Effects by Heme, Insulin, and Serum Albumin on Heme and Protein Synthesis in Chick Embryo Liver Cells Cultured in a Chemically Defined Medium, and a Spectrofluorometric Assay for Porphyrin Composition*

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Primary chick embryo liver cells, which had been previously cultured in Eagle’s medium containing 10% fetal bovine serum, had the same characteristics (inducibility of δ-aminolevulinic acid synthetase and synthesis of plasma proteins) when cultured in a completely defined Ham F-12 medium containing insulin. Insulin was active in the physiological range; 2 to 3 nM were sufficient to increase the induced δ-aminolevulinic acid synthetase to 50% of the maximum effect obtained with a saturating amount of insulin (30 nM).

Serum albumin added to the Ham-insulin medium caused protoporphyrin but not uroporphyrin, generated in the cultured liver cells, to be transferred to the medium. As little as 10 μg of human serum albumin per ml caused the transfer of one-half of the protoporphyrin. Bovine serum albumin was only about 1/10 as effective.

A spectrofluorometric method and calculation procedure are described for quantitation, in the nanomolar range, of total porphyrin and the percentage of this that is protoporphyrin or uroporphyrin plus coproporphyrin. The method is satisfactory for the measurement of porphyrins generated by 1 mg wet weight of cells in culture in 20 hours.

Heme (0.1 to 0.3 μM), when added to the medium as hemin, human hemoglobin, or chicken hemoglobin, specifically inhibited the induction of δ-aminolevulinic acid synthetase by one-half. This high sensitivity for heme was observed under conditions in which the defined medium was free of serum and where a chelator of iron was added to the medium to diminish the synthesis of endogenous heme. Heme endogenously generated from exogenous δ-aminolevulinic acid also inhibited the induction; chelators of iron prevented this inhibition.

The migration of heme from the mitochondria to other portions of the cell is discussed in terms of the affinities of different proteins for heme. A hypothesis of a steady state of liver heme metabolism, controlled by the concentration of “free” heme, is presented.

The different effects of heme on the synthesis of a number of proteins are summarized.

In 1963 (1) a method was developed in this laboratory to culture primary chick embryo liver cells in Eagle’s basal medium supplemented with 10% fetal bovine serum. A variety of chemicals, drugs, and steroids, acting on the cells in this medium was found to induce a marked (10- to 30-fold) increase in porphyrins, the increase being caused primarily by an increase in δ-aminolevulinic acid synthetase activity (2, 3).

Depending on the chemical, the induction was at the level of transcription or was post-transcriptional (4). Discussions of the induction mechanisms have been presented in several recent reviews by Granick and Sassa (5), Marks (6), and DeMatteis (7).

More recent studies from this laboratory have shown that the induced δ-aminolevulinic acid synthetase of chick embryo liver mitochondria is a dimer of molecular weight 87,000 ± 9,000 (8).

On induction, the marked increase in enzyme activity was found to be directly proportional to an increase in the amount of enzyme protein (actually equivalent to 3% of the newly synthesized mitochondrial protein) as measured with a specific antibody.1 Therefore, the induced activity of δ-aminolevulinic acid synthetase in the mitochondria may now be equated directly with an increase in amount of enzyme.

Previous studies (4, 5) led us to the assumption that heme acts as a specific repressor of δ-aminolevulinic acid synthetase.
controlling an unknown post-transcriptional step of the induc-
tion of the enzyme. Heme was found not to change the rate of
decay of the enzyme activity in the cultured cells in the
presence of acetoxycycloheximide (4). The purified enzyme
activity from chick embryo liver mitochondria was inhibited by
hemin but only at much higher hemin concentrations ($K_i = 35\mu M$). Hayashi et al. (9) have postulated that in the rat, heme
may also inhibit the transport into mitochondria of newly
synthesized $\delta$-aminolevulinic acid synthetase. However, in
chick embryo liver no evidence has been found that heme inhibits this postulated transport (Tomita et al. (10), Strand et
al. (11), see also Footnote 2). Moreover, our recent experiments have demonstrated a decrease in the amount of immuno
precipitable $\delta$-aminolevulinic acid synthetase after the treat-
ment of cells with heme. On the basis of this evidence the
decrease in $\delta$-aminolevulinic acid synthetase activity caused by
heme may be equated with a decrease in the amount of this enzyme caused by heme.

In this paper we report on a method of culturing chick
embryo liver cells in a completely chemically defined medium
of modified Ham’s F-12 (12) supplemented with insulin. This
defined medium has made two investigations possible: (a) an
inquiry into which components of fetal bovine serum, added to
Eagle’s basal medium, are required for maximal induction of
$\delta$-aminolevulinic acid synthetase. (b) A reinvestigation of the
amount of the added heme required to inhibit the induction of
$\delta$-aminolevulinic acid synthetase. The absence of fetal bovine
serum in the defined medium eliminated problems of the
binding of exogenous heme to serum proteins. The endogenous
formation of heme by the cells was decreased by adding, to the
defined medium, chelators of iron to block heme synthesis at
the ferrochelatase step. By means of these modifications, the
concentration of exogenous heme which would inhibit by 50% the
induction of $\delta$-aminolevulinic acid synthetase in the cells
could be determined.

We have used porphyrin accumulation as an indirect mea-
sure of $\delta$-aminolevulinic acid synthetase. To quantitate the
porphyrins accumulated by the cells and in the medium, a
fluorescence assay was developed of sufficient sensitivity to
determine porphyrins in the picomole range. This assay made
possible the finding that human serum albumin in the medium
causd the transport of protoporphyrin out of the cells. The
method also distinguished protoporphyrin from uroporphyrin
plus coproporphyrin), and this made possible the recognition
and quantification of the specific effect of chlorinated hydro-
carbons on causing uroporphyrin formation (13).

MATERIALS AND METHODS

Growth of Cells in Defined Medium

Culture Media and Reagents

Ham F-12 medium (12) is modified by omitting lipids, pyruvate,
and trace metals. Medium Ham-fetal bovine serum contains the
modified Ham medium plus 10% fetal bovine serum. Medium Ham-
insulin is modified Ham medium plus 0.2% of insulin per ml (30 nM).
Stock insulin is prepared by dissolving 2 mg of crystalline bovine
insulin (Sigma) in 0.3 ml of 10 mM HCI and diluting to 1 ml with water;
when kept frozen it is active for several weeks. We are grateful for gifts
of the following compounds: crystalline porcine glucagon from Lilly
Research Co.; samples, all ovine, of growth hormone, prolactin, and
thyrotropin from the Endocrinology Study Section of the National
Institute of Arthritis and Metabolic Diseases; prostaglandins from
Upjohn Co.

