to porphyrins; (3) the conversion of uroporphyrin (or its reduced derivative) to protoporphyrin; and (4) the insertion of iron into protoporphyrin to form heme.

Various techniques were utilized to inactivate enzyme systems without disrupting cofactors so that the enzymes catalyzing the above portions of heme biosynthesis could be localized in a particular cell fraction.

It was found that both cytoplasmic and nuclear fractions were required for the conversion of glycine and succinate to ALA, but the inactivation techniques indicated that the enzymes involved were in the nuclei while the cytoplasm furnished essential cofactors. Attempts to solubilize these enzymes from the nuclear fraction were unsuccessful.

All the factors necessary for the conversion of ALA to uroporphyrin were in the cytoplasmic fraction as prepared. The possibility was suggested that these enzymes might have been eluted from the surface of the nuclei during hemolysis.

Further data indicated that the enzymes catalyzing the synthesis from the uroporphyrin to protoporphyrin stage were most probably in the nucleus (more specifically, in its soluble fraction) with essential cofactors provided by the cytoplasm.

The nuclear soluble fraction also contained the enzyme(s) responsible for the insertion of iron into protoporphyrin to form heme, the cytoplasmic fraction again providing necessary cofactors.

The relationship of these results to numerous reports by other groups using erythrocyte water-hemolysates has been discussed, and the chicken erythrocyte has been compared to the human red cell as regards heme biosynthesis.

It has been suggested that isolation of nuclear fractions as described here provides convenient sources of many of the enzyme systems concerned in heme biosynthesis. In nuclear fractions, separated from the mass of cytoplasmic hemoglobin, these enzymes may be studied in purer form than in whole cells or hemolysates. In addition, study of the cytoplasmic fraction should be of value in identifying essential cofactors involved in various stages of the synthesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A. Biological Importance of Heme

The biological importance of the heme molecule is well known. Heme and derivatives of heme serve as the prosthetic groups of such vital compounds as the oxygen carriers, hemoglobin and myoglobin, and important enzymes including the cytochromes, catalase, and the peroxidases. In the Plant Kingdom the chlorophyll molecule, essential to photosynthesis, is a magnesium-porphyrin complex, and recently vitamin B_{12} has been shown to contain cobalt within a porphyrin-like structure (1,2). The importance of these heme- and other porphyrin-containing compounds has stimulated interest in the pathway of heme biosynthesis. Thus, the intermediates involved in this process have received a great deal of attention in recent years.

B. Methods of Studying Heme Biosynthesis

The biosynthesis of heme has been studied in several ways in a variety of systems. Early *in vivo* investigations (3,4,5) have been supplemented by *in vitro* studies of heme synthesis using liver preparations (6), *Chlorella* (an alga) (7), certain bacterial organisms (8,9), and avian erythrocytes (10,11). The latter system has received extensive investigation and is the object of the studies described herein.

Since heme synthesis is closely related to porphyrin formation, biochemical and clinical studies of the human disease, porphyria, have added to our knowledge (5,12,13,14,15). An experimental type of porphyria has been induced in rabbits (16), rats (17), and chicken embryos (18) by administration of allylisopropylacetylcarbamide (Sedormid) or related compounds and has been used to study various aspects of porphy-

rin formation (19,20). In addition, human and experimental porphyria have provided sources of porphyrins and porphobilinogen (PBG) for use in the study of heme formation in other systems. A few studies of heme formation have also been carried out in human subjects with certain hematological abnormalities (21,22,23).

C. Established Intermediates in Heme Synthesis

Though Hans Fischer synthesized heme chemically in 1929 (24), little was known of its synthesis in the body until 1945 when Shemin and Rittenberg found that N^{15}-labeled hemin could be isolated from the red blood cells of a normal human who had been fed N^{15}-glycine (3). Further studies by these workers indicated that the nitrogen of glycine is directly employed for the synthesis of the protoporphyrin of hemoglobin (25) and that it is the precursor for both pyrrole types in protoporphyrin (26). Glycine's role as a porphyrin precursor was also suggested by isotope studies carried out by English workers on human porphyria patients (4,5) and rabbits and rats (27,28). Further early tracer studies in the intact animal showed that the α-carbon atom of glycine is utilized in the formation of heme (29), but the carboxyl carbon is not (30), conclusions that were supported by later work using an *in vitro* system (31). The discovery of Shemin *et al.* that erythrocytes from the peripheral blood of ducks can carry out heme synthesis from glycine (10,32) provided a convenient system for study of this pathway *in vitro*. An early observation by Bloch and Rittenberg while studying acetic acid metabolism was that significant amounts of deuterium appear in hemin isolated from the red cells of rats receiving deuterium-labeled acetate (33). This finding stimulated investigation of acetate as a

possible precursor of heme, and data were obtained suggesting that most or all of the carbon atoms of heme are derived from acetate and glycine (34,35).

The elegant method of hemin degradation worked out by Wittenberg and Shemin (36) provided a means of identifying the source of each atom of the heme molecule and led to identification of glycine and succinate as heme precursors, thus linking heme formation to the tricarboxylic acid cycle (37,38,39). It was postulated that glycine and succinate might condense to form δ-aminolevulinic acid (ALA) (Figure I), and Shemin and co-workers, using dilution experiments and C^{14}-labeled ALA, showed that ALA is, in fact, a true intermediate in heme biosynthesis (40,41,42,43,44), a finding that was reported simultaneously by Neuberger and Scott (45). It was postulated that two molecules of ALA could combine in a Knorr type of condensation to form a monopyrrole. Following the isolation of porphobilinogen (PBG) from human porphyric urine and characterization of its structure (46,47,48), this compound was identified as the monopyrrole intermediate in heme synthesis (49,50, 51,52).

The synthesis beyond the monopyrrole stage has not been so clearly elucidated. The conversion of uroporphyrin III to protoporphyrin has been reported (53), but neither uroporphyrin III, coproporphyrin III, nor protoporphyrin acted as true intermediates in dilution-type experiments described by Dresel (51). No conversion of C^{14}-labeled uroporphyrin or coproporphyrin to protoporphyrin or heme was noted by Schwartz using both *in vivo* and *in vitro* systems (50). Various workers have suspected that certain colorless porphyrin derivatives or precursors are actual intermediates in heme synthesis (19,54), and Nevé *et al.* showed

that reduced uroporphyrin III behaves as a true intermediate in dilution experiments (55). Thus, it would appear that the porphyrins may merely be "side-products" of heme synthesis. Such a scheme has been proposed by Dresel and Falk (56). Shemin et al. have proposed a mechanism by which the conversion of the monopyrrole to a tetrapyrrole (reduced uroporphyrin III) via a tripyrrylmethane could occur (57).

Despite the fact that protoporphyrin has not been established as a true intermediate, its conversion to heme has been noted in bone marrow and liver preparations (19), in bacteria (58), and in chicken red cell hemolysates (59,60,61,62). Figure I summarizes briefly the most probable scheme, based on present knowledge, of the biosynthetic pathway from glycine and succinate to heme.

D. Results of Past Enzymatic Studies of Heme Synthesis

In addition to describing intermediates, studies of heme synthesis carried out in avian (chicken or duck) erythrocyte systems have revealed further information about certain segments of the pathway. Of particular pertinence to the present discussion are reports of the enzymatic reactions and conversions that occur in fractions of red cell hemolysates and some enzyme purifications that have been carried out from these fractions. Though cell fractionation on a morphologic basis, as has been done with such interesting results in liver cells (63), has not been carried out with avian red cells, various soluble systems have been prepared from erythrocyte hemolysates and some reactions involved in heme synthesis studied.

Heme is synthesized from glycine and succinate or from ALA in intact red cells or in hemolysates, but PBG is converted to porphyrins and heme

more efficiently in hemolysates, indicating that it probably does not penetrate the intact cell readily (64,65). Dresel reported that a supernate, prepared by centrifugation at 20,000 g of a water-hemolysate, could convert ALA or PBG to uro- and coproporphyrin but that there was scarcely any free protoporphyrin or heme formed (51). On the other hand, Shemin and associates prepared from an homogenized water-hemolysate a similar supernatant fluid (their cell-free extract) that would convert ALA (but not glycine) to heme (66).

Granick has prepared three fractions from the soluble portion of chicken erythrocytes by starch electrophoresis (67). Fraction 1 contains the enzyme converting ALA to PBG, fraction 2 the enzyme which converts PBG to a colorless precursor of uroporphyrin III, and fraction 3 when mixed with fraction 2 converts PBG under anaerobic conditions to a colorless precursor of coproporphyrin III.

ALA dehydrase, which converts ALA to PBG, has received the most attention and has been found in numerous tissues in addition to avian red cells (68,69). Purification and characterization of the properties of the enzyme(s) have been carried out by several groups of workers (52, 70,71,72).

The conversion of PBG to porphyrins has been studied in liver homogenates (73), Chlorella suspensions (74), and in chicken erythrocyte hemolysates (75). The enzyme converting PBG to uroporphyrin III was partially purified from a supernatant preparation of chicken erythrocyte hemolysate (75).

The conversion of protoporphyrin to heme has been shown to take place in various soluble preparations of chicken erythrocytes (59,61,62).

Thus, it is seen that certain soluble preparations of avian

erythrocytes contain enzymes catalyzing the reactions concerned in heme synthesis from ALA to heme. However, the experimental methods employed have not permitted conclusions as to the intracellular distribution of the enzymes involved. Undoubtedly the insoluble precipitate resulting from centrifugation of a water-hemolysate contains mainly nuclear material, but just how much, if any, nuclear material is present in the soluble portion as prepared is not clear from the studies to date. The work presented herein has been undertaken in an attempt to elucidate the role of the nucleus in heme biosynthesis.

It is interesting to compare the results from the above studies of heme synthesis by avian erythrocyte systems with some results obtained using human red cell systems. Morphologically the avian red cell differs from the mature human erythrocyte in that the former is elliptical in cross-section and contains a large nucleus whereas the latter is a non-nucleated biconcave disk. The two cell types also differ markedly from the standpoint of heme biosynthesis. The human red cell lacks most of the enzymes necessary to carry out the reaction sequence from glycine to heme though it does contain ALA dehydrase (52) and in addition can apparently convert ALA to certain porphyrins (76). Study of the peripheral blood from a patient with sickle cell anemia revealed that this blood can convert glycine to heme (22), but it has also been found that the mere presence of reticulocytes, which contain nuclear fragments, does not insure this ability to synthesize heme (77). These experiences with normal and pathological human erythrocytes also raise the question of the role of the nucleus, and one wonders if the differences in heme-synthesizing capability of the human and avian red cell might be related to the absence in the former and presence in the latter of a nucleus.

E. The Avian Erythrocyte

Except for the use of the chicken erythrocyte in studies of heme synthesis, neither it nor its nucleus has received much attention from biochemists in recent years. Laskowski prepared nuclei from hen erythrocytes and found that they did respire though the respiration was very low (78), and Laskowski and Ryerson studied the effects of different sodium chloride concentrations on isolated nuclei (79). Melampy studied the content and distribution (cytoplasmic versus nuclear) of amino acids in the chicken erythrocyte (80). One writer, summarizing the small amount of work done on the avian red cell, expressed the belief that, "The bird erythrocyte is a cell of such limited metabolic function that studies on the whole cell or its components may be of dubious value as far as drawing general conclusions is concerned." (81)

However, observations by Warburg in 1910 on the aerobic respiration of avian red cells and nuclear and cytoplasmic fractions thereof led him to assert that it is the nucleus and not the cytoplasm that is responsible for the respiration that occurs after hemolysis of the red cell (82). He found no measurable oxygen uptake by the cytoplasm alone. These early conclusions of Warburg would certainly seem to indicate that the avian erythrocyte nucleus is a metabolically active fraction of the cell and must contain numerous enzymes. It seems reasonable, therefore, to believe that examination of the avian erythrocyte nucleus for enzymes concerned in the biosynthesis of heme would be worthwhile and perhaps rewarding.

