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cAMP-DEPENDENT INDUCTION OF δ-AMINOLEVULINATE SYNTHASE IN ISOLATED EMBRYONIC CHICK LIVER CELLS

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SUMMARY: Isolated liver cells were prepared from 17-day old chick embryos and incubated in Eagle's basal medium. Induction of δ-aminolevulinate synthase activity occurred immediately upon addition of allylisopropylacetamide and was totally dependent on the presence of Bt_2cAMP (or cAMP) during the first 6 h of incubation. Under optimal inducing conditions in the presence of desferrioxamine mesylate and hormones, δ-aminolevulinate synthase induction occurred at rates comparable with those observed *in ovo*. The isolated liver cells provide a convenient experimental system for studying the effect of porphyrogenic drugs on porphyrin metabolism.

INTRODUCTION

Overt attacks of the disease acute intermittent porphyria are accompanied by a gross elevation of hepatic ALA synthase activity (1) and several drugs when administered to patients are known to precipitate or exacerbate such attacks (2). Granick (2) pioneered studies on the effects of such porphyrogenic drugs and developed a procedure for growing primary cultures of chick embryo liver cells. When exposed to these drugs, such cells accumulated porphyrins and ALA synthase activity. Increase in the latter involved *de novo* synthesis of the enzyme (3). More recently, procedures have been developed (3-6) for obtaining induction of ALA synthase in chick embryo liver cells cultured in chemically defined serum-free media thereby eliminating the variability observed (7) with different batches of fetal calf serum. However, no clearly defined requirement for Bt_2cAMP in the induction of either porphyrin or ALA synthase by drugs has been reported using cultured chick embryo cells.

The abbreviations used are:- ALA, δ-aminolevulinic acid; AIA, allylisopropylacetamide; DES, desferrioxamine mesylate; MIX, 3-isobutyl-1-methylxanthine; Bt_2cAMP, dibutyryl cyclic AMP.

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In this paper we report that isolated chick embryo liver cell suspensions can be used directly for studies of ALA synthase induction, without the need for culturing. Induction by AIA of protoporphyrin synthesis and ALA synthase activity is dependent on the presence of Bt_2cAMP.

EXPERIMENTAL PROCEDURES

Materials.

AIA was a gift from Hoffman-La Roche Inc., Nutley, N.J. and desferrioxamine mesylate a gift from CIBA-Geigy Australia Ltd. Crystalline bovine insulin, hydrocortisone, hyaluronidase, collagenase, Bt_2cAMP, 3-isobutyl-1-methylxanthine, cycloheximide, cordycepin and heparin were obtained from Sigma Chemical Co.

Preparation of Isolated Chick Embryonic Liver Cell Suspensions.

The procedure used was modified from that of Sassa and Kappas (4) and Edwards and Elliott (8). Livers of ten 17-day old chick embryos were removed and minced as thinly as possible with a stainless steel razor blade in 20 ml of Ca^{2+} and Mg^{2+}-free Hank's salt solution, pH 7.4, containing 0.5 mM ethylene glycol bis (β-aminoethyl ether) N,N'-tetracetic acid and 150 units of heparin. After 15 min at room temperature, the sedimented liver slices were transferred to 20 ml of Hank's salt solution, pH 7.4, containing 4 mM $CaCl_2$, 0.05% hyaluronidase and 0.05% collagenase and were shaken at 37° in a gyrotory water bath shaker (150 cycles/min) for 50 min. During this shaking the liver slices were intermittently drawn up and down in a large bore pasteur pipette. The cell suspension was centrifuged at 150 x g for 3 min and the supernatant was discarded. In order to hemolyse contaminating red blood cells, 20 ml of sterile NH_4/HCO_3/Tris solution (0.185 M NH_4Cl, 0.017 M Tris base, and 0.01 M $KHCO_3$, pH 7.4) was added to the sedimented cells. The suspension was again pipetted up and down a few times, allowed to stand for 20 min at room temperature, and then centrifuged at 175 x g for 3 min. The yield was about 1 ml of packed cells. The cell pellet was suspended in 100 ml Eagle's basal medium (Commonwealth Serum Laboratories, Melbourne) containing glutamine (2 mM) and supplemented with streptomycin (10 mg) and penicillin G (10,000 units).

Incubation of Cell Suspensions.