Glucagon, growth hormone, and prolactin are dissolved in 1 mM HCI
in a concentration of 1 mg/ml, prostaglandins in ethanol. Desferriox-
amine-B mesylate (Ciba) solution is freshly prepared from an am-
poule; the activity decreased when the powder was exposed to air but
less so in a desiccator at 4°. Aroclor-1254 is a mixture of poly-
chlorinated biphenyls containing mainly 5 and 6 chlorine atoms per
mole (Monsanto Co.).

Preparation for Culture of Chick Embryo Liver

Cell Suspension

The originally described method (3) has been modified. Livers of 16-
to 17-day-old embryos are perfused to deplete red cells and hemoglo-in. The perfusion of the livers is accomplished by slitting the embryo
open by a median ventral section, inserting a 22-gauge syringe needle
into the heart, and forcing through about 20 ml of Earle’s glucose-salt
solution minus magnesium and calcium. Four livers prepared in this
way are minced into fragments (1 to 2 mm) with a razor blade and
mixed with 5 ml of 1% sterile trypsin at 38° for 15 min. Then Varidase
(<0.1 mg free, from a mercury preservative, courtesy of Lederle
Laboratories) is added, and the incubation is continued a further 10
min during which the material is intermittently pipetted in and out of
a 2- to 3-mm pipette orifice until no lumps or strands are visible. The
suspension consists of clusters of 1 to 10 cells. Cell damage, if present,
is detected by phase microscopy as a dark bluish-like swelling on the cell
surface. Approximately 1.5 to 2 ml of this cell suspension is added to
100 ml of Ham-fetal bovine serum. Other details of the culture method
are described in a previous paper (4).

Standard Incubation Procedure

One millilitre of the above cell suspension containing 3 to 5 x 10^6
cells is transferred to a vial containing a coverslip of 16-mm diameter.
In a 5% CO2-air atmosphere at 38°, the cells become attached to the
coverslip by the 3rd to 4th hour. After the 4th to 6th hour, the medium
together with cell debris and red cells is removed by suction and
replaced by 1 ml of fresh Ham-fetal bovine serum. After 15 hour
further incubation the medium is replaced by 2 ml of Ham-insulin +
desferrioxamine (500 g/ml) and incubated for another 2 hours. This
washing serves to remove fetal bovine serum, and the presence of
desferrioxamine serves to deplete the cells of iron. Then the medium
is sucked off, and replaced by 1 ml of fresh Ham-insulin plus desferri-
oxamine; various chemicals are now added to determine their ability to
induce porphyrin accumulation. Desferrioxamine causes a small in-
crease in porphyrin concentration by itself; however, it greatly en-
harces the amount of porphyrin produced in the presence of low
concentrations of some inducers, thus greatly increasing the sensitivity
of this assay to detect inducibility (14). Other chelators of iron appear
to act similarly (15).

Determinations for porphyrins and protein are made 18 to 20 hours
later from triplicate vials. Some coverslips are examined microscopi-
cally with phase and fluorescence optics. The porphyrins in the cells on
other coverslips, and in the media, are determined in a perchloric acid/
mechanol solvent by fluorescence as described below.

Fluorometric Procedure to Determine Percentage of Protoporphyrin in
Mixture of Porphyrins, and Total Porphyrin in Cells on Coverslip and in Medium

Principle of Procedure

The double-banded emission of the porphyrin mixture is scanned with
a spectrofluorometer. From this curve two parameters are selected. One is the ratio of the heights of the two fluorescence
bands at 580 nm; another parameter is the height of the sec-
ond (600 nm) fluorescence band, from which the total of the two
fluorescence bands of uroporphyrin and of coproporphyrin is calculated.

To simplify the calculations advantage has been taken of the fact
that the positions and intensities of the maxima of the two fluorescence
bands of uroporphyrin and of coproporphyrin are almost identical but
are quite different from those of protoporphyrin (Fig. 1 and Table I). In
this way, knowing the total porphyrin and the percentage of protopor-
phyrin in the mixture, the remainder by difference will be uropor-
phyrin or coproporphyrin in a mixture of both.

Fluorometry

The porphyrins are measured in a solvent of 1 n aqueous perchloric
acid and methanol (1:1, v/v) in a glass test tube (Kimble, 6 x 50 mm)
that serves as a cuvette, with the use of a Hitachi Perkin-Elmer MPP2A fluorescence spectrophotometer. The excitation wave length is 400 nm, slit width 20 nm, and Zeiss filters BG and BGa are used. On the emission side a Corning 2-63 filter with cutoff below 587 nm is used. The emission spectrum is scanned from 580 to 680 nm with the use of a 12-nm slit. The photomultiplier is the R446UR which is specially sensitive to red light. If red-insensitive photomultipliers are used, e.g. the R106, then the second peak at 660 nm is not observed. The apparatus is standardized daily with a rhodamine B standard (40 ng/ml in ethylene glycol). Other details of the procedure are described by Sassa et al. (17).

The intensities of the fluorescence bands are expressed in fluorescence units or FU, one FU being defined as 0.01 division of the full scale recording on a 23-cm chart at maximal sensitivity. For example, for our instrument the standard rhodamine B solution gives a reading at 600 nm of 230 FU. The actual FU and ratios of the band heights are dependent on the characteristics of the photomultiplier, slits, and filters, and, therefore, each spectrophotometer must be calibrated with known porphyrin standards (See legend to Table I).

**Actual Procedure and Calculations**

We shall now detail the steps in the procedure and the calculations to determine the percentage of protoporphyrin and the total porphyrin in a mixture.

1. For determination of the porphyrins in the 1 ml of culture medium, 0.1 ml of the medium is pipetted into 0.4 ml of perchloric acid/methanol contained in the Kimble test tube used as a cuvette. If protein is present in the medium the test tube is centrifuged to pellet the protein. The emission spectrum of the supernatant layer is scanned from 580 to 680 nm.

2. A “blank” curve of the medium is made and is subtracted from the first curve.

3. The ratio of the band maxima (max, at 602 to 608 nm/max, at 654 to 662 nm) is calculated and referred to Fig. 3, Column 1 of the nomogram.

4. The nomogram is read across to Column 2 to obtain the percentage of total porphyrin that is protoporphyrin. By reading across to Column 4, a “corresponding factor” is obtained.

5. From the fluorescence curve, the FU at max, is determined. This FU value is multiplied by the “corresponding factor” in Column 4 and again by 10 to give picomoles of total porphyrin in the 1 ml of medium.

6. For the determination of the porphyrin in the cells on the coverslip, the remainder of the medium in the vial is removed, and 0.5 ml of perchloric acid/methanol is added to the coverslip in the vial. After 5 min in dim light the solution is transferred to the Kimble test tube and its fluorescence spectrum scanned. Steps 2 to 6 in the procedure are repeated except that the FU at max, is multiplied directly by the factor in Column 4 of the nomogram, to give total porphyrin in picomoles of the cells on the coverslip.

