Biochemical Pharmacology, Vol. 20, pp. 2999–3007. Pergamon Press, 1971. Printed in Great Britain

DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—III

INHIBITION OF DRUG-INDUCED PORPHYRIA BY PROTOHEMIN*

D. W. SCHNECK,† D. L. J. TYRRELL‡ and G. S. MARKS

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

(*Received* 9 *October* 1970; *accepted* 2 *April* 1971)

Abstract—Protohemin was shown to inhibit increased porphyrin synthesis induced in monolayer cultures of chick embryo liver cells by the following drugs: allylisopropylacetamide, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, griseofulvin, glutethimide, diethyl-2,3,5,6-tetramethylterephthalate and diethyl 3,3'dimethylglutarate. The nature of protohemin inhibition of porphyrin induction was studied. Chick embryo cells were incubated with allylisopropylacetamide and the porphyrin accumulation between 9 and 13 hr was measured. Porphyrin accumulation in this 9–13-hr period was depressed if actinomycin D or cycloheximide was added after 9 hr of incubation, but was not depressed by addition of protohemin.

GRANICK[1] has shown that the increased synthesis of δ-aminolevulinic acid synthetase (ALA-synthetase) induced in chick embryo liver cells by allylisopropylacetamide (AIA) can be overcome by protohemin. He has suggested that the repressor for ALA-synthetase consists of a protein aporepressor combined with protoheme, which functions as a corepressor. AIA and other porphyria-inducing drugs are thought to compete with protoheme for a site on the aporepressor so that the gene for ALA-synthetase is derepressed. The studies described in this paper were designed to test the above ideas and to extend our knowledge of the mechanism of drug-induced porphyrin biosynthesis.

Previous studies of the relationship between chemical structure and porphyria-inducing activity did not reveal an underlying common feature to which the property of porphyria induction could be ascribed.[2] For this reason, it appeared unlikely that all porphyria-inducing compounds acted at a common site, *viz.* a site on the aporepressor normally occupied by protoheme. To obtain further information on this aspect of the problem, protohemin inhibition of porphyria induction by a variety of compounds was studied. The rationale for these studies was that protohemin might inhibit porphyria induction by some but not all porphyria-inducing compounds, thus strengthening the view that these compounds act at different sites.

It is difficult to visualize a structural similarity between any porphyria-inducing drug and protohemin that would allow both compounds to act at the same site. If protohemin and porphyria-inducing drugs act at the same site, then protohemin inhibition would be competitive. If, however, protohemin acts at a site different from the porphyria-inducing drugs, then protohemin inhibition should be noncompetitive.

* This investigation was supported by a grant from the Medical Research Council, Canada.

† Medical Research Council of Canada Fellow.

‡ This work was carried out during the tenure of a Life Insurance Medical Research Fund Fellowship.

Experiments were therefore designed to study the nature of the inhibition exerted by protohemin. According to the above hypothesis,[1] protoheme by combining with an aporepressor inhibits the transcription of a segment of DNA into the messenger RNA (m-RNA) for ALA-synthetase. To test this idea, the effect of protohemin on porphyrin accumulation has been compared with that of actinomycim D and cycloheximide. It was anticipated that, if the above hypothesis were correct, then the inhibitory effect of protohemin would be found to resemble that exerted by actinomycin D rather than that exerted by cycloheximide.

EXPERIMENTAL

Culture of chick embryo liver cells on petri-dishes

The procedure of Granick[1] was used with the following modifications. The enzyme solution for preparing the cell suspension from four chick embryo livers consisted of 5 ml of 2·5% trypsin in saline (Microbiological Associates) and 5 ml magnesium-free and calcium-free Earle's solution. The cell suspension was centrifuged at low speed, the supernatant discarded and the cells resuspended in the complete medium at 37°. Fetal bovine serum (Microbiological Associates) was used for most of the experiments. In the experiments in the latter part of this study, the batches of fetal bovine serum were unsatisfactory and bovine serum (Pentex Inc., Winley-Morris Co. Ltd.) was used instead. The experiments in which bovine serum was used instead of fetal bovine serum will be indicated in the Results section.