Aliquots (50 ml) of the liver cell suspension, containing approximately 2-3 x 10^7 cells per ml were transferred to 300 ml capacity flasks. The flasks were continuously gassed by humified CO_2:air (5:95, v/v). The pH was maintained at pH 7.4 by regulating the gas flow rate and the suspension shaken at 37° in a gyrotory water bath (150 cycles per min). As judged by phase contrast microscopy the cells were essentially all dissociated from each other and retained their morphology for at least 20 h. About 97% of the cells were viable as measured by tryphan blue and fluorescein diacetate staining. Viability fell by about 1% per h under the incubation conditions.

Inducers or other compounds were added to cell suspensions in a small volume of either 0.9% NaCl solution or redistilled ethanol. Appropriate controls showed that the ethanol concentration used had no effect on induction and cell viability. In all cases a similar volume of saline or ethanol was added to control incubations.

Determination of Protoporphyrin in Cells.

After incubation for appropriate times, samples of the cell suspension were centrifuged at 500 x g for 5 min and the cell pellet rinsed with 1 ml of buffer (0.15 M NaCl, 0.02 M Tris, pH 7.4). Protoporphyrin in the cells was extracted with 1 ml of a mixture of 1 N $HClO_4$ and methanol (1:1, v/v) and quantitated using a Perkin Elmer Model 203 Fluorescence Spectrophotometer equipped with a red light sensitive photomultiplier R 446-UR as described by Granick *et al.* (3). The instrument was calibrated daily with a rhodamin B standard (40 ng/ml) in ethylene glycol. For determination of protein content, the $HClO_4$/methanol insoluble material was rinsed with 5 ml of ethanol, then with 5 ml of ethanol-ether (3:1, by volume), dried and protein determined by the method of Lowry (9).

Determination of ALA Synthase Activity in Cells.

At the end of the appropriate incubation period, aliquots (25 ml) of the cell suspension were centrifuged at 500 x g for 5 min. The cell pellet was homogenized in 0.6 ml of assay medium which also contained 5 mM EDTA in a Potter Elvhjem homogenizer with a teflon pestle and ALA synthase activity was determined by the colorimetric procedure of Sinclair and Granick (10).

Measurement of Protein Synthesis in Cells.

An aliquot (25 ml) of cell suspension was incubated with 25 μCi of L-[4,5-^{3}H]leucine (specific radioactivity 105 Ci/mol) added at zero time, and duplicate 1 ml samples were taken for the determination of radioactivity incorporated into total protein using the method of Ballard *et al.* (11).

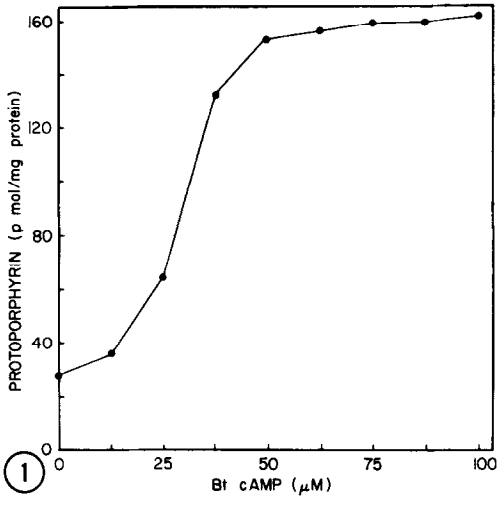
RESULTS

ALA synthase is the rate limiting step in porphyrin biosynthesis and in initial studies the accumulation of protoporphyrin by isolated chick embryo cells was used as an indirect measure of the amount of ALA synthase. Table 1 shows that isolated cells synthesized 29.1 pmol of protoporphyrin/mg cellular protein over 10 h in the presence of AIA and desferrioxamine mesylate (DES), an inhibitor of ferrochelatase, compared with 6.0 pmol/mg protein in their absence. This level of protoporphyrin was stimulated a further four fold by the addition of Bt_2cAMP (50 μM). Optimal induction of protoporphyrin accumulation by cells was observed when both insulin and hydrocortisone were present (Table 1). Individually, these hormones had only a marginal effect on protoporphyrin synthesis but together they significantly increased production.

The effect of increasing concentrations of Bt_2cAMP on protoporphyrin accumulation by isolated cells is shown in Fig. 1. Maximum synthesis occurred at 50 μM Bt_2cAMP. In the presence of this concentration of Bt_2cAMP,

Table I: Isolated liver cells were incubated in Eagle's basal medium containing glutamine. All additions were made at 0 h and protoporphyrin was determined spectrofluorometrically 10 h after incubation. Values are the mean of two to four separate determinations.

