Biochemical Pharmacology, Vol. 21, pp. 2077–2093. Pergamon Press, 1972. Printed in Great Britain.

DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—V
EFFECT OF PROTOHEMIN ON THE TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL PHASES OF δ-AMINOLEVULINIC ACID SYNTHETASE INDUCTION*

D. L. J. TYRRELL† and G. S. MARKS

Department of Pharmacology, Queen's University, Kingston, Ontario, Canada

(*Received* 26 *July* 1971; *accepted* 14 *January* 1972)

Abstract—Delta-aminolevulinic (ALA) synthetase is induced by porphyrin-inducing compounds and the induction is prevented by protohemin. To obtain information about the mechanisms involved in these processes, the following experiments were performed. Chick embryo liver cell cultures were treated with cycloheximide 20 μM and an inducer of the enzyme, *viz.* either allylisopropylacetamide (AIA), 2·1 mM or 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), 37 μM for 5 hr. The cells were then washed, reincubated in fresh medium and the ALA synthetase activity assayed at 2 hr intervals. A significant increase in ALA synthetase activity was observed indicating that a phase of induction had occurred prior to washing which required RNA synthesis but was independent of translation. This increase of ALA synthetase activity normally observed after washing was inhibited by cycloheximide, 20 μM, but not by actinomycin D, 0·8 μM. Therefore, this second phase of induction observed after washing was independent of transcription. Protohemin added during the translational (post-wash) phase prevented the increase of ALA synthetase activity. Hence, protohemin appears to exhibit some form of post-transcriptional inhibition in eukaryotic cells. It was found that transcription of induction-specific RNA could occur in the presence of concentrations of protohemin which completely inhibited the post-transcriptional process.

A LARGE number of chemicals and certain steroids are capable of inducing increased porphyrin biosynthesis in animals and in chick embryo liver cell cultures.[1] This increased porphyrin biosynthesis is mediated by increasing the activity of the first enzyme in the porphyrin biosynthetic pathway, δ-aminolevulinic acid (ALA) synthetase.[2] This enzyme is rate limiting in aerobic bacteria,[3] in differentiating erythrocytes[4] and in liver cells.[2] Using chick embryo liver cells in culture, Granick[1] was able to obtain indirect evidence that porphyria-inducing compounds increased ALA synthetase activity by increasing the synthesis of this enzyme and that hemin prevented this effect. Since the publication of this report we have sought evidence for the accumulation of induction-specific RNA and have sought to clarify the mechanism of protohemin inhibition.[5, 6] Recently, Sassa and Granick[7] have reported that ALA synthetase is induced by etiocholanolone and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl pyridine (DDC) at the level of transcription and by allylisopropylacetamide (AIA) and α-hexachlorohexane at the level of translation. Using a different experimental design, we now present further evidence for the accumulation of induction-specific RNA in the presence of AIA and DDC. This induction-specific RNA appears to

* Supported by the Medical Research Council of Canada.

† Life Insurance Medical Research Fellow.

mediate increased ALA synthetase activity by translation into either this enzyme or into some other protein involved in ALA synthetase activation. In order to carry out these experiments, it was necessary to develop a procedure for ALA synthetase assay in the chick embryo liver cell culture system of Granick.[9] This was achieved by modifying the procedure of Marver *et al.*[8] The procedure we developed has several features in common with that recently reported by Sassa and Granick.[7]

In *Rhodopseudomonas spheroides*, protoheme participates in feedback repression and feedback inhibition of ALA synthetase.[9] On the other hand, Granick[1] obtained indirect evidence for feedback repression of ALA synthetase by protohemin in chick embryo liver cell cultures, but could obtain no evidence for feedback inhibition using a crude preparation of ALA synthetase. Scholnick *et al.*[10] were able to demonstrate that protohemin (10^{-4} M) inhibited the activity of partially purified ALA synthetase from male Wistar rats by 77 per cent, but did not inhibit crude preparations of this enzyme. These investigators suggest that the failure to demonstrate protohemin inhibition of ALA synthetase in crude preparations is caused by protohemin binding to protein and therefore its inability to reach the enzyme located in mitochondria. Aoki *et al.*[11] purified ALA synthetase from rabbit reticulocytes and found that protohemin (10^{-5} M) inhibited this purified enzyme by 40 per cent. Whether this feedback inhibition of ALA synthetase occurs *in vivo* remains to be established.

Sassa and Granick[7] have recently presented evidence for protohemin inhibition of ALA synthetase induction at the level of translation of messenger RNA (m-RNA) into the enzyme. Kurashima *et al.*[12] have shown that protohemin prevents the transfer of ALA synthetase from cytoplasm into mitochondria. In the present study, the induction of ALA synthetase activity by the porphyrin-inducing compounds, AIA and DDC has been divided into two phases, a transcriptional phase independent of translation, and a translational phase independent of transcription. The action of protohemin has been studied in both phases of the induction process.

METHODS

Cell culture techniques. All experiments were performed on primary chick embryo liver cell cultures grown as monolayers. After 24–36 hr, the media were changed and replaced with fresh media containing the desired drugs. This time is the beginning of a period which has been designated "pretreatment period" in the figures of experimental results.

Chick embryo liver cell cultures were prepared using the procedure of Granick[1] with the following modifications; eight 17-day-old chick embryo livers were removed and a cell suspension was prepared in a mixture of 10 ml of 2·5% trypsin in saline (Microbiological Associates) and 10 ml magnesium- and calcium-free Earle's solution. The cell suspension was centrifuged at 250 *g* (average) for 5 min, the supernatant was discarded and the cells were resuspended in 20 ml of warm fresh growth medium. One ml of this suspension was added to each large petri dish containing 25 ml of growth medium and placed in a Napco incubator at 37° and constant air flow of 8·4 l/min. The air flow was adjusted to contain 5% CO_2.

Growth medium was prepared using powdered basal medium containing Earle's salts and glutamine (Grand Island Biological Company). To each 100 ml of freshly prepared medium was added 10 ml of pooled bovine serum (lot 30, Pentax, Inc.