It is worth pointing out that one of the major problems encountered in this study involved the variable porphyrin production with different sera and different batches of serum from the same company. In Table 1 some recent results are shown comparing

TABLE 1. EFFECT OF SERUM ON ALLYLISOPROPYLACETAMIDE-INDUCED PORPHYRIN BIOSYNTHESIS IN CHICK EMBRYO LIVER CELL CULTURE

Serum	Porphyrin* (μg/mg protein ± S.E.)
Fetal bovine, heat inactivated† (Microbiological Associates)	0·069 ± 0·002
Fetal bovine (Microbiological Associates)‡	0·088 ± 0·019
Fetal bovine (Grand Island Biological Co.)	0·446 ± 0·043
Bovine (Pentex Inc., Winley-Morris Co.)	0·635 ± 0·019

* Porphrin content was measured after a 20-hr period of incubation with AIA.

† Heat inactivation was carried out by heating at 56° for 30 min.

‡ Porphyrin production varied with different batches of serum from the same company. In the early experiments of this study, considerably larger quantities of porphyrin were obtained with different batches of this serum.

porphyrin production with cells grown under identical conditions but using different sera. We have concluded from this variability that a large batch of serum with which good porphyrin production is obtained should be stored frozen and used for a complete set of experiments.

Measurement of the inhibitory effect of protohemin on porphyrin biosynthesis induced by different drugs

The culture medium (4 ml) was removed from each 5-cm dia. Petri dish after 24 hr of incubation and replaced with 4 ml of fresh medium. The growth medium[1] was made up of Eagle's basal medium (100 ml), fetal bovine serum (10 ml) and glutamine (1 ml). Mycostatin, penicillin and streptomycin were added to protect against bacterial and fungal contamination.[1] Porphyria-inducing drugs were dissolved in 95% redistilled ethanol (5 μl) and added to the liver cells by means of disposable lambda pipettes. Protohemin (approximately 15 mg), prepared according to the procedure of Shemin,[3] was dissolved in 1 ml of 1 N KOH and distilled water (10 ml) was added.[4] The pH was adjusted to 7·8 with 0·1 N HCl (approximately 9 ml) on a pH meter with continuous stirring. Protohemin solutions (0·1 ml) added to the Petri dishes were prepared from this solution by diluting with 0·05 N KCl, pH 7·8. Protohemin was used rather than protoheme because of the following considerations. Burnham and Lascelles[4] have shown that protohemin and protoheme are essentially equal in their ability to inhibit ALA-synthetase. For this reason and because of the technical difficulties involved in keeping protoheme in the reduced state, protohemin has been used in subsequent studies[1,4] involving the actions of protoheme as a negative feedback inhibitor and a corepressor of ALA-synthetase. After the addition of porphyria-inducing drugs and protohemin, the petri dishes were returned to the incubator for approximately 24 hr. The porphyrin content of cells and media was determined and, after determining the protein content of cells, the results were expressed as micrograms of porphyrin per milligram of protein. The results of these experiments are shown in Table 2. Each drug was tested in duplicate or triplicate at a particular concentration. In addition, the amount of porphyrin in cultures to which ethanol (5 μl) alone was added is recorded. Cells were examined under the phase-contrast microscope (magnification, 640 diameters) after incubation with drugs. The criteria established by Granick[1] were used to assess possible harmful effects of drugs on cells. Thus, injury to cells could be recognized by rounding up of the parenchymal cells and inhibition of growth by retraction of the filmy edge of the colonies. No morphological changes were recognized in any of our experiments.

