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DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—XV

INDUCTION OF PORPHYRIN BIOSYNTHESIS IN CHICK EMBRYO LIVER CELLS MAINTAINED IN SERUM-FREE WAYMOUTH MEDIUM*

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Abstract—Several drugs were shown to induce porphyrin accumulation in chick embryo liver cells maintained in serum-free Waymouth MD 705/1 medium. In this system, allylisopropylacetamide (AIA) was shown to increase δ-aminolevulinic acid (ALA)-synthetase activity 5-fold over control values. Addition of insulin to the medium resulted in enhanced porphyrin accumulation. Porphyrin accumulation in response to increasing doses of AIA, propylisopropylacetamide (PIA) and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (Ox-DDC) was 3- to 4-fold greater in serum-containing than in serum-free medium. With 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), however, porphyrin accumulation in response to increasing doses was slightly greater in serum-free than in serum-containing medium. In serum-free medium coproporphyrin accumulated in response to AIA and PIA, uroporphyrin in response to Ox-DDC, and a mixture of uro-, copro-, and protoporphyrin in response to DDC. The pattern of porphyrin accumulation in response to Ox-DDC and DDC was similar in serum-free and serum-containing medium. With AIA and PIA a mixture of uro-, copro- and protoporphyrin accumulated in serum-containing medium which contrasts with the accumulation of coproporphyrin alone in serum-free medium. It appears that porphyrin-inducing drugs might inhibit a variety of steps in the heme biosynthetic pathway.

In 1966, Granick [1] introduced a procedure for maintaining chick embryo liver cells in culture. This system has been used by numerous investigators [2–4] to determine whether drugs induce porphyrin biosynthesis and to study the mechanisms of control of hepatic heme biosynthesis. In the original procedure of Granick [1], the cells were maintained in Eagle's basal medium [5], supplemented with 10% fetal bovine serum. One of the problems encountered was the variability in porphyrin production observed with different batches of sera [6]. This variability could be attributed to the presence, in serum, of free hemoglobin and of unknown amounts of various hormones; these substances could interfere with the induction of δ-aminolevulinic acid (ALA)-synthetase. In addition, the serum contains esterase activity which could inactivate drugs containing ester groups.† Clearly, it would be advantageous to have a serum-free medium available for these studies.

Recently, Sinclair and Granick [7] cultured chick embryo liver cells in a modified Ham F-12 medium in the absence of serum. These cells, however, had to be maintained in a serum-containing medium for the first 24 hr; otherwise they would not attach to the surface of the Petri dish. After this time, the medium was replaced with serum-free medium containing the porphyrin-inducing drugs. Twenty-four hr later, porphyrins were found to have accumulated. There are two disadvantages to the above procedure: (1) the modified medium is not available commercially and must be prepared by the investigator, a time-consuming task; and (2) during the initial incubation in the presence of serum, several serum components might attach to the cell surface or enter the cell. These factors which could vary from batch to batch of serum and which might persist during the serum-free phase of culture could result in a variability of responsiveness. Recently, Goodridge [8] described a procedure for maintaining chick embryo liver cells in a commercial medium (Waymouth MD 705/1) [9] in the absence of serum. The cells were cultured in plastic bacterial Petri dishes and attached themselves to the surface without preincubation in medium-containing serum. This system was used successfully to study fatty acid synthesis.

The first objective of the present study was to determine whether drug-induced porphyrin biosynthesis would occur in chick embryo liver cells maintained in serum-free Waymouth medium. Our second objective was to qualitatively and quantitatively compare drug-induced porphyrin biosynthesis in serum-containing and serum-free medium. The final objective of this study was to examine the nature of the porphyrins accumulating in serum-free and serum-containing medium.

