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DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—XVI. EFFECTS OF HYDROCORTISONE, ADENOSINE 3′:5′-CYCLIC MONOPHOSPHORIC ACID, ITS DIBUTYRYL DERIVATIVE AND A PHOSPHODIESTERASE INHIBITOR ON ALLYLISOPROPYLACETAMIDE-INDUCED PORPHYRIN BIOSYNTHESIS IN CHICK EMBRYO LIVER CELLS MAINTAINED IN SERUM-FREE WAYMOUTH MEDIUM*

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Abstract—Chick embryo liver cells were maintained in serum-free Waymouth medium containing insulin and thyroxine. Hydrocortisone (20.6 μM) enhanced allylisopropylacetamide (AIA)-induced δ-aminolevulinic acid (ALA) synthetase activity, as it was previously demonstrated to do in rat liver. It is likely that previous failures to observe a hydrocortisone effect in hepatic cell culture were due to the presence of hydrocortisone in serum added to the medium. Adenosine 3′:5′-cyclic monophosphoric acid (cAMP; 0.3 mM) and its dibutyryl derivative (61 μM), although not essential for AIA induction of porphyrin biosynthesis, were observed to enhance the accumulation of porphyrins. In contrast, cAMP has been reported to be essential for AIA induction of ALA-synthetase activity in rat liver cell suspensions. The cyclic nucleotide phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (135 μM), was shown to exert a similar effect on AIA-induced porphyrin biosynthesis as cAMP. It was concluded that cAMP enhances but does not function as a "second messenger" in AIA-induced porphyrin biosynthesis.

Hydrocortisone has been shown to exert a "permissive" effect on allylisopropylacetamide (AIA) induction of hepatic δ-aminolevulinic acid (ALA) synthetase in the rat [1, 2] and in perfused rat liver [3]. In view of the above findings, Edwards and Elliott [4] were surprised at the absence of an effect of hydrocortisone (10 μM) on AIA-induced ALA-synthetase activity in suspensions of isolated rat liver cells and suggested that the apparent absence of a glucocorticoid requirement might have been due to the presence of glucocorticoids in the serum component of their medium. Granick *et al.* [5] were similarly unable to demonstrate a hydrocortisone requirement for AIA-induced porphyrin biosynthesis in a culture of chick embryo liver cells. In the experiments of Granick *et al.* [5], cells were maintained for the first day in a serum-containing medium and on the second day, when AIA was added, in a serum-free medium. Therefore, it is possible that the failure to demonstrate a hydrocortisone effect with hydrocortisone (5.5 to 27.6 μM) was due to the uptake of hydrocortisone by the cells on the first day of incubation. Goodridge [6] developed a procedure for maintaining chick embryo liver cells in a serum-free Waymouth medium and used the method to study lipid biosynthesis. We have shown that, when insulin and thyroxine were added to chick embryo liver cells maintained in serum-free Waymouth medium, drug-induced porphyrin biosynthesis occurred in a manner comparable to that observed in serum-containing medium [7, 8]. In this procedure, the use of serum is avoided throughout, and the effects of hormones may be studied without interference from unknown hormone components of serum. The first objective of the present study was to use this serum-free culture system to determine whether hydrocortisone exerts a permissive effect on AIA-induced porphyrin biosynthesis and ALA-synthetase activity in chick embryo liver cells as it does in rat liver.

Neither adenosine 3′:5′-cyclic monophosphoric acid (cAMP) nor its dibutyryl derivative produced an elevation of hepatic ALA-synthetase activity in 18-day-old chick embryos [9]. However, dibutyryl cAMP was shown to enhance the AIA-induced hepatic ALA-synthetase activity by 10–15 per cent [10]. The effects of cAMP and/or its dibutyryl derivative on AIA-induced hepatic ALA-synthetase have been studied in the rat. Some workers report no effect [11], while others report an inhibition of AIA induction [12]. Kim and Kikuchi [13, 14] have demonstrated that the result (inhibition, enhancement, or no observable effect) depends upon the time of administration of the cyclic nucleotide after AIA administra-

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tion and the time of measurement of ALA-synthetase activity. Edwards and Elliott [4] who used rat liver cells in suspension showed that AIA-induced ALA-synthetase activity was dependent on the presence in the medium of cAMP (1.2 mM) or its dibutyryl derivative (50 μM). The second objective of this study was to determine whether cAMP and its dibutyryl derivative would enhance AIA-induced ALA-synthetase activity in chick embryo liver cells maintained in serum-free Waymouth medium (containing insulin and thyroxine) as it does in rat liver cell suspensions. Inhibitors of cyclic nucleotide phosphodiesterase would be expected to produce elevated levels of cAMP. Thus, one would anticipate that the effect on AIA-induced porphyrin biosynthesis of a cyclic nucleotide phosphodiesterase inhibitor would be similar to that of cAMP or its dibutyryl derivative; our third objective was to determine if this was so.