7. For protein determination of the cells on the coverslips, the remainder of the perchloric acid/methanol is sucked off. Then the protein is dissolved in 0.5 ml of 0.5 M NaOH (60°, 1 hour), diluted to 1 ml with H2O, and determined by the Lowry procedure. Crystalline bovine serum albumin, with nitrogen determined by the Kjeldahl method, is used as colorimetric standard. To determine the porphyrin and protein of cells cultured in the presence of fetal bovine serum, the procedure is repeated except that the FU at max, is multiplied directly by the factor in Column 4.

8. In routine determinations where the fluorescence is due to porphyrins only, it is not necessary to scan the spectrum; rather, one can merely measure the intensities at 605 and 660 nm and use these values for the calculations.

**Procedures for Determining Curves of Fig. 2 and for Deriving Nomogram of Fig. 3**

As noted above, it is necessary to standardize each spectrophotometer individually and adjust the sensitivity of the instrument with a fluorescence standard, e.g. rhodamine B in ethylene glycol or coproporphyrin in 0.1 N HCl. The following calculations are specific for constants obtained with our spectrophotometer.

**Determination of Percentage of Protoporphyrin—In a porphyrin mixture of uroporphyrin plus protoporphyrin (Fig. 2, left ordinate) ratios of the band heights from the FU values (max, /max, ) derived from the curves of Fig. 1 for 1 nm porphyrin are plotted from 0.84 for 100% uroporphyrin to 1.27 for 100% protoporphyrin. To obtain intermediate points on the curve the contribution of each component at each peak height has to be calculated. For example, for a 50:50 mixture of uroporphyrin and protoporphyrin at a total concentration of 1 nm porphyrin the FU (for uroporphyrin at max, = 50% of 23 FU) plus (for protoporphyrin at max, = 50% of 23 FU) 26.2 FU. At max, the total FU (for uroporphyrin = 50% of 35) plus (for protoporphyrin = 50% of 18.1) = 26.5 FU. Then the ratio (FU max/FU max = 26.2/26.5 = 0.987).

**Determination of FU of Nanomolar Porphyrin Mixture Given Percentage of Porphyrin—Fig. 2 (right ordinate) shows the relation between the fluorescence intensity of a 1 nm porphyrin solution to the percentage of protoporphyrin in the uroporphyrin plus protoporphyrin mixture. This relation is a straight line extrapolation between FU 35.0 (at 100% uroporphyrin) and FU 18.1 (at 100% protoporphyrin).**

**Table I**

Fluorescence units and ratios of two peak fluorescences for 1 nm porphyrins* in 1 N (aqueous) perchloric acid/methanol (1:1 v/v)

| Porphyrin | λmax, | FU | λmax, | FU |
|-----------|-------|-----|-------|-----|
| Uroporphyrin | 604 | 29.5 | 608 | 23.0 |
| Coproporphyrin | 635 | 19.8 | 604 | 18.1 |
| Protoporphyrin | 600 | 39.0 | 604 | 19.6 |

**Ratio, λmax, / λmax,**

|          | 0.84 | 0.79 | 1.27 |

*The concentrations of the porphyrins were determined in a Cary 15 spectrophotometer in 1 M HCl with the use of the extinctions given by Caughey (16).

†Isomers have identical fluorescence and absorption values. For calibrations, amounts of uroporphyrin, coproporphyrin, and protoporphyrin at concentrations of 1 μM may be obtained from Dr. R. F. Burnham, Porphyrin Products, 1244 E. Third North, Logan, Utah, 84321.

**FU, see text for definition.**

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**FIG. 1.** Fluorescence spectra of porphyrins in perchloric acid/methanol mixture.
Values for fluorescence intensities and for ratios of the two bands for substances other than porphyrins in, or added to the medium. The determined and the presence of some non-porphyrin fluorescence. Especially because of the smaller amounts of porphyrin that are cells on the coverslip. However, for the determination of porphyrin in errors present in a mixture with protoporphyrin, is an overestimate of the two fluorescence intensities in FU of 1 nM solutions containing different proporphyrin plus protoporphyrin mixture (Cl---O). Right ordinate, uroporphyrin plus protoporphyrin mixture (w) and for a co-

Convert FU (λmax) to Picomoles of Total Porphyrin—In Fig. 3, Column 3, the FU/nM refers to the FU of a nanomolar solution corresponding to a particular percentage of protoporphyrin. To convert to picomoles of total porphyrin the volume of the extracted porphyrin must be considered. Example: for total porphyrins in the cells on the coverslip, the porphyrins are extracted into a total volume of 0.6 ml of perchloric acid/methanol. Assume that only protoporphyrin is present, then the fluorescence emitted by a 1 nM solution at λmax (or 662 nm) is 18.1 FU. Then the picomoles of protoporphyrin in 0.6 ml = (0.6/18.1) x FU = 0.0276 x FU. This factor of 0.0276 is shown in the nomogram Fig. 3, Column 4. Similar factors can be calculated for solutions containing different percentages of protoporphyrin to give the values in Column 4.

For total porphyrin in the 1-ml culture medium, 0.1 ml of medium is mixed with 0.4 ml of perchloric acid/methanol. Because of the dilutions made, the factor to be used is 10 times the factor in Column 4.

Errors

The fluorescence blank of perchloric acid/methanol is only 4 to 5 FU and may be neglected when determinations are made of porphyrin in cells on the coverslip. However, for the determination of porphyrin in the culture medium, a blank correction for the medium is necessary especially because of the smaller amounts of porphyrin that are determined and the presence of some non-porphyrin fluorescence.

The fluorescence in the 600 to 680 nm region may be due to substances other than porphyrins in, or added to the medium. The non-porphyrin fluorescence may be recognized by the shape of the fluorescence curve or by the fact that the ratio of the two fluorescence peaks may be greater than 1.3 (Fig. 3, Column 1), i.e. greater than a value for protoporphyrin alone.

Simplifications in the calculations contribute certain inherent errors. By selection of a 20-nm excitation slit and a 12-nm emission slit to increase sensitivity, the fluorescence peaks of uroporphyrin, coproporphyrin, and protoporphyrin overlap, and the band peaks are broadened about 6 to 8 nm. With the use of a series of mixtures of uroporphyrin plus protoporphyrin the maximum deviation of the experimental from the theoretical was found to be ± 8%.

We have also simplified the calculations by assuming identical values for fluorescence intensities and for ratios of the two bands for uroporphyrin and coproporphyrin (Table I). The maximum calculated errors introduced, when coproporphyrin rather than uroporphyrin is present in a mixture with protoporphyrin, is an overestimate of protoporphyrin by 20% and an underestimate in total porphyrin by 10%.

