THE OCCURRENCE OF SUBSTANCES IN HUMAN PLASMA CAPABLE OF INDUCING THE ENZYME δ-AMINOLEVULINATE SYNTHETASE IN LIVER CELLS

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Communicated June 13, 1969

Abstract.—We have demonstrated the presence, in the plasma of several patients with acute intermittent porphyria, of a substance which strongly induced the synthesis of porphyrins in chick embryo liver cells growing in primary culture. The induction response evoked by this humoral agent was in all respects similar to that elicited by drugs and hormones which are known to enhance porphyrin production by inducing the *de novo* formation of δ-aminolevulinate synthetase, the rate-limiting enzyme in this pathway. Inducing properties were not found in the plasma of normal individuals or in that from porphyric patients in remission. Significant inducing activity was, however, found in the plasma of some normal subjects ingesting drugs or contraceptive steroid mixtures.

The occurrence of a potent inducer of δ-aminolevulinate synthetase in the plasma of certain porphyric patients may have clinical significance for these genetically susceptible individuals. It will also permit chemical characterization of those humoral agents which may be related to the episodic exacerbations of this hereditary liver disease.

The mitochondrial enzyme δ-aminolevulinate (ALA)-synthetase controls the rate-limiting step in heme formation.[1] Hepatic levels of ALA-synthetase are substantially higher than normal in patients with the hereditary disease, acute intermittent porphyria,[2–5] and the enzyme is readily inducible in the liver by natural steroids, drugs, and foreign chemicals.[6–11] The ability of these agents to provoke clinical and chemical exacerbations of porphyria in man may be related to such an inducing action.

The present study was undertaken to examine the possibility that there might be a humoral substance (or substances) present in the plasma of some porphyric subjects which was capable of stimulating the formation of porphyrins by the liver. We present evidence in this report of the occurrence of such a substance in several patients with acute intermittent porphyria in relapse. The characteristics of the porphyric response produced by the agent in the plasma of these patients in liver cell culture are in all respects similar to those elicited by known drug or hormonal inducers of ALA-synthetase.

The chemical nature and origin of the inducing substance in the plasma of these porphyric subjects are not known; moreover, as we note in this report, this finding is not unique to porphyria since the plasma of some normal individuals ingesting drugs or hormones may display significant porphyrin-inducing properties as well. Nevertheless, the occurrence of a potent inducer of hepatic ALA-

synthetase in the peripheral circulation of individuals carrying the genetic lesion of porphyria is of evident clinical interest, since such a humoral agent could be related to the episodic exacerbations of the porphyric process in these susceptible subjects. Preliminary reports of these findings have been presented earlier.[12, 13]

Methods.—The tissue culture method used in this study has been described in detail.[11] The porphyrinogenic response in the liver cells was evaluated semi-quantitatively by fluorescence microscopy and quantitatively by a method to be reported later. Briefly, this method involves lyophilization of the cells and culture medium in each vial, extraction of the lyophylate with an appropriate solvent, and analysis of the porphyrins contained in the extracts in a Hitachi MPF-2A fluorescence spectrophotometer. Extracts of plasma were prepared by precipitation of plasma proteins with 9 vol of absolute ethanol, evaporation of the supernatants *in vacuo* at room temperature, and reconstitution of the residues to original plasma volumes with culture medium.

Examination of the cultured cells by fluorescence microscopy and quantitative analysis of formed porphyrins was performed at various intervals after the start of the incubation period as noted. Each culture preparation (comprising 200–300 vials) included various controls for untreated and solvent-treated cells, cells treated with allylisopropylacetamide, a chemical inducer of ALA-synthetase of known potency, etc. The quantitative values reported are based on the means of assays of 4 to 12 vials, and are representative of replicate experiments in each instance. In the case of the highly active F1 and M1 plasmas, experiments were extensively repeated in various forms (dose response, time course, etc.) over the two year period since the samples became available for study.