MATERIALS AND METHODS

Cell culture techniques. Unincubated fertilized eggs from White Leghorn chickens were obtained from a commercial supplier. They were incubated at 38° with a relative humidity of 68 per cent. After 18 days of incubation, the embryos were sacrificed by decapitation. The livers of 15–20 chick embryos were

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† Unpublished data.

removed under aseptic conditions and washed in sterile modified Krebs–Ringer bicarbonate buffer (modified KRB), pH 7·4, with one-half the recommended amount of $CaCl_2$ [10]. All subsequent preparations were carried out aseptically, and all solutions were sterilized by millipore filtration. The livers were placed on filter paper discs, freed of connective tissue and blood clots, and sliced as thinly as possible with a stainless steel razor blade. The sliced liver was transferred to a 500-ml Erlenmeyer flask containing 75 ml of 0·05% collagenase and 0·1% hyaluronidase in modified KRB [11]. The flask was stoppered and shaken at 150 oscillations/min at 37° for 40 min. The resulting cell suspension was filtered through a double layer of nylon mesh, and the cells were sedimented by centrifugation at $300\,g_{av}$ for 5 min. The cells were washed twice with modified KRB and once with 2% bovine serum albumin in modified KRB. The cells were then suspended in 20 vol of Waymouth MD 705/1 medium containing 60 mg penicillin G, 100 mg streptomycin sulfate and 1·0 mg insulin/l. Cell suspension (0·3 ml containing approximately 1·2 mg protein) was added to 4·7 ml Waymouth medium in 5-cm plastic Petri dishes. These quantities were doubled when 10-cm dishes were used. During the first 24 hr of incubation in an atmosphere of 5% CO_2 in air at 40°, the hepatocytes formed monolayer colonies on the surface of the Petri dishes. After 24 hr of incubation, the medium was replaced with fresh medium. This procedure removed most of the hemoglobin from the lysed red blood cells, as well as the intact red cells. Plastic Petri dishes were used for this method, since they resulted in better cell attachment than did glass.

The method used to culture cells in serum-containing medium has already been described [12]. These cells were grown on glass Petri dishes. The medium was supplemented with 10% Donor Calf serum, Flow Laboratories Inc., Cat. No. 4-023 M, control 4023018.

Assay of porphyrins and ALA-synthetase. After 24 hr of preincubation, the medium was changed and AIA, DDC, Ox-DDC or PIA was added to the fresh medium. The cells were then returned to the incubator for various periods of time. The porphyrins present in both the medium and the cells were determined and the combined values reported [1]. To assay ALA-synthetase, cells were grown in 10-mm plastic Petri dishes and the cells from three dishes were pooled in order to obtain sufficient material for the assay [12].

Chromatographic separation of porphyrins. The type of porphyrins that were formed by cells grown in the serum-free and serum-containing mediums after induction with AIA, PIA, DDC or Ox-DDC was determined by the method of Doss [13]. The porphyrins were esterified and applied to glass thin-layer plates coated to a thickness of 250 μm with Silica gel "H." After development, the plates were scanned with a Turner model 111 fluorometer, equipped with a CAMAG TLC scanner. The individual porphyrins were identified by comparison to uro-, copro- and protoporphyrin standards run on the same plate. The relative concentrations of the porphyrins were determined by integration of the area under the curve.

Source of chemicals. AIA was a gift from Hoffmann-La Roche, Montreal, Quebec. DDC, Ox-DDC and PIA were synthesized in this laboratory according to procedures previously described [14–16]. Waymouth MD 705/1 medium and Eagle's basal medium were purchased in powdered form from Grand Island Biological Co. Penicillin G, streptomycin sulfate, insulin, bovine serum albumin, collagenase and hyaluronidase were obtained from Sigma Chemical Co.

RESULTS

The response of chick embryo liver cells, maintained in serum-free Waymouth medium, to increasing doses of AIA in the presence and absence of insulin (1 mg/l.) is shown in Fig. 1A. Porphyrin accumulation is observed to be significantly higher in the insulin-containing medium. The effect on porphyrin induction of varying cell concentration in serum-free medium is shown in Fig. 1B. When 0·5 ml of cell suspension (2·0 mg protein)/5 ml medium was used,

