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THE ROLE OF CYCLIC-AMP IN THE REGULATION OF STEROID METABOLISM IN ISOLATED RAT HEPATOCYTES

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Abstract—*In vivo* experiments have shown that hepatic steroid metabolism is under hormonal control but the intracellular mechanism of action of the hormones has been little studied. One pathway of hormone action known to be active in the liver is the alteration of cyclic-AMP levels. To investigate the role played by cyclic-AMP in the control of hepatic steroid metabolism, we raised intracellular cyclic-AMP by a number of methods and studied the resultant changes in the metabolism of androst-4-ene-3,17-dione after various periods of time. Results indicate that cyclic-AMP levels are raised to their maximal levels (2–20-fold stimulation) 2–20 min following the additions but that the effects on steroid metabolism were seen later and depended on the initial change in cyclic-AMP levels. At lower rises in cyclic-AMP (up to 5-fold stimulation), a marked inhibition of steroid metabolism is seen at ½–1 hr post-treatment, whereas at higher stimulations of cyclic-AMP (>10-fold stimulation), a significant stimulation of steroid metabolism is observed at later time periods (after 1 hr), sometimes following a slight inhibition at ½ hr. This indicates that acute rises in intracellular cyclic-AMP produced by hormonal stimulation may play a role in regulating steroid metabolism in the rat liver both in an inhibitory and a stimulatory direction.

The liver is largely responsible for the metabolism of both endogenous substances (e.g. steroid hormones) and exogenous compounds (xenobiotics). The same enzymes are thought to be responsible for the biotransformation of both steroids and xenobiotics [1]. It has been found that the activities of the various enzymes are not constant but are regulated by the endocrine system and a number of hormones are known to affect the enzyme activities *in vivo* (e.g. growth hormone [2], insulin and glucagon [3], thyroxine [4] and adrenaline [5]). The intracellular mechanism by which these hormones regulate the activities of the enzymes has, however, been little studied. It is known that many of these hormones affect the turnover of cyclic-AMP within the cell with adrenaline and glucagon activating adenylate cyclase and leading to a marked increase in intracellular cyclic-AMP [6] and thyroxine elevating cyclic-AMP by potentiating the action of circulating catecholamines [7, 8]. These three hormones all decrease hepatic steroid and drug metabolism. Insulin conversely decreases intracellular cyclic-AMP content [9–11] and stimulates the activity of many steroid biotransforming enzymes [12]. It has also been seen that *in vivo* treatment with a cyclic-AMP analogue, dibutyryl-c-AMP can inhibit the subsequent metabolism of aminopyrine by a liver microsomal preparation [13] but it should be remembered in this study that an indirect effect of the treatment cannot be ruled out. The weight of evidence suggests, however, that raised cyclic-AMP levels in the liver are correlated to decreased drug/steroid metabolism.

Pyerin *et al.* [14] have recently shown that the catalytic subunit of cyclic-AMP-dependent protein kinase can phosphorylate cytochrome P-450, the terminal oxidase of the enzyme system responsible for much of the drug and steroid metabolism in the liver. Phosphorylation of cytochrome P-450 resulted in its conversion to cytochrome P-420 [15] with a concomitant decrease in enzyme activity [16, 17].

It could be postulated, therefore, that cyclic-AMP, by activating the appropriate protein kinase, causes phosphorylation of hepatic drug and steroid metabolising enzymes and, thus, decreases their activity. Hormonal control of this metabolic pathway could then be via the association of the hormone and its receptor leading to changes in the activity of adenylate cyclase.

There are a number of problems, however, with extrapolating the above data to what happens in the liver *in vivo*: (1) are the hormones injected into the animal acting directly on the liver? and (2) is the *in vitro* phosphorylation of cytochrome P-450 a physiological occurrence?

In order to investigate the feasibility of the above postulate and attempt to overcome the problems discussed above, we have performed the present study. We have investigated the effects of various methods of raising intracellular cyclic-AMP in isolated rat hepatocytes on the metabolism of a steroid substrate, androst-4-ene-3,17-dione. The timing and percentage rise in cyclic-AMP has been correlated to altered steroid metabolism.