Measurement of effects of cycloheximide, actinomycin D and protohemin on AIA-induced porphyrin biosynthesis

The culture medium was removed from each Petri dish after 24 hr of incubation and replaced with 5 ml of fresh medium. AIA (1500 μg) in 95% redistilled ethanol (5μl) was added to each Petri dish and the cells were reincubated at 37°. After 9 hr, the Petri dishes were removed from the incubator and five dishes were set aside for porphyrin and protein analysis. Actinomycin D (5 μg) in 95% redistilled ethanol (5 μl) was added to each of five dishes to give a concentration of $7{\cdot}8 \times 10^{-4}$ μmoles/ml, and cycloheximide (0·28 μg) in 95% redistilled ethanol (5 μl) was added to a different

TABLE 2. INHIBITORY EFFECT OF PROTOHEMIN ON A VARIETY OF PORPHYRIA-INDUCING DRUGS*

Drugs	Concn. of porphyria-inducing drug (μmole/ml)	Porphyrin accumulation (μg/mg protein)
AIA	0·028	0·093
	2·1	0·581
	4·3	0·531
AIA + protohemin (0·024 μmole/ml)	0·028	0·025
	2·1	0·062
	4·3	0·123
DDC	0·009	0·035
	0·018	0·071
	0·036	0·190
DDC + protohemin (0·021 μmole/ml)	0·009	0·012
	0·018	0·032
	0·036	0·075
Griseofulvin	0·006	0·023
	0·029	0·072
	0·057	0·475
Griseofulvin + protohemin (0·026 μmole/ml)	0·006	0·009
	0·029	0·017
	0·057	0·022
Diethyl 2,3,5,6-tetramethylterephthalate	0·014	0·020
	0·025	0·509
	0·25	0·963
Diethyl 2,3,5,6-tetramethylterephthalate + protohemin (0·026 μmole/ml)	0·014	0·013
	0·025	0·078
	0·25	0·205
Glutethimide	0·009	0·019
	0·046	0·482
	0·46	1·040
Glutethimide + protohemin (0·026 μmole/ml)	0·009	0·010
	0·046	0·029
	0·46	0·142
Controls		0·009

* Each value represents the mean of two determinations, except in the case of DDC where values represent the mean of three determinations.

set of five dishes to give a concentration of 2×10^{-4} μmoles/ml. Protohemin, prepared as described above and in the concentration shown in Table 3, was added to each of five dishes. To a further set of five dishes no addition was made. The cells were reincubated for a further 4 hr. The porphyrin content of cells and media was determined and, after determining the protein content of cells, the results were expressed as micrograms of porphyrin per milligram of protein. The results are shown in Table 3.

TABLE 3. EFFECTS OF ACTINOMYCIN D, CYCLOHEXIMIDE AND PROTOHEMIN ON ALLYLISOPROPYLACETAMIDE-INDUCED PORPHYRIN BIOSYNTHESIS IN CHICK EMBRYO LIVER CELLS*

Period of incubation after addition of AIA (300 μg/ml) (hr)	Drug added after 9-hr incubation period	Drug concn (μmole/ml)	Porphyrin (μg/mg protein ± S.E.)†	
			Experiment I	Experiment II
9			0·097 ± 0·002	0·081 ± 0·002
13			0·295 ± 0·007	0·212 ± 0·005
13	Actinomycin D	$7{\cdot}8 \times 10^{-4}$	0·248 ± 0·010‡	0·177 ± 0·005‡
13	Cycloheximide	2×10^{-4}	0·216 ± 0·008‡	0·138 ± 0·010‡
13	Protohemin	$6{\cdot}1 \times 10^{-2}$	0·346 ± 0·010	
13	Protohemin	$12{\cdot}2 \times 10^{-2}$		0·203 ± 0·010

* Bovine serum was used in this experiment.
† Each value represents the mean ± S.E. of five determinations.
‡ Significant at 0·01 level using a one-tailed *t*-test.

Control experiments showed that the solvents used for dissolving drugs did not affect porphyrin accumulation.