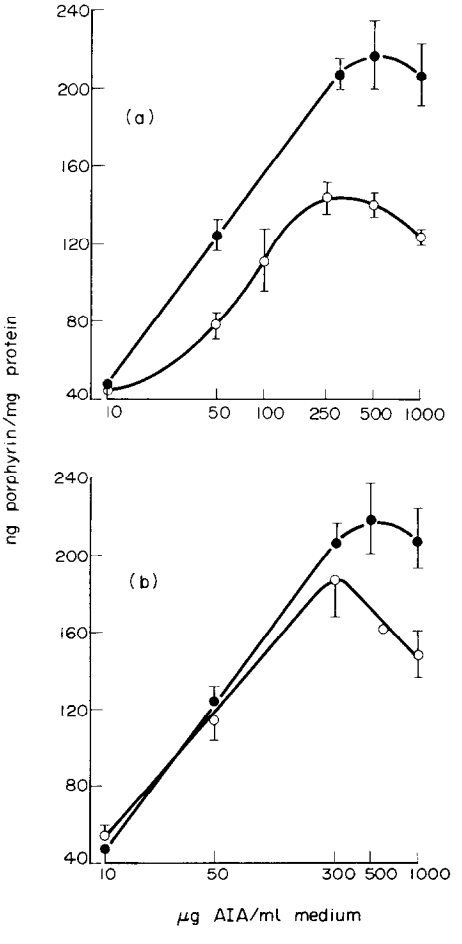

Fig. 1. (a) Porphyrin biosynthesis by cells, exposed to different doses of AIA for 24 hr. The cells were maintained in serum-free Waymouth MD 705/1 medium in the presence (●——●) and absence (○——○) of insulin (1 mg/l.). Each point represents the mean of four to eleven determinations ± S.E.M. (b) Porphyrin biosynthesis by different concentration of cells [1·2 mg protein (●——●) and 2 mg protein (○——○)/dish] exposed to increasing doses of AIA. The cells were maintained in serum-free Waymouth MD 705/1 medium for 24 hr. Each point represents the mean of four to nineteen determinations ± S. E. M.

optimum induction occurred with 300 μg AIA/ml. Increasing the dose of AIA resulted in decreased porphyrin accumulation. When 0·3 ml of cell suspension (1·2 mg protein)/5 ml medium was used, the induction continued to increase to a maximum at about 500 μg AIA/ml. This was significantly above the induction observed with this dose using the higher cell concentration. The increase in ALA-synthetase activity at various times after administration of 300 μg AIA/ml of serum-free medium is shown in Fig. 2. There was a rapid increase in activity in the first 6 hr. After this time, the activity increased less rapidly reaching a maximum at about 16 hr. The porphyrin accumulation in the serum-free and serum-containing systems at different times after the administration of AIA (300 μg/ml) is shown in Fig. 3. During the first 10 hr, porphyrin accumulation in both media was similar. After this time, porphyrin accumulation was markedly greater in the serum-containing than in the serum-free medium.

The accumulation of porphyrins in response to various doses of AIA in chick embryo liver cells maintained in Waymouth MD 705/1 and Eagle's basal media is compared in Fig. 4. Maximum accumulation in both systems is observed at a dose of 600 μg AIA/ml of medium. The maximum accumulation observed in the serum-containing medium was approximately four times greater than that observed in the serum-free medium. The accumulation of porphyrins in response to PIA in serum-containing and serum-free media is compared in Fig. 5. The accumulation observed in the two systems was very similar to that observed with the same doses of AIA. With PIA, as with AIA, the accumulation of porphyrins was approximately four times greater in the serum-containing medium as compared to the serum-free medium. The accumulation of porphyrins in response to DDC in the two cell culture systems is compared in Fig. 6. Maximum accumulation in both systems was obtained with a dose of 2 μg DDC/ml of medium. With DDC, in contrast to the other inducers studied, a slightly higher maximum accumulation was obtained in the serum-free than in the serum-containing medium. The accumulation of porphyrins in response to Ox-DDC in the two systems is shown in Fig. 7; a greater accumulation occurred in serum-containing medium. The difference in the maximum accumulation attained was, however, not as large as that seen with AIA and DDC. Maximum accumulation occurred with Ox-DDC (50 μg/ml) in both systems.