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MATERIALS AND METHODS

(i) *Chemicals*. Collagenase and bovine serum albumin were obtained from BCL (Lewes, East Sussex, U.K.) and adrenaline, 3-isobutyl-1-methyl- xanthine (IBMX), 8-bromo-cyclic-AMP, forskolin, propranolol and androst-4-ene-3,17-dione from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Prazocin was supplied by Pfizer (Sandwich, Kent, U.K.) and Ham's F-10 medium and penicillin/streptomycin solution by Gibco BRL Ltd. (Paisley, Scotland). Adenosine-3′5′-cyclic phosphoric acid 2′-*O*-succinyl-3-(^{125}I)-iodotyrosine methyl ester and 4-(4-^{14}C)-androstene-3,17-dione were purchased from Amersham International p.l.c. (Aylesbury, Bucks, U.K.). All other chemicals were of the highest grade available commercially.

(ii) *Animals*. Mature, male Wistar rats, bred in the Department and weighing 250–300 g, were used throughout the study. The animals were housed in light- and temperature-controlled conditions (lights on 0700–1900; 19 ± 1°) prior to use. The animals were allowed free access to food (CRM Nuts, Labsure, Croydon, U.K.) and tap water.

(iii) *Isolation and culture of hepatocytes*. Hepatocytes were prepared by the two-step perfusion technique of Seglen [18]. This method yielded approximately 5×10^7 cells/g wet weight liver with a viability, assessed by trypan blue exclusion, of greater than 97%. Following isolation, the cells were washed in calcium-free Hank's balance salt solution (BSS) and resuspended in the same buffer to give a cell density of 2×10^7 cells/ml. The cells were plated into 10 cm dia., sterile culture dishes (Nunclon, Denmark) with 10^7 cells in 10 ml of Ham's F-10 medium supplemented with 0.1% bovine serum albumin and 100U penicillin/streptomycin per ml [19]. The cultures were placed in an incubator set at 5% CO_2, 98% humidity and 37° and equilibrated for 30 min before proceeding with the experiment.

(iv) *Preincubation of hepatocytes*. The following additions: (a) 8-bromo-cyclic AMP 10^{-4} M; (b) adrenaline 10^{-7} M; (c) IBMX 10^{-3} M; (d) adrenaline and IBMX 10^{-7} M/10^{-3} M; (e) forskolin 10^{-5} M; (f) forskolin and IBMX 10^{-5} M/10^{-3} M, were added to the cells in culture and left for varying periods of time from 2 min to 24 hr. Adrenaline was dissolved as the bitartrate in ascorbic acid solution (10^{-7} M) and forskolin in dimethylsulphoxide (DMSO). All other compounds were dissolved in distilled water. Control cells received vehicle alone except for those used as control for the addition of adrenaline when sodium tartrate solution (10^{-7} M) was used. Following preincubation the cells were scraped from the plates and washed with Hank's BSS supplemented with 1 g/l glucose, 100 mg/l each of magnesium chloride and sulphate and 185 mg/l calcium chloride (incubation medium). Viability, as assessed by trypan blue exclusion, was in excess of 90% following each incubation period. The cells were centrifuged at 300 *g* for 3 min in a Damon-IEC DPR-60 00 centrifuge and resuspended in incubation medium (for assay of enzyme activities) or sodium acetate buffer (50 mM; pH 5.0) (for cyclic-AMP assay).

(v) *Assays*. For the assay of androst-4-ene-3,17-dione metabolism, approximately 3×10^7 hepatocytes were suspended in 3 ml incubation medium to which was added ^{14}C-labelled substrate (0.1 μCi; 500 μg). The incubation was kept for 30 min at 37° in a shaking waterbath. The metabolites were separated and assayed according to Berg and Gustafsson [20] and results expressed as pmoles product/min/10^6 cells.