Winley–Morris Co. Ltd.), 10,000 units penicillin G (Ayerst), 2000 units nystatin (Squibb) and 10 mg streptomycin sulfate (Pfizer). In a previous communication we have discussed the importance of careful selection of the serum for these experiments.[13]

The porphyrin-inducing compounds, cycloheximide and actinomycin D, were dissolved in 95% redistilled ethanol (25 μl) and added to the liver cells in culture when required. Protohemin was dissolved in 0·5 ml of 1 N KOH and 4·5 ml of distilled water. The pH was adjusted to 7·8 with 0·1 N HCl on a pH meter with continuous stirring. A portion of this solution (0·5 ml) was added to each petri dish requiring protohemin. Control dishes received the appropriate drug solvents. The cells in culture were examined under phase contrast microscopy (640×) and the *criteria* of Granick[1] were used to assess possible harmful effects of the drugs. The cells did not undergo any recognizable morphological changes.

When the cells were washed, the old medium was aspirated off the cells using a sterile suction apparatus. Fresh warm medium (25 ml) was added to the cells and the petri dishes were returned to the incubator for 5 min. This procedure was repeated to complete cell washing.

Enzyme assay. The procedure used was a modification of the method of Marver *et al.*[8] To obtain a sufficient quantity of cells for the determination of ALA synthetase activity, cells were grown in petri dishes of 150 mm diameter and 15 mm depth containing 25 ml of growth medium. To assay the enzyme, the medium was removed and 5 ml of 0·9% NaCl, Tris (10 mM) and EDTA (0·5 mM), pH 7·4, were added to each dish. The cells were scraped from the surface of the petri dishes using a rubber policeman and cells from two identically treated dishes were combined and centrifuged. The supernatant was removed and the cells were suspended in the same solution (0·8 ml) and homogenized. Aliquots of this homogenate (0·3 ml) were added to each of two Erlenmeyer flasks (25 ml) containing 0·6 ml of a solution of glycine (133 mM), Tris (100 mM), EDTA (13·3 mM), pH 7·2. To one of these flasks 0·3 ml of 25% trichloroacetic acid (TCA) was added, and this served as a tissue blank. Both Erlenmeyer flasks were placed in a shaker bath at 37° and the reaction was terminated in the test flask after 1 hr by the addition of 0·3 ml of 25% TCA. The precipitates were removed by centrifugation and 0·8 ml of the supernatant was added to a test tube containing 0·6 ml of 1 M phosphate buffer, pH 6·8, and 0·1 ml of 5 N NaOH. Ethylacetoacetate (0·1 ml) was added and the solution heated in a boiling water bath for 7 min. Modified Erhlich's reagent[14] (1·5 ml) was added and after 20 min the absorbancy measured from 600 to 490 nm in a Unicam model SP 800 recording spectrophotometer using microcuvettes of 20 mm pathlength. A Perkin–Elmer model 165 slave recorder was used to expand the scale as required. Using a solvent-partition technique similar to that employed by Granick[1] and standard reference pyrroles, it was found that at least 90 per cent of the aminoketone generated was ALA. Hence, extraction of the aminoacetone was not considered necessary in these experiments. Two samples of the cell homogenate (50 μl) were used for protein determination.[15] The enzyme activity was expressed as millimicromoles of ALA/hr/100 mg at 37°.

Counting of radioactive samples. All radioactive samples were counted in a Nuclear Chicago Mark II liquid scintillation system. Scintillation fluid was prepared by dissolving 6 g 2,5-diphenyloxazole (PPO; Amersham–Searle) and 100 mg 1,4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP; Amersham–Searle) in 1 l. of toluene. The samples were counted for a sufficient time to insure less than 1 per cent standard error.

Determination of appropriate concentrations of cycloheximide and actinomycin D. Since both actinomycin D and cycloheximide in high concentrations exert toxic effects on cells in culture, it was desirable to select a small but effective concentration of each drug to inhibit ALA synthetase induction.

Actinomycin D. The media were removed from 12 petri dishes (150 × 20 mm) after 24 hr of incubation and the cells were reincubated in fresh warm medium. To ten of the dishes, AIA (2·1 mM) was added and actinomycin D (0, 8×10^{-4}, 8×10^{-3}, 8×10^{-2} and 8×10^{-1} μM) was added to two dishes at each concentration. To the media of two dishes, only drug solvents were added. After 16 hr the ALA synthetase activity was measured (Fig. 1).

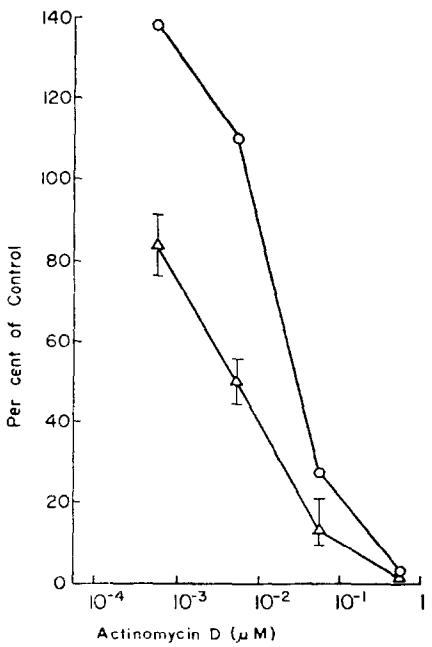

FIG. 1. Dose–response curve of actinomycin D inhibition of ALA synthetase induction and ^{3}H-uridine incorporation into RNA. ALA synthetase activity (○—○) was estimated 16 hr after the cells were exposed to AIA and actinomycin D. Control cells for ALA synthetase received AIA (2·1 mM) and the solvent for actinomycin D (ethanol; 25 μl). ^{3}H-uridine incorporation (△—△) into RNA was estimated 12 hr after the cells were incubated in media containing ^{3}H-uridine and actinomycin D. Control cells for ^{3}H-uridine incorporation received the solvent for actinomycin D (ethanol; 5 μl). Each point on the ALA synthetase curve represents a single determination and each point on the ^{3}H-uridine incorporation curve represents the average of three determinations and the range of values is shown.

The incorporation of ^{3}H-uridine into RNA in the presence of actinomycin D was determined as follows. After 24 hr of incubation, the media were removed from petri dishes (50 × 10 mm) and the cells were reincubated in media containing ^{3}H-uridine (1 μc/ml; Amersham–Searle) and actinomycin D at the above concentrations. After 12 hr of incubation, the media were removed, the cells fixed with 95% ethanol and washed three times with 4 ml of 0·5 N perchloric acid precooled to 4°. The residue

was solubilized with NCS tissue solubilizer (Amersham–Searle), transferred to counting vials and the radioactivity was measured. The protein content of cells grown in petri dishes under identical conditions was determined and the specific activity expressed as disintegrations/min/mg of protein. The results in Fig. 1 indicate that actinomycin D at a concentration of 0·8 μM adequately inhibited ALA synthetase induction and ^{3}H-uridine incorporation into RNA. For this reason this dose of actinomycin D was used in subsequent experiments.