	Additions (μg/ml)	Protoporphyrin
		pmol/mg protein/10 h
1.	No addition	6.0
2.	DES(500) alone	16.4
3.	DES(500) + AIA(500)	29.1
4.	DES(500) + AIA(500) + Bt_2cAMP(50 μM)	117.4
5.	DES(500) + AIA(500) + Bt_2cAMP(50 μM) + insulin(1.0)	103.2
6.	DES(500) + AIA(500) + Bt_2cAMP(50 μM) + hydrocortisone(0.05)	127.1
7.	DES(500) + AIA(500) + Bt_2cAMP(50 μM) + insulin(1.0) + hydrocortisone(0.05)	146.7
8.	As (7) DES omitted	115.1
9.	As (7) AIA omitted	16.8



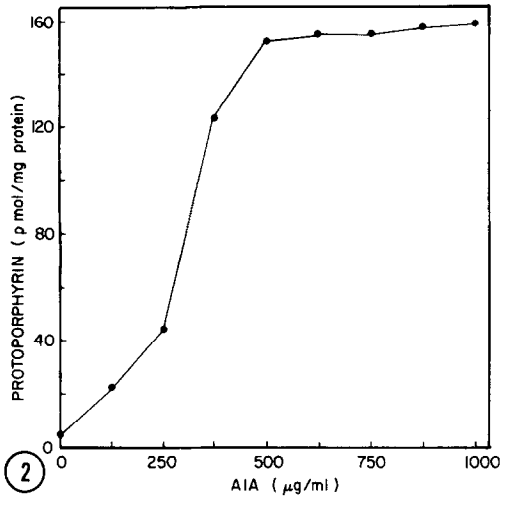
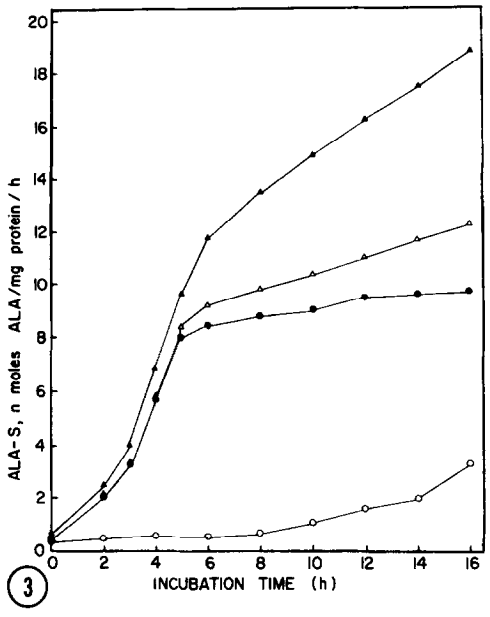


Fig. 1: Protoporphyrin accumulation in response to increasing concentrations of Bt_2cAMP in the chick embryo liver cell suspensions. Liver cells were prepared and incubated in Eagle's basal medium containing AIA (500 μg/ml), insulin (1.0 μg/ml), hydrocortisone (0.05 μg/ml) and DES (500 μg/ml). Protoporphyrin in the cells was determined spectrofluorometrically 10 h after incubation. Values are the mean of four determinations.

Fig. 2: Protoporphyrin accumulation in response to increasing concentrations of AIA in the chick embryo liver cell suspensions. Liver cells were incubated in Eagle's basal medium containing Bt_2cAMP (50 μM), insulin (1.0 μg/ml), hydrocortisone (0.05 μg/ml) and DES (500 μg/ml). Protoporphyrin in the cells was estimated spectrofluorometrically 10 h after incubation. Values are the mean of two determinations.