Cyclic-AMP content of the cells was assayed essentially by the method of Brooker *et al.* [21]. Hepatocytes were suspended in sodium acetate buffer pH 5 at a concentration of 1×10^7 cells/ml and protein denatured by placing in a boiling waterbath for 10–15 min. The mixture was subsequently centrifuged at 4000 *g* for 5 min in a Damon-IEC DPR-6000 centrifuge and the supernatant taken for cyclic-AMP assay. All samples (including standards) were acetylated by the addition of 10 μl of a freshly-prepared mixture of triethylamine and acetic anhydride (2:1). To this acetylated sample was added 150 μl of an anti-cyclic-AMP antibody (raised in goats against human serum albumin conjugated to succinyl-cyclic-AMP) and 100 μl (3000 c.p.m.) of the ^{125}I-labelled cyclic-AMP analogue. The sample was mixed and incubated at 4° for 16 hr following which 0.5 ml of activated charcoal suspension (in 100 mM phosphate buffer containing 0.25% bovine serum albumin) was added. The mixture was centrifuged at 4000 *g* for 4 min and a 0.6 ml aliquot of the supernatant taken for counting in an LKB Gamma counter. Standard curves were constructed for the assay on each occasion. The results were expressed as pmoles cyclic-AMP/10^6 cells. The specificity of the antiserum was tested by measuring the ability of a range of adenine and guanine nucleotides to compete with ^{125}I-cyclic AMP for binding to the antiserum. The antiserum was relatively specific for cyclic AMP with 10^3-fold (cyclic GMP) and 10^4-fold (AMP, ADP, ATP) higher concentrations of the other nucleotides required to produce 50% displacement of the ^{125}I-cyclic AMP.

Statistics. Results were calculated as mean ± SD of at least 4 different cell samples and converted to percentage of relevant control. Significance was tested using Student's *t*-test and the level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Using the substrate, androst-4-ene-3,17-dione, at least 5 different enzyme activities can be calculated; 7α-, 6β- and 16α-hydroxylase, 17-oxosteroid oxidoreductase (17-OHSD) and 5α-reductase. It will be appreciated that enzyme activities as measured in this study do not necessarily represent enzyme amount. As shown in Table 1, the addition of 8-bromo-cyclic-AMP (10^{-4} M) caused the reduction in activity of all of the enzymes studied after 2 hr. This was the case for all of the additions studied and thus, to simplify the discussion, only the 6β- and 16α-hydroxylase and the 5α-reductase activities will be shown and discussed in subsequent experiments.

Figure 1 shows the time course of the effect of 8-bromo-cyclic-AMP (10^{-4} M) and it is seen that maximal inhibition (30–50% of control) occurs at 2 hr following addition and the activities have

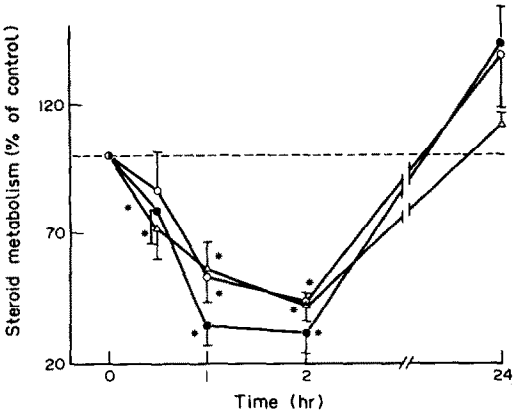

Fig. 1. Change in 6β- (—○—) and 16α-hydroxylase (—△—) and 5α-reductase activities (—●—) with time in isolated hepatocytes following administration of 8-bromo-cyclic-AMP (10^{-4} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

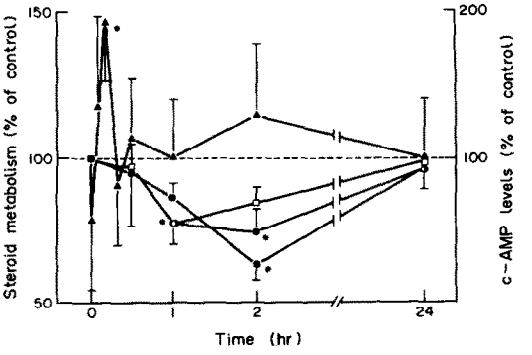

Fig. 2. Change in cyclic-AMP content (—▲—), 6β- (—○—) and 16α-hydroxylase (—□—) and 5α-reductase (—●—) activities with time in isolated hepatocytes following addition of adrenaline (10^{-7} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