Cycloheximide. After 24 hr of incubation, the media were removed from 12 petri dishes (150 × 20 mm) and the cells were reincubated in fresh medium. To ten dishes, AIA (2·1 mM) was added and cycloheximide (0, 0·1, 1, 10 and 100 μM) was added to two dishes at each concentration. Two petri dishes received only the drug solvents. After 16 hr the ALA synthetase activity was assayed (Fig. 2).

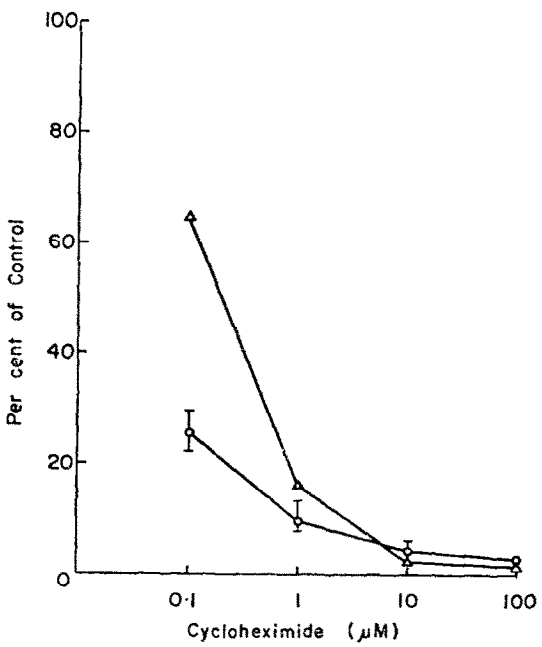

FIG. 2. Dose-response curve of cycloheximide inhibition of ALA synthetase induction and ^{14}C-protein hydrolysate incorporation into protein. ALA synthetase activity (△—△) was estimated 16 hr after the cells were exposed to AIA and cycloheximide. Control cells for ALA synthetase received AIA (2·1 mM) and the solvent for cycloheximide (ethanol; 25 μl). ^{14}C-protein hydrolysata incorporation into protein (○—○) was estimated 12 hr after the cells were incubated in medie containing ^{14}C-protein hydrolysate and cycloheximide. Control cells for ^{14}C-protein hydrolysate received the solvent for cycloheximide (ethanol; 5 μl). Each point on the ALA synthetase curve represents a single determination and each point on the ^{14}C-protein hydrolysate incorporation curve represents the average of three determinations and the range of values is shown.

After 24 hr of incubation, the media were removed from petri dishes (50 × 10 mm) and the cells were incubated in fresh warm media containing ^{14}C-protein hydrolysate (0·1 μc/ml; Amersham–Searle). After 12 hr the media was removed and the cells washed twice with 4 ml of normal saline. The monolayer of cells was washed three times with 4 ml of 25% TCA, twice with 4 ml of distilled water and the cell residue

was solubilized with 2 ml of NCS tissue solubilizer. The solutions were transferred to counting vials and the radioactivity was determined. The protein content of cells grown in petri dishes under identical conditions was measured and the specific activity expressed as disintegrations per minute per milligram of protein. The results in Fig. 2 indicate that cycloheximide at a concentration of 20 μM adequately inhibited ALA synthetase induction and ^{14}C-protein hydrolysate incorporation into protein. For this reason this dose was used in subsequent experiments.

Demonstration of "induction-specific" RNA for ALA synthetase

After 30 hr of incubation, the media were removed from 34 large petri dishes. The cells were treated with fresh warm medium containing AIA (2·1 mM) and divided into four alphabetically labeled groups. Group A (eight dishes) was reincubated and a set of two dishes was removed after 0, 1, 5 and 8 hr for ALA synthetase assay. The dishes in groups B (ten), C (eight) and D (eight) received cycloheximide (20 μM). After 5 hr of incubation two dishes were removed from group B for ALA synthetase assay. The remaining dishes in group B and the dishes in groups C and D were washed and the cells treated with fresh warm medium. Cycloheximide (20 μM) and actinomycin D (0·8 μM) were added to the media of groups D and C respectively. After 1, 3, 5 and 7 hr of reincubation, sets of two dishes were removed from each of groups B, C and D for ALA synthetase assay. The results are shown in Fig. 3.

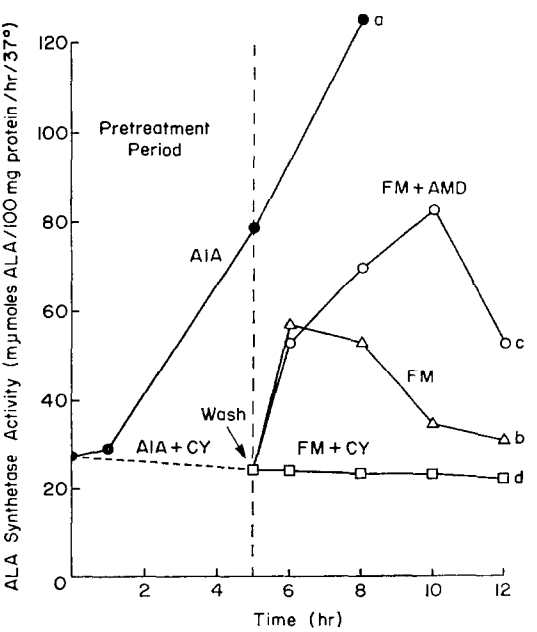

FIG. 3. Cells were exposed to 2·1 mM AIA and ALA synthetase activity was measured at various intervals over 8 hr (curve a, ●—●). Cells were exposed to 2·1 mM AIA and 20 μM cycloheximide (CY) for 5 hr (● - - - □), washed and reincubated in fresh medium (FM) (curve b, △——△), fresh medium and 0·8 μM actinomycin D (AMD; curve c, ○——○), or fresh medium and 20 μM cycloheximide (curve d, □——□).

The following control experiments were performed. Cells were exposed to media containing both AIA (2·1 mM) and actinomycin D (0·8 μM) for 5 hr, washed and reincubated in fresh medium or fresh medium containing AIA (2·1 μM). ALA synthetase activity was assayed at 1, 3, 5 and 7 hr after reincubation. Cells were incubated in medium containing cycloheximide (20 μM) for 5 hr, washed and reincubated in fresh medium. ALA synthetase activity was assayed 1, 3, 5 and 7 hr after reincubation. The results are shown in Fig. 4. These experiments were repeated with DDC (37 μM) instead of AIA and the results are shown in Figs. 5 and 6.