Selection of appropriate concentrations of actinomycin D and cycloheximide

Since actinomycin D and cycloheximide exert toxic effects on cells, it was of importance to select the smallest concentration of actinomycin D that would completely inhibit transcription of DNA into the m-RNA for ALA-synthetase and the smallest concentration of cycloheximide that would completely inhibit translation of the m-RNA for ALA-synthetase into ALA-synthetase.

The culture medium (5 ml) was removed from each Petri dish after 24 hr of incubation and replaced with 5 ml of fresh medium. AIA (1500 μg) in 95% redistilled ethanol was added to each Petri dish. Actinomycin D dissolved in 95% redistilled ethanol (5 μl) was added to Petri dishes to give the series of concentrations shown in Table 4.

TABLE 4. ACTINOMYCIN D INHIBITION OF ALLYLISOPROPYLACETAMIDE-INDUCED PORPHYRIN BIOSYNTHESIS*

Actinomycin D (μmole/ml)	Porphyrin (μg/mg protein)†	Percentage inhibition
0	1·22	0
$3{\cdot}9 \times 10^{-6}$	1·34	0
$3{\cdot}9 \times 10^{-5}$	0·296	78
$3{\cdot}9 \times 10^{-4}$	0·022	98
$3{\cdot}9 \times 10^{-3}$	0·016	99

* Porphyrins were estimated 20 hr after addition of allylisopropylacetamide (300 μg/ml) to cells. Actinomycin D was added at the same time as allylisopropylacetamide. Bovine serum was used in this experiment.

† Mean of three determinations.

Similarly, cycloheximide dissolved in 95% redistilled ethanol was added to Petri dishes to give the concentrations shown in Table 5. The Petri dishes were returned to the incubator for 24 hr. The porphyrin content of cells and media was determined and, after determining the protein content of cells, the results were expressed as micrograms of porphyrin per milligram of protein (Tables 4 and 5).

Analysis of Petri dishes for porphyrin and protein

The procedure of Granick[1] was used without modification.

RESULTS AND DISCUSSION

The results shown in Table 2 clearly show that protohemin inhibits the action of porphyria-inducing drugs of diverse chemical structure and provides no evidence for drugs acting at different sites. This result is in accord with one hypothesis of the mechanism of protohemin action.[1] However, this experiment does not prove that this hypothesis is correct. This follows from the fact that protohemin might act at a site distinct from that of the porphyria-inducing drugs, causing an inhibition of a

TABLE 5. CYCLOHEXIMIDE INHIBITION OF ALLYLISOPROPYLACETAMIDE-INDUCED PORPHYRIN BIOSYNTHESIS*

Cycloheximide (μmole/ml)	Porphyrin (μg/mg protein)†	Percentage inhibition
0	0·719	0
1×10^{-6}	0·625	13
1×10^{-5}	0·463	36
1×10^{-4}	0·020	97
1×10^{-3}	0·014	98
1×10^{-2}	0·009	99

* Porphyrins were estimated 20 hr after addition of allylisopropylacetamide (300 μg/ml) to cells. Cycloheximide was added at the same time as allylisopropylacetamide. Bovine serum was used in this experiment.
† Mean of three determinations.

step which is necessary for all porphyria-inducing drugs, even though they may initially act at different loci.

In order to explore the nature of protohemin inhibition, the concepts of Ariens *et al.*[5] were used. The porphyria-inducing drug was considered as the agonist, protohemin as the antagonist, the aporepressor as the receptor, and porphyrin accumulation as the biological effect. If protohemin acted as a competitive inhibitor, then in the presence of protohemin the log-dose–response curve for the porphyria-inducing drug should shift in a parallel way to higher concentrations. Moreover, competitive inhibition would be demonstrable by the fact that the influence of protohemin could be overcome by an increase of the dose of porphyria-inducing drug. If protohemin acted as a noncompetitive inhibitor, a parallel shift in the dose–response curve for the porphyria-inducing drug would not be observed. Instead, a gradual decline in the maximum porphyrin accumulation would be observed with increasing doses of protohemin and the antagonistic action of a sufficiently high dose of protohemin would be insurmountable by the highest dose of porphyria-inducing drug. Granick[1] reported that the degree of inhibition exerted by protohemin depended on the concentration of protohemin relative to one fixed dose (15 μg/ml) of AIA. On this basis, he suggested that protohemin inhibition was competitive in nature. However, the same result would be anticipated if the inhibition exerted by protohemin were noncompetitive. In both competitive and noncompetitive inhibition, a reduction in the concentration of protohemin relative to a fixed concentration of porphyria-inducing drug will result in a larger porphyrin accumulation. In order to differentiate competitive from noncompetitive inhibition, it is necessary to study the effect of the antagonist over a wide range of agonist concentrations. Moreover, the effects of a range of antagonist concentrations should be explored.