returned to control levels by 24 hr. 8-Bromo-cyclic-AMP is known to pass freely across the hepatocyte membrane and directly activate cyclic-AMP-dependent protein kinase [22]. This effect of the cyclic-AMP analogue is similar to that seen for the *in vivo* treatment with dibutyryl-cyclic-AMP [13] and suggests that the latter effect may, in part at least, be due to a direct effect of the cyclic-AMP analogue on the liver and further suggests that cyclic-AMP may be involved in the inhibition of drug/steroid metabolism caused by some hormones [23]. This is further illustrated by the effects of adrenaline (10^{-7} M) shown in Fig. 2, which elevated cyclic-AMP to 194% of control at 10 min and subsequently inhibited steroid metabolism (down to 60–75% of control) at ½–2 hr. The level of cyclic-AMP had returned to control at 20 min and the steroid metabolism by 24 hr post-treatment. This hormone is known to inhibit drug metabolism when injected *in vivo* [5] and this would, thus, appear to be a direct effect on the liver.

It appears, therefore, that there is a time lag between the raised cyclic-AMP levels and the effects on steroid metabolism suggesting that there are one or more intermediate steps in the inhibitory process. The inhibitory effects of adrenaline were seen to be blocked by propranolol (10^{-5} M), a β_2-antagonist, but not by prazosin (10^{-5} M), and α_1-antagonist (Table 2). This would suggest that adrenaline is acting on a β_2-receptor in the liver to elevate cyclic-AMP which subsequently inhibits steroid metabolism by some mechanism. The hepatic β_2-receptor is, indeed, thought to be linked to cyclic-AMP generation [6] whereas the α_1-receptor is linked to phosphatidylinositol turnover [24].

Table 1. The effect of preincubation for 30 min with 8-bromo-cyclic-AMP (10^{-4} M) on the metabolism of androst-4-ene-3,17-dione in isolated rat hepatocytes

	7α-Hydroxylase	6β-Hydroxylase	16α-Hydroxylase	17-OHSD	5α-Reductase
Control	47.8 ± 4.4	76.1 ± 2.4	117.5 ± 5.0	87.9 ± 6.1	320.6 ± 17.5
Test	25.8 ± 1.5	33.3 ± 2.0	49.3 ± 5.7	50.4 ± 3.6	100.8 ± 29.0

17-OHSD = 17-oxosteroid oxidoreductase.
Results are expressed as pmoles product/min/10^6-cells and as mean ± SD of at least 4 different cultures. All tests are significantly different from the relevant control ($P < 0.05$).

Table 2. The effect of prazosin (10^{-5} M) and propranolol (10^{-5} M) on the effect of adrenaline (10^{-7} M) on the metabolism of androst-4-ene-3,17-dione by isolated rat hepatocytes

Addition	6β-Hydroxylase	16α-Hydroxylase	5α-Reductase
Adrenaline	76.7 ± 5.6*	77.4 ± 6.4*	63.1 ± 4.9*
Adrenaline + prazosin	70.6 ± 3.9*	71.4 ± 9.5*	59.8 ± 3.3*
Adrenaline + propranolol	95.4 ± 2.4	99.3 ± 8.2	95.9 ± 3.7

Results are expressed as percentage of relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$ compared to control.

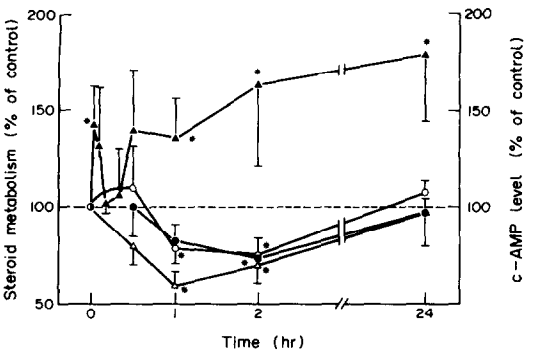

Fig. 3. Change in cyclic-AMP content (—▲—), 6β- (—○—) and 16α-hydroxylase (—△—) and 5α-reductase (—●—) activities with time in isolated hepatocytes following administration of IBMX (10^{-3} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