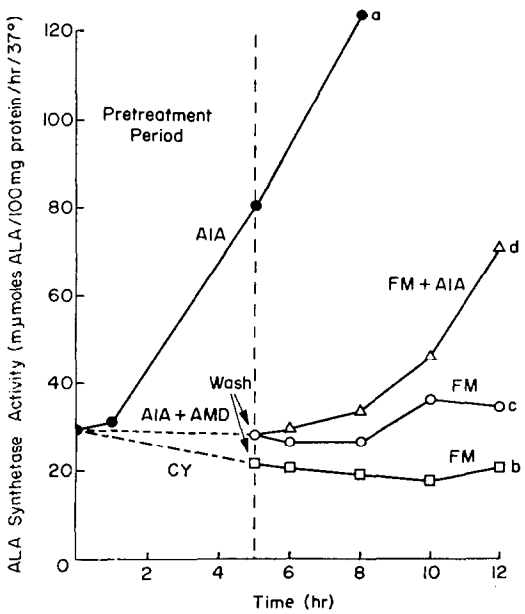

FIG. 4. Cells were exposed to 2·1 mM AIA, and ALA synthetase activity was measured (curve a, ●——●). Cells were exposed to 20 μM cycloheximide (CY) for 5 hr (● - - - □), washed and reincubated in fresh medium (curve b, □——□). Cells were exposed to 2·1 mM AIA and 0·8 μM actinomycin D (AMD) for 5 hr (● - - - ○), washed and reincubated in fresh medium (curve c, ○——○) or fresh medium and AIA (curve d, △——△).

After 24 hr of incubation, the medium was removed from 14 large petri dishes. The drug solvents were added to two dishes and ALA synthetase activity was measured after 7 hr of incubation. The remaining dishes were treated with fresh warm medium containing AIA (2·1 mM). Four dishes were reincubated and ALA synthetase activity was determined after 5 and 7 hr. To four dishes, cycloheximide (20 μM) was added and the level of ALA synthetase measured after 5 and 7 hr of incubation. The remaining four dishes received cycloheximide (20 μM) and were reincubated. After 5 hr, the cells were washed and two dishes reincubated with fresh medium for 2 hr. The remaining two dishes were reincubated with fresh medium containing actinomycin D (0·8 μM) for 2 hr. ALA synthetase activity was determined after the 2-hr incubation period. The experiment was repeated four times and the results are shown in Table 1. Experiments were carried out in the same manner with DDC and the results are also shown in Table 1.

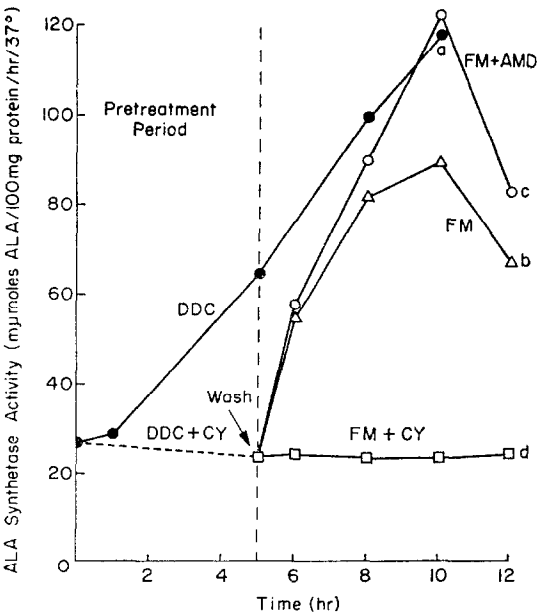

FIG. 5. Cells were exposed to 37 μM DDC and ALA synthetase was measured over 8 hr (curve a, ●——●). Cells were exposed to 37 μM DDC and 20 μM cycloheximide (CY) for 5 hr (● - - - □), washed and reincubated in fresh medium (curve b, △——△), or fresh medium and 0·8 μM actinomycin D (AMD; curve c, ○—○), or fresh medium and 20 μM cycloheximide/curve d, □—□).

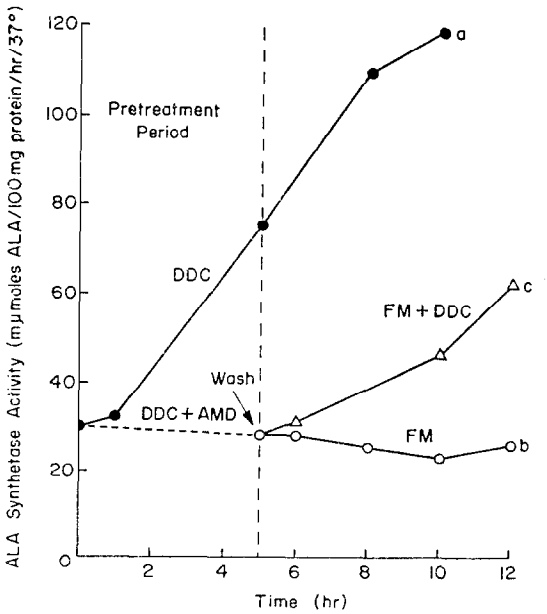

FIG. 6. Cells were exposed to 37 μM DDC and ALA synthetase activity was measured (curve a, ●——●). Cells were exposed to 37 μM DDC and 0·8 μM actinomycin D (AMD) for 5 hr (● - - - ○), washed and reincubated in fresh medium (curve b, ○——○) or fresh medium containing DDC (curve c, △——△).

TABLE 1. DEMONSTRATION OF THE INDUCTION OF RNA FOR ALA SYNTHETASE BY PORPHYRIN-INDUCING COMPOUNDS

Preincubation medium	Wash after 5 hr	Drugs in post-incubation medium	ALA synthetase activity (nmoles ALA/100 mg/hr at 37°) 5 hr	7 hr
Drug solvents				25·9 ± 4·6*
AIA			62·6 ± 12·3	78·4 ± 12·9
AIA + cycloheximide			23·2 ± 3·4	23·2 ± 4·8
AIA + cycloheximide	Yes			54·8 ± 13·4†
AIA + cycloheximide	Yes	Actinomycin D		69·0 ± 11·0†
DDC			58·0 ± 12·8	73·6 ± 7·2
DDC + cycloheximide			19·6 ± 3·1	21·5 ± 3·5
DDC + cycloheximide	Yes			75·8 ± 12·2†
DDC + cycloheximide	Yes	Actinomycin D		63·8 ± 10·7†

* The values shown represent the mean of five determinations ± standard deviation.
† Indicates significance at the 0·01 level using one-tail Student's *t*-test.