We obtained a set of dose–response curves for AIA in the presence of protohemin in a concentration which was constant for each curve but which varied for the various curves. Ideally it would have been desirable to construct a complete set of curves in one experiment. However, from a practical point of view this was not possible. For this reason, in most experiments the log-dose–response curve for AIA in the absence

and presence of one given dose of protohemin was obtained. The results of this experiment indicate that protohemin at a concentration of 0·002 μmoles/ml has a slight inhibitory effect on porphyria induction (Fig. 1a), although the results are inconclusive. In Fig 1b, and d are shown the results obtained from experiments in which protohemin was present at 0·005, 0·01 and 0·015 μmole/ml respectively. The pattern of inhibition observed is compatible with that of noncompetitive inhibition, but incompatible with that of competitive inhibition as described by Ariens *et al.*[5] It is noteworthy that with very high doses of AIA, porphyrin accumulation decreased for reasons that are not readily apparent. For this reason, only a limited range of doses of AIA could be explored and the results have therefore to be treated with some reservation.

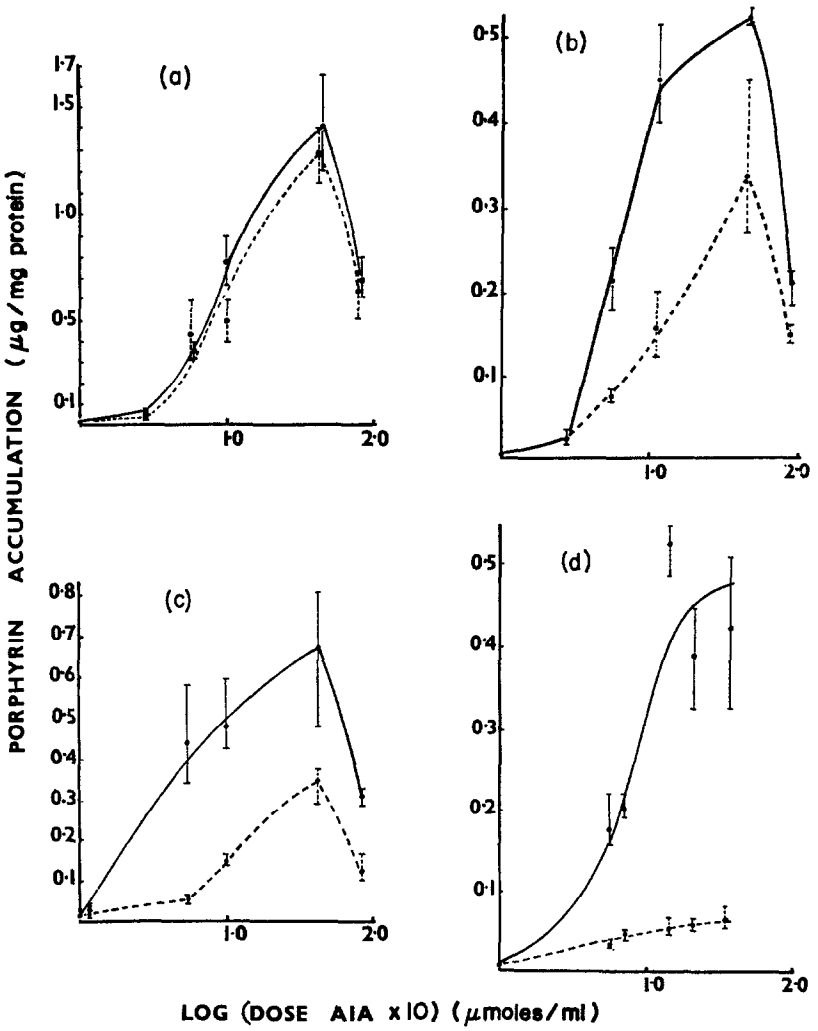

FIG. 1. Dose–response curves for AIA in the absence of protohemin (●—●) and in the presence of protohemin (●— — — —●). The protohemin concentrations were 0·002 μmole/ml (a), 0·005 μmole/ml (b), 0·01 μmole/ml (c), and 0·015 μmole/ml (d). Each point represents the mean of triplicate samples. The bars represent the range.

After addition of allylisopropylacetamide to chick embryo liver cells grown as a monolayer culture, porphyrin formation was shown to increase rapidly after a lag period of approximately 9 hr (Table 3). Cells were incubated with allylisopropylacetamide and the porphyrin accumulation between 9 and 13 hr was measured. The porphyrin accumulation in this 9–13-hr period was depressed if actinomycin D or cycloheximide was added after 9 hr of incubation (Table 3). The effect of actinomycin D was small, but statistically significant, in repeated experiments. This small depression is a problem of the experimental design which produces limitations in comparing