with higher corpus allatum activity in the last instar larvae of *Melanoplus*, *Pyrrhocoris*, *Anisolabis*, and *Locusta*[18–21].

The periodic functioning of the corpus allata seems, therefore, to continue in last instar larvae, although perhaps at a reduced rate. The role of fluctuations in the rate of enzymatic breakdown of JH with respect to the changes in JH titer has still to be determined in both *Pieris* and *Barathra*.

The morphogenetic and physiological significance of higher JH titers in the second half of the last larval instar is as yet not clear. In *Pieris* and *Barathra*, possibly also in other Holometabola, it occurs at a time when the critical period for the determination of metamorphosis is over and the larvae prepare themselves for pupation (e.g. spinning of webs or cocoons). The presence of JH may be related to its inhibitory effect on growth and differentiation of imaginal discs and other developing structures. Their precocious differentiation would be unfavourable for the insect until an appropriate environment for pupation has been ensured. In this connection it is very suggestive to compare our idea with a few data from the literature referring to a significant corpus allatum activity during larval diapause in *Chilo*, *Plodia*, and *Diatraea*[22–24]. It seems that in larval diapause the JH peak, typical also for non-diapause development, is temporarily stabilized. It is also worthwhile mentioning that in *Pieris brassicae* the JH peak found in the last instar coincides with the critical period of light sensitivity for pupal morphological colour change[25]. This peak cannot bear a relation to 'girdle' spinning behaviour itself, as, according to BENZ[16], the endocrine induction of this behaviour occurs within the first 60 hours of the instar.

In conclusion we can state that in *Pieris* and *Barathra*, the complex processes leading to metamorphosis are accompanied by subtly timed changes in JH titer, which may partly explain the species specific variations found in morphogenetic and physiological responses to exogenous hormone supply.

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Monolayer Cultures of Normal Adult Rat Adrenocortical Cells: Steroidogenic Responses to Nucleotides, Bacterial Toxins and Antimicrotubular Agents

M. J. O'HARE[1]

Division of Pathology, Chester Beatty Research Institute, Fulham Road, London, SW3 6JB (England), 22 August 1975.

Summary. Monolayer cultures of normal rat adrenocortical cells were treated with agents which stimulate steroidogenesis by Y-1 adrenal tumour cells. Choleragen was active, whereas cyclic nucleotides other than cyclic AMP, bacterial endotoxins and antimicrotubular agents were inactive.

Normal adult rat adrenocortical cells can be maintained in vitro for several months as primary monolayer cultures, while retaining their morphological and steroidogenic responses to ACTH and cyclic AMP[2–4]. Cultures of differentiated adrenal tumour cells, particularly the Y-1 established cell-line[5], have also been extensively used in studies of adrenal-specific functions[6]. These tumour cells, however, respond to a number of substances other than ACTH and cyclic AMP. They are stimulated by other cyclic nucleotides such as cyclic CMP[7], by bacterial toxins including choleragen (*V. cholerae* enterotoxin) and unpurified endotoxins[8,9] and by microtubule-disrupting agents such as colchicine[10,11].

The long-term responses of cultured normal adrenal cells to these agents have therefore been examined, because the validity of tumour cells as models of adrenal function evidently depends on the extent to which they have retained normal responses.

Materials and methods. Tissue culture. Confluent monolayer cultures of approximately $0.5–1.0 \times 10^6$ cortical cells were prepared from the zona fasciculata-reticularis of adrenal glands from 8-week-old (200 g) male Wistar rats, using the collagenase-hyaluronidase disaggregation procedure described previously[2]. Cultures were maintained at 37 °C in 25 cm² polystyrene culture flasks (Falcon) with 5 ml Dulbecco's Eagle's medium containing 15% fetal calf serum (GIBCO), plus 100 µg/ml each of penicilllin and streptomycin with a gas-phase of 10% CO_2 in air. Culture medium was changed every 24 h and retained for measurement of steroid content, and the number of cortical cells in each culture was determined by direct counting of cells in randomly-chosen fields of known area under an inverted phase-contrast microscope.