Effect of protohemin on the post-transcriptional phase of induction

After 30 hr of incubation, the media were removed from 26 petri dishes and the cells were treated with fresh warm medium containing AIA (2·1 mM). Eight dishes were incubated and sets of two dishes were removed for ALA synthetase assay after 0, 1, 5 and 8 hr. Cycloheximide (20 μM) was added to the remaining 18 dishes. After a 5-hr incubation period, two dishes were removed for ALA synthetase assay and the remaining 16 dishes were washed. Eight dishes were then incubated in fresh warm medium and eight dishes were incubated in fresh warm medium containing protohemin (10 mM). ALA synthetase activity was assayed 1, 3, 5 and 7 hr later. The experiment was repeated using DDC (37 μM) instead of AIA. The results are shown in Figs. 7 and 8.

The effect of increasing the concentration of protohemin during the post-transcriptional phase of induction was examined as follows; after 24 hr of incubation, the media were removed from 14 petri dishes, and the cells incubated in fresh medium containing DDC (37 μM) and cycloheximide (20 μM). After 5 hr, two dishes were removed for ALA synthetase assay. The remaining cells were washed and reincubated in fresh warm medium. To the medium of two dishes, the solvent for protohemin was added. To sets of two dishes, protohemin was added in the following concentrations: 5, 11, 22, 45 and 90 μM. Two hr later the ALA synthetase activity was estimated. The results are shown in Table 2.

Effect of protohemin on the transcriptional phase of induction

After 30 hr of incubation, the medium was removed from 28 petri dishes and the cells were treated with fresh warm medium containing AIA (2·1 mM). The dishes were divided into three groups. Group A (eight dishes) was reincubated and sets of two dishes were removed after 0, 1, 5 and 8 hr for assay of ALA synthetase activity. To the medium in the dishes of group B (ten dishes), cycloheximide (20 μM) was added and the dishes were returned to the incubator. After 5 hr, a set of two dishes was removed for assay of ALA synthetase activity. The remaining dishes of group B were washed, reincubated in fresh medium and ALA synthetase activity was assayed

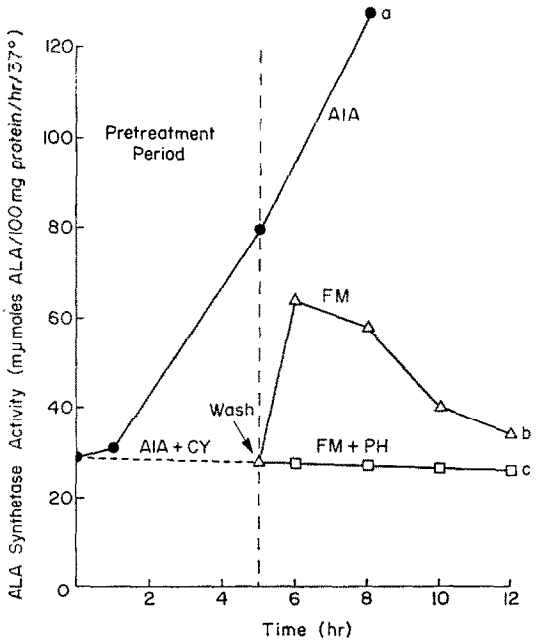

FIG. 7. Cells were exposed to 2·1 mM AIA and ALA synthetase activity was measured over 8 hr (curve a, ●——●). Cells were exposed to 2·1 mM AIA and 20 μM cycloheximide (CY) for 5 hr, washed and reincubated in fresh medium (curve b, △——△) or in fresh medium containing 10 μM protohemin (PH; curve c, □——□).

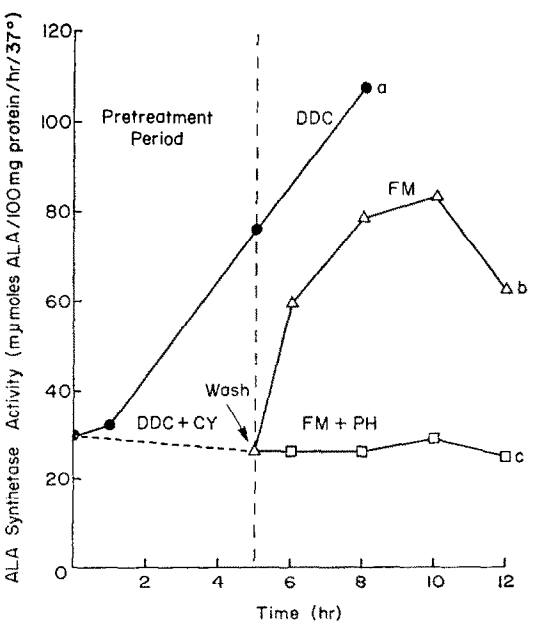

FIG. 8. Cells were exposed to 37 μM DDC and ALA synthetase activity was measured over 8 hr (curve a, ●——●). Cells were exposed to 37 μM DDC and 20 μM cycloheximide (CY) for 5 hr, washed and reincubated in fresh medium (curve b, △——△), or in fresh medium containing 10 μM protohemin (PH; curve c, □——□).

TABLE 2. PROTOHEMIN INHIBITION OF POST-TRANSCRIPTIONAL PHASE OF ALA SYNTHETASE INDUCTION*

Protohemin (μM)	% Inhibition
0	0
5	85
11	89
22	100
45	100
90	100

* Cells were incubated in medium containing 37 μM DDC and 20 μM cycloheximide for 5 hr, washed and reincubated in fresh media containing protohemin at different concentrations. Two hr after washing, ALA synthetase activity was estimated.

1, 3, 5 and 7 hr later. To the medium of the dishes in group C (ten dishes), cycloheximide (20 μM) and protohemin (20 μM) were added and the cells returned to the incubator. After 5 hr, a set of two dishes was removed for ALA synthetase assay. The remaining dishes were washed, reincubated in fresh medium and the ALA synthetase activity was assayed 1, 3, 5 and 7 hr later. The experiment was repeated using DDC (37 μM) instead of AIA. The results are shown in Figs. 9 and 10.