The present study was undertaken in an attempt to define the role of the avian erythrocyte nucleus in the biosynthesis of heme, that is, to determine which of the enzymes, if any, concerned in heme synthesis

are located in the nucleus. The results indicate that the nucleus does play a very important role in heme synthesis, that the enzymes which catalyze several of the steps of the pathway are, in fact, in the nucleus, and that fractionation of the cell as described here may provide convenient systems for the further study of these enzymes as well as furnishing information about structure-function relationships.

METHODS AND MATERIALS

A. Fractionation of Chicken Red Cells and Isolation of Nuclei

For all experiments, chicken red blood cells were utilized in an *in vitro* system. Each collection of blood represented a pooled sample from at least 100 birds.* Experiments were carried out on the same day (except where noted) with a lapse of less than 12 hours between collection of blood and incubation of samples.

The heparinized blood was centrifuged and the plasma and leukocyte layers removed. The red cells were washed twice with either Red Blood Cell Salt Solution (see below) or 0.9 percent sodium chloride. All steps following washing of the red cells were carried out in the cold (4-5° C.). Several methods of lysing the red cells were investigated. Saponin, 3 mg./ml., and cationic detergent, 1.5 mg./ml., (both in 1.5 volumes of RBC Salt Solution) were effective in lysis (of one volume of red cells) but inhibited heme synthesis from glycine and succinate so were discarded. London *et al.* also observed this inhibitory action of saponin (77). Water, which has been used for lysis in most previous studies with red cell hemolysates (60,62,65,69,83), disrupts the nuclei so as to make their isolation in a relatively unchanged condition impossible. The nuclear residue isolated after water-hemolysis consists of a jelly-like unworkable mass. Lysis with the Waring Blendor is effective but also alters the structure of the nucleus and, as will be shown later, releases certain important soluble fractions of the nucleus into solution. Nuclei isolated after Waring Blendor-lysis tend to aggluti-

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*Chicken blood was obtained, mainly from White Leghorn and Rhode Island Red chickens, through the courtesy of Northwest Poultry and Dairy Products Co. and Fresh Foods Co., Portland, Oregon.

nate in a gummy mass and have a stringy appearance microscopically. The method of lysis finally chosen for these experiments was addition to the red cells of 1.5 volumes of a hypotonic sodium chloride solution (0.02 to 0.1 percent depending on cellular fragility) added slowly with constant stirring. When hemolysis, as judged microscopically, was reasonably complete (usually within 30 minutes), solid potassium chloride was added to the lysis mixture to restore approximate isotonicity. The freed nuclei were then centrifuged at 9000 g for 10 minutes in a Servall Angle Centrifuge. The dark red supernatant fluid, consisting essentially of the cell cytoplasm diluted with the added saline was carefully pipetted off and preserved for addition to the appropriate samples for incubation. This fraction will be referred to as "cytoplasm" in all following discussion. Any remaining unlysed red cells formed an obvious red layer below the lighter-colored nuclear layer and could be removed. The nuclei were then washed a variable number of times (indicated in the tables of data) with RBC Salt Solution and subjected in certain experiments to other treatments that will be described.

B. Preparation of Samples for Incubation

The various erythrocyte preparations were pipetted into fifty-milliliter Erlenmeyer flasks for incubation. Substrates, iron, and coenzymes dissolved in water or saline were added to the flasks and the final volumes brought to a given amount with water or saline. The samples were incubated in air in the dark at 37° C. with shaking for the periods of time indicated in the tables. The volumes of samples at incubation were usually between 10 and 20 ml. In the early experiments it was attempted to make the amount of a given cell fraction roughly

equivalent to the amount that would be present in 20 ml. of blood. In later experiments specific volumes of the various cell fractions were added. Following incubation, the samples were frozen to arrest enzyme activity, and at a later time the isolation of hemin and/or the determination of porphyrins was carried out.

C. Waring Blendor Treatment

All Waring Blendor treatment was carried out in the cold (4-5° C.) using a stainless steel water-jacketed Waring Blendor cup so that the temperature of the treated material remained low. In several experiments the lysis mixture was blendorized at high speed for 30 or 40 minutes (in 5 or 10 minute intervals). After centrifugation for 10 minutes at 9000 g, two fractions were obtained. The supernatant fluid now contained the "cytoplasm" plus soluble portions of the nucleus ("nuclear soluble fraction") while the residue was the insoluble remainder of the nucleus ("nuclear residue"). In certain experiments the isolated, washed nuclei, suspended in an equal volume of RBC Salt Solution or water, were treated for 3 to 10 minutes at high speed in the Waring Blendor. Centrifugation then separated the "nuclear soluble fraction" from the "nuclear residue". Waring Blendor treatment was also used in one experiment to inactivate the enzymes concerned in the formation of δ-aminolevulinic acid from glycine and succinate.

D. Heating Cell Fractions

In several experiments various cell fractions were heated in attempts to inactivate certain of the enzyme systems concerned in different stages of heme synthesis. The materials were heated in water baths at the specified temperatures for thirty-minute periods. In cases

where the entire heated fraction was utilized, it was diluted to its original volume with water after heating. In other cases the material was centrifuged and the supernatant fluid removed. Sufficient water was then added with stirring to the precipitate to make up the original volume, and the precipitate was again spun down. This water-extract was then added to the original supernatant fluid to bring it to the original volume of the cell fraction heated. Solutions prepared in this way will be referred to as "extracts" of a heated cell fraction.

E. Homogenization

In one experiment isolated nuclei were homogenized with an equal volume of water in a Potter-Elvejhem homogenizer in an attempt to solubilize certain enzyme systems. Following centrifugation for 10 minutes at 9000 g, aliquots of the residue were re-homogenized with equal volumes of various salt solutions and again centrifuged to yield various supernatant and residue fractions.

F. Microscopic Observations of Cell Fractions

Wet smears of the various cell fractions were examined microscopically throughout each experiment as a rough check on the homogeneity of these fractions. On one occasion smears of the various cell fractions were stained with Wright's stain and examined microscopically under an oil immersion lens.

G. Protoporphyrin Preparation

Protoporphyrin was prepared by the method of Grinstein (84). Whole blood, cells, or clotted blood was thoroughly mixed with 2 to 3 volumes of acetone. The resultant precipitate was filtered through a Buchner funnel and washed 2 or 3 times with acetone. Suction was applied till

a grayish dry powder, mostly hemoglobin, was obtained. The powder was suspended in 10 percent oxalic acid in methanol and shaken for 10 minutes. The hematin solution was separated by filtration using a Buchner funnel and #5 Whatman paper. Extraction was repeated with several portions of oxalic acid solution until the extracted solution was colorless (4 or 5 extractions). Three to five grams of ferrous sulfate per 100 ml. were dissolved in the combined solutions. Precipitation occasionally took place here. The solution was then saturated with a strong current of gaseous hydrochloric acid (generated from sodium chloride and concentrated sulfuric acid). The hematin solution became clear, and the temperature rose. The color changed to a red-brown and then to a red-violet. At this point the protoporphyrin had become esterified (methyl ester), and the addition of hydrochloric acid was stopped. The solution was mixed with chloroform and a large excess of water and let stand 15 to 30 minutes till the ester was concentrated in the chloroform. The chloroform solution was washed 3 times with distilled water, once with 10 percent ammonia solution, and 3 times again with water. A white precipitate sometimes formed with the ammonia wash and was removed. The final chloroform solution was concentrated (preferably by vacuum) and mixed with excess methanol. Crystals of the methyl ester of protoporphyrin precipitated out on standing. The protoporphyrin could then be stored in this esterified state. Saponification of the ester to obtain free protoporphyrin was carried out in the dark at room temperature for a period of 4 to 20 hours using 7.5 N hydrochloric acid (about 1 mg. ester per ml. acid). Ten percent sodium hydroxide was added dropwise to precipitate the protoporphyrin. If a white precipitate (sodium chloride) formed, it was removed by the sub-

sequent water wash. The precipitated protoporphyrin was washed with water, redissolved in 0.1 N potassium hydroxide, and reprecipitated with concentrated acetic acid. The reprecipitation step was then repeated. The protoporphyrin was dissolved in M/7 sodium bicarbonate, adjusted cautiously to physiologic pH with dilute hydrochloric acid, and an aliquot diluted with 1.5 N hydrochloric acid for fluorimetric determination of concentration. The protoporphyrin thus obtained was diluted with water or physiological saline to the desired concentration for addition to the appropriate samples for incubation.

H. Reduced Uroporphyrin Preparation

The methyl ester of uroporphyrin III* which had been obtained from the urine of a porphyric patient (85) was hydrolyzed overnight in 7.5 N hydrochloric acid and the porphyrin precipitated by the addition of sodium hydroxide. The precipitate was washed with water and dissolved in a small volume of 0.1 N potassium hydroxide. At this point the concentration was determined fluorimetrically in a Farrand fluorimeter by dilution of a small aliquot with 1.5 N hydrochloric acid and comparison with a coproporphyrin standard also in 1.5 N hydrochloric acid. Reduced uroporphyrin was prepared as described by Nevé (86). To the alkaline porphyrin solution was added a small amount of ammonium chloride and ascorbic acid. Two or three grams of sodium amalgam were added, and the solution was shaken until the fluorescence had decreased markedly. A second fluorimetric determination of concentration was carried out at this point to determine the percent of the uroporphyrin that had been reduced. The solution was then filtered through a fine sintered glass filter, carefully adjusted to approximately pH 8 with dilute hydrochloric

*Generously supplied by Dr. Richard Nevé.

acid, diluted to the desired volume with water, and equal aliquots added to each incubation flask. An aliquot of reduced uroporphyrin was incubated for 3 hours and the uroporphyrin concentration again determined. This reduced uroporphyrin was also allowed to stand in the light for a further period to see if additional re-oxidation would occur.

I. Hemoglobin Determination

Hemoglobin was determined by conversion to cyanmethemoglobin with Drabkin's solution and reading on a spectrophotometer (Bausch and Lomb, Spectronic 20) at 540 mμ (87). This method provides a simple and direct analysis for total hemin (88) and utilizes standards that are stable for long periods (89). Drabkin's solution is prepared as follows from reagent grade chemicals (87): 1.0 gm. sodium bicarbonate, 50 mg. potassium cyanide (in these experiments 37.6 mg. sodium cyanide was used in place of potassium cyanide), 200 mg. potassium ferricyanide, and distilled water to one liter. The solution was stored in a brown bottle in the cold and made up fresh every month. The cyanmethemoglobin method described by Crosby et al. (87) was modified as follows: One ml. of the material to be analyzed was diluted to 25 ml. with water. One-half ml. of this solution was pipetted into 4.5 ml. of Drabkin's solution in a cuvette, the pipette rinsed 3 times with the solution, and the tube swirled to obtain thorough mixing. The blank consisted of 4.5 ml. of reagent plus 0.5 ml. water. The cuvette was allowed to stand at least 10 minutes before reading. The instrument was calibrated using standards provided by the clinical laboratory of the University of Oregon Medical School Hospital, and a permanent sub-standard was prepared and used as a check on the instrument before each series of determinations. Calibration of the spectrophotometer with accepted

standards made possible calculation of a factor to convert the optical density reading directly to mg./ml. hemin. For this calculation it was assumed that hemin represents 3.92 percent of hemoglobin.