RESULTS

Factors in Defined and Serum-containing Culture Media for Optimizing Induction for δ-Aminolevulinic Acid Synthetase

The increase of δ-aminolevulinic acid synthetase caused by inducing chemicals, e.g. allylisopropylacetamide, and the consequent porphyrin accumulation require continuing RNA and protein synthesis in the primary cultured chick embryo liver cells (3). In this paper we used the criterion of maximum induction of porphyrin accumulation as a measure of the ability of substances of the medium to support hepatocyte protein synthesis.

Defined Medium—The completely defined medium consisted of Ham F-12 free of lipids, pyruvate, and trace metals but supplemented with bovine insulin (0.2 μg/ml). The lipids and pyruvate were found not to be essential. Sufficient amounts of the trace metals were presumably supplied as contaminants. The cells cultured in this medium exhibited the same characteristics as those cultured in the presence of Eagle's medium containing 10% fetal bovine serum: induction of the synthesis of δ-aminolevulinic acid synthetase by various chemicals, drugs, and steroids; inhibition by heme of the synthesis of δ-aminolevulinic acid synthetase; and the synthesis and secretion of plasma proteins into the medium (18).

Functions of Fetal Bovine Serum—Several functions were noted. (a) The proteins of the serum protected the cells against damage by the enzymes, trypsin and Variadase, which were used to separate the liver cells in preparing the cell inoculum. The effect of fetal bovine serum was seen in the satisfactory attachment of the cells to the coverslip, with a cell inoculum of 3 x 10⁴ cells per ml of medium, when the medium contained 10% fetal bovine serum, but the attachment was poorer in the presence of 3% fetal bovine serum. Once the cells had become attached, then Ham-insulin without fetal bovine serum was sufficient to support the cell culture. It is important to note that the addition of serum was not essential for cell attachment.
since cells could attach in the absence of fetal bovine serum if the cell suspension was washed twice with Eagle's minus calcium and magnesium to remove the trypsin and Varidase, and Ham-insulin then added to the cells; however, this procedure required that 2 to 4 times more cells be used to yield an equivalent cell density on the coverslip. In general, better attachment and heavier growth was always obtained on Falcon plastic surfaces than on glass. (b) Another function of fetal bovine serum was to increase the protoporphyrin production to a level equivalent to that obtained when certain amino acids and vitamins were added to enrich the Eagle's medium and make it equivalent to the Ham. No individual amino acid, but rather a combination of them increased porphyrin production. Of the vitamins, B12 alone increased porphyrin production slightly, but the combination of vitamins was somewhat better. (c) In addition, the fetal bovine serum supplied a component with insulin-like activity which could be replaced by low concentrations of insulin.

**Insulin Effect to Increase Porphyrin Production**—A number of investigators have reported that, depending on the cell type and clone used, insulin could replace the requirement of serum entirely or in part to support cell function or cell multiplication (19-21). However, multiplication of HeLa or L cells in suspension did not require serum or insulin.

In the case of chick embryo liver cells, insulin added to Ham F-12 medium but not to Eagle's medium, completely replaced the requirement of serum for maximum porphyrin production. Commercial insulin (Sigma) was equally as effective as purified bovine insulin free of glucagon (courtesy of Dr. Lyman Craig, Rockefeller University). Addition of insulin (0.2 μg/ml) to the Ham medium in the presence of allylisopropylacetamide increased the induction of δ-aminolevulinic acid synthetase as shown by the increase in protoporphyrin yield 2- to 5.5-fold (Fig. 4). Plasma protein synthesis was increased by the insulin almost 2-fold; over-all plasma protein synthesis comprises about 50% of the total liver protein synthesis.  

The effect of insulin to increase the protoporphyrin yield (Fig. 4) was maximal at 8 to 15 nM, 50% of the maximum was at 2 to 3 nM, and even 0.3 to 1 nM caused a doubling over the control value. The physiological range of insulin in the hepatic portal vein of the rat is about 1 nM (29). Although δ-aminolevulinic acid synthetase was increased by induction in the presence of allylisopropylacetamide the induction of δ-aminolevulinic acid synthetase was not of insulin on the conversion of δ-aminolevulinic acid to porphyrin. Similar dose response curves were obtained in the presence of allylisopropylacetamide and the iron chelator desferrioxamine (760 μM). The dose response curves for insulin to increase plasma protein synthesis similarly were similar to those for increasing protoporphyrin accumulation.

Insulin appeared to be more effective when glucose was absent from the medium, i.e. the half-maximal increase of protoporphyrin required 0.3 to 2 nM insulin. However, lower concentrations of insulin (0.01 nM) have been reported to be sufficient for half-maximal stimulation of glucose oxidation to CO₂, or for the conversion of glucose to lipid in the liver cells or fat cells of the rat (23).

**Other Hormones**—These were also tested for their effect on the accumulation of porphyrins in the presence of allylisopropylacetamide (20 μM) plus desferrioxamine (760 μM) and insulin (30 nM). The addition of ovine growth hormone (2 μg/ml) to the insulin-containing medium enhanced the protoporphyrin yield about 40%, and ovine prolactin (2 μg/ml) enhanced it 10 to 20%.

No effect on protoporphyrin accumulation was observed with reduced insulin (0.2 to 2 μg/ml), reduced at pH 7 with 0.1 M mercaptoethanol for 2 days under N₂. Nor was there an effect of N,0'-dibutyryl adenosine 3':5'-monophosphate (10 to 30 μg/ml), cortisol (2 to 10 μg/ml), or thyroxin (1 to 5 μg/ml) in the presence or absence of insulin. In the presence of insulin the following hormones were slightly inhibitory at 10 μg/ml but not at 1 μg/ml: porcine glucagon, ovine follicle-stimulating hormone, ovine thyrotropin, and the prostaglandins. Prostaglandin F₂α was least and prostaglandin F₂β was most inhibitory. None of these hormones damaged the cells, for they did not cause a loss of porphyrins from the cells as judged by measurement of the ratio of porphyrin in the cells to porphyrin in the medium.

The 60% inhibitory effect of hemin (0.1 μg/ml) on the induction of porphyrin by allylisopropylacetamide was not overcome by insulin (0.2 μg/ml) whether the hemin or insulin was added first and a 2-hour period intervened. This result suggests that hemin and insulin act independently and that hemin does not affect the insulin receptor site on the surface of the cell membrane.

**Effect of Serum Albumins on Porphyrin Accumulation**

To study the effects of other constituents of fetal bovine serum as they affect protoporphyrin yield, we chose to test the effect of the major protein constituent of fetal bovine serum, serum albumin.