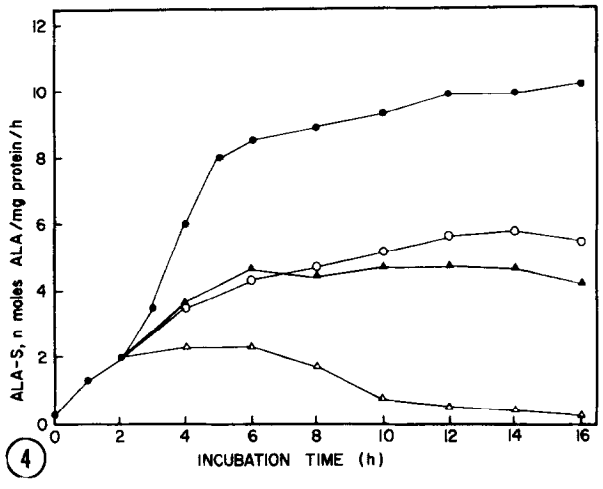


Fig. 3: Effect of Bt_2cAMP on ALA synthase synthesis. The liver cells were incubated in Eagle's basal media containing AIA (500 µg/ml), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml), DES (500 µg/ml) and ALA synthase was estimated in cell homogenates prepared after various time intervals as described in the experimental procedures. Further additions were: no Bt_2-cAMP (○); 50 µM Bt_2cAMP at 0 h (●); 50 µM Bt_2cAMP at 0 h and extra 50 µM cAMP at 4 h (△); 50 µM Bt_2cAMP with MIX (30 µg/ml) at 0 h (▲).

Fig. 4: Effect of inhibitors of RNA and protein synthesis on induction of ALA synthase. The liver cells were incubated in Eagle's basal media containing AIA (500 µg/ml), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml), DES (500 µg/ml), Bt_2cAMP (50 µM) and ALA synthase was estimated in cell homogenates prepared after various times as described in Experimental Procedures. Further additons after 2 h incubation were: cycloheximide (100 µg/ml) (△); actinomycin D (2.5 µg/ml) (▲); cordycepin (12.5 µg/ml) (○) and no addition (●).

protoporphyrin accumulation was dependent on AIA with the optimal level of the latter being about 500 µg/ml (Fig. 2).

ALA synthase induction by isolated cells was investigated using the conditions optimal for protoporphyrin accumulation. In the presence of Bt_2-cAMP, an immediate induction of ALA synthase by AIA was seen and continued for about 6 h; with control suspensions incubated in the absence of Bt_2cAMP there was almost no increase in the level of ALA synthase for 8 h (Fig. 3). Separate experiments showed that cAMP (50 µM) was nearly as effective as Bt_2-cAMP (results not shown) in permitting this induction by AIA. Addition of

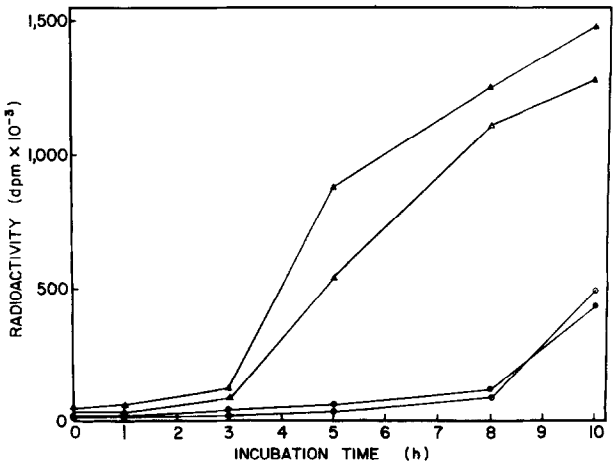


Fig. 5: Effect of Bt_2cAMP, hydrocortisone and insulin on general protein synthesis. The liver cells were incubated in Eagle's basal media supplemented with AIA (500 µg/ml), DES (500 µg/ml) and the incorporation of L-[4,5-^{3}H]leucine into total cellular protein after various intervals was determined as described in the Experimental Procedures. Further additions at 0 h were: hydrocortisone (0.05 µg/ml) (○); insulin (1.0 µg/ml) (●); Bt_2cAMP (50 µM) (△); hydrocortisone (0.05 µg/ml), insulin (1.0 µg/ml) and Bt_2cAMP (50 µM) together (▲).

3-isobutyl-1-methylxanthine (MIX), an inhibitor of cyclic nucleotide phosphodiesterase activity and hence cAMP breakdown, caused a further increase in the accumulation of ALA synthase with the level reaching about 19 nmol ALA/mg cellular protein/h measured after 16 h (Fig. 3). When additional Bt_2cAMP was added at 4 h (in the absence of MIX), only a small increase was seen (Fig. 3).

Fig. 4 shows that ALA synthase induction by AIA apparently requires continuing RNA and protein synthesis since induction was prevented by either cordycepin, actinomycin D or cycloheximide.