The porphyrins produced in response to AIA (100 μg/ml) in serum-containing medium were separated by thin-layer chromatography (Fig. 8). Protoporphyrin, coproporphyrin and uroporphyrin were present in similar amounts. Smaller amounts of pentacarboxylic, hexacarboxylic and heptacarboxylic porphyrin were present. In serum-free medium, coproporphyrin made up almost 90 per cent of the total porphyrin, with protoporphyrin forming most of the remainder. The results obtained with PIA (Fig. 9) were very similar to those observed with AIA. The porphyrins, formed after administration of DDC (1·5 μg/ml), are shown in Fig. 10. In the serum-containing medium, uroporphyrin makes up about 50 per cent

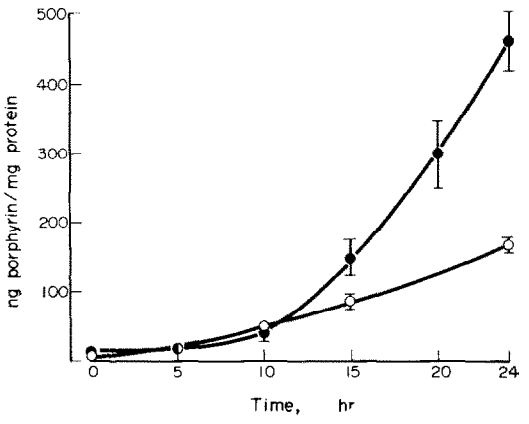

Fig. 3. Time course of the biosynthesis of porphyrins by cells maintained in serum-free Waymouth MD 705/1 medium (○——○) and serum-containing Eagle's basal medium (●——●) after the administration of AIA (300 μg/ml). Each point represents the mean of six to twelve determinations ± S. E. M.

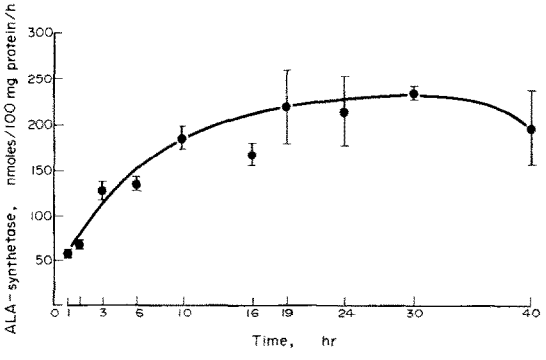

Fig. 2. Time course of the induction of ALA-synthetase by cells maintained in serum-free Waymouth MD 705/1 medium after the administration of AIA (300 μg/ml). Each point represents the mean of four to seven determinations ± S. E. M.

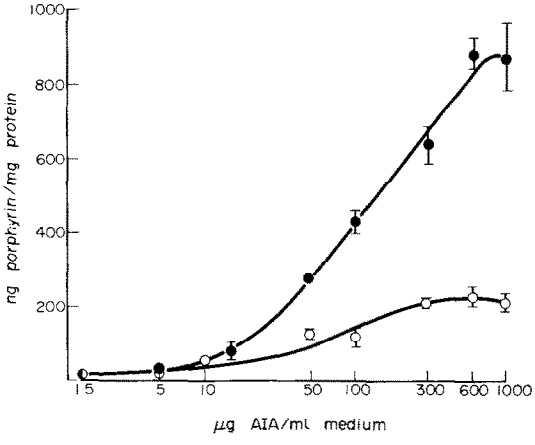

Fig. 4. Biosynthesis of porphyrins by cells maintained in serum-free Waymouth MD 705/1 medium (○——○) and serum-containing Eagle's basal medium (●——●) 24 hr after the addition of increasing doses of AIA. Each point represents the mean of four to twenty determinations ± S. E. M.