**Effect of Different Serum Albumins, Added to Ham-insulin Medium, on Porphyrin Yield**—These are listed in Table II. A maximum change of +30% in protoporphyrin yield was obtained, depending on the particular preparation of serum albumin and its species of origin. With defatted, purified monomer (half-cystinyl) human serum albumin (100 μg/ml) (24), an increase of 30% in protoporphyrin yield was observed, a commercial untreated preparation (Mann, 100 μg/ml) caused an inhibition of 30% in protoporphyrin yield, and a crystallized preparation, Pentex (Miles), even at 1000 μg/ml did not affect the yield. Bovine serum albumin (Armour, 1000 μg/ml), also
TABLE II
Effects of serum albumins on production and location of porphyrins

Standard incubation procedure as described under “Methods.” Last change is to 1 ml of medium containing modified Ham F-12 plus insulin (0.2 μg/ml) plus allylisopropylacetamide + desferrioxamine and additive. Incubation 20 hours.

| Experiment | allylisopropylacetamide | Desferrioxamine | Additives in 1 ml medium | Amount serum albumin (μg) | Protoporphyrin ratio, cell to medium | Total protoporphyrin (pmol) |
|------------|-------------------------|-----------------|-------------------------|--------------------------|-------------------------------------|----------------------------|
| 1          | 3                       | 500             | Control                 | 3.0                      | 259                                 |                            |
|            |                         |                 | Fetal bovine serum unheated | 1600*                    | 0.3                                 | 187                        |
|            |                         |                 | 30 μl                   | 600*                     | 0.6                                 | 266                        |
|            |                         |                 | 10                      | 180*                     | 1.1                                 | 273                        |
|            |                         |                 | Fetal bovine serum heated |                         |                                     |                            |
|            |                         |                 | 55°C 30 min            | 1600*                    | 0.4                                 | 246                        |
|            |                         |                 | 30 μl                   | 600*                     | 0.6                                 | 299                        |
|            |                         |                 | 10                      | 180*                     | 1.0                                 | 268                        |
|            |                         |                 | 3                       | 100                      | 0.7                                 | 341                        |
|            |                         |                 | Human serum albumin (defatted, half-cystin) | 100                      | 0.7                                 | 316                        |
|            |                         |                 |                          | 10                       | 1.1                                 | 301                        |
| 2          | 3                       | 500             | Control                 | 3.0                      | 122                                 |                            |
|            |                         |                 | Human serum albumin fraction V (Mann) white | 1000                    | 0.1                                 | 11                         |
|            |                         |                 |                          | 300                      | 0.1                                 | 22                         |
|            |                         |                 |                          | 100                      | 0.3                                 | 84                         |
|            |                         |                 | Bovine serum albumin fraction V (Armour) | 1000                    | 0.7                                 | 122                        |
|            |                         |                 | Hemin, 0.1 μg          | 3.3                      | 47                                  |                            |
| 3          | 50                      | 0               | Control                 | 3.2                      | 110                                 |                            |
|            | 50                      | 0               | Human serum albumin (Pentex) | 250                      | 0.3                                 | 125                        |
|            | 50                      | 0               | Aroclor (3 μg)          | 4.4*                     | 90*                                 |                            |
|            | 50                      | 0               | Aroclor (3 μg) + human serum albumin | 250                      | 3.2*                                | 105*                       |

* Sum of porphyrins of coverslip plus porphyrins in 1 ml of medium. Average of triplicate vials. Maximum deviation was ±15% from mean. Porphyrins determined as described under “Methods.”

- 60 μg of serum albumin = 1 μl of serum.
- Uroporphyrin.

did not affect the yield. Fetal bovine serum (30 μl/ml), i.e. 3% serum, decreased the protoporphyrin yield by 30%, but fetal bovine serum heated at 50°C for 30 min was not inhibitory. One explanation for these results may be that variations in protoporphyrin are due to differences in impurities adhering to or accompanying the different preparations of serum albumin.

**Release of Protoporphyrin from Cells by Serum Albumin**—A second effect of serum albumin was to cause protoporphyrin generated in the cells to be released into the medium (Table II). (In the presence of desferrioxamine, the porphyrin formed is all protoporphyrin.) The effect was most marked with purified human serum albumin where even 10 μg/ml caused one-half of the protoporphyrin to leave the cells. In the absence of serum albumin, the ratio of protoporphyrin in the control cells to that in the medium was 3:1. Hemin (0.1 μg/ml) added to the medium in the absence of serum albumin did not affect this ratio (Table II, Experiment 2, last line), although this hemin concentration was sufficient to decrease the protoporphyrin formed to 40% of the control. The loss of protoporphyrin from the cells to the medium in the presence of human serum albumin was not due to cell damage because the total protoporphyrin formed was greater by 30% than the control, i.e. in absence of human serum albumin. Also supporting the idea that cell damage is not involved is the experiment (Table II, Experiment 2) with human serum albumin (Mann, 100 μg/ml); the human serum albumin caused inhibition of protoporphyrin synthesis yet produced a protoporphyrin ratio (cell to medium) similar to that of the defatted human serum albumin. This result suggests that the property of human serum albumin involved in transport of protoporphyrin out of the cells is not related to the inhibitory properties of certain human serum albumin preparations (e.g. Mann human serum albumin).

Fetal bovine serum in a concentration of 3% (v/v) of the medium effectively caused the release of protoporphyrin from the cells. However, on the basis of serum albumin content, fetal bovine serum was 1/20 as effective and bovine serum albumin alone was 1/30 as effective as human serum albumin.

The protoporphyrin distribution between cells and media in the presence of human serum albumin was independent of the kind of inducer, whether allylisopropylacetamide or secobarbital, and of their concentrations (5 to 50 μg/ml). It was independent of the source of the δ-aminolevulinic acid, whether generated in the cell by δ-aminolevulinic acid synthetase or from exogenous δ-aminolevulinic acid. Neither the presence nor absence of the chelators, desferrioxamine or (Ca + Mg)-EDTA affected the protoporphyrin distribution caused by human serum albumin.
Human serum albumin caused the release of protoporphyrin rather than uroporphyrin from the cells. In the presence of Aroclor-1254 the cells accumulated mainly uroporphyrin (13). In the presence of human serum albumin, uroporphyrin remained in the cells as indicated by the high ratio of 3.2 of porphyrin in cells to medium (Table II, Experiment 3). The decrease in ratio from 4.4 to 3.2 caused by human serum albumin was probably due to the release of the small amount of protoporphyrin made by the cells.

Control by Heme of Heme Synthesis

Repression by Hemin of Synthesis of &Aminolevulinic Acid Synthetase—It had previously been found (3) that the addition of 2 μM hemin to the medium was required to inhibit the synthesis of induced porphyrins by 90% in the primary chick embryo liver cells cultured in the presence of 10% fetal bovine serum. The significance of this value was difficult to assess because of the following considerations: serum was present which had some affinity for hemin; the serum was contaminated with variable amounts of hemoglobin heme; in the cell inoculum were red cells which tended to hemolyze; and in addition heme was produced within the hepatocytes.