J. Isolation and Purification of Hemin

Hemin was labeled with either Fe^{59} (from ferrous citrate Fe^{59}) or C^{14} (from α-C^{14}-glycine). In the experiments in which Fe^{59} was used, between 0.5 and 1.0 μcurie was added per sample. In experiments utilizing α-C^{14}-glycine as substrate 13.3 microcuries were added to each sample (except in one experiment in which 6.6 microcuries were used). Hemin was prepared by a new method (90). To the appropriate incubated samples was added the necessary volume of "cytoplasm" to make all samples of the experiment equivalent in hemin content and to act as a source of carrier hemin. One ml. was removed in some experiments for the hemoglobin determination, and the remainder was added with swirling to about 10 volumes of a 3:1 mixture of acetone:glacial acetic acid, the glacial acetic acid containing 2 percent (weight/volume) strontium chloride. An additional 2 volumes of the acetone-acetic acid mixture were used to rinse out the incubation flask and were added to the above mixture. The total mixture was allowed to stand with occasional swirling for at least 30 minutes to allow protein precipitation. The precipitated protein was removed by gravity filtration on paper or with suction through a medium sintered glass filter covered with filter paper to prevent clogging. The filtrate was then heated on a hot plate to 105° C. after which the hemin crystallized out immediately. The hemin was centrifuged and washed twice each with 50 percent acetic acid, distilled water, and 95 percent ethanol. If immediate recrystallization was contemplated, the hemin was also washed once or twice with

commercial ether to aid in drying. The crystallized material was dried and then purified by recrystallization. The hemin was triturated thoroughly with 0.3 to 0.5 ml. of pyridine till it had dissolved. Ten to fifteen ml. of chloroform were added, and the solution was filtered through fluted #2 Whatman filter paper. The filter paper was washed carefully until colorless with an additional volume of chloroform (10 to 15 ml.). Ten to twelve ml. of glacial acetic acid containing 2 percent (w/v) strontium chloride were added, and the solution was allowed to stand 30 minutes. The chloroform was removed by heating on a steam bath until boiling ceased. Crystallization of the hemin then occurred in the acetic acid. The crystals were washed as before and dried. Previous work in this laboratory indicated that the recrystallized hemin is pure and that a second recrystallization produces no significant change in specific activity (86). Furthermore, it was found with this method for preparing hemin that the specific activity did not even change markedly after the first recrystallization. Consequently a few of the results reported here were obtained from hemin that was not recrystallized (noted in appropriate tables), though in most of the experiments recrystallization was carried out. In the former cases the differences between samples to be compared were generally of such magnitude as to make of little significance the 10 to 15 percent change in activity that might have occurred after recrystallization.

K. Determination of Radioactivity

For Fe^{59}-labeled hemin an appropriate amount (usually 5 to 10 mg.) was weighed out and dissolved in 0.1 N potassium hydroxide to a concentration of 1 mg./ml. A two-milliliter aliquot was then pipetted into a tube for counting. Fe^{59} activity was measured in a Nuclear well

type scintillation counter attached to a Berkeley Decimatic Scaler. The time required for 4000 (in some experiments 3000) counts was recorded. For C^{14}-labeled hemin the dry recrystallized material was weighed out into aluminum planchets and distributed in a uniform layer. C^{14} activity was measured with an end window Geiger-Müller tube and Berkeley Decimatic Scaler. The time required for 1000 counts was recorded. Results on C^{14} hemin are expressed in counts per minute after background with correction to infinite thickness. Fe^{59} hemin results are expressed in some experiments as counts per minute after background of the two-milligram sample in 0.1 N potassium hydroxide, in other experiments, in which the hemoglobin concentration and the specific activity of the Fe^{59} added to each sample were determined, as the percent of Fe^{59} incorporation into heme.

L. Porphyrin Quantitation

The method of quantitation of uro-, copro-, and protoporphyrin used is a modification of that described by Schwartz (85). With use of this modification recoveries from red cell systems of 90 to 100 percent were obtained on copro- and protoporphyrin and 85 percent on uroporphyrin. Other methods of tissue porphyrin assay gave much poorer recoveries of uroporphyrin (91).

1. Extraction of Uroporphyrin

To a 5 ml. aliquot of the incubated sample were added 10 volumes of acetone containing 2 percent (by volume) concentrated hydrochloric acid to precipitate the protein and solubilize the porphyrins. The solution was filtered through a medium sintered glass filter and the precipitate washed with the acetone-hydrochloric acid until the filtrate was colorless. Two-thirds volume of ethyl acetate

was added and the solution put in a separatory funnel. Ten percent sodium acetate was added until the phases separated, and further extraction was carried out with 5 ml. aliquots of 10 percent sodium acetate until the aqueous phase did not fluoresce under a Wood's lamp (usually 4 to 5 aliquots). The uroporphyrin was now in the aqueous phase and was put aside (in the dark) for later purification.

2. Extraction of Coproporphyrin

Coproporphyrin was extracted from the ethyl acetate with 0.1 N hydrochloric acid (4 to 5 5-ml. aliquots). With higher levels of uroporphyrin some appeared here with the coproporphyrin. Protoporphyrin is not extracted by this dilute acid. The coproporphyrin was also put aside for later purification.

3. Extraction of Protoporphyrin

The protoporphyrin was extracted from the ethyl acetate with 5 ml. aliquots of 3 N hydrochloric acid. The volumes added and taken off were checked because the aqueous phase (hydrochloric acid) apparently extracts some acetone from ethyl acetate. The volume of the solution thus obtained was adjusted to contain the equivalent of 1.5 N hydrochloric acid for fluorimetric determination of the protoporphyrin concentration against a coproporphyrin standard in 1.5 N hydrochloric acid.

4. Purification of Coproporphyrin

Using the pH meter the coproporphyrin solution (in 0.1 N hydrochloric acid) was adjusted to pH 4.5 to 5.0 with 6 N potassium hydroxide. At this pH coproporphyrin and protoporphyrin (already removed) can be extracted by ethyl acetate while uroporphyrin cannot be extracted. Sodium acetate is necessary for quantitative extraction of the porphyrin into ethyl acetate, but excessive amounts should be avoided. Generally

a few drops of 10 percent sodium acetate were added. The coproporphyrin solution was added to 2 volumes of fresh ethyl acetate. Any fluorescence remaining in the aqueous phase was due to uroporphyrin which was then added to the uroporphyrin fraction. The ethyl acetate was washed once with water and the coproporphyrin extracted with 1.5 N hydrochloric acid (4 to 5 5-ml. aliquots). The concentration of coproporphyrin was then determined fluorimetrically.

5. Purification of Uroporphyrin

Uroporphyrin can be extracted by ethyl acetate at pH 3.0 to 3.2. The aqueous uroporphyrin solution was adjusted to this pH (pH meter) with hydrochloric acid and extracted with 2 volumes of ethyl acetate. The uroporphyrin was then extracted from the ethyl acetate with 3 N hydrochloric acid. As in all cases, extraction was continued with 5 ml. aliquots until there was no visible fluorescence in the extract. The ethyl acetate was washed with 5 ml. of 6 N potassium hydroxide and then with 10 ml. water. This washed ethyl acetate was used to extract the rest of the uroporphyrin out of the aqueous solution. The uroporphyrin was in turn extracted out of the ethyl acetate with 3 N hydrochloric acid and the whole process repeated again if necessary. The uroporphyrin in 3 N hydrochloric acid was diluted to 1.5 N and the concentration determined fluorimetrically.

M. Other Materials

1. α-C^{14}-Glycine

Obtained commercially.

2. Succinate

The desired amount of succinic acid was weighed out, dissolved in water, and adjusted to approximate neutrality with solid sodium bicarbonate.

3. δ-Aminolevulinic Acid Hydrochloride

Generously supplied by Dr. John R. Dice, Research Department, Parke, Davis and Company, Detroit, Michigan.

4. Ferrous citrate - Fe^{59}

Purchased from Abbott Laboratories, North Chicago, Illinois.

5. Carrier Iron

Prepared as a solution of ferrous sulfate heptahydrate ($FeSO_4 \cdot 7\ H_2O$) in water.

6. Glutathione (GSH)

A stock solution of 0.4 M glutathione in 0.01 N hydrochloric acid was prepared and preserved in the frozen state. For the appropriate experiments this stock solution was diluted to the desired concentrations and adjusted to approximate neutrality with solid sodium bicarbonate.

7. Diphosphopyridine nucleotide (DPN)

A stock solution of 0.02 M DPN in water was prepared and preserved in the frozen state. For the appropriate experiments this stock solution was diluted and its pH adjusted as with glutathione.

8. Red Blood Cell Salt Solution

The composition of this salt solution approximates that within the human red cell. It contains the following concentrations of the specified salts in water: potassium chloride, 120 meq./L.; sodium bicarbonate, 24 meq./L.; magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 6 meq./L.; and 85 percent phosphoric acid, 1.2 meq./L. The solution has a pH of 7.3 to 7.4.

RESULTS AND CONCLUSIONS

A. Observations on Isolated Nuclei

The nuclei isolated following hemolysis of the chicken red cells with hypotonic saline formed a grossly homogeneous fraction and did not agglutinate or form a jelly-like mass as occurred with other methods of lysis. The nuclei were readily resuspended in "cytoplasm", salt solutions, or water. Wet smears examined microscopically during the course of the experiments indicated that there was minimal contamination of the nuclear fraction with intact red cells. Repeated washings with RBC Salt Solution removed most of the "cytoplasm" as judged by the lighter color of successive washings and as shown by the experimental results on heme synthesis using nuclei washed a varying number of times.

Stains of the various fractions (erythrocytes before lysis, lysis mixture, nuclei, and "cytoplasm") with Wright's stain showed the following: (1) Nuclei in the lysis mixture and isolated nuclear fraction were noticeably swollen and stained lighter than those seen within the intact red cells. The swelling is assumed to be due to the treatment with hypotonic saline, and the relatively lighter staining with Wright's stain, which stains the nuclei blue, may be due to dilution within the nucleus or possibly to an eluting out of some of the nucleoprotein. According to Laskowski and Ryerson dilute sodium chloride solutions cause disappearance of internal nuclear structure and partial precipitation and extraction of nucleoprotein without rupturing the nuclear membrane (79). (2) The supernate was almost completely devoid of blue-staining nuclear material.

It is impossible to say with certainty from these studies that the method of hemolysis used did not result in dissolution of soluble

material from the surface of or from within the nucleus, this material then appearing in the "cytoplasm". However, the results of the biochemical studies of heme synthesis made with these fractions were extremely consistent throughout many experiments.

B. Division of Heme Biosynthesis into Four Segments

It will be convenient in discussing the experimental results to organize the data according to its application to specific segments of the heme biosynthetic pathway. Figure I summarizes heme synthesis, picturing in sequence the known and postulated precursors and intermediates. As previously cited, these have been convincingly worked out by Shemin and others up to the stage of the monopyrrole, porphobilinogen. In addition Nevé has shown that reduced uroporphyrin is a direct intermediate, probably lying close to PBG on the pathway. The subsequent tetrapyrrole intermediates are not clearly defined, but copro- and protoporphyrin or derivatives thereof are probably involved.

In the following discussion the heme biosynthetic pathway will be divided into four stages and the role of the nucleus in each discussed: (1) the condensation of glycine and succinate (with decarboxylation of the α-amino-β-keto adipic acid formed) to yield δ-aminolevulinic acid (ALA); (2) the conversion of ALA through PBG to porphyrins; (3) the conversion of uroporphyrin to protoporphyrin; and (4) the insertion of iron into protoporphyrin to form heme.

C. The Formation of δ-Aminolevulinic Acid from Glycine and Succinate

Table 1 shows the radioactivity of the hemin isolated after incubation of α-C^{14}-glycine with red cell fractions and combinations of fractions. The lysis mixture was active in forming heme from glycine,

but heating it for 30 minutes at 56° C. inhibited the synthesis completely, presumably due to inactivation of essential enzymes. This is in agreement with the observation of Goldberg et al. that heating at 56° C. produces 99 percent inhibition of heme formation from glycine in a chicken red cell system (60). Neither the nuclei nor the "cytoplasm" alone formed a significant amount of heme from glycine. The reconstituted system of nuclei plus "cytoplasm" was active but to a lesser degree than the lysis mixture, probably reflecting losses during isolation and washing of the nuclei. Thus, it is seen that both nuclei and "cytoplasm" must be present for heme to be synthesized from glycine.