Results.—Control cultures: Untreated cultures synthesized trace amounts of porphyrins. These varied over a narrow range for each weekly culture preparation; the over-all variation for all control cultures in the two-year period of study was larger, however (see Table 1), due to differences in amounts of cells growing on the cover slips, variations in the bovine fetal serum of the medium used for growth, etc. Controls for specific experimental groups are indicated where relevant.

Studies with normal subjects: The normal control group contained 60 subjects, ages 21–63, with an approximately equal sex ratio. Precautions were taken to exclude individuals with a history of recent drug ingestion of any kind. Normal sera did not stimulate excess porphyrin formation when added directly to vials in volumes of 0.01–0.10 ml. A few normal plasma extracts did elicit a "trace"[11] of fluorescence at 20 hours when examined by microscopy but quantitative analysis of porphyrins in these cultures showed values (Table 1) within the limits of the untreated controls (mean ± 2 SD). In volumes of 0.2 ml per vial, normal sera and plasma extracts produced considerable damage to the cultures; 1 out of 24 plasma extracts studied nevertheless produced a small but significant increase of porphyrins.

Studies with extracts of plasma from women ingesting contraceptive steroids: Plasma extracts from several individuals receiving barbiturates or certain tranquilizing agents known to have strong inducing activity for ALA-synthetase in chick embryo liver evoked significant porphyrin induction in the cultures. Because of this observation and the known ability of natural steroids to induce this enzyme,[8–11] a group of 13 women receiving contraceptive steroid mixtures for a minimum of 3 months was studied. In volumes of 0.1 ml/vial, a number of plasma extracts of these women showed substantial inducing activity by fluorescence microscopy as well as by quantitative spectrofluorometry (Table 1). It is

TABLE 1. *Effects of extracts of human plasma on porphyrin synthesis in chick embryo liver cell culture.*

Group	Plasma extract added per vial (ml)	Coproporphyrin formed 10^{-9} M/vial Range	Mean	Comment
Over-all control (untreated cultures)	—	2.6–10.3	5.9	Over-all control for all experimental groups over a two-year period. Intra-experimental controls vary through a much narrower range*
Normal subjects	0.1	3.4–12.5	6.5	No significant activity*
Porphyric subjects (in remission)	0.1	3.1–12.2	6.5	No significant activity*
Women receiving contraceptive steroids	0.1	5.2–42.6	16.1	8 out of 13 cases showed significant inducing activity compared with specific intra-experimental control groups*

* See *Results*.

not clear yet whether such inducing ability derives directly from trace amounts of the synthetic hormones contained in plasma or is due to a secondary product or biological effect of the drug in these subjects.

Studies with porphyric patients in remission: The sera of 28 patients with various forms of hepatic porphyria in remission were added directly to the cultures in volumes of 0.01–0.10 ml/vial, and none induced porphyrin synthesis in excess of untreated control cultures. Plasma extracts of these patients were also inactive. At 0.2 ml/vial, both extracts and sera causes serious cell damage but, nevertheless, two extracts added in this volume showed inducing activity slightly but significantly in excess of controls.

Studies with porphyric patients in relapse: The sera and plasma extracts of 5 patients with acute intermittent porphyria in relapse were studied. *Subjects F1* and *M1* were a 30-year-old female and a 51-year-old male, respectively, whose clinical exacerbations apparently began spontaneously, four to six weeks prior to referral here. Blood samples were obtained for study during the acute illness, convalescence (three months later) and remission (one year later). *Subject M2* was a 23-year-old male narcotic addict who died 1 week after admission to a local municipal hospital. His attack of porphyria may have been precipitated one week earlier by barbiturate ingestion; however, barbiturates could not be identified in his blood. Blood samples were obtained for study on each of the six days prior to death. *Subject F2* was a 28-year-old female prone to severe symptomatic attacks of porphyria in relation to her menses. Sera and plasma extracts were studied on five occasions including one relapse associated with transient neurological defects. The porphyrinogenic activity displayed by a plasma extract obtained during the latter episode is reported here. *Subject F3* was a 28-year-old female whose porphyric relapse had begun four days earlier. The attack quickly responded to glucose treatment.[14] The blood sample sent to us for study was obtained shortly after therapy had been initiated.