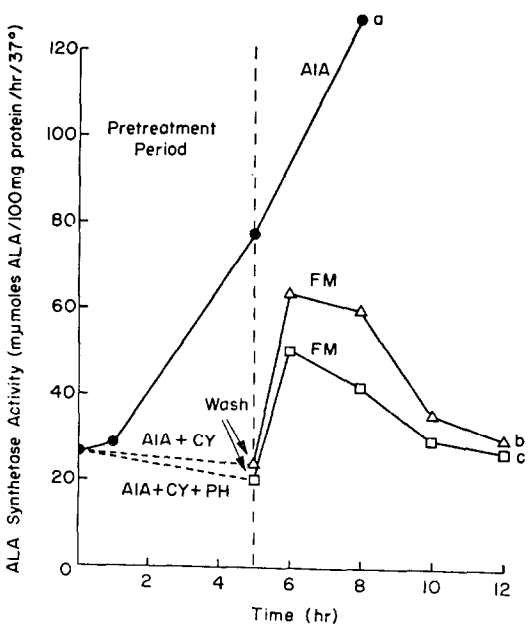

FIG. 9. Cells were exposed to 2·1 mM AIA and ALA synthetase activity was measured over 8 hr (curve a, ●——●). Cells were exposed to 2·1 mM AIA and 20 μM cycloheximide (CY) for 5 hr, washed and reincubated in fresh medium (curve b, △——△). Cells were exposed to 2·1 mM AIA, 20 μM cycloheximide and 20 μM protohemin (PH) for 5 hr, washed and reincubated in fresh medium (curve c, □——□).

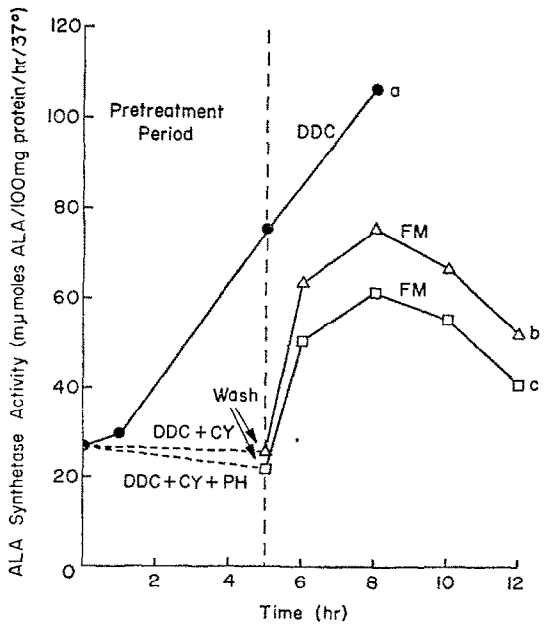

FIG. 10. Cells were exposed to 37 μM DDC and ALA synthetase activity was measured over 8 hr (curve a, ●——●). Cells were exposed to 37 μM DDC and 20 μM cycloheximide for 5 hr, washed and reincubated in fresh medium (curve b, △——△). Cells were exposed to 37 μM DDC, 20 μM cycloheximide and 20 μM protohemin (PH) for 5 hr, washed and reincubated in fresh medium (curve c, □——□).

After 24 hr of incubation, the media were removed from 24 large petri dishes. Sixteen dishes (group A) were incubated with medium containing DDC (37 μM) and cycloheximide (20 μM). Eight dishes (group B) were incubated with medium containing DDC (37 μM), cycloheximide (20 μM) and protohemin (20 μM). After 5 hr of incubation, eight dishes from group A were removed for ALA synthetase estimation. The remaining dishes in group A and the dishes in group B were washed and reincubated in fresh medium. Two hr later, the ALA synthetase activity was estimated and the results are shown in Table 3.

TABLE 3. EFFECT OF PROTOHEMIN ON THE TRANSCRIPTIONAL PHASE OF ALA SYNTHETASE INDUCTION

Drugs in pre-wash medium (μM)	Wash after 5 hr	ALA synthetase activity (nmoles ALA/100 mg protein/hr at 37°)
DDC (37) + cycloheximide (20)		30·8 ± 2·9* (5 hr)
DDC (37) + cycloheximide (20)	Yes	82·6 ± 18·9 (7 hr)
DDC (37) + cycloheximide (20) protohemin (20)	Yes	76·5 ± 4·0† (7 hr)

* Each value represents the mean of four samples ± standard deviation.
† There is no statistical difference between the two groups of values measured at 7 hr.

The effect of increasing concentrations of protohemin during the transcriptional phase of induction was examined as follows: after 24 hr of incubation, the media were removed from 16 petri dishes and the cells were incubated in fresh medium containing DDC (37 μM) and cycloheximide (20 μM). Protohemin was added to sets of two petri dishes in the following concentrations: 5, 10, 20, 40, 80 and 160 μM. The solvent for protohemin was added to four petri dishes. After 5 hr of incubation, two dishes which had received DDC, cycloheximide and the solvent for protohemin were removed and the ALA synthetase activity was assayed. The remaining dishes were washed and reincubated in fresh medium. Two hr later, ALA synthetase activity was measured (Table 4).

TABLE 4. PROTOHEMIN REPRESSION OF TRANSCRIPTION*

Protohemin (μM)	% Repression
0	0
5	48
10	50
20	54
40	72
80	78
160	100

* Cells were incubated in media containing 37 μM DDC, 20 μM cycloheximide and protohemin of indicated concentrations. After 5 hr, the cells were washed and reincubated in fresh medium. Two hr after washing, ALA synthetase activity was estimated.

Estimation of the ability of the washing process to remove protohemin from cell cultures

The media were removed from 20 large petri dishes after 24 hr of incubation. Ten dishes (group A) were incubated in fresh medium containing the solvent for protohemin and ten dishes (group B) were incubated in fresh medium containing protohemin (20 μM). After 5 hr of incubation, the cells were washed and reincubated in media containing DDC (37 μM). After 5 hr, ALA synthetase activity was estimated (Table 5).

RESULTS AND DISCUSSION

When chick embryo liver cells were incubated in medium containing AIA or DDC, ALA synthetase activity increased during the 8-hr measurement period after a delay of approximately 1 hr (curve a, Figs. 3 and 5). If cycloheximide was added with AIA or DDC, the enzyme induction was suppressed. If the cells were pretreated with AIA or DDC and cycloheximide for 5 hr, washed and reincubated in fresh medium, ALA synthetase activity increased markedly without a lag period (curve b, Figs. 3 and 5).