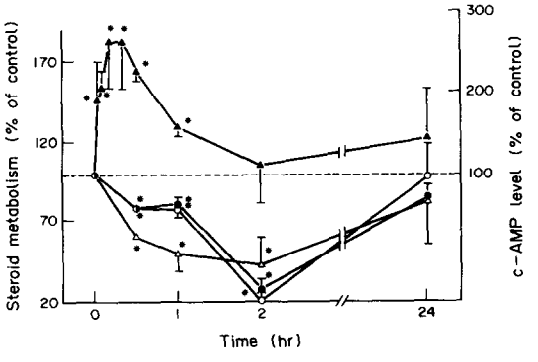

Fig. 4. Change in cyclic-AMP content (—▲—), 6β- (—○—) and 16α-hydroxylase (—△—) and 5α-reductase (—●—) activities with time in isolated hepatocytes following addition of adrenaline (10^{-7} M) and IBMX (10^{-3} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

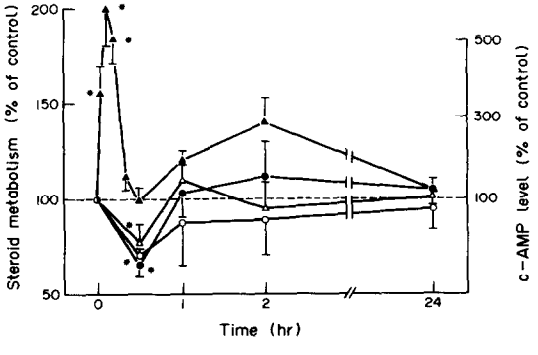

Fig. 5. Change in cyclic-AMP content (—▲—), 6β- (—○—) and 16α-hydroxylase (—△—) and 5α-reductase (—●—) activities with time in isolated hepatocytes following the addition of forskolin (10^{-5} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

Figure 3 shows the effect of the phosphodiesterase inhibitor, IBMX (10^{-3} M), on steroid metabolism. This compound inhibits the degradation of cyclic-AMP in the cell and, thus, may be expected to cause an increase in cellular cyclic-AMP levels. This is seen to be the case, with cyclic-AMP rising to 186% of control after 2 min but rapidly dropping back to control levels only to give a sustained rise (to 295% of control) from 30 min to the end of the time period studied. Steroid metabolism showed a pattern of inhibition very similar to that for the addition of 8-bromo-cyclic-AMP and adrenaline with maximal inhibition (60–75% of control) at 1–2 hr. The later sustained rise in cyclic-AMP levels is most probably attributable to the inhibition of phosphodiesterase but the initial rise is more likely related to the antagonistic action of IBMX at the hepatic adenosine receptor [25]. It is also likely that the effects on steroid metabolism are related to this initial rise in cyclic-AMP and not the later sustained increase (cf. cyclic-AMP after adrenaline addition; Fig. 2). The combination of adrenaline (10^{-7} M) and IBMX (10^{-3} M) caused a more marked increase in cyclic-AMP than either agent alone (Fig. 4), rising to 266% of control at 10 min. This sharp rise was followed by a slower decline in cyclic-AMP concentration than seen with adrenaline alone, indicating an inhibition of the phosphodiesterase. The combination of these two agents also led to a marked inhibition of steroid metabolism (to 20–40% of control values after 1–2 hr). This was a greater effect than either adrenaline or IBMX alone. This would indicate that the degree of inhibition of steroid metabolism is related to the height of the cyclic-AMP peak in the first 10 min.

Intracellular cyclic-AMP levels can also be raised by directly stimulating adenylate cyclase by the addition of forskolin (10^{-5} M). This compound bypasses the need for receptor activation [26] and was seen to greatly elevate cyclic-AMP levels in the hepatocytes (to 607% of control; Fig. 5). If steroid metabolism was measured following this treatment, however, a less marked inhibition was observed than with adrenaline and IBMX (Figs 4 and 5) (65–75% of control). This is an unexpected finding and could be explained in two different ways; either forskolin is having another effect (other than stimulating adenylate cyclase) on the cells or the higher than physiological level of cyclic-AMP is having a different effect. Further experiments with forskolin, where different levels of cyclic-AMP were recorded in the cells, gave a possible answer. When an increase of 488% in cyclic-AMP was seen, the inhibition of steroid metabolism was similar to that seen for adrenaline and IBMX (which increased cyclic-AMP to 266% of control) (results not shown). This suggests that the excessive cyclic-AMP levels may be revealing a second effect on steroid metabolism (i.e. a stimulation). This was further tested by the addition of forskolin and IBMX which increased cyclic-AMP levels to 1256% of control after 5 min (Fig. 6). In this experiment cyclic-AMP levels remained elevated throughout the time period studied. Steroid metabolism was seen to be slightly inhibited at ½ hr but this was followed by a significant stimulation of activity at 1 hr. The enzyme activities had returned to control levels by 2 hr. As no secondary stimulation of this