The results of typical experiments with the plasma extracts of these five patients are shown in Table 2. The *F1* and *M1* sera induced strongly even when added directly to the cultures. The *F1* and *M1* plasma extracts showed potent

TABLE 2. *Induction of porphyrin synthesis in liver cell culture by plasma extracts of 5 patients with acute intermittent porphyria during exacerbation of the disease.*

Patient	Plasma extract added per vial (ml)	Coproporphyrin formed in culture treated with patients extract 10^{-9} M/vial Range	±	SE	Coproporphyrin formed in intra-experimental control group cultures 10^{-9} M/vial Range	±	SE
Subject F1	0.1	99.0 (60.8 — 182.5)*		5.7	8.3		0.8
Subject F2	0.1	54.2		4.3	3.5		0.2
Subject F3	0.1	5.9		0.3	3.5		0.2
Subject M1	0.1	75.1 (51.0 — 129.5)*		2.5	7.6		1.1
Subject M2	0.1	16.5		0.5	8.6		0.8

* Range of amounts of porphyrins induced by 0.1 ml of these extracts over a series of experiments on the acute phase sera carried out over a 2-year period. Remission plasmas showed inducing activities within the normal range.

inducing properties as well. The results of the specific experiments recorded in Table 2 approximate the mean amounts (*ca.* 7–20 times normal) of excess porphyrins whose formation was stimulated by these extracts in repeated studies carried out over a two-year period. The full range of values for these experiments are shown in parentheses. The value shown for the *M2* extract was typical for each of the six daily plasma samples obtained before death. The *F2* value is the highest of the assays of five different plasma extracts studied over six months; of these five plasma samples, three had significant porphyrin-inducing activity. The *F3* plasma extract in four series of experiments has shown activity which was slightly higher than that of its specific experimental controls, as typified by the value in Table 2; however, these values remain within the overall control group range (Table 1).

Evidence for an ALA-synthetase inducing agent in the F1 and M1 plasma extracts: 1. *Induction response:* The induction of excess porphyrins with both extracts was detectable by microscopy with volumes as small as 0.01 ml/vial and was proportional to volume (as determined quantitatively) up to 0.1 ml (*F1*) and 0.2 ml (*M1*). Larger volumes damaged cultures and diminished porphyrinogenesis.

The pattern of induction response evoked by the extracts was typical of that elicited by drug and hormone inducers of ALA-synthetase, i.e., there was a prolonged latent period of six to eight hours followed by a rapid synthesis and accumulation of porphyrins in the cells, reaching a plateau at 20 to 24 hours. Examples of the responses produced by the extracts, and a comparison with the time-course of conversion of added ALA to porphyrins, are shown in Figure 1. Porphyrinogenesis was not observed by fluorescence microscopy before four to six hours with the extracts; at this time, however, added ALA over a wide range of concentrations had been extensively transformed to porphyrins.

The fluorescence emission spectrum of the porphyrins induced by one of the extracts (*F1*) is depicted in Figure 2, together with those of (*a*) the extract alone which contained no porphyrins, (*b*) the untreated cultures, which contained very small amounts of porphyrins, and (*c*) a standard solution of coproporphyrin III. The induction of large amounts of coproporphyrin formation by the ex-