We succeeded in controlling these complications by growing the cells in a defined medium devoid of serum, by perfusing the chick embryo livers to remove red cells before preparing the hepatic cell suspension, and by adding high concentrations of chelators of iron (14) to diminish the endogenous level of heme production.

With these modifications it now became possible to repress the synthesis of δ-aminolevulinic acid synthetase with 1/10 to 1/20 of the previous heme concentration. As seen in Fig. 5 (arrows) the activity was repressed to one-half when the medium contained 0.1 to 0.2 μM hemin. The activity was followed by determining the amount of protoporphyrin accumulated in the intact cells in culture.

Inhibition of Protoporphyrin Synthesis by Hemoglobin—Because of the question of solubility of hemin chloride even below 1 μM in the defined medium, the experiments (see “Repression by Hemin of Synthesis of δ-Aminolevulinic Acid Synthetase”) were repeated with the use of, as source of soluble heme, supernatants of adult human and chick embryo red cell lysates and adult human hemoglobin crystallized by the modified Drabkin procedure as described in Ref. 25. It was found that these preparations, in terms of heme concentration, were as inhibitory as hemin itself. The hemoglobins became denatured in the medium and released hemin. After the 20-hour incubation, denatured globin was observed as a deposit on the coverslip, even in the absence of hepatocytes. It is of interest that administration to rats of hemin, ferric hemoglobin, ferric heme albumin, but apparently not hemoglobin, was found by Marver et al. (26) to block the allylisopropylacetamide-stimulated increase of both δ-aminolevulinic acid synthetase activity and of microsomal cytochromes. In our experiments in which no serum or globin or hemopexin (27) was present in the culture medium, hemin appeared to get into the cells as judged by the hemin causing a decreased induction of δ-aminolevulinic acid synthetase.

Repression of Induction of δ-Aminolevulinic Acid Synthetase by Endogenous Heme Synthesized from Added δ-Aminolevulinic Acid—As seen in Table III (Experiment 1) the addition of δ-aminolevulinic acid (60 μM) to the culture medium containing allylisopropylacetamide (350 μM) decreased the induced activity of δ-aminolevulinic acid synthetase to 40% of the control without δ-aminolevulinic acid. This experiment suggests that the added δ-aminolevulinic acid was converted to heme which inhibited the induction of synthesis of δ-aminolevulinic acid synthetase. A similar inhibition of the induction of this enzyme by δ-aminolevulinic acid was observed by Strand et al. (11) in cultured chick embryo liver cells and by Padmanaban et al. in rats (28). As expected, the addition of hemin (8 μM) to the medium (Experiment 1) greatly inhibited the induction of δ-aminolevulinic acid synthetase (i.e. to 15% of control activity). However, added δ-aminolevulinic acid (Experiment 2) did not inhibit the induction of this enzyme when the cells were grown in a medium containing desferrioxamine (750 μM). In this latter case,

![Graph showing inhibition of protoporphyrin formation](http://example.com/graph.png)

Fig. 5. Inhibition by exogenous hemin of protoporphyrin formation induced by allylisopropylacetamide (AIA) plus desferrioxamine. The cells were incubated by the standard procedure as described under “Methods.” After leaving the cells for 2 hours in Ham-insulin containing desferrioxamine (500 μg/ml), the medium was changed to 1 ml of the same composition, and to this was added allylisopropylacetamide (21 or 210 μM) plus increasing concentrations of hemin. The hemin was dissolved in a 1:1 (v/v) mixture of 10 mM KOH/ethanol, and 2 μl was added to 1 ml of the medium. Arrows indicate the hemin concentration that inhibited protoporphyrin yield by 50% of the maximal. The basal protoporphyrin formed is 0.1 to 0.2 nmol.

### Table III

| Experiment | Additives | Relative activity, δ-aminolevulinic acid synthetase in homogenate |
|------------|-----------|---------------------------------------------------------------|
| 1 | Allylisopropylacetamide (350) | 100 |
| | Allylisopropylacetamide (350) + δ-aminolevulinic acid (60) | 40 |
| | Allylisopropylacetamide (350) + hemin (8) | 16 |
| 2 | Allylisopropylacetamide (350) + desferrioxamine (750) | 100 |
| | Allylisopropylacetamide (350) + desferrioxamine (750) + δ-aminolevulinic acid (60) | 110 |
desferrioxamine is presumed to have prevented the formation of heme from added δ-aminolevulinic acid, and in the absence of endogenously formed heme no decrease in δ-aminolevulinic acid synthetase activity occurred. As noted in the introduction to the text, the induced activity may now be equated with induced synthesis of the enzyme.

Only Hemin among Tetrapyrroles Inhibited Induction of δ-Aminolevulinic Acid Synthetase—Rilinrin (8.5 μM) in the presence of desferrioxamine (750 μM) plus allylisopropylacetamide (200 μM) did not affect the yield of protoporphyrin, as determined by the method described in the legend to Fig. 5. Similarly, manganese protoporphyrin (0.04 to 4 μM) which is water-soluble did not affect the yield of protoporphyrin. When δ-aminolevulinic acid was incubated with the cells in the presence of desferrioxamine (750 μM), protoporphyrin accumulated at a constant rate for 20 hours, indicating that the accumulation of protoporphyrin did not make rate-limiting any of the enzymes converting δ-aminolevulinic acid to protoporphyrin.

**Repression of Synthesis of δ-Aminolevulinic Acid Synthetase by Exogenous Hemin as Measured by Assay for δ-Aminolevulinic Acid Synthetase Activity**—In order to show more directly that hemin inhibition of protoporphyrin accumulation was due to the repression of the synthesis of δ-aminolevulinic acid synthetase, the enzyme activity was measured directly in homogenates of cells grown with and without heme. Levulinate (10 mM) was added to the assay mixture to block the activity of δ-aminolevulinic acid dehydratase so that δ-aminolevulinic acid accumulated and was determined colorimetrically. In this experiment cells were cultured on plastic dishes in a final culture medium of Ham's F-12 containing allylisopropylacetamide (35 μM) plus desferrioxamine (750 μM) ± heme (0.3 μM) for 20 hours.

