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.

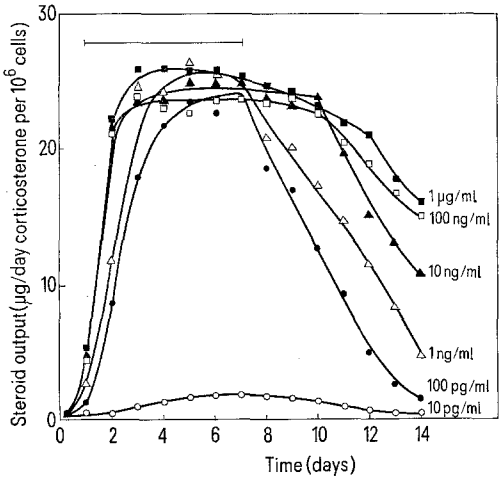

Fig. 3. Steroidogenic response of 3-week-old monolayer cultures of adult rat adrenal cells to *V. cholerae* enterotoxin (choleragen). Cultures were treated with toxin-containing medium at the concentration shown over the period indicated by the bar.

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]

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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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Materials and methods. Male rats (110–120 days old) of the S_1 Berkeley strain were used, in the first experiment to evaluate the possible occurrence of 24-hour rhythmicity in adrenomedullary DBH. They were acclimated for a minimum of 15 days in similar temperature-controlled (21 °C), window-less and artificially-lighted (LD: 12:12) rooms, with food and water available continuously. Entry into these rooms was extremely restricted and care was taken to leave the animals undisturbed. Animals were killed by quick decapitation at 6 times (noted in the Figure), and the paired adrenals were immediately frozen and stored (−20 °C) until enzymatic assay.

With the same conditions and procedures as above, the effect of pinealectomy was studied in 2 concurrent experiments. Pineal glands were surgically removed[6] under ether anaesthesia from animals of the experimental group. Unoperated, sham and pinealectomized rats were sacrificed 15 days later and the adrenal medullae were similarly treated. Autopsies were all at 23.00 h ± 30 min. Approximately 2–3 min elapsed from decapitation to tissue freezing.

In the second experiment, using unoperated, sham and pinealectomized animals, on the day of sacrifice the light was left on beyond the usual hour (22.00) (Figure). These animals were sacrificed in light and the glands were collected so as to represent DBH activity level at 23.00 h as above. At autopsy the brains from the sham and pinealectomized animals of both experiments were dissected open and examined for completion of pinealectomy and/or other post-operative physical signs of injury, if any.

DBH activity in the adrenal medulla was estimated by the method of MOLINOFF, WEINSHILBOUM and AXELROD[7]. Paired adrenals were homogenized in 400 volume of 0.005 *M tris* buffer, pH 7.5, containing 0.1% Triton X-100. Homogenates were centrifuged at 10,000 × *g* for 10 min and lipids removed by aspiration. Supernatant fluid (200 µl) was added to a 15 ml glass centrifuge tube containing tyramine as substrate. The final reaction mixture (310 µl) contained fumarate, ascorbic acid, MAO-inhibitor (pargyline), catalase, *tris* buffer and copper sulphate in appropriate quantity and pH to obtain maximal activity. The reaction was allowed to proceed for 20 min at 37 °C with added tyramine converted to octopamine by sample DBH. The PNMT part of the assay was then initiated by changing pH (from 6.0 to 8.6) and adding S-adenosylmethionine-methyl-^{14}C (New England Nuclear) and phenylethanolamine-N-methyl-transferase (Miles Labs.). The radioactive synephrine formed was extracted into toluene-isoamyl alcohol (6 ml; 3:2 v/v), and after centrifugation, 4 ml of the organic phase was transferred to vials and dried (80 °C). Ethanol (1 ml) and phosphor (10 ml) were added and the radioactivity was determined in a liquid scintillation counter (Unilux II, Nuclear Chicago). Blanks (200 µl) consisting of tissue homogenates heated to 95 °C for 5 min and heated homogenates (200 µl) with 40 ng of octopamine added to the entire reaction mixture were run with assay of each sample. From each paired-adrenal homogenate representative of each sample, triplicates of blank, standard and samples were run. Results were expressed as nmoles of octopamine formed per g adrenal wet weight per 20 min. Significance of differences between group means were based upon the Student-Fisher-*t*.

Results. A significant 24-hour periodicity in adrenal dopamine-β-hydroxylase activity was found (Figure). Throughout the light phase DBH activity declined gradually, reaching a nadir at 21.00 h. A sharp rise in enzyme activity by 1 h after the lights went off at 22.00 h was highly significant ($p < 0.001$). This peak activity presumably continued through midnight followed by another well-defined fall towards the later part of the night. There was also another significant ($p < 0.001$) rise from this late night low to the early day activity level.

Surgical extirpation of the pineal led to very significant reduction in adrenal DBH activity compared with either unoperated or sham-operated animals (Figure, experiment 1). In the absence of the pineal the rise in adrenal DBH activity that normally occurs near the start of the dark phase failed to appear. When light was left on beyond 22.00 h, an inhibition in rise of DBH activity was noted at 23.00 h (Figure, experiment 2). Although there was a suggested trend of further loss of activity ($p < 0.01$) in