the inhibitory effects of protohemin relative to actinomycin D. If the hypothesis of Granick[1] were correct, addition of protohemin after 9 hr of incubation should inhibit transcription of DNA into the m-RNA for ALA-synthetase and depress porphyrin accumulation in the 9–13-hr period in a manner similar to that observed with actinomycin D. This could not be demonstrated (Table 3). It is noteworthy that protohemin exerts an inhibitory effect on porphyrin biosynthesis similar to that observed with actinomycin D and cycloheximide when added at the same time as the porphyrin-inducing drug. However, when added 9 hr after the porphyrin-inducing drug, this inhibitory effect could not be demonstrated. Thus, by means of this experiment we are unable to demonstrate protohemin inhibition of transcription. It is noteworthy that Kurashima *et al.*[6] have recently suggested that protohemin inhibits transport of ALA-synthetase from the cytosol into mitochondria. Moreover, Sassa and Granick[7] have recently suggested that protohemin inhibits ALA-synthetase induction at the level of translation. Our experiment does not provide support for either of these hypotheses. If protohemin inhibits at the level of translation, it should exert an effect similar to that of cycloheximide, which is not the case (Table 3). If protohemin inhibits ALA-synthetase movement into mitochondria, formation of ALA and consequently of porphyrin would fall in the absence of the mitochondrial succinyl-CoA-generating system. This is not observed. Further studies are in progress to delineate the mode of action of protohemin.

REFERENCES

1. S. Granick, *J. biol. Chem.* **241**, 1359 (1966).
2. D. W. Schneck, W. J. Racz, G. H. Hirsch, G. L. Bubbar and G. S. Marks, *Biochem. Pharmac.* **17**, 1385 (1968).
3. D. Shemin, *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 4, p. 643. Academic Press, New York (1957).
4. B. F. Burnham and J. Lascelles, *Biochem. J.* **87**, 462 (1963).
5. E. J. Ariens, A. M. Simonis and J. M. van Rossum, *Molecular Pharmacology* (Ed. E. J. Ariens), Vol. 1, pp. 148–290. Academic Press, New York (1964).
6. Y. Kurashima, N. Hayashi and G. Kikuchi, *J. Biochem., Tokyo* **67**, 863 (1970).
7. S. Sassa and S. Granick, *Proc. natn. Acad. Sci. U.S.A.* **67**, 517 (1970).