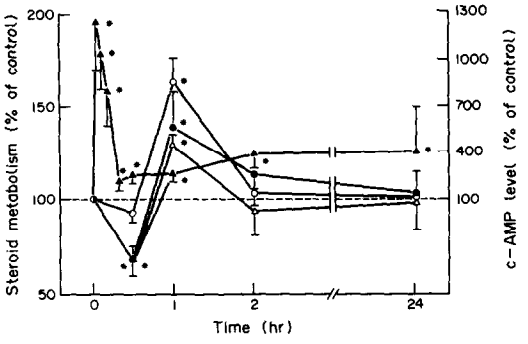

Fig. 6. Change in cyclic-AMP content (—▲—), 6β- (—○—) and 16α-hydroxylase (—△—) and 5α-reductase (—●—) activities with time in isolated hepatocytes following the addition of forskolin (10^{-5} M) and IBMX (10^{-3} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

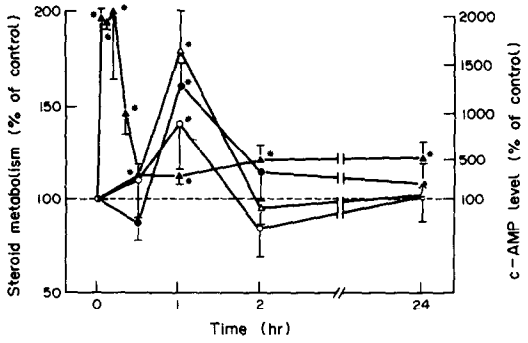

Fig. 7. Change in cyclic-AMP content (—▲—), 6β- (—○—) and 16α-hydroxylase (—△—) and 5α-reductase (—▲—) activities with time in isolated hepatocytes following the addition of forskolin (10^{-5} M) and IBMX (10^{-3} M). Results are expressed as percentage of the relevant control and as mean ± SD of at least 4 different cultures. * $P < 0.05$.

kind was seen with IBMX (which also caused a marked rise in cyclic-AMP levels at later time periods), it seems safe to conclude that both the inhibition and stimulation are related to the initial rise in cyclic-AMP level seen in the first 10 min. In another experiment where cyclic-AMP levels were raised to 1912% of control by forskolin and IBMX treatment (Fig. 7), there was no inhibition at ½ hr and only the stimulatory effect of the cyclic-AMP remained. It thus appears that the effects of elevating cyclic-AMP on hepatic steroid metabolism can be separated into two phases; at lower, physiological levels (2–3-fold stimulation), an inhibition is seen whereas at higher levels (>10-fold stimulation) a stimulatory effect is seen, possibly at a later time point than the inhibition.

The inhibitory phase correlates well with the effects seen *in vivo* by Kato and Gillette [5] with adrenaline and Ross *et al.* [13] with dibutyryl-cyclic-AMP. The intracellular mechanism of cyclic-AMP action is under investigation but appears to involve a cascade of effects probably involving the cyclic-AMP-dependent protein kinase and subsequent phosphorylation of one or more intracellular proteins. It is unlikely that the stimulatory response is a physiological action but it is interesting to note that induction of drug metabolism by phenobarbitone or 3-methylcholanthrene is accompanied by a marked increase in cyclic-AMP levels in the liver [27]. The data presented in this paper may, therefore, help to clear up the contradictory evidence that elevation of cyclic-AMP has been correlated to both inhibition and induction of drug metabolism in the liver.

In summary, elevation of intracellular cyclic-AMP in isolated rat hepatocytes leads to a biphasic effect depending on the degree of elevation. At lower levels of cyclic-AMP, a marked inhibition of enzyme activity is seen whereas at higher levels, a significant stimulation of enzyme activity is observed. Steroid metabolism in hepatocytes is, thus, regulated by cyclic-AMP and this may explain the effects of some of the hormones which are known to regulate steroid metabolism in the liver.

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