This increase was not prevented by the addition of actinomycin D to the fresh medium (curve c, Figs. 3 and 5), but instead was augmented. On the other hand,

TABLE 5. EFFECTIVENESS OF THE WASHING PROCESS IN REMOVING PROTOHEMIN FROM THE CELLS*

Drugs prior to washing (μM)	Drugs after washing (μM)	ALA synthetase activity (nmoles ALA/100 mg protein/hr at 37°)
Solvent	Solvent	30·8 ± 2·9
Solvent	DDC (37)	216 ± 28
Protohemin (20)	DDC (37)	181 ± 39†

* The cells were exposed to protohemin or the solvent for protohemin for 5 hr prior to washing. ALA synthetase activity was estimated 5 hr after the cells were washed.
† Indicates significant inhibition using one-tail Student's *t*-test at the 0·05 level.

cycloheximide added to fresh medium completely blocked the increase in ALA synthetase activity normally seen on reincubation (curve d, Figs. 3 and 5). Treatment of cells with cycloheximide alone prior to washing did not result in increased ALA synthetase activity after washing (curve b, Fig. 4). Therefore, the response observed after washing cells pretreated with AIA or DDC and cycloheximide was clearly caused by the porphyrin-inducing drug. If cells were exposed to actinomycin D and a porphyrin-inducing drug for 5 hr, washed and reincubated in fresh medium, no significant increase in ALA synthetase was observed (curve c, Fig. 4 and curve b, Fig. 6). This suggests that RNA synthesis was required in the pretreatment period for the increase in ALA synthetase activity after washing. When cells were pretreated with a porphyrin-inducing compound and actinomycin D for 5 hr, washed and reincubated in fresh medium containing AIA or DDC, the ALA synthetase activity increased slowly after a lag period of about 1 hr. This indicates that the inhibitory effect of actinomycin D was at least partially reversed by the washing process (curve d, Fig. 4 and curve c, Fig. 6).

The results in these experiments were best interpreted[16, 17] by assuming that RNA had accumulated prior to washing in cells treated with AIA or DDC and cycloheximide. The induction-specific RNA which had accumulated in the translation-independent period prior to washing was thought to mediate increased ALA synthetase activity after washing by translation into either this enzyme or into some other protein responsible for ALA synthetase activation. Since the addition of actinomycin D to the medium after washing cells pretreated with AIA or DDC and cycloheximide did not prevent the increase in ALA synthetase activity normally observed after washing, this second phase of induction is independent of transcription. The second phase of induction was inhibited by cycloheximide and therefore was dependent on translation.

We have interpreted the increase in ALA synthetase activity in the second phase of induction to be a result of RNA accumulation in the first phase. However, the possibility existed that residual inducing drug which had not been removed by washing was responsible for this increase in enzyme activity. Two observations suggest that this is not the case. First, when a porphyrin-inducing compound is added to cells in culture, only a very small increase in ALA synthetase activity is observed after 1 hr. On the other hand, cells exposed to ALA or DDC and cycloheximide exhibited markedly increased ALA synthetase activity 1 hr after washing (curve b, Figs. 3 and 5). The magnitude of this increased enzyme activity ruled out the possibility that it was caused by residual inducer. Second, when actinomycin D was added with AIA or DDC, the increase in ALA synthetase activity was prevented (Figs. 4 and 6). This

increased enzyme activity cannot be due to residual inducer, since actinomycin D did not prevent it (curve c, Figs. 3 and 5).

Our results have been presented in graphical form rather than in tables to facilitate interpretation. It was desirable to obtain statistical evidence for the increased ALA synthetase activity after washing cells pretreated with AIA or DDC and cycloheximide for 5 hr. For this reason, five experiments were carried out in which cells were pretreated with AIA and cycloheximide and the ALA synthetase activity was measured 2 hr after washing. Similarly, five experiments were carried out in which AIA was replaced by DDC. The results shown in Table 1 demonstrate the significance of the increase in enzyme activity observed.

Our results indicate that treatment of chick embryo liver cell cultures with DDC or AIA results in increased levels of "induction-specific" RNA for ALA synthetase. These results are consistent with one of the main points of the model proposed by Granick[1] for the control of protoheme biosynthesis, *viz.* the porphyrin-inducing compounds cause increased synthesis of m-RNA for ALA synthetase. However, our results are also consistent with the concept that the porphyrin-inducing compounds prevent the breakdown of specific RNA for ALA synthetase induction or increase the transport of induction-specific RNA from the nucleus to the cytoplasm.

Sassa and Granick,[7] using similar experimental methods but different experimental designs, obtained results indicating that DDC acts at the level of transcription and AIA acts at the level of translation to increase ALA synthetase activity. In their system, the half-life of ALA synthetase activity is measured. After the addition of cycloheximide, the half-life is 3·0 hr. If AIA or DDC is added with cycloheximide, the half-life of the enzyme remains unchanged at 3·0 hr, indicating that these drugs do not affect the rate of degradation of ALA synthetase. After the addition of actinomycin D, the half-life of the enzyme activity is 5·2 hr. If AIA is added with actinomycin D, the half-life increases to 10·6 hr. On the other hand, if DDC is added with actinomycin D, the half-life of ALA synthetase activity is 5·2 hr. It is possible to reconcile the results which we have obtained with those obtained by Sassa and Granick[7] in the following manner: DDC increases the amount of induction-specific RNA for ALA synthetase by increasing the rate of its transcription. AIA, on the other hand, might increase the level of induction-specific RNA in the cytoplasm by slowing its breakdown or facilitating its movement out of the nucleus.

The effect of protohemin on the post-transcriptional phase of induction was examined and the results are shown in Figs. 7 and 8. The normal induction of ALA synthetase activity by porphyrin-inducing compounds is shown in curve a, Figs. 7 and 8. When cells pretreated with a porphyrin-inducing compound and cycloheximide for 5 hr were washed and reincubated in fresh medium, ALA synthetase activity increased (curve b, Figs. 7 and 8). On the other hand, when cells pretreated with a porphyrin-inducing compound and cycloheximide for 5 hr were washed and reincubated with fresh medium containing protohemin, no increase in ALA synthetase activity was observed (curve c, Figs. 7 and 8). These results were not anticipated on the basis of the model for the control of protoheme biosynthesis suggested by Granick,[1] since the model limits the ability of protoheme to prevent ALA synthetase induction to repression of transcription. However, the results are consistent with the more recent observations of Sassa and Granick[7] indicating that protohemin prevents ALA synthetase induction at the level of translation.