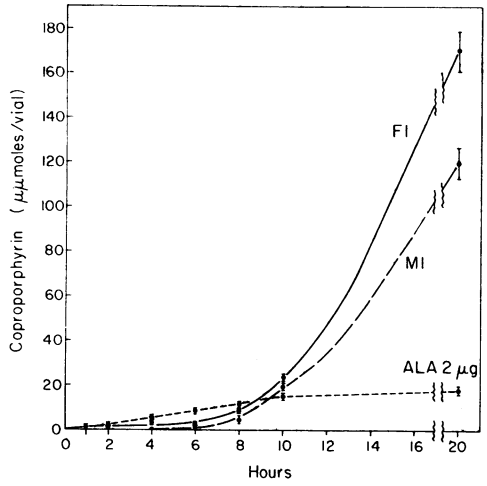

FIG. 1.—Kinetics of the porphyrin-induction response elicited by the *F1* and *M1* plasma extracts compared with those of the conversion in the cultures of added ALA to porphyrins. The amounts of porphyrins shown are those in excess of the amounts found in control (untreated) cultures at each time period.

tract is consistent with the response described earlier for drugs, although synthesis of small amounts of other porphyrins probably occurs as well.[11]

2. *Effects of inhibitors of nucleic acid and protein synthesis:* The maximum (*F1*) or near-maximum (*M1*) porphyrinogenic response produced by both extracts was markedly inhibited by small amounts of agents which block nucleic acid and protein synthesis. The results of typical experiments with actinomycin D and puromycin are depicted in Figure 3. The concentrations of actinomycin D and puromycin shown in Figure 3 did not significantly alter the conversion of added ALA to porphyrins; larger concentrations of these agents blocked the

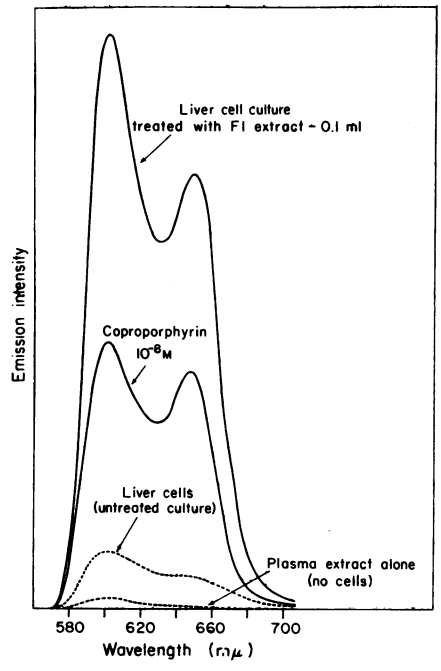

FIG. 2.—The fluorescence emission spectrum of porphyrins produced by the *F1* plasma extract in liver cell cultures compared with a coproporphyrin standard and other controls.

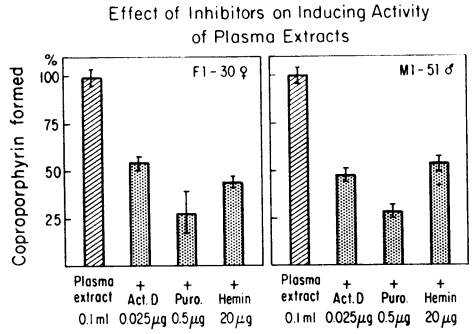

FIG. 3.—Inhibitory effects of actinomycin D, puromycin, and hemin on the porphyrin-induction response produced by the *F1* and *M1* plasma extracts. Comparison made against the amounts of porphyrins formed (100%) by the extracts alone.

inducing effects of the extracts entirely, but they then showed some inhibition of the conversion of ALA to porphyrins. This presumably reflects the toxic action of larger concentrations of the antibiotics on the liver cells.

3. *Effects of heme and uridine diphosphate glucuronic acid:* Heme which has been postulated to act as a co-repressor in the regulation of ALA-synthetase formation[11] blocks drug and hormone induction of the enzyme in liver cells.[9, 11] Heme also significantly inhibited porphyrinogenesis by the plasma extracts (Fig. 3), in amounts which did not alter the conversion of ALA to porphyrins. Amounts of uridine diphosphate glucuronic acid (UDPGA) in the range of 250 μg/vial also substantially inhibited the inducing effect of the extracts, without influencing proportionally the latter transformation. We have previously speculated that the ability of UDPGA to block steroid induction of ALA-synthetase may result in part from enhanced inactivation of the inducer[9]; however, other explanations of this effect may be formulated and the proximate mode of action of this agent, like that of glucose,[14] in inhibiting porphyrinogenesis remains obscure.