Cyclic nucleotides, analogs thereof, nucleotides, nucleosides and purine and pyrimidine bases (Sigma Chemical Co.) were added directly to the cultures dis-

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solved in culture medium, as were unpurified bacterial lipopolysaccharides (Boivin) from Difco. Purified *Vibrio cholerae* enterotoxin (choleragen) prepared under contract to NIAID by the method of FINKELSTEIN[12] (lot 0172) was obtained through Dr. R. NORTHRUP (SEATO Cholera Research Program), and added to cultures dissolved in 50 µl sterile phosphate-buffered saline with 0.1% gelatin. Vinblastine sulfate, colchicine and 100 mU/ml ACTH (91.5 U/mg porcine) from Sigma were added in 50 µl sterile 9 g/l NaCl solution, and podophyllotoxin (Aldrich) in 20 µl ethanol.

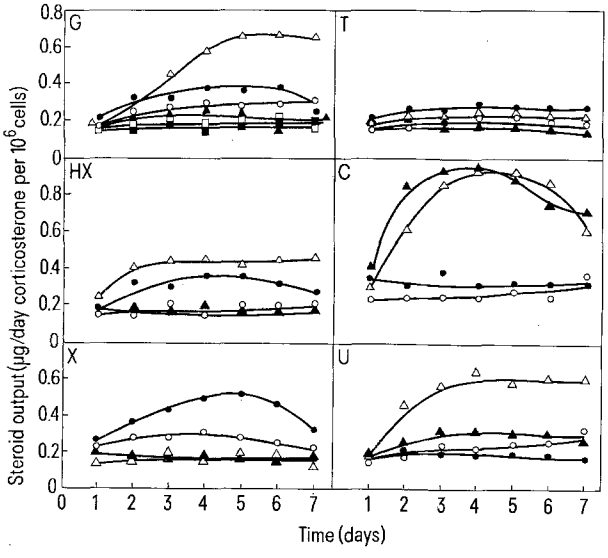

Fig. 1. Steroidogenic response of 3-week-old monolayer cultures of adult rat adrenal cells to the 3′,5′-cyclic nucleotides (○—○), monobutyryl (■—■) and 8-bromo cyclic nucleotides (□—□), ribonucleoside-5′-monophosphates (●—●), ribonucleosides (△—△) and bases (▲—▲) of guanine (G), hypoxanthine (HX), xanthine (X), thymine (T), cytosine (C) and uracil (U). All compounds were tested at 2 m*M* except cyclic nucleotide analogs at 0.5 m*M*.

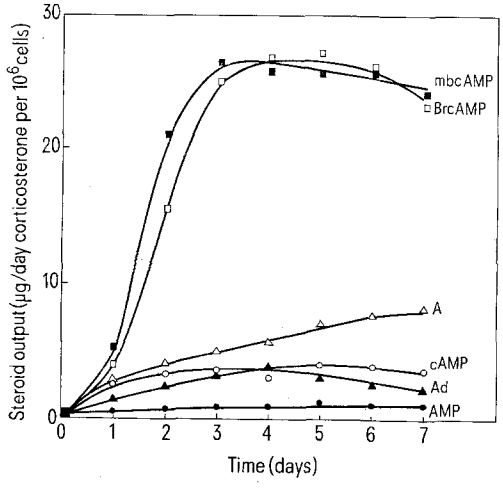

Fig. 2. Steroidogenic response of 3-week-old monolayer cultures of adult rat adrenal cells to 3′,5′-cyclic AMP (cAMP), N^6-monobutyryl cyclic AMP (mbcAMP), 8-bromo cyclic AMP (BrcAMP), adenosine-5′-monophosphate (AMP), adenosine (A) and adenine (Ad). All compounds were tested at 2 m*M*, except cyclic nucleotide analogs at 0.5 m*M*.