The ability of protohemin to repress the transcriptional phase of ALA synthetase induction was examined and the results are shown in Figs. 9 and 10. The normal induction of ALA synthetase activity by porphyrin-inducing compounds is shown in curve a, Figs. 9 and 10. When cells pretreated with a porphyrin-inducing compound and cycloheximide for 5 hr were washed and reincubated in fresh medium, ALA synthetase activity increased (curve b, Figs. 9 and 10). When cells pretreated for 5 hr with medium containing a porphyrin-inducing compound, cycloheximide and protohemin were washed and reincubated in fresh medium, ALA synthetase activity increased (curve c, Figs. 9 and 10). However, this increase was not as marked as when protohemin was omitted from the pre-wash medium. It was important to determine if the inclusion of protohemin in the pre-wash medium significantly decreased the rise in ALA synthetase activity in the post-wash phase. For this reason, additional studies were carried out which showed that the inclusion of protohemin in the pre-wash medium did not significantly inhibit the increase in ALA synthetase activity in the post-wash phase (Table 3). However, it is possible that if the number of dishes in each group had been larger, then a significant inhibition might have been revealed. It is important to point out that the dose of protohemin used in this study was twice the dose required to inhibit completely the rise in ALA synthetase activity when added in the post-wash phase (Figs. 7 and 8). With higher concentrations of protohemin in the pre-wash medium, the increase in ALA synthetase activity normally observed in the post-wash phase can be prevented (Table 4).

The model proposed by Granick[1] requires that protohemin added to the medium during the transcriptional phase should prevent transcription of m-RNA for ALA synthetase and therefore prevent the increase in activity normally observed during the post-transcriptional phase. The small effect (curve c, Figs. 9 and 10) of protohemin on the transcriptional phase of induction could be a result of repression by the above mechanism. However, it may also be explained by incomplete removal of protohemin from the cells by the washing process. The protohemin remaining in the cells after washing may be sufficient to inhibit partially the post-transcriptional phase that is sensitive to small amounts of protohemin.

It was thus of importance to determine whether protohemin was completely removed by the washing process. For this reason, cells were exposed to protohemin, washed and reincubated with DDC. Induction of ALA synthetase in these cells was compared to induction in cells which had not been exposed to protohemin. The results shown in Table 5 indicate that protohemin pretreatment decreased the ability of cells to produce increased ALA synthetase activity when exposed to DDC and suggest that protohemin was not completely removed by washing.

On the basis of our results, we are unable to rule out the possibility that protohemin exerts an effect on the transcriptional process. However, low doses of protohemin do not effectively repress the transcriptional process. On the other hand, our results show that the post-transcriptional phase of induction is extremely sensitive to inhibition by low doses of protohemin (Table 2).

Recently Hayashi *et al.*[18] have shown that AIA induced ALA synthetase activity in the cytoplasm of rat liver, and the newly formed enzyme was transferred into the mitochondria by an unknown mechanism. Protohemin has been shown to inhibit this transfer[12]. Since our assay for ALA synthetase activity measures only the intramitochondrial enzyme activity, protohemin in the post-transcriptional phase may be

preventing the enzyme from being transferred from the cytoplasm into the mitochondria.

Sassa and Granick[7] have presented evidence that protohemin inhibits ALA synthetase induction at the level of translation. In their study, the half-life of ALA synthetase activity in the presence of actinomycin D alone is 5·2 hr. However, the half-life of the enzyme activity decreased to 3·6 hr in the presence of protohemin and actinomycin D. Sassa and Granick[7] have interpreted these results as suggesting that protohemin inhibits the synthesis of ALA synthetase at the translational level. However, since their assay procedure also measures the intramitochondrial enzyme, we believe these results could also be explained by protohemin preventing the movement of ALA synthetase from the cytoplasm into mitochondria.

Further experiments are required to differentiate between the mechanisms of protohemin inhibition suggested by Sassa and Granick[7] and by Hayashi *et al.*[18]

Acknowledgements—We thank Mrs. F. Taylor for her technical assistance and Mr. Kwan Oei for his assistance in developing the method for ALA synthetase estimation in cell culture.

REFERENCES

1. S. Granick, *J. biol. Chem.* **241,** 1359 (1966).
2. S. Granick and G. Urata, *J. biol. Chem.* **238,** 821 (1963).
3. J. Lascelles, in *Tetrapyrrole Biosynthesis and Its Regulation* (Ed. B. D. Davis), p. 90. W. A. Benjamin, New York (1964).
4. R. D. Levere and S. Granick, *Proc. natn. Acad. Sci. U.S.A.* **54,** 134 (1965).
5. D. W. Schneck and G. S. Marks, *Pharmacologist* **11,** 285 (1969).
6. J. M. Creighton, W. J. Racz, D. L. J. Tyrrell, D. W. Schneck and G. S. Marks, *S. Afr. J. Lab. clin. Med.* **17,** 79 (1971).
7. S. Sassa and S. Granick, *Proc. natn. Acad. Sci. U.S.A.* **67,** 517 (1970).
8. H. S. Marver, D. P. Tschudy, M. G. Perlroth and A. Collins, *J. biol. Chem.* **241,** 2803 (1966).
9. J. Lascelles, in *Tetrapyrrole Biosynthesis and Its Regulation* (Ed. B. D. Davis), p. 87. W. A. Benjamin, New York (1964).
10. P. L. Scholnick, L. E. Hammaker and H. S. Marver, *Proc. natn. Acad. Sci. U.S.A.* **63,** 65 (1969).
11. Y. Aoki, O. Wada, G. Urata, F. Takaku and K. Nakao, *Biochem. biophys. Res. Commun.* **42,** 568 (1971).
12. Y. Kurashima, N. Hayashi and G. Kikuchi, *J. Biochem.* **67,** 863 (1970).
13. D. W. Schneck, D. L. J. Tyrrell and G. S. Marks, *Biochem. Pharmac.* **20,** 2999 (1971).
14. D. Mauzerall and S. Granick, *J. biol. Chem.* **219,** 435 (1956).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
16. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **245,** 160 (1970).
17. B. Peterkofsky and G. M. Tomkins, *Proc. natn. Acad. Sci. U.S.A.* **60,** 222 (1968).
18. N. Hayashi, B. Yoda and G. Kikuchi, *Archs Biochem. Biophys.* **131,** 83 (1969).