Preformed porphyrins or their precursors contained in the plasma extracts have been excluded as contributing significantly to the induction responses evoked by these extracts. The potent *F1* and *M1* extracts did not contain detectable porphyrins (see also Fig. 2). Porphobilinogen in very high concentration (2 μg/vial) was transformed to porphyrins in amounts which were less than 10 per cent of those induced by 0.1 ml of these extracts in parallel experiments. Moreover, this conversion occurred spontaneously even in the absence of liver cells in the culture vials; no similar transformation with the plasma extracts was observed.

ALA enters the liver cells readily and is transformed to porphyrins in the culture. This conversion occurs rapidly, evidence of porphyrinogenesis being detectable by microscopy within one to two hours after addition of ALA in amounts greater than 1 μg/ml medium in the vials. A typical time course of this transformation to porphyrins is shown in Figure 2 with 2 μg ALA/vial. Even with this large amount of ALA (equivalent to a serum ALA concentration of 2000 μg/100 ml in relation to the amounts of extracts added) porphyrinogenesis was only a fraction of that elicited by the *F1* and *M1* extracts. Direct estimation of ALA in the plasma extracts by the Haeger-Aronsen technique[15] or a related method being developed here showed concentrations of this aminoketone of

less than 50 μg per cent. These are within the range of "serum ALA" concentrations reported earlier in man[15, 16] and are insufficient, by a factor of 20 to 50 times, to yield porphyrins in the cultures in the amounts elicited by the extracts (Fig. 1).

The rate or degree of conversion of added ALA to porphyrins in the cultures over the time period one to eight hours was not influenced by concomitant addition of plasma extracts. Two potent endotoxin preparations prepared from *E. coli* did not elicit porphyrinogenesis in the cultures when added in amounts ranging from 1 to 100 μg/vial. Studies on the physical and chemical characterization of the inducing substance in human plasma are in progress.

Discussion.—This study demonstrates that there is in the peripheral circulation of certain individuals a substance (or substances) which is capable of stimulating porphyrin synthesis in chick embryo liver cells growing in primary culture. This substance has the properties of an agent which induces the *de novo* formation of ALA-synthetase, the rate-limiting enzyme in the porphyrin-heme pathway.

The plasmas of normal subjects, selected to exclude those receiving medications of any kind, did not contain an inducing agent in a concentration sufficient to stimulate excess porphyrin synthesis in our tissue culture preparation. The possibility that larger volumes of such plasmas might be shown to contain some inducing activity for ALA-synthetase, however, cannot be excluded (see *Results*); the toxic effects of larger volumes of plasma extracts on the cultures prevented a rigorous examination of this question.

The occurrence of inducing activity in normal human plasma can, on the other hand, be readily demonstrated following the ingestion of drugs and hormones known to induce ALA-synthetase in the liver. In the case of women receiving contraceptive steroids, quite potent inducing action may be displayed by plasma extracts despite the relatively small amounts of hormones ingested. It is not clear whether such biological activity reflects the direct action of trace amounts of hormones contained in the plasmas, or whether it is due to other secondary effects or products of the hormones *in vivo*.

The plasmas of patients with hepatic porphyria in remission did not display significant inducing activity; however, the same consideration relating to normal subjects, concerning the possible occurrence of small amounts of activity in larger volumes of plasma, may also apply to these individuals. Patients with porphyria excrete variable and sometimes quite large amounts of porphyrins and precursors in their urine even during remission. It is evident, therefore, that, as is the case with clinical symptoms in these subjects, a direct correlation between the inducing activity in plasma and the urinary output of intermediates in heme synthesis cannot be made.