MATERIALS AND METHODS

Chemicals. Hydrocortisone-21-sodium succinate, adenosine 3′:5′-cyclic monophosphoric acid (cAMP), N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (dibutyryl cAMP), and L-thyroxine sodium pentahydrate (T_4) were purchased from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co., Inc. Allylisopropylacetamide (AIA) was a gift from Hoffmann-La Roche (Montreal), and Waymouth MD 705/1 medium was obtained from Grand Island Biological Co.

Cell culture technique. The details of the cell culture technique have been described previously [6–8]. The cells were maintained in either 60-mm diameter disposable plastic Petri dishes (Falcon Plastics), containing 5 ml of Waymouth MD 705/1 medium supplemented with 60 mg penicillin G, 100 mg streptomycin sulfate, 1.0 mg insulin and 1.0 mg T_4/litre, or 100-mm disposable plastic Petri dishes (Fisher Scientific Co.), containing 15 ml of the medium. After an initial incubation period of 24 hr, the medium was discarded, replaced with fresh medium, and re-incubated for a further 24 hr. The T_4 (1.0 mg) was dissolved in 0.01 N NaOH (2 ml) prior to addition to 1 litre of medium. Hydrocortisone, cAMP and dibutyryl cAMP were dissolved in medium and added as required. 3-Isobutyl-1-methylxanthine and AIA were dissolved in 10 μl of 95% ethanol for addition to the 60-mm dishes and in 30 μl of 95% ethanol for addition to the 100-mm dishes. T_4, hydrocortisone, cAMP and dibutyryl cAMP, when used, were added in both incubation periods, while AIA and 3-isobutyl-1-methylxanthine were added only during the second 24-hr incubation period.

Assay of porphyrins and ALA-synthetase. Porphyrins and ALA-synthetase were assayed as previously described [15, 16]. To assay ALA-synthetase, cells were grown in 100-mm dishes and the cells from two dishes were pooled in order to obtain sufficient material for the assay. To assay porphyrins, cells were grown in 60-mm dishes.

RESULTS AND DISCUSSION

The response of chick embryo liver cells, maintained in Waymouth MD 705/1 medium, containing insulin and T_4, to increasing doses of AIA is shown in Fig. 1. The addition of hydrocortisone (10 μg/ml; 20.6 μM) to the medium produced a significant enhancement of AIA-induced porphyrin biosynthesis at lower doses of AIA (2–7 μg/ml) but not at higher doses (10–100 μg/ml); hydrocortisone (10 μg/ml) did not have a significant effect on porphyrin biosynthesis in the absence of AIA. To determine whether the hydrocortisone enhancement of porphyrin biosynthesis was due to enhanced ALA-synthetase activity, the effect of hydrocortisone (10 μg/ml) on AIA-induced ALA-synthetase activity was determined. The results in Fig. 2 demonstrate a significant enhancement by hydrocortisone of ALA-synthetase activity 6 and 12 hr after the administration of AIA (5 μg/ml). The

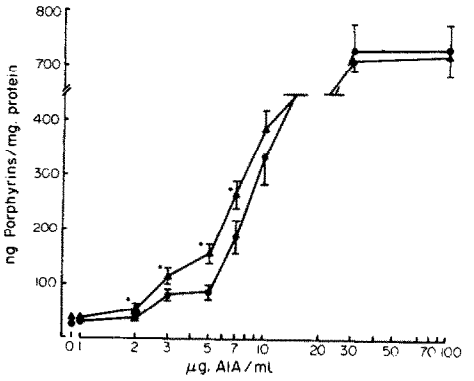

Fig. 1. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of hydrocortisone (10 μg/ml). The dots indicate a significant different ($P < 0.05$) in porphyrin accumulation between cells maintained in the presence of hydrocortisone and those maintained in its absence at the same dose of AIA. Each point represents the mean of four determinations ± S. E. M.