The data in Table 2 summarize the results of a similar experiment in which it was again demonstrated that neither nuclei nor "cytoplasm" alone could synthesize heme from glycine. The "cytoplasm" could, however, be replaced by lysed human red cells or by "cytoplasm" heated at 56° C. without loss of the heme-synthesizing capacity of the system. The lysed human red cells actually stimulated synthesis more than "cytoplasm" in this experiment. Heating at 56° C. would not be expected to destroy cofactors but might very well inactivate enzyme systems. Heat treatment was shown in the previous experiment to inhibit the synthesis, so the ability of nuclei plus heated "cytoplasm" (sample 5, Table 2) to synthesize heme implies that the enzymes catalyzing the conversion of glycine and succinate to ALA are located in the nucleus.

The fact that lysed human red cells can replace the "cytoplasm" would seem to indicate that the enzymes catalyzing the reactions to ALA are in the nucleus because although human red cells have been reported to contain enzymes probably involved in certain steps of heme synthesis, i.e. the conversion of ALA to PBG (52) and the conversion of ALA to

certain porphyrins (76), normal human red cells cannot synthesize heme from glycine (77). Thus, human red cells probably do not contain the enzymes necessary to convert glycine and succinate to ALA.

The glycine-succinate condensing enzymes could be studied directly if it were possible to stop heme synthesis at the ALA stage and determine ALA concentrations. However, since an ALA-determination investigated (92) gave inconsistent results, a further experiment was designed to demonstrate more directly the site of the enzymes concerned in the conversion of glycine and succinate to ALA. It was found (in experiments to be described later) that when a lysis mixture was treated for 30 to 40 minutes in the Waring Blendor and centrifuged, the supernatant fluid, consisting of "cytoplasm" plus the "nuclear soluble fraction", was capable of converting ALA to heme but could not carry out the synthesis from glycine; i.e. the glycine-succinate condensing enzymes were lacking or, more likely, inactivated. This provided then a very convenient method to study the conversion of glycine to ALA. Various preparations could be added to this supernatant fluid from Waring Blendor-treated lysis mixture (WB). Only if the added preparation contained the glycine-succinate condensing enzymes would labeled heme be formed from C^{14}-glycine. The results of such an experiment are shown in Table 3. It is seen that added "cytoplasm" was inactive (sample 2), but added nuclei stimulated the synthesis (sample 3). It might be argued, however, that addition of nuclei merely supplied necessary cofactors for enzymes present in the other material (WB). To rule out this possibility samples 4-6 were included. Samples 4 and 5 show that heating, which may denature enzymes but should not destroy cofactors, inhibited the synthesis only when applied to the nuclei. Shemin, Abramsky, and Russell noted that

"homogenization" disrupts the functional activity of only those enzymes that are involved in the condensation of succinate with glycine (66). Homogenization would not be expected to destroy cofactors. Sample 6 shows that homogenization of the nuclei (5 minutes in the Waring Blendor) inhibited the synthesis completely.

It seems quite clear then that the enzymes responsible for the glycine-succinate condensation and conversion of the product, α-amino-β-keto adipic acid, to ALA are in the nuclear fraction of the chicken red cell. Knowledge of this fact should simplify further studies greatly since considerable purification of these enzymes can be achieved by fractionation of the cell and isolation of the nuclei. A particular advantage to nuclear studies is the fact that the great mass of hemoglobin contained in the cytoplasm has been separated from the enzymes. In addition, further study of the cytoplasmic fraction may reveal the essential factors which it contains and lead to discovery of the coenzymes involved in this reaction sequence.

Unpublished results from this laboratory on the conversion of protoporphyrin to heme in a fractionated rat liver system indicated that glutathione stimulated this conversion (93). An experiment was set up to determine if glutathione (GSH), which is found in high concentrations in erythrocytes, could replace the "cytoplasm" in forming heme from glycine in the fractionated chicken red cell system. The results, presented in Table 4, show that glutathione alone (at the concentrations tested) is inactive in this respect.

Schulman and Richert reported that the rate of heme synthesis from glycine in the red cells of both vitamin B_6- and pantothenic acid-deficient ducklings was below that in normal ducklings (94,95). Synthesis

from ALA, however, was unaffected. They concluded that both pyridoxine and pantothenic acid deficiencies exerted their effects on heme synthesis in the utilization of glycine and succinate for the formation of ALA. Similar conclusions were reached by Lascelles who studied porphyrin synthesis in cell suspensions of Tetrahymena vorax (9). Laver and Neuberger have prepared a supernatant and two particulate fractions by centrifugation of an homogenized lysate of chicken erythrocytes (96). Their supernatant fraction alone was inactive in forming ALA from glycine and succinate, and the particulate fractions alone showed only low activity. However, the activity of the top particulate fraction was increased 3.5 times by addition of the supernate and 2.5 times by addition of pyridoxal phosphate and coenzyme A. These results are in remarkable agreement with the results reported herein and support the assertion that the cytoplasm contributes only cofactors in the conversion of glycine and succinate to ALA, the enzymes being in the nuclear fraction. Pyridoxal phosphate and coenzyme A may well be the cytoplasmic cofactors in question and should be investigated further in conjunction with nuclear preparations.

An attempt was made to extract and solubilize the glycine-succinate condensing enzymes from the nuclear fraction by treatment in the Potter-Elvejhem homogenizer. Though the enzymes are inactivated by Waring Blendor-treatment, it was thought that treatment as mild as this homogenization should not damage them, and it is known that soluble enzymes can sometimes be extracted from a cell fraction by a salt solution (97). The results of this experiment are shown in Table 5. The various supernate and residue fractions were added to the supernatant fluid resulting from centrifugation of Waring Blendor-treated lysis mixture (WB) to see

which contained the enzymes to convert glycine and succinate to ALA.

It is seen that homogenization with water did not in itself inhibit the synthesis (samples 2 and 3). After centrifugation of the water-homogenate the residue consisted of a gummy layer of agglutinated nuclei covered with a filmy, more easily-resuspended layer. This filmy layer may be equivalent to Laver and Neuberger's top particulate (P_1) fraction (96). Subsequent homogenization of the pellet with salt solutions gave a more agglutinated mass after centrifugation with less or none of the filmy layer overlying it. Samples 4 and 5 show that after homogenization with water, the activity still resided in the residue. Rehomogenization of the residue with 0.9 percent NaCl (samples 6 and 7), 0.1 percent NaCl (samples 8 and 9), or RBC Salt Solution (samples 10 and 11) was also ineffective in solubilizing the enzymes, and the synthesis was somewhat decreased in the residues. A combination of two of the supernates (sample 12) was also inactive.

Thus, although homogenization of the nuclear fraction in the Potter-Elvejhem homogenizer does not destroy the enzymes that convert glycine and succinate to ALA, none of the attempted treatments was successful in solubilizing these enzymes. Extraction with other, perhaps more concentrated, salt solutions or other treatment should be tried. Sonic oscillation has been effective in liberating certain enzymes from liver cell fractions and yeast suspensions (97,98,99,100) and might be used in this case.

D. The Conversion of ALA to Porphyrins

In early experiments, ALA was incubated with various chicken red cell fractions. An extract of a portion of the incubated sample was prepared by adding an equal volume of 10 percent trichloroacetic acid

(TCA) and centrifuging. The visible fluorescence of this TCA-extract under a Wood's lamp was then observed as an estimate of porphyrin formation. Fluorescence was graded subjectively according to its intensity from 0 (no fluorescence) to 10 (very strong fluorescence). In most of these experiments in which fluorescence was graded, Fe^{59} was added in order to observe also heme synthesis from ALA in the various cell fractions. The data on heme formation will be only briefly alluded to at present and will be discussed more fully in a later section.

The concentration of ALA used in all incubations was approximately equivalent to the optimal level reported by Falk in studying porphyrin and heme formation in chicken red cell hemolysates (101) and four to five times the optimal level reported by Goldberg et al. studying only heme formation (60).

It is seen from Table 6 that in every case "cytoplasm" alone (sample 1) was very active in converting ALA to porphyrins while the nuclei alone and fractions thereof (samples 2-4) were in most cases relatively inactive. The cases where some fluorescence did appear after incubation of nuclear fractions with ALA may have resulted from contamination with "cytoplasm" due to inadequate washing or from other sources of error in this very approximate determination. In this regard it is noted in two cases that the combined nuclear washings were active in converting ALA to porphyrins (samples 5 and 6). In cases where washed nuclei or nuclear fractions were recombined with "cytoplasm" (samples 7-9), porphyrins were also formed, as would be expected. The smaller values in some cases may be due to the fact that these systems were active in forming heme from ALA (as will be shown later). Thus there may have been less tendency for the porphyrins, which are probably

by-products of intermediates in the pathway to heme, to accumulate. For example, the one zero value (sample 8) was recorded in an experiment in which that sample was very active in forming heme. Intact red cells (sample 10) and lysis mixture (sample 11) contain cytoplasm of course, so the reason for their lack of activity in forming porphyrins from ALA is not clear. Perhaps either the amount of heme formation or possibly permeability difficulties are responsible. The one experiment with lysis mixture in which definite fluorescence was noted was one in which the red cells were lysed with the Waring Blendor rather than hypotonic saline.

Though the data presented in Table 6 are somewhat variable and were obtained using a rough, very subjective quantitation of porphyrin formation, there are rather definite indications that the enzymes converting ALA via PBG to porphyrins appear in the cytoplasmic fraction of the red cell prepared by these procedures and that the nucleus is inactive in this respect. It is interesting to note at this point that these enzymes were found to be stable for long periods of time. When "cytoplasm" that was stored in the refrigerator for over a month (up to 43 days) was incubated with ALA, the TCA-extract still showed very strong fluorescence.

More precise data on porphyrin formation from ALA were obtained by quantitation of the uro-, copro-, and protoporphyrin formed in various cell fractions incubated with ALA. The results of such an experiment quantitating porphyrins and measuring Fe^{59} incorporation into heme are recorded in Table 7. The data on heme formation will be discussed more fully later. All determinations were done after 3 and again after 6 hours of incubation. Since the length of incubation made little differ-

ence in the gross nature of the results, only the 3 hour values are recorded. Several facts stand out: (1) Significant amounts of protoporphyrin were formed in only those samples in which heme was synthesized. (2) Generally little coproporphyrin was formed, but, except for sample 6, there were relatively greater amounts of coproporphyrin formed in those samples in which protoporphyrin was also synthesized. (3) Uroporphyrin was formed in all samples containing "cytoplasm" with especially great quantities formed in blendorized samples (5-7). Since a significant amount of uroporphyrin was formed by "cytoplasm" alone (sample 2), it can be concluded that the nucleus is not required for the conversion of ALA to uroporphyrin and that the enzymes exist in the cytoplasmic fraction. Added nuclei (sample 4) did not stimulate this conversion. The reason for the higher uroporphyrin values in Waring Blendor-treated samples is not entirely clear, but in sample 7, at least, the extremely high value may represent an accumulation at this intermediate stage due to lack of factors (enzymes) necessary to carry the conversion on to coproporphyrin, protoporphyrin, and heme. It might be pointed out here (and will be discussed later) that the nucleus or its soluble fraction was apparently necessary to carry the reaction past the uroporphyrin stage.

In connection with these results, a report by Granick is of interest (67). He separated three fractions from the soluble portion of erythrocytes by starch electrophoresis. Fraction 1 contained the enzyme which converts ALA to PBG, fraction 2 the enzyme converting PBG to a colorless precursor of uroporphyrin, and fractions 2 and 3 together under anaerobic conditions converted PBG to a colorless precursor of coproporphyrin.