The plasmas which contained the most potent porphyrin-inducing activities in this study were derived from several patients with acute intermittent porphyria during acute relapses of their disease. The characteristics of the induction response produced by the acute phase plasmas were in all respects similar to those characterizing the action of drugs and hormones known to be potent inducers of ALA-synthetase in the liver. In the two porphyric patients who were studied

over a two-year period of time, the inducing activity in plasma was noted to disappear when the disease was in remission; and in the young woman who was subject to recurrent symptomatic attacks of porphyria in relation to some of her menstrual periods, inducing activity in plasma of considerable potency was displayed on several but not all occasions when she was ill.

The specific chemical nature and origin of the ALA-synthetase inducing substance in the plasmas of the acute porphyric subjects studied remain speculative. It could derive from endogenous sources in these individuals and may include certain of the natural steroids which have been shown to have potent porphyrinogenic activity.[8, 9, 17] The inducing substance could also originate exogenously through an unrecognized exposure of the patients to active chemicals derived from the environment via dietary or other means. In both these respects, the further possibility must also be considered that defective disposal of inducing chemicals or abnormal metabolism of ordinarily nonporphyrinogenic endogenous and exogenous substances to active metabolites could account for the presence of an inducing agent in the plasma of these patients. Whatever its derivation, the occurrence in certain porphyric subjects of a humoral agent capable of strongly inducing ALA-synthetase in liver cells may have clinical significance since such an agent could be related to some of the episodic exacerbations of the porphyric process in these genetically susceptible individuals.

The authors are indebted to Drs. C. J. Watson, G. Ludwig, N. Kosower, and A. Redeker for providing blood samples from their patients; and to Drs. J. Hirsch and Z. Cohen for gifts of the endotoxin preparations.

* Supported in part by USPHS grants H.D.-04313, AM09838, and General Clinical Research Center grant FR-00102.

[1] Granick, S., and G. Urata, *J. Biol. Chem.*, **238**, 821 (1963).

[2] Tschudy, D. P., M. G. Perlroth, H. S. Marver, A. Collins, G. Hunter, Jr., and M. Rechcigl, Jr., these Proceedings, **53**, 841 (1965).

[3] Nakao, K., O. Wada, T. Kitamura, K. Uono, and G. Urata, *Nature*, **210**, 838 (1966).

[4] Watson, C. J., *New England J. Med.*, **263**, 1205 (1960).

[5] Schmid, R., "The Porphyrias," in *The Metabolic Basis of Inherited Disease*, eds. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson (New York: McGraw-Hill, 1966), pp. 813–870.

[6] De Matteis, F., *Biochim. Biophys. Acta*, **82**, 641 (1962).

[7] Marver, H. S., A. Collins, D. P. Tschudy, and M. Rechcigl, Jr., *J. Biol. Chem.*, **241**, 4323 (1966).

[8] Granick, S., and A. Kappas, *J. Biol. Chem.*, **242**, 4587 (1967).

[9] Kappas, A., and S. Granick, *J. Biol. Chem.*, **243**, 346 (1968).

[10] Kappas, A., C. S. Song, R. D. Levere, R. A. Sachson, and S. Granick, these Proceedings, **61**, 509 (1968).

[11] Granick, S., *J. Biol. Chem.*, **241**, 1359 (1966).

[12] Kappas, A., C. S. Song, S. Sassa, and R. D. Levere, *Clin. Res.*, **17**, 464 (1969).

[13] Kappas, A., C. S. Song, S. Sassa, and R. D. Levere, *Trans. Ass. Am. Physicians*, **82**, in press (1969).

[14] Tschudy, D. P., F. H. Welland, A. Collins, and G. Hunter, Jr., *Metabolism*, **13**, 396 (1964).

[15] Haeger-Aronsen, B., *Scand. J. Clin. Lab. Invest.*, **12**, Suppl. 47 (1960).

[16] Chisolm, J. J., *Analyt. Biochem.*, **22**, 54 (1968).

[17] Goldberg, A., M. R. Moore, A. D. Beattie, P. E. Hall, J. McCallum, and J. K. Grant, *Lancet*, **1**, 115 (1969).