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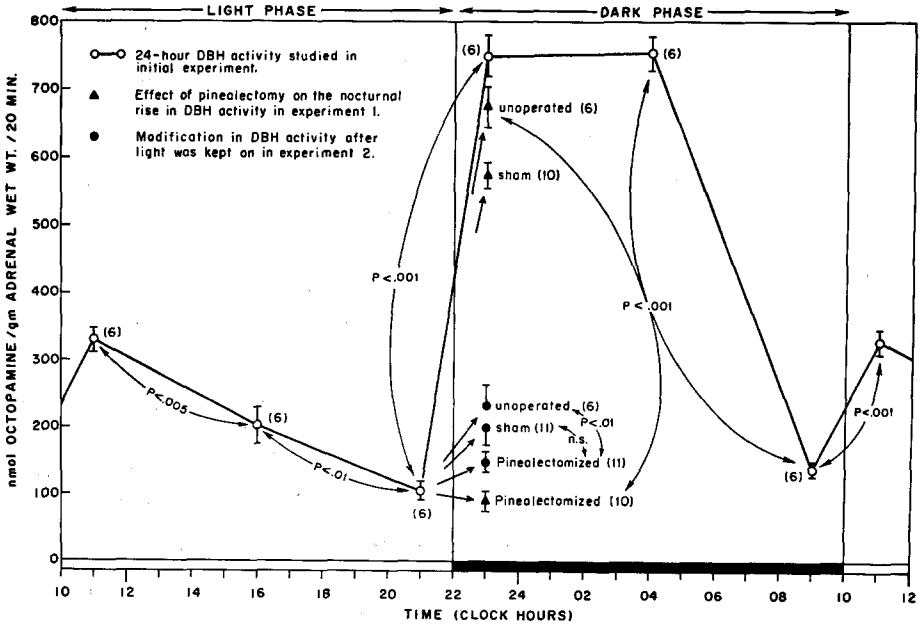

Graphic representation of the results, showing: 1. a 24-hour rhythmicity in adrenal DBH activity in normal male rats (○—○), 2. the loss of the rise in DBH activity in pinealectomized rats (▲) that normally occurs near onset of darkness, and 3. the failure of the rise in DBH activity in control animals as well as pinealectomized ones (●) when the lights remain on. Means ± standard errors are plotted in relation to time of sacrifice; numbers of animals are noted within parentheses. Probability (p) values are based on the Student-Fisher-*t*.

DBH in the pinealectomized group as compared with that in the unoperated control, the difference between pinealectomized and sham groups was not statistically significant in this second experiment with the lights left on.

Discussion. LEW and QUAY[8] have shown that adrenal norepinephrine (NE) content, in the same rat strain (S_1), is highest at middark, which is consistent with our early night time tise in DBH activity. However our results for midday DBH activity level do not parallel the high midday adrenal NE content observed by the above authors. Nor do they explain the findings of DUNN and LIN[9] who reported that adrenomedullary NE content is lowest during night when epinephrine (E) store is very high[9,10]. It might be noted, however, that there are at least several reasons why one should not expect necessarily close correlations in time between levels of adrenomedullary DBH activity and of NE and E: 1. Adrenomedullary NE, in addition to being the precursor of E, is also secreted as an independent hormone. 2. NE and E in the medulla are available interchangeably in storage-vesicles in bound form and in the cytoplasmic sap in a free pool. 3. They undergo a continuous and concomitant catabolic process of oxidative deamination (intra-vesicularly) and *O*-methylation (free cytoplasmic)[11].

Our results suggest that the nocturnal rise in adrenal DBH activity is both pineal- and darkness-dependent (Figure). However, we can not yet rule out the possibility that in the pinealectomized animal a phase shift in DBH rise occurred rather than necessarily an abolishing of the DBH periodicity. Although an effect of pinealectomy on an adrenomedullary activity or function has not been presented previously, physiological interrelations of the rat pineal gland and catecholaminergic and stress-related systems have long been suggested by other kinds of evidence[12,13].

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THEORIA

Molecular Superdelocalizability. A Correlation with Diamagnetic Susceptibility

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Summary. Diamagnetic susceptibilities of 44 divers aromatic molecules were successfully predicted from molecular superdelocalizabilities calculated from Hückel molecular orbital theory.

In the past, diamagnetic susceptibilities for organic compounds were estimated through a set of PASCAL constants[2–5] which permitted the calculation of a molar susceptibility on an additive basis (a given contribution for each species of atom) provided that appropriate constitutive corrections were made. A more recent approach assumed that a molar susceptibility may be written as a sum of bond contributions and of correction terms that represented interactions between adjacent bonds[6–10]. Efforts to prove the validity of each of these methods from molecular orbital theory have been made[6–8] and on this basis an argument has been presented in favor of the bond over the atom contribution approach[6,9,10]. These additive procedure are fundamentally empirical despite their theoretical rationales. Thus it should not matter in a practical application which scheme is followed so long as the empirically derived contributions and associated corrections are used in a consistent manner.

We prefer an alternative correlative approach in relating diamagnetic susceptibilities to values calculated from indices obtained from Hückel molecular orbital theory[11]. Attempts to correlate experimentally determined diamagnetic susceptibilities by linear multiple regression analysis with molecular orbital indices: energy of highest

Table I. HMO parameters found suitable for correlations with diamagnetic susceptibility

Atom	h	Bond	k
C	0.0	C_{ar}-C_{ar}	1.0
C(≡N)	0.0	C_{ar}-C_{al}	0.9
$\dot{C}H_3$	2.0	C_{ar}-C(≡N)	0.9
$\dot{O}$	1.0	C_{ar}-CH_3	0.7
$\ddot{O}$	3.0	C≡N	1.2
O(nitro)	2.0	C—$\dot{O}$	1.0
$\dot{N}$	0.4	C—$\ddot{O}$	1.0
$\ddot{N}$	2.0	C—$\dot{N}$	1.0
N(≡C)	0.4	C—$\ddot{N}$	0.7
N(nitro)	1.0	C—N(nitro)	3.0
F	3.0	C—$\ddot{S}$	1.0
Cl	1.1	C—F	0.7
Br	0.8	C—Cl	0.4
I	0.5	C—Br	0.4
$\ddot{S}$	1.5	C—I	0.3
		N—O(nitro)	0.2

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