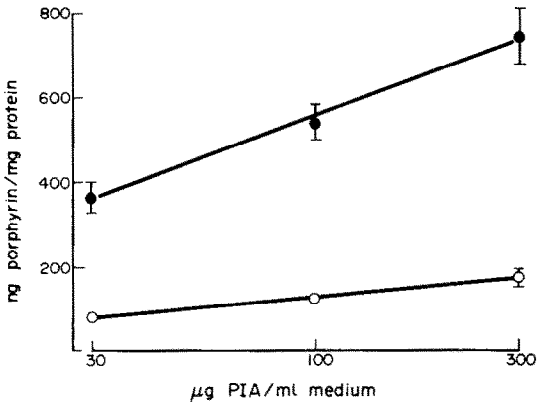

Fig. 5. Biosynthesis of porphyrins by cells maintained in serum-free Waymouth MD 705/1 medium (○——○) and serum-containing Eagle's basal medium (●——●) 24 hr after the addition of increasing doses of PIA. Each point represents the mean of eight determinations ± S. E. M.

of the total porphyrins, with smaller amounts of proto-, tricarboxylic, copro- and heptacarboxylic porphyrin. The serum-free system has protoporphyrin as its major porphyrin with lesser amounts of uro-, copro-, tricarboxylic and heptacarboxylic porphyrin. The distribution of porphyrins after Ox-DDC administration is shown in Fig. 11. Similar results were obtained in serum-free and serum-containing medium with the major porphyrin being uroporphyrin and with minor amounts of other porphyrins.

DISCUSSION

In the chick embryo liver cell culture system introduced by Granick [1], Eagle's basal medium supplemented by 10% fetal bovine serum is used. We have found this system to be valuable in the study of control mechanisms in porphyrin biosynthesis. The major limitation of this procedure is the variability encountered with different batches of serum. When a batch of serum is exhausted after a series of experiments, a search for a suitable batch of new serum must be

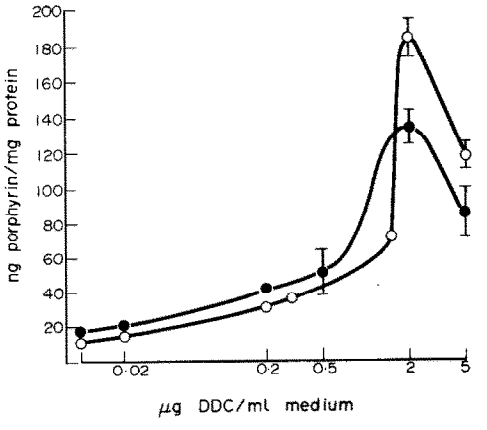

Fig. 6. Biosynthesis of porphyrins by cells maintained in serum-free Waymouth MD 705/1 medium (○——○) and Eagle's basal medium (●——●) 24 hr after the addition of increasing doses of DDC. Each point represents the mean of four to eight determinations ± S. E. M.

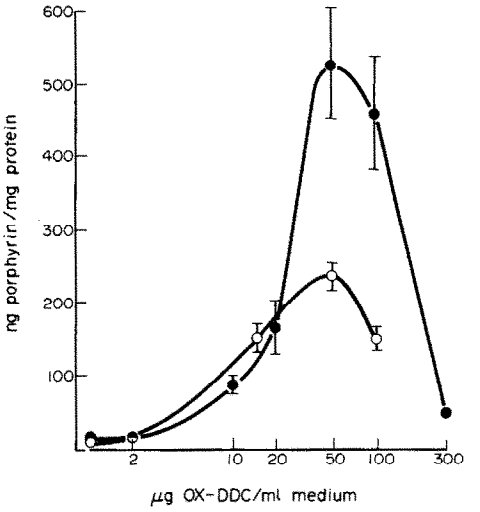

Fig. 7. Biosynthesis of porphyrins by cells maintained in serum-free Waymouth MD 705/1 medium (○——○) and Eagle's basal medium (●——●) 24 hr after the addition of increasing doses of Ox-DDC. Each point represents the mean of four to twelve determinations ± S. E. M.

undertaken. Frequently this is a time-consuming and laborious process in view of the variability observed with different sera. This variability has been reported previously and reconfirmed in the present study (Table 1). Our first objective was to investigate the possibility of inducing ALA-synthetase and porphyrin accumulation in chick embryo liver cells maintained in serum-free medium and thus overcome the problem of variability. Goodridge [8] developed a procedure for maintaining chick embryo liver cells in ser-