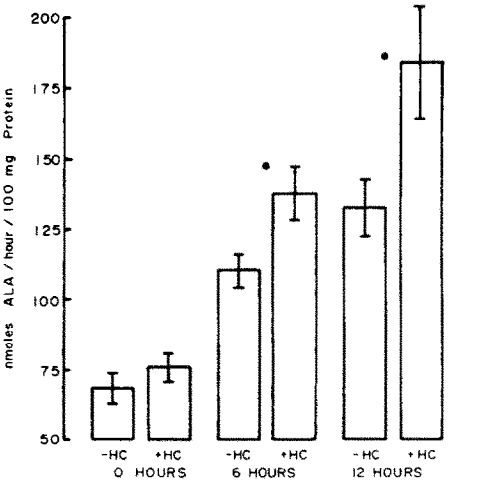

Fig. 2. ALA-synthetase activity in the presence and absence of hydrocortisone (10 μg/ml) at 0, 6 and 12 hr after the administration of AIA (5 μg/ml). The dots indicate a significant difference ($P < 0.05$) between the ALA-synthetase activity in cells maintained in the presence of hydrocortisone (HC) and those maintained in its absence. The bars represent the mean of nine determinations ± S. E. M.

concentration of hydrocortisone used (10 μg/ml) is higher than the physiological concentration of corticosteroids in 17-day-old chick embryo plasma which has been reported to be 80 ng/ml [17]. Corticosteroids, however, are rapidly metabolized and in man a synthetic rate of 25 mg/day is necessary to maintain a plasma level of 100 ng/ml [18]. Thus, assuming that the plasma volume of a 70-kg man is 2.8 litres, 8.9 μg corticosteroid is synthesized/ml of plasma over a 24-hr period. This figure is similar to the amount of hydrocortisone (10 μg/ml) to which our chick embryo liver cells were exposed over a 24-hr period.

A hormone is thought to play a permissive role in a process when a stimulus produces a specific effect in the presence of that hormone but not in its absence [19]. Marver *et al.* [1] concluded that the hydrocortisone exerted a permissive effect on AIA induction of hepatic ALA-synthetase in the rat and this conclusion was apparently supported by the results of Matsuoka *et al.* [2] and Bock *et al.* [3]. The data in Fig. 2 show that AIA can produce an increase in ALA-synthetase activity in the absence of hydrocortisone and, therefore, it is clear that in chick embryo liver cells hydrocortisone does not have a permissive effect on ALA-synthetase induction by AIA. However, it is also clear that at low doses of AIA (2–7 μg/ml) hydrocortisone (10 μg/ml) does enhance AIA-induced porphyrin biosynthesis and ALA-synthetase activity. A careful evaluation of the original data of Marver *et al.* [1], Matsuoka *et al.* [2] and Bock *et al.* [3] reveals that hydrocortisone does not exert a permissive effect on AIA-induced ALA-synthetase activity if the strict definition of the term is adhered to. This follows from the fact that AIA is able to produce a small but significant induction of ALA-synthetase even in the absence of hydrocortisone. Thus, it would appear that these authors have shown an enhancing effect of hydrocortisone rather than a permissive effect. This enhancing effect of hydrocortisone was not observed by Edwards and Elliott [4] in rat liver cells nor by Granick *et al.* [5] in chick embryo liver cells. A possible reason for the difference between our results and those of Granick *et al.* [5] was the presence of T_4 in our medium and the absence of T_4 in the serum-free medium used by Granick *et al.* [5]. For this reason, it was decided to explore the role of hydrocortisone on AIA-induced porphyrin biosynthesis in the presence and absence of insulin and T_4. The results in Fig. 3A demonstrate that none of the hormones (T_4, insulin and hydrocortisone) or combinations of these hormones produced a significant elevation of porphyrin biosynthesis. In the presence of AIA (10 μg/ml), hydrocortisone (10 μg/ml) produced an enhancement of AIA-induced porphyrin biosynthesis when added to a medium containing insulin and T_4 (Fig. 3C-2 and 3C-6). If T_4 were omitted from the medium, hydrocortisone (10 μg/ml) did not produce an enhancement of AIA-induced porphyrin biosynthesis (Fig. 3C-4 and 3C-8; 3B-4 and 3B-8). Similarly, the enhancing effect of hydrocortisone was not observed when insulin was omitted from the medium (Fig. 3C-3 and 3C-7; 3B-3 and 3B-7). Since the hydrocortisone enhancement could not be observed in the absence of T_4, the failure of Granick *et al.* [5] to detect the hydrocortisone effect is understandable. In summary, our results show that hydrocortisone (~ 10 μg/ml; 20.6 μM) when added to media containing insulin and T_4 will enhance porphyrin biosynthesis induced by low doses of AIA. Since the requirements for the enhancement are relatively specific with respect to concentration of hydrocortisone, dose of AIA, and concentration of other hormones in the medium, it is not surprising that this effect could not be observed previously in cell culture. A question arising from our study is the following: Why is the enhancing effect of hydrocortisone observed at low doses of AIA but not at higher doses? In order to consider this question, it is necessary to review modern ideas on the mechanism of hydrocortisone and other glucocorticoid actions on the liver [20]. The glucocorticoids, which exert an anabolic effect on liver, are believed to bind to specific cytoplasmic receptor proteins, and the complex moves to the nucleus where it increases the rate of transcription of some genes. This results in the formation of several mRNAs which are transported to the cytoplasm for translation to protein on ribosomes. The proteins synthesized are in the main those involved in gluconeogenesis. AIA has been shown to stimulate enhanced synthesis of ALA-synthetase [21], and this is believed to result from enhanced formation of the mRNA for ALA-synthetase [18, 22]. A possible explanation for the enhancement of ALA-synthetase activity at low doses of AIA is that the stimulation of protein synthesis produced by hydrocortisone provides one or other factors (e.g. tRNA, initiation factors, ribosomes) which are limiting in the synthesis of ALA-synthetase. At high doses of AIA these factors would presumably be synthesized in optimum amounts in the absence of hydrocortisone. Alternatively, it is possible that the