Schwartz and Watson obtained a "supernatant suspension" from rat liver homogenate that converts PBG to porphyrins (mainly coproporphyrin and protoporphyrin with smaller amounts of uroporphyrin) (6). They obtained similar results with chicken blood hemolysates, but a much higher proportion of the PBG was recovered as uroporphyrin, which was in agreement with the results being reported here.

Since the "cytoplasm" was shown to contain the enzymes converting ALA to uroporphyrin, further study was directed at this fraction, and the ability of heated "cytoplasm" to carry out this conversion was examined. The results of two such experiments are tabulated in Tables 8 and 9. Table 8 shows again that the presence of nuclei is required to carry the conversion past uroporphyrin to protoporphyrin (samples 1 and 3), and again very little coproporphyrin was formed in any fraction. The relatively low values for coproporphyrin may merely be due to the kinetics and equilibria of the reaction sequence causing any coproporphyrin or precursor of coproporphyrin formed to be converted rapidly to protoporphyrin or heme.

It is seen that the cytoplasmic enzymes catalyzing the formation of uroporphyrin from ALA are stable up to 70° C., and uroporphyrin synthesis is actually stimulated by heating to this temperature. "Cytoplasm" heated at 100° C. is, however, inactive.

Booij and Rimington have reported that heating chicken or human red cell hemolysates 30 minutes at 60° C. before adding substrate alters the synthesis as follows: (1) The amount of uroporphyrin formed increases (an observation supported by the data in Tables 8 and 9). (2) The amounts of coproporphyrin and protoporphyrin decrease. (3) Uroporphyrin I and coproporphyrin I replace the type III isomers (102). Similar

results are reported by Bogorad and Granick using suspensions of Chlorella to study porphyrin synthesis (74). Boiling the Chlorella destroyed its porphyrin-synthesizing capacity. Chlorella heated at 55° C. for 60 minutes converted PBG to porphyrin, which was mainly uroporphyrin I rather than uroporphyrin III, as was formed in the unheated preparation. In their heated preparation the porphyrin formed accounted for 90 percent of the PBG that disappeared as compared with only 55 percent in the unheated preparation, an interesting observation in light of the fact that the heated "cytoplasm" samples in the present study also yielded increased amounts of uroporphyrin (Tables 8 and 9). Isomer determinations were not done on the samples reported here, but some type III isomers must have been formed since heated "cytoplasm" (56° C.) added to nuclei could stimulate heme synthesis from glycine (Table 2).

The question might be raised whether these soluble enzymes of the cytoplasm that convert ALA to uroporphyrin may not exist in the nucleus in the intact cell and merely have been extracted from within the nucleus or eluted from its surface by the treatment used in fractionating the red cell. This possibility cannot be entirely ruled out by the results of the above experiments, but it can certainly be said with assurance that these enzymes do exist in solution in the cytoplasmic fraction as prepared, and isolation of that fraction yields a system in which this segment of the heme biosynthetic pathway may be studied apart from the enzyme systems of the nuclear fraction.

One step in the conversion of ALA to porphyrins, the condensation of two molecules of ALA to form a molecule of PBG, has been studied extensively by several groups. Purification and partial characterization of the enzyme, ALA dehydrase, that catalyzes this condensation, has been

carried out by Shemin *et al.* using a cell-free extract of duck erythrocytes (41,52), by Granick using a chicken erythrocyte extract (69), by Schulman using extracts of pigeon liver acetone powder (70), and by Gibson, Neuberger, and Scott using a soluble fraction of ox liver cytoplasm (71). That it is a soluble cytoplasmic enzyme in the chicken erythrocyte is supported by the results reported here.

The conclusion that ALA is converted to uroporphyrin by the cytoplasmic fraction of the chicken red cell is in agreement with the reports of Dresel and associates that a supernatant preparation of a chicken erythrocyte water-hemolysate formed significant amounts of uroporphyrin from either ALA or PBG (51,65,103). In that system, however, coproporphyrin (but not protoporphyrin) was also formed. The formation of coproporphyrin may be a result of the different method of hemolysis since nuclei are definitely disrupted in a water-hemolysate. Addition of a particulate preparation from rat liver to Dresel's supernatant preparation supplied the factor necessary for protoporphyrin to be formed, and the activity of this liver preparation appeared to be associated with the mitochondria. This is an interesting observation in that a mitochondrial fraction from rat liver has been shown by Nishida to catalyze the conversion of protoporphyrin to heme (93). Dresel and Falk's supernatant preparation did not convert uroporphyrin itself to coproporphyrin or protoporphyrin (56), but this is not surprising since uroporphyrin is not a true intermediate in heme synthesis. Shemin and co-workers have prepared a supernatant fraction from an homogenized water-hemolysate of duck red cells that is capable of converting ALA all the way to heme (66). The apparent disagreement between the data of these two groups is clarified by results, alluded to above and to be discussed further, which show that

homogenization in the Waring Blendor releases from the nucleus certain soluble enzymes vital to the later stages of heme biosynthesis.

The work of Rimington and Booij indicates that human red cells contain the enzymes found in chicken erythrocyte supernate, i.e. those catalyzing the conversion of ALA to uroporphyrin III (76). This explains the ability of a system of lysed human red cells and chicken erythrocyte nuclei to convert glycine to heme (Table 2).

An enzyme, PBG deaminase, which converts PBG to a precursor of uroporphyrin has been prepared from extracts of spinach leaf acetone powder by Bogorad (54), and the enzyme(s) converting PBG to uroporphyrin III have been purified from chicken erythrocyte hemolysates by Lockwood and Rimington (75).

E. The Conversion of Uroporphyrin to Protoporphyrin

As mentioned in the previous section, other workers have obtained supernatant preparations from chicken red cell water-hemolysates that convert ALA to uro- and coproporphyrin or their precursors (51,65,67, 103). The cytoplasmic fraction in the present study carried the conversion of ALA only as far as uroporphyrin. Thus it seemed reasonable to examine the nucleus as a source of the enzymes necessary to carry the synthesis of heme past the uroporphyrin stage to copro- and protoporphyrin. That the addition of nuclei or the "nuclear soluble fraction" to "cytoplasm" is essential for the formation of protoporphyrin from ALA is shown by the results already presented in Tables 7 and 8. It could, of course, be asserted that the nuclear fractions were contributing only essential cofactors and not enzymes.

Since uroporphyrin is probably not a true intermediate in heme synthesis, it would not be expected that uroporphyrin itself would

necessarily be converted to copro- or protoporphyrin in red cell systems. Falk, Dresel, and Rimington reported 52 percent conversion of uroporphyrin III to protoporphyrin in red cell hemolysate (103), but in a later report Dresel states that the maximum protoporphyrin obtainable from uroporphyrin III was less than 10 percent of that obtainable from PBG under identical conditions (51).

The elucidation by Nevé et al. of reduced uroporphyrin III as a true intermediate in the biosynthesis of heme (55) pointed to this material as a good substrate with which to study the role of the nucleus in the uroporphyrin-to-protoporphyrin portion of the pathway of heme synthesis. The results of an experiment in which reduced uroporphyrin III was incubated with various cell fractions in the presence of Fe^{59} are presented in Table 10. Hemin was isolated and its radioactivity determined, and porphyrins were quantitated. The results of this experiment were very inconclusive as really significant amounts of copro- or protoporphyrin were not formed in any sample, and heme was synthesized only in sample 5 to any extent.

It may be that permeability barriers to the reduced uroporphyrin substrate were presented by intact nuclei so that only after Waring Blendor treatment and preparation of the "nuclear soluble fraction" were the necessary nuclear enzymes sufficiently accessible. Even then the nuclear soluble fraction alone did not convert reduced uroporphyrin to heme (sample 4) due perhaps to lack of cytoplasmic cofactors.

The results that were obtained are, however, in agreement with Shiffmann and Shemin's report that a soluble system (cell-free extract) of duck erythrocytes contains the enzymes responsible for the decarboxylation of porphyrin side chains (44).

Concrete conclusions cannot be drawn from these data, but it can safely be asserted that the nucleus (more specifically, the "nuclear soluble fraction") contains essential factors (quite possibly enzymes) for catalyzing the uroporphyrin-to-protoporphyrin stages of heme synthesis and that further study of the "nuclear soluble fraction", perhaps in combination with heated "cytoplasm" or added cofactors, is warranted.

F. The Conversion of Protoporphyrin to Heme

The first information about the final stage of heme synthesis, that is, the incorporation of iron into the porphyrin ring, was obtained in experiments similar to those already described in which ALA and Fe^{59} were incubated with various cell fractions and the radioactivity of isolated hemin determined. As would be expected from the results already discussed, both nuclear and cytoplasmic fractions were required in order for heme to be formed from ALA. Results of such an experiment are expressed in Table 11. It is seen that the lysis mixture was more active than intact red cells, perhaps due to decreased permeability barriers, and that the reconstituted system (sample 7) was less active than lysis mixture but still definitely capable of carrying out the conversion. When washed nuclei and "cytoplasm" were stored separately in the cold, their activity in synthesizing heme from ALA when recombined decreased about 20 percent in one day and 45 percent in two days.

Furthermore, in agreement with the report of Shemin, Abramsky, and Russell that a cell-free extract can convert ALA to heme (66), it was found that centrifugation (at 9000 g) following Waring Blendor treatment of intact red cells yielded a supernatant preparation capable of this conversion (Table 12, sample 3). A similar preparation obtained

from Waring Blendor treatment of lysed red cells gave similar results (Table 7). Since "cytoplasm" alone could not convert ALA to heme, it was assumed that the Waring Blendor treatment liberated soluble materials (probably including enzymes) from the nucleus. To substantiate this assumption, a "nuclear soluble fraction" was prepared directly by blendorizing isolated nuclei. As seen in Table 13, when this nuclear soluble fraction was added to "cytoplasm", heme was synthesized from ALA. Some of the activity, however, still resided in the "nuclear residue".

Since these preliminary observations on iron insertion hinted that the enzyme(s) involved might be in the "nuclear soluble fraction", further experiments were undertaken using protoporphyrin as a substrate to study iron insertion specifically.

As yet it has not been shown that protoporphyrin is a true intermediate in heme biosynthesis, and certain workers have presented evidence that it is not (51,86). On the other hand, several workers have described biological systems which can insert iron into protoporphyrin to form heme (19,58,59), and work of this type carried out in chicken red cell systems indicates that this iron insertion is, in fact, enzyme dependent (60,61, 62).

The results of two of several similar experiments in which protoporphyrin and Fe^{59} were incubated with various cell fractions are given in Tables 14 and 15. In the latter table results are expressed in terms of percent incorporation of Fe^{59}. It is seen that maximal insertion of iron into protoporphyrin was obtained when both nuclear and cytoplasmic fractions were present, and that although some of the stimulation provided by nuclei was retained in the "nuclear residue", most of the nuclear

activity was solubilized by the Waring Blendor treatment.

Tables 14 and 15 also show that nuclei or the "nuclear soluble fraction" alone catalyzed some degree of iron insertion into protoporphyrin while "cytoplasm" alone was inactive. The tentative conclusion was made that the enzyme catalyzing iron insertion was in the nucleus (more specifically, the "nuclear soluble fraction") while the "cytoplasm" contributed cofactors stimulating this reaction. Further studies were carried out to try to confirm this hypothesis.

To determine the optimal protoporphyrin level for iron incorporation, "nuclear soluble fraction" added to "cytoplasm" was incubated with varying amounts of protoporphyrin. As shown in Table 16 and Figure II, the level of protoporphyrin that produced maximal uptake of Fe^{59} was 0.1 μmoles or a concentration in the incubated sample of approximately 0.5×10^{-5}M. This is only one-tenth as great as the optimal concentration reported by Goldberg et al. (60) and as that used by Schwartz et al. (62) in their studies of iron incorporation into protoporphyrin in chicken red cell hemolysates, and was also less than the concentration used by Krueger, Melnick, and Klein (61). The optimal iron concentration was not determined, but the level used in this and subsequent experiments was about 0.01 μmoles per sample or a concentration of added iron of approximately 0.5 to 0.6×10^{-6}M. depending on the volume of the sample.