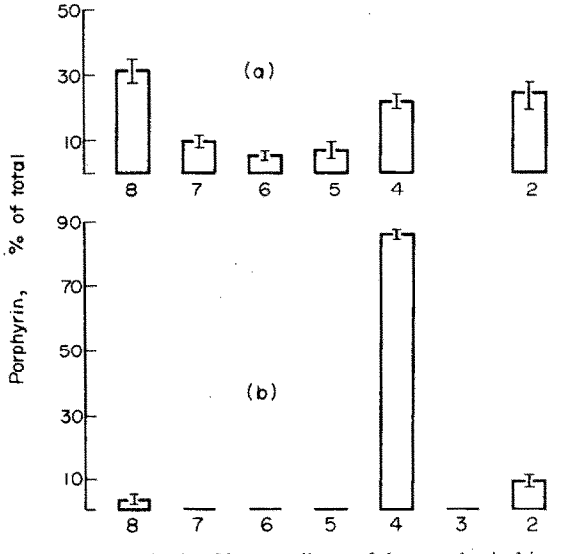

Fig. 8. Biosynthesis of intermediates of the porphyrin biosynthetic pathway by cells maintained in serum-containing Eagle's basal medium (a) and serum-free Waymouth MD 705/1 medium (b) in response to AIA (100 μg/ml). The accumulation of the intermediates is expressed as the percentage of total porphyrins formed ± S. E. M. The intermediates are: 8, uro-; 7, heptacarboxylic; 6, hexacarboxylic; 5, pentacarboxylic; 4, copro-; 3, tricarboxylic; 2, protoporphyrin.

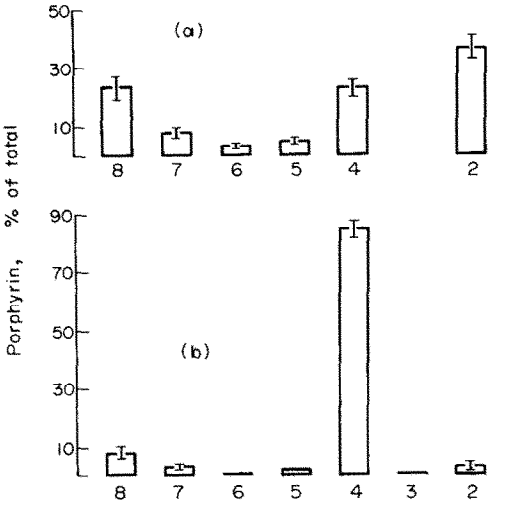

Fig. 9. Biosynthesis of intermediates of the porphyrin biosynthetic pathway by cells maintained in serum-containing Eagle's basal medium (a) and serum-free Waymouth MD 705/1 medium (b) in response to PIA (100 μg/ml). The results are expressed as described in the legend to Fig. 8.

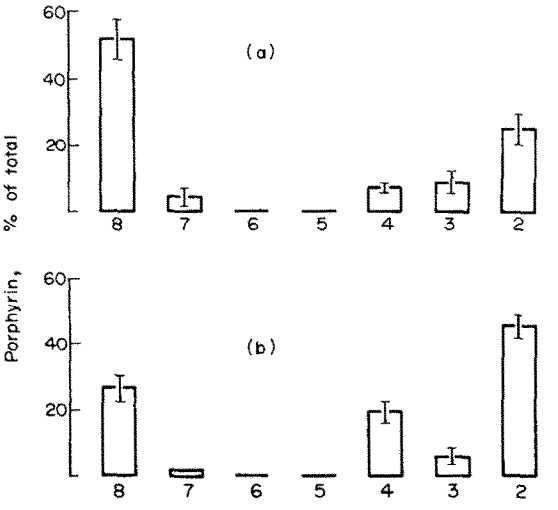

Fig. 10. Biosynthesis of intermediates of the porphyrin biosynthetic pathway by cells maintained in serum-containing Eagle's basal medium (a) and serum-free Waymouth MD 705/1 medium (b) in response to DDC (1·5 μg/ml). The results are expressed as described in the legend to Fig. 8.