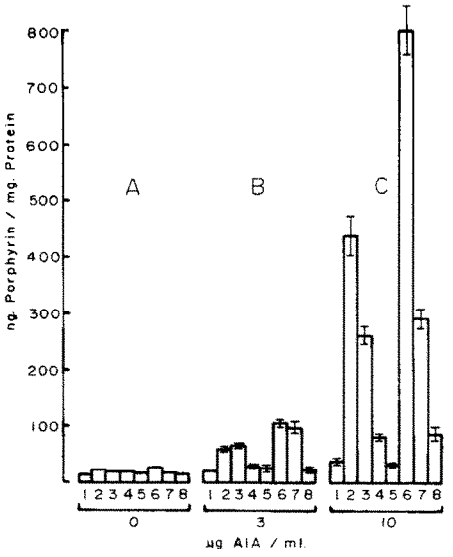

Fig. 3. Porphyrin accumulation in response to the presence of various hormones after administration of 10 μl ethanol (A) or AIA dissolved in ethanol (3 μg (B) or 10 μg (C)/ml of medium). Key: (1) no hormones; (2) T_4 (1 μg/ml) and insulin (1 μg/ml); (3) T_4 (1 μg/ml); (4) insulin (1 μg/ml); (5) hydrocortisone (10 μg/ml); (6) T_4 (1 μg/ml), insulin (1 μg/ml) and hydrocortisone (10 μg/ml); (7) T_4 (1 μg/ml) and hydrocortisone (10 μg/ml); and (8) insulin (1 μg/ml) and hydrocortisone (10 μg/ml). Each bar represents the mean of four determinations ± S. E. M.

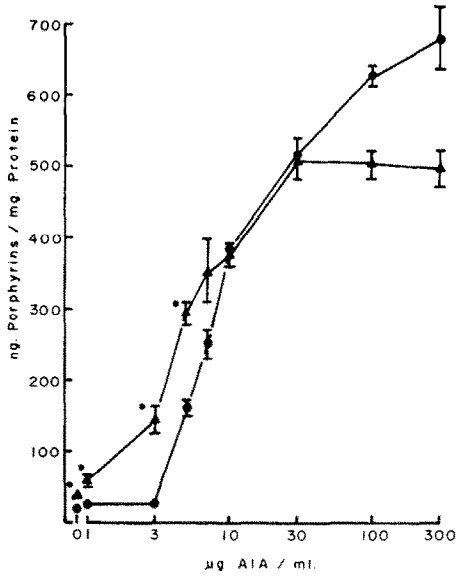

Fig. 4. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of dibutyryl cAMP (30 μg/ml). The dots indicate a significant difference ($P < 0.05$) in porphyrin accumulation between cells maintained in the presence of dibutyryl cAMP and those maintained in its absence at the same dose of AIA. Each point represents the mean of four determinations ± S. E. M.

enhancing effect of hydrocortisone on AIA-induced porphyrin biosynthesis is exerted on the cell membrane or an intracellular membrane [20] and results from the alteration of the transport of either an important metabolite or an intermediate in heme biosynthesis or of AIA. Thus, by utilizing a serum-free medium we have shown that hydrocortisone exerts an enhancing effect on AIA-induced ALA-synthetase activity in chick embryo liver as it has been demonstrated to do in rat liver [1–3]. Other workers utilizing serum-containing media were unable to demonstrate this effect in hepatic cell culture [4, 5] presumably because of the presence of hydrocortisone in the serum.