Steroid Assay. Steroids secreted into the culture medium were measured as corticosterone using the sulphuric acid-induced fluorescence method[3]. None of the substances used in the present study interfered significantly with this assay.

Results. The monolayer cultures of adult rat adrenal cells were routinely maintained in medium without ACTH for 3 weeks before testing, to allow endogenous steroidogenesis to fall to the very low steady baseline levels[3], following which individual cultures were treated for 7 days consecutively with medium containing one of the potential steroidogenic agents,

Cyclic nucleotides and derivatives. The cyclic 3′,5′-nucleotides, ribonucleoside-5′-monophosphates and ribonucleosides of adenine, guanine, hypoxanthine and xanthine, and of thymine, cytosine and uracil, and the purine and pyrimidine bases themselves were individually tested for steroidogenic potential at a concentration of 2 m*M*, which represented the limits of aqueous solubility for some of these compounds. The objective of this experiment was a) to determine the relative potencies of the cyclic nucleotides and their analogs themselves, and b) to compare them with those of their potential catabolic products at equimolar concentrations, since cyclic nucleotides are readily degraded in serum-containing culture medium[13].

The majority of the cyclic nucleotides (with the exception of cyclic AMP) were without significant long-term steroidogenic effects, whereas a number of the non-cyclic compounds did produce detectable responses. Thus cyclic CMP was inactive, while both cytosine and cytidine gave responses equivalent to 4% of the maximum with 100 mU/ml ACTH (Figure 1). Similar results were obtained with cyclic IMP, XMP and UMP, while all thymine derivatives were without effect at 2 m*M* (Figure 1).

Cyclic GMP produced a just detectable steroidogenic response at 2 m*M*, equivalent to 2% of maximum. GMP and guanosine, however, were both more effective, and the less readily degraded analogs N^2-monobutyryl and 8-bromo cyclic GMP were completely inactive at 0.5 m*M* (Figure 1). In marked contrast the N^6-monobutyryl and 8-bromo analogs of cyclic AMP both resulted in a maximal stimulation of steroidogenesis, greatly in excess of that obtained with cyclic AMP itself, while adenosine was slightly more and adenine slightly less effective than the cyclic nucleotide (Figure 2).

Bacterial toxins. Choleragen was an extremely effective long-term stimulator of steroidogenesis by the cultured normal cells, with maximal responses obtained with 1 ng/ml (Figure 3). Removal of choleragen from the medium, however, resulted in a very slow decline in steroidogenesis compared with ACTH[3].

Unpurified bacterial lipopolysaccharides from *E. coli* (0111:B4), *Serratia marcescens* and *Salmonella typhosa* (0901), on the other hand, were completely inactive when applied to unstimulated cultures from 1 ng/ml to 1 mg/ml. At the higher concentrations the endotoxins in fact reversibly inhibited the morphological and steroidogenic responses of the cultured cells to ACTH, without any overtly toxic effects, but did not inhibit the response to monobutyryl cyclic AMP.

Antimicrotubular agents. Colchicine, vinblastine sulfate and podophyllotoxin were all without detectable steroidogenic effects when applied to normal adrenal cells

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at concentrations up to 5 μM. Neither did they further increase the steroid output of ACTH-stimulated cultres.

Discussion. These results demonstrate clearly that normal adult rat adrenal cells in monolayer culture possess considerable specificity in their steroidogenic responses. The cultured adrenal tumour cells of the Y-1 cell-line, on the other hand, appear to possess a greater latitude in their responses, reminiscent to some extent of the latitude in receptor specificity seen in other adrenal tumours[14]. Thus, considerable caution must be exercized when adrenal tumour cells are used as models of adrenal function, unless comparable studies of normal cells are available.