It was found that the δ-aminolevulinic acid synthetase activity of the control homogenate was 4.3 n mole of δ-aminolevulinic acid/mg of cell protein/hour, 37°. The presence of 0.3 μM heme in the culture medium during the induction decreased the enzyme activity to 1.9 or about one-half the control activity. The corresponding 50% inhibition of protoporphyrin synthesis (Fig. 5) required 0.1 to 0.2 μM of heme. These similar experiments together with those of Sassa and Granick (4) suggest that inhibition by heme of protoporphyrin accumulation reflects the inhibition by heme of the synthesis of δ-aminolevulinic acid synthetase. Furthermore, the decrease in activity after heme treatment correlates with a decrease in amount of immunoprecipitable δ-aminolevulinic acid synthetase.

**Effect of Exogenous Hemin in Repressing Protoporphyrin Production in Presence of Allylisopropylacetamide**—As seen in Fig. 5, protoporphyrin production was inhibited by 50% when heme at 90 to 210 nM (arrows) was present in the culture medium. An inhibitory effect of heme was detectable at concentrations as low as 50 nM. (None of the concentrations of heme used in these experiments quenched the porphyrin fluorescence in the perchloric acid/methanol solvent.) In the presence of higher concentrations of allylisopropylacetamide (e.g. 212 μM), more exogenous heme (210 nM) was required to inhibit protoporphyrin accumulation by 50% than at the lower concentrations of allylisopropylacetamide (21 μM). These results may be explained by assuming that allylisopropylacetamide at higher concentrations was causing a more effective destruction of heme in the cells (6, 7, 29). Therefore, to compensate for this loss of heme, higher concentrations of exogenous heme had to be supplied to the cells in order for the assumed repression of the synthesis of δ-aminolevulinic acid synthetase to take place. Because of the changing ratio of allylisopropylacetamide to heme at 50% inhibition, it is unlikely that heme and allylisopropylacetamide were acting competitively at the same site.

**DISCUSSION**

**Effect of Insulin and Related Hormones on Synthesis of δ-Aminolevulinic Acid Synthetase by Cultured Chick Embryo Liver Cells in Defined Medium**

In previous studies Eagle's medium containing 10% fetal bovine serum was used to determine the effect of various chemicals that induced the formation of δ-aminolevulinic acid synthetase. In this paper we have reported that a chemically defined medium consisting of Ham F-12 plus insulin can replace the previously used medium which contained fetal bovine serum. The use of the completely defined medium made it possible to inquire into the functions of fetal bovine serum and the activity of a number of hormones on the induction of δ-aminolevulinic acid synthetase.

We found that the requirement for fetal bovine serum could be replaced by additional amino acids, vitamins, and insulin. The effect of insulin appears to be anabolic. It promoted several cell functions: the increase in δ-aminolevulinic acid synthetase in response to inducers, the increased synthesis and secretion of plasma proteins, and the increase in amino acid incorporation into general proteins; concomitantly the monolayer colonies appeared healthier, with more cell extensions. In the presence of inducers, insulin, in the physiological range of 1 nM, increased porphyrin production up to 5.5-fold (e.g. Fig. 4). Porphyrin production was enhanced somewhat more by added growth hormone or prolactin in the medium containing insulin (0.2 μg/ml). It is of interest that these three hormones which increased porphyrin production appear to be related. The NH₂-terminal region of growth hormone exhibits insulin-like activity (30), and prolactin is immunologically similar to growth hormone. None of the other polypeptide hormones tested, such thyroid, cyclic adenosine 3':5'-monophosphate, and prostaglandins enhanced porphyrin production.

The general anabolic effect of insulin may in part be explained by insulin causing an increase in the surface area of the plasma membrane as a result of an increase in microvilli formation. Insulin receptors have been shown by Cuatrecasas to be present on membranes of liver and fat cells (23). Evans et al. (31) have found that within 1 hour after insulin was added to mouse fibroblasts, microvilli had increased on the cell surface, while simultaneously the uptake of uridine, leucine, and glucose was increased. The insulin effect may become most apparent when the surface area of the cells becomes limiting for metabolism, as when the cells become confluent. Griffiths (32) observed that insulin stimulated protein synthesis and glucose uptake in WI-38 human cells when the surface areas of the cells exposed to the medium were minimal, a condition similar to that in our induction experiments.

**Effect of Serum Albumin in Causing Release of Protoporphyrin from Hepatocyte**

This effect was most marked with human serum albumin and appeared to depend on the presence of one high affinity site (Kd = 10 nM) on the human serum albumin that is
identical for heme and protoporphyrin (33, 34). Heme was suggested to bind through the two propionic acid side chains. In the presence of as little as 10 μg of human serum albumin added to 1 ml of the defined medium we found that one-half of the protoporphyrin migrated out of the hepatocytes or approximately 1 mol of protoporphyrin per mol of human serum albumin. Bovine serum albumin, with a much weaker binding site, was only 1/20 to 1/30 as effective in causing protoporphyrin to be released from the cells.

Fluorescence microscope examination of the hepatocytes indicated that protoporphyrin generated in the cells was localized in the cytosol, and uroporphyrin (Table II) mainly in the nucleus. Uroporphyrin was not released by the action of exogenous serum albumin into the medium.

Studies by others have indicated that although serum albumin is produced and secreted by hepatocytes, this protein does not appear to re-enter the cells (35, 36). The release of lipophilic protoporphyrin, but not hydrophilic uroporphyrin, from the cells most probably occurs in a manner similar to one that has been postulated for serum albumin-fatty acid transport exchange across the plasma membrane.

The release of protoporphyrin from the cells by human serum albumin provides a simple and highly sensitive assay for use in the study of the mechanism of action of serum proteins for transfer of lipophilic anionic molecules across the plasma membrane of the hepatocyte.

Control of Liver Heme Metabolism by Heme

We had shown before that δ-aminolevulinic acid synthetase was the rate-limiting enzyme in the biosynthesis of heme. The enzyme had a relatively short half-life of 3 hours in the chick embryo liver cells in culture (5) and 1 hour in adult rat liver (37). A major control on the enzyme was by heme acting as a repressor at the post-transcriptional level, presumably on the synthesis of this enzyme (4). The repression of the enzyme by exogenous hemin was as rapid as the inhibition of the synthesis by acetoxycycloheximide, and the rate of decay of activity of the enzyme in the presence of acetoxycycloheximide was not changed by the addition of hemin (4). The rapid repression by hemin indicated that the hepatic cell was readily permeable to heme.

By culturing the chick embryo liver cells in a completely defined Ham-insulin medium and by blocking the formation of endogenous heme with a chelator of iron, it became possible to determine several parameters of liver heme metabolism and formulate a hypothesis on the steady state control of heme in the liver cell.