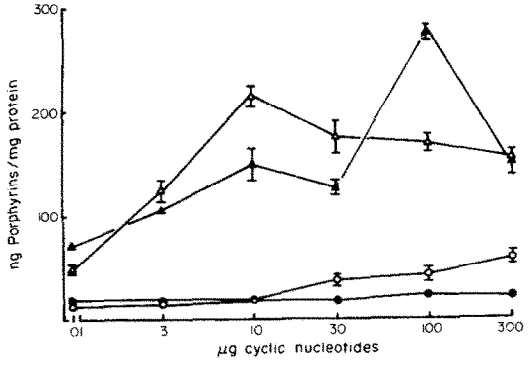

Fig. 5. Porphyrin accumulation in response to increasing doses of cyclic nucleotides in the presence and absence of AIA. Key: dibutyryl cAMP alone (○), dibutyryl cAMP plus 3 μg AIA/ml (△), cAMP alone (●), and cAMP plus 3 μg AIA/ml (▲). Each point represents the mean of four determinations ± S. E. M.

The response of chick embryo liver cells, maintained in Waymouth MD 705/1 medium, containing insulin and T_4, to increasing doses of AIA is shown in Fig. 4. The addition of dibutyryl cAMP (30 μg/ml) to the medium (Fig. 4) resulted in a significant enhancement of AIA-induced porphyrin biosynthesis at lower doses of AIA (1–5 μg/ml), no effect at intermediate doses (10–30 μg/ml) and a decrease at higher doses (100–300 μg/ml). The response of chick embryo liver cells to increasing doses of cAMP and dibutyryl cAMP are shown in Fig. 5. In the absence of AIA, large doses of dibutyryl cAMP produce a small elevation of porphyrin levels, while cAMP has no significant effect. In the presence of AIA (3 μg/ml), dibutyryl cAMP and cAMP produce a marked enhancement of porphyrin accumulation.

It is of interest that the maximum effect occurs with 100 μg/ml of cAMP which is ten times the concentration needed for a maximum effect with dibutyryl cAMP. This is possibly due to a slower rate of degradation of the dibutyryl derivative coupled with greater ease of penetrability into the hepatocytes [23]. When AIA (100 μg/ml) was used, cAMP had no significant enhancing effect on porphyrin biosynthesis. The minimum concentration of cAMP (8.5 nmoles/ml) needed for an enhancement of porphyrin accumulation (Fig. 5) is higher than the level reported [24] in chicken blood (0.23 nmole/ml). The cyclic nucleotide, however, is rapidly metabolized by hepatocytes [25] and, consequently, it is likely that actual concentrations in the cell culture system are close to physiological levels.

Edwards and Elliott [4] using rat liver cell suspensions showed that AIA-induced ALA-synthetase activity was dependent on the presence in the medium of cAMP or its dibutyryl derivative. In contrast, our results show that, while cAMP and its dibutyryl derivative will enhance AIA-induced porphyrin biosynthesis in cultures of chick embryo liver cells at low doses of AIA, the induction produced by AIA is not dependent upon the presence of either compound. Investigators, who studied the effect of cAMP or its dibutyryl derivative on AIA-induced hepatic ALA-synthetase activity in the rat, reported either no effect, an inhibition of activity, or enhancement of activity [11–14]. It is possible that the discrepancy between the results *in vivo* and *in vitro* is complicated by the effects of cAMP on organs other than the liver resulting in secondary effects on the liver. Thus, hepatic cell culture appears to be a useful system for the study of a possible role of cAMP on drug-induced hepatic porphyrin biosynthesis. We have demonstrated that cAMP and its dibutyryl derivative enhanced AIA-induced porphyrin biosynthesis (and, therefore, presumably ALA-synthetase activity) in chick embryo liver cells as they do in rat liver cell suspensions. However, in contrast to the results reported in rat liver cells [4], the cyclic nucleotide is not essential for AIA-induced porphyrin biosynthesis.