Although induction of ALA synthase by cells under optimal inducing conditions occurred immediately (Fig. 3), the incorporation of L-[4,5-^{3}H]leucine into total cellular protein showed a pronounced lag for 3 h (Fig. 5). In the absence of Bt_2cAMP, this lag increased to about 8 h in the presence of either hydrocortisone or insulin (Fig. 5).

DISCUSSION

This paper establishes that freshly prepared isolated chick embryo liver cells can be used directly for studying the effect of AIA (and other

porphyrogenic drugs) on the porphyrin biosynthetic pathway. The system has advantages in that it is simple, it eliminates the need for tissue culture and perhaps most important it permits pipetting of cell suspensions; since the cells from mahy livers can be pooled this eliminates variability in cell number or sensitivity which may occur in tissue culture experiments.

Induction of protoporphyrin synthesis (and hence ALA synthase) by isolated cells was increased by, but not dependent upon, the presence of both insulin and hydrocortisone. By comparison, in chick embryo tissue culture studies, cells inoculated in the presence of these hormones remained poorly responsive to AIA for about 24 h (3,4). When hormones were added to cells cultivated for 24 h in serum-free media lacking hormones, it took a further 24 h before maximal induction of ALA synthase could be obtained (4).

Perhaps the most important finding in the present work is that induction by AIA of ALA synthase is completely dependent on the presence of Bt_2cAMP over the first 6 h of incubation. (Some induction begins after about 8 h in the absence of Bt_2cAMP but only to a small extent.) The induction of ALA synthase in the presence of Bt_2cAMP is immediate and reaches a maximum level of about 9 nmol of ALA/mg protein/h. This value doubled in the presence of MIX, an inhibitor of cAMP breakdown. These values compare favourably with the maximum value of 15 nmol ALA/mg of protein/h observed by Sassa and Kappas (4) for chick embryo culture cells and with that found in *in ovo* (12).

However, although ALA synthase induction by AIA in the presence of Bt_2cAMP was immediate, the isolated cells showed a lag of about 3 h in total cellular protein synthesis, as measured by the incorporation of L-[4,5-^{3}H]leucine into trichloroacetic acid precipitable material. The reason for this lag is not known but it is extended in the absence of Bt_2cAMP.

The observed dependence on Bt_2cAMP for ALA synthase induction by isolated chick embryo liver cells correlates well with the previous report from this laboratory (8) of a similar requirement in adult rat liver isolated cell suspensions. Previous workers (2-6) using chick embryo liver cultures have

found no clearly defined requirement for Bt_2cAMP in induction of ALA synthase and protoporphyrin synthesis.

It seems that isolated cells and not cultured cells have a requirement for cAMP before they can be induced to synthesize ALA synthase. Perhaps, isolated cells cannot make enough of the compound, whereas cultured cells can or perhaps isolated cells have a peculiar requirement for cAMP.

REFERENCES

1. Schmid, R., in J.B. Stanbury, J.B. Wyngarden and D.S. Fredrickson (Editors), *Metabolic Basis of Inherited Disease*, McGraw-Hill Book Company Inc., New York, 1960, p.939.
2. Granick, S. (1966). *J. Biol. Chem. 241*, 1359-1375.
3. Granick, S., Sinclair, P., Sassa, S. and Grieninger, G. (1975). *J. Biol. Chem. 250*, 9215-9225.
4. Sassa, S. and Kappas, A. (1977). *J. Biol. Chem. 252*, 2428-2436.
5. Morgan, R.O., Stephens, J.K., Fischer, P.W.F. and Marks, S. (1977). *Biochem. Pharmac. 26*, 1389-1394.
6. Tomita, Y., Ohashi, A. and Kikuchi, G. (1976). *Tohoku J. Exp. Med. 120*, 239-250.
7. Honn, K.V., Singley, J.A. and Chavin, W. (1975). *Proc. Soc. Exp. Biol. Med. 149*, 344-347.
8. Edwards, A.M. and Elliott, W.H. (1974). *J. Biol. Chem. 249*, 851-855.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). *J. Biol. Chem. 193*, 265-275.
10. Sinclair, P. and Granick, S. (1977). *Anal. Biochem. 79*, 380-393.
11. Ballard, F.J., Hopgood, M.F., Reshef, L., Tilghman, S. and Hanson, R.W. (1974). *Biochem. J. 144*, 199-207.
12. Whiting, M.J. and Granick, S. (1976). *J. Biol. Chem. 251*, 1340-1346.