Table 17 shows that lysed human red cells, when added to nuclei, could stimulate increased iron incorporation into protoporphyrin (sample 5) though not as markedly as could "cytoplasm" (sample 4). Since it is reported that human red cells cannot form heme from protoporphyrin (60), this was an indication that the "cytoplasm" was furnishing only cofactors

and not enzymes. "Cytoplasm" heated at 56° C. also stimulated synthesis when added to nuclei (sample 6) but again less than unheated "cytoplasm". At any rate the 86 percent inhibition of protoporphyrin conversion to heme in chicken red cell hemolysates caused by heating at 56° C. that was reported by Goldberg (60) was certainly not noted here. This then lends further weight to the hypothesis that the enzyme is nuclear.

However, attempts to verify Goldberg's heat-inhibition results and apply this inhibition separately to nuclear and cytoplasmic fractions to localize the enzyme gave results that are not entirely clear-cut. It was found in such an experiment (Table 18) that when the nuclei were heated at 56° C. and added to "cytoplasm", iron incorporation was decreased 66 percent but not completely inhibited (sample 4). This much inhibition would certainly be unlikely, though, if the nuclei were furnishing only cofactors. On the other hand similar treatment of "cytoplasm" gave 29 percent inhibition (sample 5). The greater heat-sensitivity of the nuclei is consistent with the hypothesis of a nuclear enzyme. However, heating either the nuclei or the "cytoplasm" at 70° C. inhibited iron incorporation practically completely (samples 6 and 7). If, as the results imply, the enzyme is in the nucleus, a reason for inhibition when the "cytoplasm" is heated must be sought.

The data from another experiment, in which the "nuclear soluble fraction" and extracts of heated cell fractions were prepared, are presented in Table 19. The results are similar to those in Table 18. It is seen again that heating either "cytoplasm" or the "nuclear soluble fraction" at 56° C. caused some inhibition. In this case, inhibition was 88 percent when the "nuclear soluble fraction" was heated and 53 percent when the "cytoplasm" was so treated. Again the greater heat-

sensitivity of the nuclear fraction is evident. Heating at 62° C. caused marked inhibition similar to that achieved at 70° C. in the previous experiment (Table 18).

These results, showing much greater heat-sensitivity of the nuclear fractions and replacement of "cytoplasm" by lysed human red cells, were interpreted as being compatible with a nuclear localization of the enzyme that catalyzes insertion of iron into protoporphyrin. The explanation for the decrease in iron incorporation when the "cytoplasm" was heated is not obvious, but it may be that this treatment caused formation of substances which could bind iron and thus decrease its incorporation into protoporphyrin. Data in support of this explanation were obtained in one experiment (Table 20) in which the nuclear fraction alone, due perhaps to inadequate washing, stimulated a significant degree of iron incorporation into protoporphyrin, but when heated "cytoplasm" (especially at or above 70° C.) was added to these active nuclei, iron incorporation was decreased markedly. In this experiment it was also demonstrated again that the nuclei (sample 6) were much more sensitive to heating at 56° C. than was the "cytoplasm" (sample 3). That the inhibition might be related to iron-binding is also indicated by the fact that no such inhibition was noted when the cytoplasm-containing fraction (WB) was heated in the experiments that utilized labeled glycine as substrate (Table 3, sample 4).

Because of the instability of the succinate-glycine condensing enzymes to Waring Blendor homogenization, the effect of such treatment on the iron-incorporating fraction was examined. This fraction was stable to prolonged blendorization (Table 21).

Nuclear localization of this enzyme would be demonstrated quite

conclusively if the "cytoplasm" could be replaced by pure cofactors. Studies of the conversion of protoporphyrin to heme in liver cell fractions indicated that glutathione (GSH) and diphosphopyridine nucleotide (DPN) stimulated iron insertion (93). The results of adding various levels of these two substances to isolated nuclei are shown in Tables 22,23, and 24, together with the comparable values for nuclear fractions alone and nuclear fractions plus "cytoplasm". The figures in Table 23 indicate that, at low concentrations DPN was able to increase iron incorporation more than 100 percent over nuclei alone, showing that it can, to some extent, replace the "cytoplasm". This then lends further weight to the hypothesis that the cytoplasm furnishes only cofactors and that the enzyme catalyzing iron insertion is in the nucleus. Tables 22 and 24 show that glutathione added to intact nuclei, and DPN added to the "nuclear soluble fraction" did not stimulate iron incorporation significantly. Combinations of these and perhaps other materials might be tried to see if the "cytoplasm" cannot be more completely replaced by known cofactors.

It may be concluded, then, from the above results that the enzyme catalyzing iron insertion into protoporphyrin to form heme is almost certainly in the nucleus of the chicken erythrocyte and can be solubilized by Waring Blendor treatment.

DISCUSSION

The conclusions drawn from the data presented can be briefly summarized as follows: (1) The enzymes catalyzing the conversion of glycine and succinate to ALA are in the nuclear fraction of the chicken erythrocyte. (2) The nuclear fraction is not required for the conversion of ALA to uroporphyrin. The enzymes and cofactors involved are in the cytoplasmic fraction as prepared here. (3) The enzymes carrying heme biosynthesis from the uroporphyrin to protoporphyrin stage are most probably in the nuclear fraction and can be rendered soluble by treatment of that fraction in the Waring Blendor. (4) Iron insertion into protoporphyrin also depends upon a nuclear enzyme (or enzymes) which can be solubilized by Waring Blendor treatment. (5) The enzymes in the nuclear fraction require certain cytoplasmic cofactors to manifest their activity.

It is seen, then, that the avian erythrocyte nucleus plays a vital role in heme biosynthesis and apparently contains all the enzymes involved except those converting ALA to uroporphyrin. It would perhaps be more reasonable from a structure-function standpoint to expect all the enzymes of this biosynthetic pathway to exist in the nucleus, and, as pointed out previously, this possibility cannot be ruled out by the studies presented here. If, for example, these enzymes existed on the surface of the nucleus in the intact cell or if they were very soluble, they might easily have been eluted into the cytoplasmic fraction during the procedures of hemolysis and fractionation.

Several implications for further study may be drawn from the conclusions reached in this report. The cell fractions described here provide sources of enzyme systems necessary for heme biosynthesis,

sources in which these enzymes may be studied in purer form than in intact or hemolyzed cells. This cell fractionation achieves separation of most of the enzymes from the mass of hemoglobin contained in the cytoplasm and also from certain essential cofactors. Study of the cytoplasm should be of value in attempts to identify these cofactors.

It is interesting to compare the chicken erythrocyte to the human red cell as regards the enzymes concerned in heme biosynthesis. The human red cell has been shown to contain the enzymes converting ALA both to PBG (52) and to certain porphyrins, in particular uroporphyrin III (76). This synthetic capability of the human red cell is remarkably similar to that of the chicken erythrocyte cytoplasmic fraction as described here. Thus the ability of the peripheral blood of humans with certain hematologic abnormalities to catalyze heme formation from glycine and succinate (22) may be due to the presence in that blood of red cells with intact nuclei or nuclear fragments containing those enzyme systems found in the chicken erythrocyte nucleus.

SUMMARY

1. The role of the chicken erythrocyte nucleus in heme biosynthesis has been investigated.

2. Fractionation of chicken erythrocytes into cytoplasmic and nuclear fractions was achieved by hemolysis of the washed red cells with hypotonic sodium chloride solution followed by centrifugation at 9000 g. The isolated nuclei were further fractionated into a "nuclear soluble fraction" and a "nuclear residue" by Waring Blendor treatment and centrifugation.

3. Microscopic study and remarkably consistent experimental results indicated that the cell fractions thus prepared were relatively homogeneous.

4. Isolated cell fractions and combinations thereof were incubated with various heme precursor substrates. Heme formation was evaluated by tracer methods using C^{14}-labelled glycine or Fe^{59}, and in certain experiments the uro-, copro-, and protoporphyrin formed were also quantitated.

5. The heme biosynthetic pathway was divided into four segments for study: (1) the condensation of glycine and succinate (with decarboxylation of the α-amino-β-keto adipic acid formed) to yield δ-aminolevulinic acid (ALA); (2) the conversion of ALA through porphobilinogen (PBG) to porphyrins; (3) the conversion of uroporphyrin (or its reduced derivative) to protoporphyrin; and (4) the insertion of iron into protoporphyrin to form heme.

6. Various techniques were utilized to inactivate enzyme systems without disrupting cofactors so that the enzymes catalyzing these portions of heme biosynthesis could be localized in a particular cell

fraction.

7. It was found that both cytoplasmic and nuclear fractions were required for the conversion of glycine and succinate to ALA, but inactivation techniques indicated quite clearly that the enzymes involved were in the nuclei while the cytoplasm furnished essential cofactors. Attempts to solubilize these enzymes from the nuclear fraction were unsuccessful.

8. All the factors necessary for the conversion of ALA to uroporphyrin were in the cytoplasmic fraction as prepared. The possibility was suggested that these enzymes might have been eluted from the nuclei during hemolysis.

9. Data were interpreted as indicating that the enzymes catalyzing the synthesis from the uroporphyrin to protoporphyrin stage were most probably in the nucleus, (more specifically the "nuclear soluble fraction") with essential cofactors provided by the cytoplasm.

10. The "nuclear soluble fraction" also contained the enzyme(s) responsible for the insertion of iron into protoporphyrin to form heme, the cytoplasmic fraction again providing necessary cofactors.

11. The relationship of these results to numerous reports by other groups using erythrocyte water-hemolysates has been discussed.

12. It has been suggested that isolation of nuclear fractions as described here provides convenient sources of many of the enzyme systems concerned in heme biosynthesis. In nuclear fractions separated from the mass of cytoplasmic hemoglobin these enzymes may be studied in purer form than in whole cells or hemolysates. In addition, study of the cytoplasmic fraction should be of value in identifying essential cofactors involved in various stages of the synthesis.

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TABLE 1

Heme Synthesis from α-C^{14}-Glycine

Heat Inactivation of Lysis Mixture and Effect of Washing Nuclei

Samples	Hemin Radioactivity (CPM)
1) 15 ml. lysis mixture	552
2) 15 ml. heated lysis mixture (56°C.)	3
3) 15 ml. "cytoplasm"	6
4) 5 ml. nuclei (washed 2X) + 5 ml. RBC Salt Solution	2
5) 5 ml. nuclei (unwashed) + 10 ml. "cytoplasm"	339
6) 5 ml. nuclei (washed 1X) + 10 ml. "cytoplasm"	261
7) 5 ml. nuclei (washed 2X) + 10 ml. "cytoplasm"	228
8) 5 ml. nuclei (washed 4X) + 10 ml. "cytoplasm"	233

Incubation for 4 hours.

Added to each sample: 13.3 μmoles (1.0 millicurie/millimole) α-C^{14}-glycine, 0.14 millimoles succinate, and 1.0 μmole iron.

TABLE 2

Heme Synthesis from α-C^{14}-Glycine

Effect of Heated "Cytoplasm" and Lysed Human Red Cells

Samples	Hemin Radioactivity (CPM)
1) 15 ml. lysis mixture	185
2) 15 ml. "cytoplasm"	2
3) 5 ml. nuclei + 5 ml. water	1
4) 5 ml. nuclei + 10 ml. "cytoplasm"	51
5) 5 ml. nuclei + 10 ml. heated "cytoplasm" (56°C.)	52
6) 5 ml. nuclei + 10 ml. lysed human red cells	111

Incubation for 4 hours.