Our next experiments were carried out with a potent inhibitor of cyclic nucleotide phosphodiesterases, viz. 3-isobutyl-1-methylxanthine (MIX) [26]. At low doses (1–30 μg/ml) 3-isobutyl-1-methylxanthine was devoid of porphyrin-inducing activity, while porphyrin levels were elevated four times over control

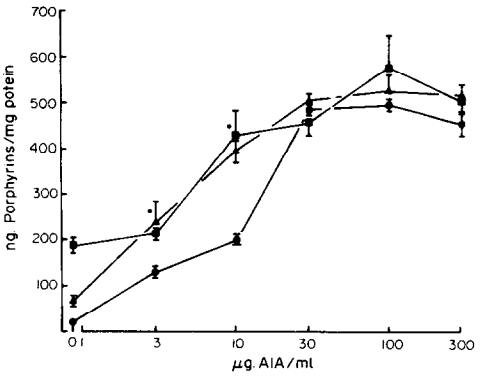

Fig. 6. Porphyrin accumulation in response to increasing doses of AIA in the presence [30 μg/ml (▲), 100 μg/ml (■)] and absence (●) of 3-isobutyl-1-methylxanthine. The dots indicate a significant difference ($P < 0.05$) in porphyrin accumulation between cells maintained in the presence of 3-isobutyl-1-methylxanthine (30 μg/ml) and those maintained in its absence at the same dose of AIA. Each point represents the mean of four determinations ± S. E. M.

values (11 ± 0.7 ng porphyrin/mg of protein) at 100 μg/ml and twenty times at 300 μg/ml. A dose of 30 μg/ml was selected for use in subsequent experiments. The response of chick embryo liver cells to increasing doses of AIA is shown in Fig. 6. The addition of 3-isobutyl-1-methylxanthine (30 μg/ml) to the medium resulted in a significant enhancement of AIA-induced porphyrin biosynthesis at lower doses (3 and 10 μg/ml of AIA) but not at higher doses (30–300 μg/ml of AIA). Increasing the dose of MIX from 30 to 100 μg/ml did not result in a further enhancement of activity. MIX would be expected to produce elevated levels of cAMP and, therefore, one would anticipate that the effect of MIX on AIA-induced porphyrin biosynthesis would be similar to that of cAMP or its dibutyryl derivative. A comparison of Figs. 4 and 6 shows that dibutyryl cAMP and MIX produce an enhancement of AIA-induced porphyrin biosynthesis at low doses but not at high doses. Thus, we have demonstrated that the effect on AIA-induced porphyrin biosynthesis of a cyclic nucleotide phosphodiesterase inhibitor (MIX) is similar to that of cAMP and its dibutyryl derivative. It must be kept in mind that methylxanthines possess a variety of additional pharmacological properties [27], and it is possible that the effect of MIX may be related to one or several other pharmacological properties.

According to current concepts [28], a protein phosphokinase participates in all well-documented examples of cAMP action in animal cells. It is believed that the interaction of cAMP with an inactive protein phosphokinase results in activation of the protein. The response of a particular cell to a rise in cAMP concentration is apparently determined by the nature of the protein phosphokinase substrates. Therefore, in order to elucidate the mechanism of the cAMP effect we have observed, it will be necessary to study the activation of protein phosphokinase and the substrates of the enzyme in chick embryo liver cells. cAMP has been shown to induce tyrosine aminotransferase and phsophoenolpyruvate carboxykinase in rat liver cells [29]. Wicks [29] has concluded that cAMP has the capacity to influence protein synthesis at both the transcriptional and translational levels in eukaryotic cells, in both a positive and negative manner. Our findings differ from those of others [29] in that cAMP enhances AIA-induced porphyrin biosynthesis but is ineffective as an enzyme inducer when used alone. Despite this difference, the basic mechanism by which cAMP exerts its effect on porphyrin biosynthesis and, therefore, presumably on ALA-synthetase induction might be related to the mechanism by which cAMP causes enzyme induction [29]. Sutherland [30] has listed four criteria for determining whether or not cAMP functions as a second messenger to a hormone. The same criteria would be applicable to determine whether cAMP functioned as a second messenger to a drug. One of these criteria is the demonstration that the drug effect is mimicked by exogenous cAMP or one of its acyl derivatives. Since the effect of AIA is not mimicked by either cAMP or its dibutyryl derivative, it is clear that cAMP is not a second messenger of AIA in our system.

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