um-free Waymouth medium and used this system successfully for studying fatty acid synthesis. The results in Figs. 1 and 2 demonstrate porphyrin accumulation and inducibility of ALA-synthetase, respectively, in the serum-free medium. Both Goodridge [8] and Sinclair and Granick [7] added insulin to serum-free medium in order to obtain optimal results. The addition of insulin to our serum-free Waymouth medium resulted in increased porphyrin accumulation (Fig. 1A). It has been reported that insulin is one of the serum macromolecules required to maintain the ribosomal integrity of primary cultures of chick embryo fibroblasts [17]. It is, therefore, possible that insulin might produce its effect in chick embryo hepatocytes by helping to maintain ribosomal integrity and thereby facilitating the increased synthesis of ALA-synthetase in response to porphyrin-inducing drugs. Porphyrin accumulation at high doses of AIA was greater in Petri dishes incubated with 0·3 ml of cell suspension (1·2 mg protein) than in dishes incubated with 0·5 ml of cell suspension (2·0 mg protein). It is possible that the decreased porphyrin accumulation observed with the higher cell population might be attributed to the exhaustion of a key nutrient required for porphyrin biosynthesis.

Livers were dissociated by means of collagenase and hyaluronidase and the dispersed cells maintained in Waymouth medium. When the Waymouth medium was replaced with Eagle's basal medium the cells appeared morphologically similar to those in Waymouth medium but were unresponsive to porphyrin-inducing drugs. Consequently, there must be constituents present in Waymouth medium, but absent in Eagle's basal medium, necessary for drug-induced porphyrin accumulation. It might be possible to identify these constituents by adding to the Eagle's basal medium components of the Waymouth medium which are absent from Eagle's basal medium. When livers were dissociated with trypsin and placed in serum-free Waymouth medium only a few of the cells attached to the Petri dish.

Our second objective was to compare qualitatively and quantitatively drug-induced porphyrin biosynthesis in serum-free and serum-containing medium. The time course of porphyrin accumulation in response to AIA (300 μg/ml) is shown in Fig. 3. Considerably larger amounts of porphyrins accumulated

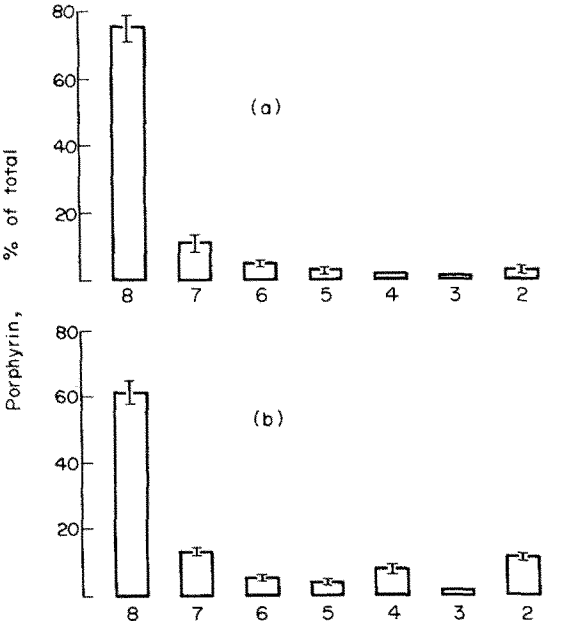

Fig. 11. Biosynthesis of intermediates of the porphyrin biosynthetic pathway by cells maintained in serum-containing Eagle's basal medium (a) and serum-free Waymouth MD 705/1 medium (b) in response to Ox-DDC (50 μg/ml). The results are expressed as described in the legend to Fig. 8.