Added to each sample: 13.3 μmoles (1.0 millicurie/millimole) α-C^{14}-glycine, 0.14 millimoles succinate, and 1.0 μmole iron.

Human red cells washed 3X with 0.9% NaCl and lysed with 1.5 volumes distilled water.

All nuclei washed 4X.

TABLE 3

Heme Synthesis from α-C^{14}-Glycine

Enzyme Inactivation by Heating and Blendorizing Nuclei

Samples	Hemin Radioactivity (CPM)
1) 10 ml. WB + 10 ml. water	2
2) 10 ml. WB + 10 ml. "cytoplasm"	1
3) 10 ml. WB + 5 ml. nuclei + 5 ml. water	237
4) 10 ml. heated WB (56°C.) + 5 ml. nuclei + 5 ml. water	329
5) 10 ml. WB + 5 ml. heated nuclei (56°C.) + 5 ml. water	2
6) 10 ml. WB + 5 ml. blendorized nuclei + 5 ml. water	2

Incubation for 4 hours.

Added to each sample: 13.3 μmoles (1.0 millicurie/millimole) α-C^{14}-glycine, 0.2 millimoles succinate, and 1.0 μmole iron.

WB = supernatant fluid resulting from Waring Blendor treatment of lysis mixture followed by centrifugation for 10 minutes at 9000 g.

All nuclei washed 2X.

TABLE 4

Heme Synthesis from α-C^{14}-Glycine

Effect of Glutathione

Samples	Hemin Radioactivity (CPM)
1) 5 ml. nuclei + 10 ml. water	11
2) 5 ml. nuclei + 10 ml. "cytoplasm"	136
3) 5 ml. nuclei + 10 ml. GSH solution (final conc. 0.03 M)	1
4) 5 ml. nuclei + 10 ml. GSH solution (final conc. 0.01 M)	2
5) 5 ml. nuclei + 10 ml. GSH solution (final conc. 0.003 M)	1
6) 5 ml. nuclei + 10 ml. GSH solution (final conc. 0.001 M)	2

Incubation for 4 hours.

Added to each samples: 13.3 μmoles (1.0 millicurie/millimole) α-C^{14}-glycine, 0.14 millimoles succinate, and 1.0 μmole iron.

All nuclei washed 2X.

TABLE 5

Attempt to Solubilize Glycine-Succinate Condensing Enzymes from Nuclear Fraction

Samples	Hemin Radioactivity (CPM)
1) 15 ml. WB	1
2) 10 ml. WB + 5 ml. nuclei + 5 ml. water	88
3) 10 ml. WB + 5 ml. homogenized nuclei + 5 ml. water	96
4) 10 ml. WB + supernate from (3)	2
5) 10 ml. WB + residue from (3)	63
6) 10 ml. WB + supernate after rehomogenization with 5 ml. 0.9% NaCl	1
7) 10 ml. WB + residue from (6)	35
8) 10 ml. WB + supernate after rehomogenization with 5 ml. 0.1% NaCl	1
9) 10 ml. WB + residue from (8)	21
10) 10 ml. WB + supernate after rehomogenization with 5 ml. RBC Salt Solution	2
11) 10 ml. WB + residue from (10)	28
12) (4) + (10)	0

Incubation for 4 hours.

Added to each sample: 13.3 μmoles (approximately 0.5 millicuries/millimole) α-C^{14}-glycine, 0.14 millimoles succinate, and 1.0 μmole iron.

WB = supernatant fluid resulting from Waring Blendor treatment of lysis mixture followed by centrifugation for 10 minutes at 9000 g.

All nuclei washed 2X.

TABLE 6

Porphyrin Formation from ALA

Samples	Fluorescence after 6 Hours' Incubation with 6 μmoles ALA	Average
1) "Cytoplasm"*	10,10,10,10,10,10,10,10,10	10.0
2) Nuclei	4,0,0,5,6,0,3,2,5,4,0,0,0	2.2
3) "Nuclear soluble fraction"	0	0.0
4) "Nuclear residue"	0	0.0
5) Combined first washings of nuclei	10	10.0
6) Combined third washings of nuclei	4	4.0
7) Nuclei + "cytoplasm"	8,10,5,10,8,3,8,10,6,10	7.8
8) "Nuclear soluble fraction" + "cytoplasm"	10,0	5.0
9) "Nuclear residue" + "cytoplasm"	10,10	10.0
10) Washed chicken red cells	0,0,0,0,0	0.0
11) Lysis mixture	1,0,0,8,0,0,0,0,0,0	1.0

0 = no visible fluorescence; 10 = very strong fluorescence.

Each figure in the fluorescence column represents the result of a separate experiment in which ALA was incubated with the indicated cell fraction.

All nuclei were washed at least 2X before incubation.

*A TCA-extract of unincubated cytoplasm shows no visible fluorescence.

TABLE 7

Porphyrin and Heme Biosynthesis from δ-Aminolevulinic Acid

Samples	Porphyrin Formation (μgm./sample) Uro	Copro	Proto	Hemin Radioactivity (CPM)
1) Lysis mixture	18	0.7	40	3800
2) "Cytoplasm"	41	0.2	7	20
3) Nuclei	3	0.2	3	110
4) Nuclei + "cytoplasm"	43	5.4	126	3020
5) Waring Blendor-treated lysis mixture	154	4.5	388	5340
6) "Nuclear soluble fraction" + "cytoplasm"	243	0.2	174	6900
7) "Nuclear residue" + "cytoplasm"	934	0.3	16	260

Incubation for 3 hours.

Added to each sample: 6 μmoles ALA and approximately 1 μcurie Fe^{59}.

Samples 6 and 7 prepared by centrifugation of Waring Blendor treated lysis mixture.

Nuclei and nuclear residues washed 4X.

TABLE 8

Porphyrins Formed from δ-Aminolevulinic Acid

Samples	Porphyrin Formation (μgm./sample) Uro	Copro	Proto
1) Lysis mixture	54	1.3	193
2) Nuclei	7	0.1	11
3) Nuclei + "cytoplasm"	122	0.8	158
4) "Cytoplasm" (without ALA substrate)	1	0.2	15
5) "Cytoplasm"	149	0.3	6
6) "Cytoplasm" heated at 56°C.	173	0.6	8
7) "Cytoplasm" heated at 60°C.	336	0.4	4
8) "Cytoplasm" heated at 64°C.	398	0.7	6

Incubation for 3 hours.

Added to each sample: 6 μmoles ALA (except sample 4).

Nuclei washed 2X.

TABLE 9

Effect of Heating "Cytoplasm" on Porphyrin Formation from δ-Aminolevulinic Acid

Samples	Porphyrin Formation (μgm./sample) Uro	Copro	Proto
1) "Cytoplasm" (unheated)	144	0.9	28
2) "Cytoplasm" heated at 60°C.	351	0.3	9
3) "Cytoplasm" heated at 70°C.	418	0.7	9
4) "Cytoplasm" heated at 100°C.	3	0.2	6

Incubation for 3 hours.

Added to each sample: 6 μmoles ALA.

TABLE 10

The Conversion of Reduced Uroporphyrin III to Heme and Other Porphyrins

Samples	Percent of Fe^{59} Incorporation
1) 10 ml. "cytoplasm" + 10 ml. water	0.5
2) 5 ml. nuclei + 15 ml. water	0.1
3) 5 ml. nuclei + 5 ml. water + 10 ml. "cytoplasm"	2.8
4) 10 ml. "nuclear soluble fraction" + 10 ml. water	0.3
5) 10 ml. "nuclear soluble fraction" + 10 ml. "cytoplasm"	35.5
6) 5 ml. nuclei + 5 ml. water + 10 ml. extract of heated "cytoplasm" (100°C.)	0.4
7) 5 ml. extract of heated nuclei (100°C.) + 5 ml. water + 10 ml. "cytoplasm"	0.4
8) 5 ml. nuclei + 5 ml. water + 10 ml. "cytoplasm" (uroporphyrin substrate)	3.2

Incubation for 3 hours.

Added to samples 1-7: 272 µgm. reduced uroporphyrin III and 0.62 µgm. iron containing 0.82 µcurie Fe^{59}.

Added to sample 8: 240 µgm. uroporphyrin III and iron as in other samples.

Reduced uroporphyrin: Solution of 332 µgm. uroporphyrin III/ml. reduced. Concentration after reduction was 60 µgm./ml. 1 ml. added to each sample. Thus 272 µgm. reduced uroporphyrin theoretically available for reaction. After incubation, concentration of uroporphyrin had increased to 130 µgm./ml., and after standing in the light, the red color returned, indicating that further re-oxidation of reduced uroporphyrin had occurred.

Neither copro- nor protoporphyrin formed in significant amounts in any sample.

TABLE 11

Heme Formation from δ-Aminolevulinic Acid in Erythrocyte Fractions

Samples	Hemin Radioactivity (CPM)
1) Washed red cells	2400
2) Lysis mixture	4270
3) "Cytoplasm"	20
4) Nuclei (washed 1X)	120
5) Nuclei (washed 2X)	90
6) Nuclei (washed 4X)	50
7) Nuclei (washed 4X) + "cytoplasm"	2700

Incubation for 6 hours.

Added to each samples 6 μmoles ALA and approximately 0.69 μcurie Fe^{59}.

TABLE 12

Heme Formation from δ-Aminolevulinic Acid in a Soluble System

Samples	Heme Radioactivity (CPM)
1) Washed red cells	6400
2) Lysis mixture	6760
3) "Cytoplasm" + "nuclear soluble fraction"	5100
4) "Nuclear residue" (washed 2X)	50

Incubation for 6 hours.

Added to each samples 6 μmoles ALA and 0.76 μcurie Fe^{59}.

Red cells lysed in Waring Blendor (sample 2) and centrifuged 10 minutes at 9000 g to obtain samples 3 and 4.

TABLE 13

Heme Formation from δ-Aminolevulinic Acid

Preparation of Nuclear Soluble Fraction from Isolated Nuclei

Samples	Hemin Radioactivity (CPM)
1) Nuclei + "cytoplasm"	6420
2) "Cytoplasm"	40
3) "Nuclear soluble fraction" + "cytoplasm"	4520
4) "Nuclear residue" (washed 2X) + "cytoplasm"	2280

Incubation for 3 hours.

Added to each sample: 6 μmoles ALA and approximately 0.8 μcurie Fe^{59}.

All nuclei washed 4X. Nuclei fractionated by treatment in Waring Blendor for 3 minutes.

Isolated hemin was not recrystallized.

TABLE 14

Heme Formation from Protoporphyrin

Samples	Hemin Radioactivity (CPM)
1) Nuclei + RBC Salt Solution	170
2) Nuclei + water	600
3) Nuclei + "cytoplasm"	5190
4) "Nuclear soluble fraction"	1260
5) "Nuclear residue" + water	380
6) "Nuclear soluble fraction" + "cytoplasm"	6300
7) "Nuclear residue" + "cytoplasm"	1100

Incubation for 3 hours.

Added to each sample: 0.14 μmoles protoporphyrin and approximately 1.0 μcurie Fe^{59}.

All nuclei washed 3X and residues 2X.

Isolated hemin was not recrystallized.

TABLE 15

Heme Formation from Protoporphyrin

Samples	Percent of Fe^{59} Incorporation
1) Lysis mixture	24.5
2) "Cytoplasm"	0.5
3) Nuclei + water	5.0
4) Nuclei + "cytoplasm"	24.9
5) "Nuclear soluble fraction"	4.7
6) "Nuclear residue"	2.6
7) "Nuclear soluble fraction" + "cytoplasm"	18.3
8) "Nuclear residue" + "cytoplasm"	2.4

Incubation for 3 hours.

Added to each sample: 0.18 μmoles protoporphyrin and 0.02 μmoles iron containing 0.52 μcurie Fe^{59}.

All nuclei washed 3X and residues 2X.