The limited steroidogenic activity of the non-adenine cyclic nucleotides and their derivatives can probably be accounted for in the known activity of these compounds as phosphodiesterase inhibitors[15]. Unlike some preparations of freshly-isolated adrenal cells[16] the cultured normal cells are directly responsive to phosphodiesterase inhibitors such as theophylline (15% of maximum ACTH response to 2 mM). A direct role of cyclic GMP in adrenal steroidogenesis is not suggested by the present results in so far as this can be determined by the application of exogenous nucleotides, since the catabolic products GMP and guanosine were more active than the native cyclic nucleotide, while the analogs N^2-monobutyryl and 8-bromo cyclic GMP were completely ineffective, in contrast to the greatly enhanced activity of the corresponding analogs of cyclic AMP.

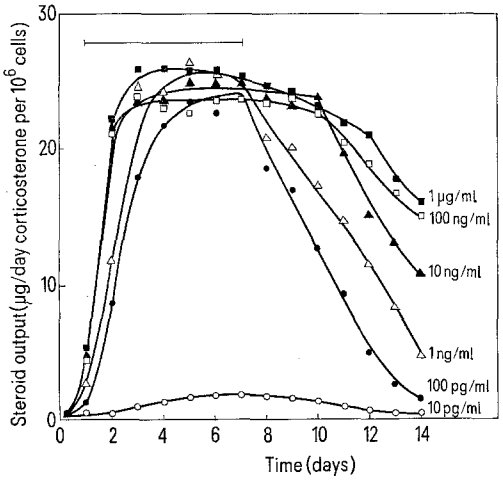

Fig. 3. Steroidogenic response of 3-week-old monolayer cultures of adult rat adrenal cells to *V. cholerae* enterotoxin (choleragen). Cultures were treated with toxin-containing medium at the concentration shown over the period indicated by the bar.

While the normal cultured adrenal cells evidently share the response of Y-1 cells to choleragen (which acts via activation of membrane adenylyl cyclase), they are not stimulated by bacterial lipopolysaccharide endotoxins. The inhibitory effects of the latter on ACTH- but not cyclic AMP-stimulated steroidogenesis are in marked contrast to the stimulatory effects of these endotoxins on cultured adrenal tumour cells[9]. The mechanism of this inhibition has not been determined, but a direct inactivation of ACTH by the lipopolysaccharide preparation has not been excluded.

The failure of the normal adrenal cells to respond to antimicrotubular agents such as colchicine, however, stands in marked contrast to the response of cultured tumour cells, where these agents apparently increase the supply of pregnenolone available for corticosteroidogenesis in the cells[11]. Exogenous pregnenolone itself has been shown to increase the steroid output of normal adrenal cells in culture[3], but since the antimicrotubular agents are inactive, the cultured adrenal tumour cells are evidently not a reliable model of normal adrenal function in this respect.

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Adrenal Dopamine-β-Hydroxylase Activity: 24-Hour Rhythmicity and Evidence for Pineal Control[1]

T. K. Banerji and W. B. Quay

Endocrinology-Reproductive Physiology Program, Department of Zoology, and The Waisman Center on Mental Retardation and Human Development, University of Wisconsin, 2605 Marsh Lane, Madison (Wisconsin 53706, USA),
21 August 1975.

Summary. Adrenal medullary dopamine-β-hydroxylase activity was found in male rats to have a 24-hour rhythm, with an approximately 6-fold increase at about the time of the onset of darkness. This nocturnal rise in enzyme activity did not occur when lights were kept on, nor did it occur in animals that had been pinealectomized.

Dopamine-β-hydroxylase (DBH) catalyses the conversion of dopamine to the neurotransmitter norepinephrine[2] and is released with catecholamines following adrenal stimulation[3]. 24-hour rhythmicities in plasma DBH activity in rat[4], and serum DBH activity in man[5] have been described. We report here a 24-hour rhythm in adrenomedullary DBH and its modification by either change in timing of daily onset of darkness or removal of the pineal gland.

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