Repression by Hemin and $K_r$—The present experiments showed by two independent methods that heme is a highly effective repressor. At a concentration of exogenous heme of 0.1 μM, the synthesis of δ-aminolevulinic acid synthetase was repressed by one-half, a value which we define as $K_r$ (Fig. 6 and “Repression by Hemin of Synthesis of δ-Aminolevulinic Acid Synthetase” and “Repression of Induction of δ-Aminolevulinic Acid Synthetase by Endogenous Heme Synthesized from Added δ-Aminolevulinic Acid”). Not only heme but heme added as hemoglobin (see “Inhibition of Protoperoporphyrin synthesis by Hemoglobin”) repressed the synthesis of the enzyme. Also heme endogenously produced from exogenous δ-aminolevulinic acid repressed the synthesis (see “Repression of Induction of δ-Aminolevulinic Acid Synthetase by Endogenous Heme Synthesized from Added δ-Amino-levulinic Acid”) suggesting that heme acts intracellularly rather than via a change in the plasma membrane. The repression by heme is rapid (4) and also specific; neither manganese protoporphyrin, bilirubin, protoporphyrin, nor intermediates of the heme biosynthetic chain were found to repress (see “Only Hemin among Tetrapyrroles Inhibited Induction of δ-Aminolevulinic Acid Synthetase”).

Within the limits of error of the determinations, the S-shaped curve of heme inhibition of protoporphyrin formation (Fig. 5) is that expected of a binding curve for a 1:1 heme-apoprotein complex. One hypothesis that may be suggested to explain repression by heme at the post-transcriptional level is that heme combines with some apoprotein to modulate the translation of δ-aminolevulinic acid synthetase.

The affinity of this postulated apoprotein for repressor heme may be approximated if it is assumed that the concentration of “free” or loosely bound heme in the cell is equal to the concentration of free heme in the relatively large volume of the medium bathing the cell. Then the dissociation constant, $K_r$ for this heme-apoprotein would be equal to the $K_r$ value obtained experimentally (Fig. 5, arrows) of 0.1 to 0.2 μM heme.

This free heme is only a minute amount (0.1%) of the total hemoprotein-heme of the hepatic parenchyma cell, but as described below it can govern the rate of synthesis of δ-aminolevulinic acid, the rate-limiting compound for heme synthesis.

Affinities of Cell Constituents and Hemoproteins for Hemin —Hemin formed in the mitochondria may be considered to move along a gradient of increasing affinity for heme. The affinities of hemoproteins competing for heme may be compared in terms of their dissociation constants, or $K_d$, where the $K_d$ is the concentration of hemoprotein at 50% dissociation. The $K_d$ value depends not only on the kind of hemoprotein, but also on the oxidation-reduction state of the heme and the modes of ligands to the iron (38).

Two hemoproteins have been used as reference standards to estimate the relative affinities of other hemoproteins for heme: these are hemopexin-ferric heme, with a $K_d$ of 1 to 10 nM (39, 40), and human serum albumin-ferric heme, with a $K_d$ of 10 nM (33). From our studies we may define sites of loose attachment, i.e. sites of readily exchangeable or free heme in the cell as those having $K_d$ greater than 10 nM.

In Fig. 6, the movement of heme from the mitochondria to the microsomes is summarized. Israels et al. (41, 42), on the basis of in vitro studies, found that the presence of cytosol proteins, which had relatively low, nonspecific affinity sites for heme, were required to contact the mitochondria in order for the heme to move out readily from the mitochondria (A) into the cytosol (B). Hemin then passes to the microsomal fraction which has a somewhat tighter binding for heme (C). In the microsomes, heme binds very tightly to some newly synthesized apoproteins, such as cytochrome P-450, with high affinity for heme (43), thus forming hemoproteins (D). Of the total heme synthesized and turning over in the rat hepatic cell 80% is attributed to two hemoproteins, cytochrome P-450 and catalase (29). Hemin in cell-free systems also appears to accelerate the synthesis of other proteins (E) as discussed below. In addition heme acts as a specific inducer to induce the synthesis of heme oxygenase when heme begins to exceed 100 nM (F). Hemin is broken down by heme oxygenase to biliverdin (G). The high $K_d$ of heme oxygenase (5 μM) indicates that it has a relatively low affinity for heme as substrate. Also of importance for the control of the basal level of heme in the cell is the effect of hemin, even at the low
concentration of 100 nM in causing 50% repression of the synthesis of δ-aminolevulinic acid synthetase, i.e. $K_r$ (H).

**Hypothesis of Control of Liver Heme Metabolism by Heme**—A steady state level of free or readily exchangeable heme in the hepatic parenchyma cell poised at the $K_r$ (Fig. 6, H) of 100 nM heme, would be most suitable to control the rate of formation of δ-aminolevulinic acid synthetase and to control the rate of heme metabolism for the following reasons. If the heme generated in the mitochondria caused the “free heme” to increase beyond 100 nM there would be three effects, each of which would tend to result in a net decrease of heme concentration. (a) The increased heme would repress the formation of δ-aminolevulinic acid synthetase. (b) The increased heme would be degraded more rapidly by the heme oxygenase (14). (c) More slowly, the increase in heme would cause an induced increase (2- to 7-fold) in heme oxygenase (Fig. 6, F) (45).

In contrast, when “free heme” decreased below 50 nM, the synthesis of δ-aminolevulinic acid synthetase would scarcely be repressed, more of this enzyme would be made, and therefore more heme would be made because this enzyme is the rate limiting enzyme of the heme biosynthetic chain. However if “free heme” decreased well below 50 nM, heme enzymes would still continue to form because of the great affinity of these apoproteins for heme, providing the rate of supply of the heme was adequate.

According to this hypothesis the concentration of free heme will normally not become low enough to limit cytochrome synthesis. Nor is cytochrome synthesis increased by increasing heme concentrations; δ-aminolevulinic acid given to rats to increase heme did not change the total amount of cytochromes in liver microsomes or mitochondria (46, 47). However, this situation differs from that in differentiating blastoderm of the chick embryo, where heme, controlled by the activity of δ-aminolevulinic acid synthetase, limited globin synthesis (48).

**Heme Control on Protein Synthesis**

As seen in Fig. 6, there are a number of actions of heme on the synthesis of different proteins. (a) In liver, heme inhibits induced synthesis of some proteins (49), e.g. δ-aminolevulinic acid synthetase (3). (b) Hemin induces an increase of heme oxygenase (45). (c) In cell-free systems hemin has been found to increase the synthesis of a number of proteins such as globin, crystalline lens protein, and viral protein (50-52). (d) Heme is without effect on the synthesis of yet other proteins (e.g. the major plasma proteins produced by chick embryo liver cells in culture). These various effects may be explained by the hypothesis that heme may have several post-transcriptional activities such as the mediation of different initiation factors in translation (53), the inhibition of translational inhibitors (50), etc. Whether these controls of heme on protein synthesis represent entirely new mechanisms for heme action or whether heme acts in a manner similar to known functions of hemoproteins such as electron transport, peroxidation, or $O_2$ activation, is not known.

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