TABLE 16

Determination of Optimal Protoporphyrin Level for Iron Incorporation

Samples	µmoles of Added Protoporphyrin	Percent of Fe^{59} Incorporation
1) 10 ml. "cytoplasm"	0.2	0.2
2) 5 ml. nuclei + 10 ml. "cytoplasm"	0.2	27.0
3) 8 ml. "nuclear soluble fraction" + 10 ml. "cytoplasm"	0.02	10.2
4) Same as (3)	0.1	23.2
5) Same as (3)	0.2	16.5
6) Same as (3)	0.4	12.1
7) Same as (3)	0.8	8.2

Incubation for 3 hours.

Added to each samples: Protoporphyrin (amounts indicated above) and 0.01 µmoles iron containing 0.57 µcurie Fe^{59}.

All nuclei washed 3X.

TABLE 17

Heme Formation from Protoporphyrin

Effect of Lysed Human Red Cells and Heated "Cytoplasm"

Samples	Percent of Fe^{59} Incorporation
1) 15 ml. lysis mixture	25.8
2) 15 ml. "cytoplasm"	1.8
3) 15 ml. nuclei + 5 ml. water	4.1
4) 5 ml. nuclei + 10 ml. "cytoplasm"	24.5
5) 5 ml. nuclei + 10 ml. lysed human red cells	11.0
6) 5 ml. nuclei + 10 ml. heated "cytoplasm" (56°C.)	16.5

Incubation for 4 hours.

Added to each samples 0.09 μmoles protoporphyrin and 0.01 μmoles iron containing 0.36 μcurie Fe^{59}.

Nuclei washed 4X.

TABLE 18

Heme Formation from Protoporphyrin

Effect of Heating Cell Fractions

Samples	Percent of Fe^{59} Incorporation
1) 5 ml. nuclei + 10 ml. water	6.9
2) 10 ml. "cytoplasm" + 5 ml. water	0.7
3) 5 ml. nuclei + 10 ml. "cytoplasm"	42.0
4) 5 ml. heated nuclei (56°C.) + 10 ml. "cytoplasm"	14.3
5) 5 ml. nuclei + 10 ml. heated "cytoplasm" (56°C.)	29.8
6) 5 ml. heated nuclei (70°C.) + 10 ml. "cytoplasm"	1.3
7) 5 ml. nuclei + 10 ml. heated "cytoplasm" (70°C.)	1.9

Incubation for 3 hours.

Added to each samples 0.12 μmoles protoporphyrin and 0.01 μmoles iron containing 0.72 μcurie Fe^{59}.

Nuclei washed 4X.

TABLE 19

Heme Formation from Protoporphyrin

Effect of Heating Cell Fractions

Samples	Percent of Fe^{59} Incorporation
1) 10 ml. "nuclear soluble fraction" + 10 ml. water	0.5
2) 10 ml. "nuclear soluble fraction" + 10 ml. "cytoplasm"	16.4
3) 10 ml. extract of heated "nuclear soluble fraction" (56°C.) + 10 ml. "cytoplasm"	1.9
4) 10 ml. "nuclear soluble fraction" + 10 ml. extract of heated "cytoplasm" (56°C.)	7.7
5) 10 ml. extract of heated "nuclear soluble fraction" (62°C.) + 10 ml. "cytoplasm"	0.7
6) 10 ml. "nuclear soluble fraction" + 10 ml. extract of heated "cytoplasm" (62°C.)	1.8

Incubation for 3 hours.

Added to each sample: 0.15 μmoles protoporphyrin and 0.01 μmoles iron containing 0.82 μcurie Fe^{59}.

Nuclei washed 4X.

TABLE 20

Heme Formation from Protoporphyrin

Effect of Heating Cell Fractions

Samples	Percent of Fe^{59} Incorporation
1) 5 ml. nuclei + 10 ml. water	24.1
2) 5 ml. nuclei + 10 ml. "cytoplasm"	27.8
3) 5 ml. nuclei + 10 ml. heated "cytoplasm" (56°C.)	24.5
4) 5 ml. nuclei + 10 ml. heated "cytoplasm" (70°C.)	3.0
5) 5 ml. nuclei + 10 ml. heated "cytoplasm" (100°C.)	1.0
6) 5 ml. heated nuclei (56°C.) + 10 ml. "cytoplasm"	12.1
7) 5 ml. heated nuclei (70°C.) + 10 ml. "cytoplasm"	0.6
8) 5 ml. heated nuclei (100°C.) + 10 ml. "cytoplasm"	0.3

Incubation for 3 hours.

Added to each sample: 0.12 μmoles protoporphyrin and 0.01 μmoles iron containing 0.8 μcurie Fe^{59}.

Nuclei washed 2X.

TABLE 21

Heme Formation from Protoporphyrin

Effect of Prolonged Waring Blendor Treatment

Samples	Percent of Fe^{59} Incorporation
1) 5 ml. nuclei + 10 ml. water	4.9
2) 5 ml. nuclei + 10 ml. "cytoplasm"	23.5
3) 5 ml. nuclei + 10 ml. blendorized "cytoplasm"	16.0
4) 5 ml. blendorized nuclei + 10 ml. "cytoplasm"	28.1

Incubation for 3 hours.

Added to each sample: 0.14 μmoles protoporphyrin and 0.01 μmoles iron containing 0.65 μcurie Fe^{59}.

Blendorization was for a total period of 40 minutes (in 10-minute intervals).

Nuclei washed 4X.

TABLE 22

Effect of Glutathione (GSH) on Iron Insertion into Protoporphyrin

Samples	Percent of Fe^{59} Incorporation
1) 5 ml. nuclei + 10 ml. water	6.9
2) 5 ml. nuclei + 10 ml. "cytoplasm"	42.0
3) 5 ml. nuclei + 10 ml. GSH (final conc. 0.001 M)	6.4
4) 5 ml. nuclei + 10 ml. GSH (final conc. 0.002 M)	6.2
5) 5 ml. nuclei + 10 ml. GSH (final conc. 0.004 M)	7.8

Incubation for 3 hours.

Added to each sample: 0.12 μmoles protoporphyrin and 0.01 μmoles iron containing 0.72 μcurie Fe^{59}.

Nuclei washed 4X.

TABLE 23

Effect of Diphosphopyridine Nucleotide (DPN) on Iron Insertion into Protoporphyrin

Samples	Percent of Fe^{59} Incorporation
1) 5 ml. nuclei + 10 ml. water	4.9
2) 5 ml. nuclei + 10 ml. "cytoplasm"	23.5
3) 5 ml. nuclei + 10 ml. DPN (final conc. 0.0004 M)	3.8
4) 5 ml. nuclei + 10 ml. DPN (final conc. 0.0002 M)	3.6
5) 5 ml. nuclei + 10 ml. DPN (final conc. 0.0001 M)	5.6
6) 5 ml. nuclei + 10 ml. DPN (final conc. 0.00005 M)	9.9

Incubation for 3 hours.

Added to each sample: 0.14 μmoles protoporphyrin and 0.01 μmoles iron containing 0.65 μcurie Fe^{59}.

Nuclei washed 4X.

TABLE 24

Effect of Diphosphopyridine Nucleotide (DPN) on Iron Insertion into Protoporphyrin

Samples	Percent of Fe^{59} Incorporation
1) 10 ml. "nuclear soluble fraction" + 10 ml. water	0.5
2) 10 ml. "nuclear soluble fraction" + 10 ml. "cytoplasm"	16.4
3) 10 ml. "nuclear soluble fraction" + 10 ml. DPN (final conc. 0.00005 M)	0.7
4) 10 ml. "nuclear soluble fraction" + 10 ml. DPN (final conc. 0.00002 M)	0.4
5) 10 ml. "nuclear soluble fraction" + 10 ml. DPN (final conc. 0.00001 M)	0.4
6) 10 ml. "nuclear soluble fraction" + 10 ml. DPN (final conc. 0.000005 M)	0.4

Incubation for 3 hours.

Added to each sample: 0.15 μmoles protoporphyrin and 0.01 μmoles iron containing 0.82 μcurie Fe^{59}.

Nuclei washed 4X.

Figure I

Proposed Pathway of Heme Biosynthesis

Key: M = - CH_3

A = - CH_2COOH

P = - CH_2CH_2COOH

V = - $CH = CH_2$

Fig. I

PROPOSED PATHWAY OF HEME BIOSYNTHESIS

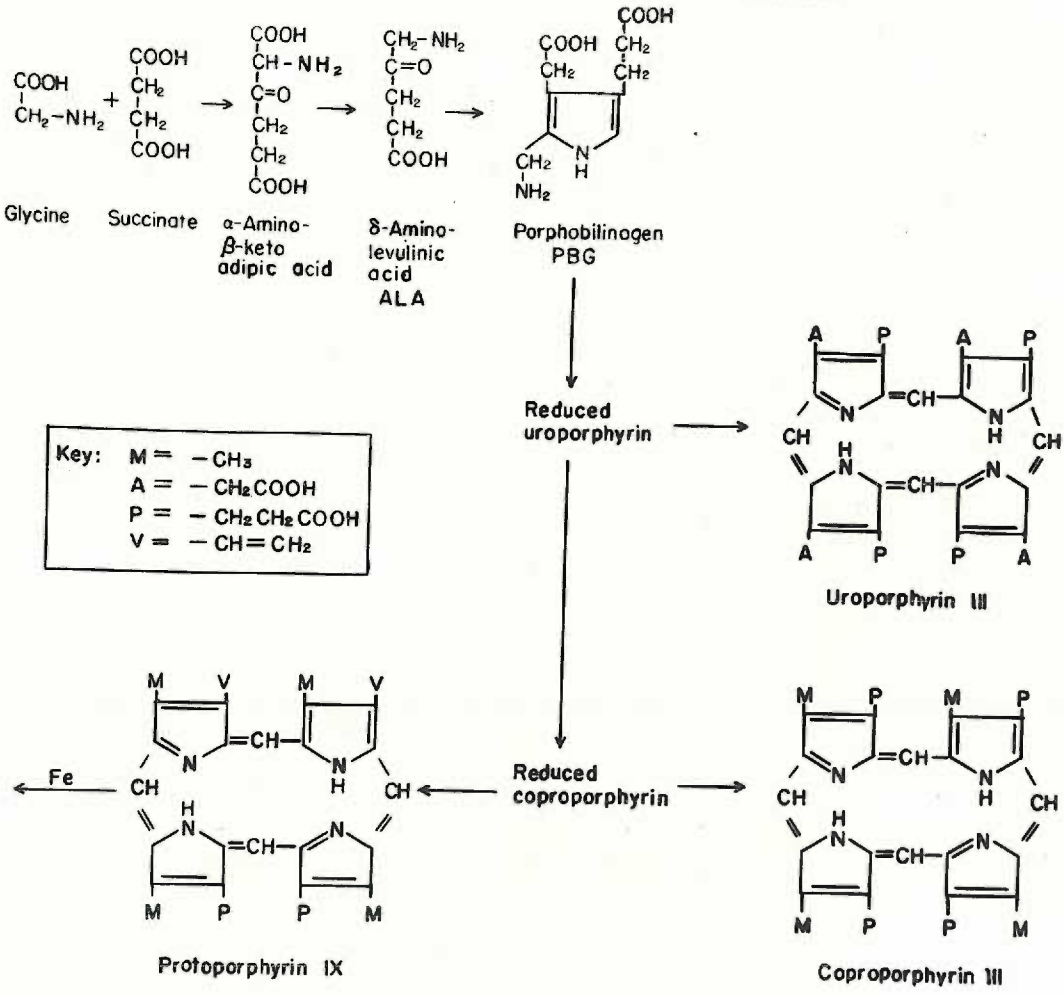

Figure II

Optimal Protoporphyrin Concentration

for Iron Incorporation

Fig. II

OPTIMAL PROTOPORPHYRIN CONCENTRATION FOR IRON INCORPORATION