Table 1. Effect of different sera on AIA (300 μg/ml)-induced porphyrin biosynthesis 24 hr after drug administration

Serum used to prepare medium	Porphyrin formed (ng/mg protein ± S. E.)
Bovine (Pentex Inc., Winley–Morris Co.)	816·9 ± 65·2
Bovine (Grand Island Biological Co.)	690·2 ± 96·3
Fetal bovine (Flow Laboratories, Inc.)	109·4 ± 7·8
Donor calf (Flow Laboratories, Inc.)	637·1 ± 26·9

after 10 hr in the serum-containing medium than in the serum-free medium. Porphyrin accumulation in response to increasing doses of AIA (Fig. 4), PIA (Fig. 5) and Ox-DDC (Fig. 7) was considerably greater in serum-containing than in serum-free medium. With DDC (Fig. 6), however, porphyrin accumulation in response to increasing doses was greater in serum-free than in serum-containing medium. Despite the fact that there is less porphyrin accumulation in the serum-free medium in response to three out of the four drugs, we believe that the advantages of the serum-free medium outweigh this disadvantage. The adoption of the serum-free medium has enabled us to obtain reproducible data and has eliminated the need for continuous screening of new batches of serum. Since serum is the most expensive constituent of cell culture systems, elimination of this component greatly decreases the expense associated with these experiments.

The final objective of our study was to examine the nature of the porphyrins which accumulate in serum-free and serum-containing medium. Since porphyrin-inducing chemicals induce a rapid increase in ALA-synthetase activity in chick embryo liver cells, it has been assumed that porphyrins accumulate as a result of the overproduction of ALA and the inability of the enzymes of the heme biosynthetic pathway to cope with an increased flux of intermediates through the pathway. If this is the correct explanation, one would expect (1) the accumulation of the same porphyrins in response to all porphyrin-inducing chemicals, and (2) the pattern of porphyrin accumulation in response to drugs should be the same as that observed when exogenous ALA is added to the chick embryo liver cell culture medium. Doss and Kaltepoth [18] have recently compared the porphyrins accumulating in response to two porphyrin-inducing drugs and to exogenous ALA in chick embryo liver cells grown in serum-containing medium. The pattern of accumulation, proto- > copro- > uroporphyrin, was found to be the same whether porphyrin-inducing drugs or ALA was added. However, Sinclair and Granick [19] have recently shown that uroporphyrin accumulates in response to several chlorinated hydrocarbons, while protoporphyrin is the major porphyrin accumulating in response to AIA. Sinclair and Granick [19] have suggested that a metabolite of the chlorinated hydrocarbons inhibits uroporphyrinogen decarboxylase, thus causing uroporphyrin to accumulate. When exogenous ALA was added, protoporphyrin accumulated. Clearly all porphyrin-inducing agents cannot be acting by the same mechanisms. This conclusion is supported by our present results. Thus, in serum-free medium, coproporphyrin accumulates in response to AIA (Fig. 8B) and PIA (Fig. 9B), while uroporphyrin is the major porphyrin to accumulate in response to Ox-DDC (Fig. 11B) and a mixture of proto-, copro- and uroporphyrin in response to DDC (Fig. 10B). These results suggest that Ox-DDC inhibits uroporphyrinogen decarboxylase and that AIA and PIA inhibit coproporphyrinogen oxidase. DDC has previously been shown to inhibit ferrochelatase [20] and the fact that protoporphyrin is the predominant porphyrin to accumulate in the serum-free medium is consistent with this interpretation. It would appear that one of the mechanisms by which porphyrin-inducing drugs act is by inhibiting a step in the porphyrin biosynthetic pathway. This would result in decreased levels of protoheme which in turn would release ALA-synthetase from feedback repression. The pattern of porphyrin accumulation in response to Ox-DDC was similar in serum-free and serum-containing medium (Fig. 11). With DDC the pattern of porphyrin accumulation was similar qualitatively but not quantitatively. In the case of AIA and PIA, the pattern of porphyrin accumulation was markedly different in serum-containing and serum-free medium (Figs. 8 and 9). Thus, while coproporphyrin was the principle porphyrin in serum-free medium, a mixture of uro-, proto- and coproporphyrins accumulated in serum-containing medium. This experiment suggests that factors in serum exert control on the activity of some of the enzymes of the heme biosynthetic pathway. The serum-free medium may, therefore, be useful in a study of factors controlling the activity of enzymes of the heme biosynthetic pathway.

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