Table III. Response of C57Bl/6 mouse thymus cells separated on BSA discontinuous gradients to LPS

Cell fraction	Unstimulated cells (counts/min)	Stimulated cells (counts/min)	Stimulation index
Control[a]	370 ± 49[b]	398 ± 50	1.08
	116 ± 09	173 ± 44	1.48
	224 ± 04	243 ± 30	1.08
Band A	1522 ± 225	1336 ± 108	0.88
	4405 ± 359	1994 ± 451	0.45
	702 ± 99	295 ± 62	0.42
Band B	253 ± 19	528 ± 15	2.09
	158 ± 20	311 ± 78	1.96
	166 ± 35	395 ± 32	2.38
Band C	93 ± 18	99 ± 12	1.09
	48 ± 06	74 ± 15	1.55
	173 ± 13	221 ± 40	1.28
Band D	136 ± 24	167 ± 30	1.23
	84 ± 11	89 ± 22	1.05
	249 ± 52	184 ± 29	0.74
Pellet	276 ± 105	192 ± 07	0.69
	160 ± 21	159 ± 48	0.99
	129 ± 14	124 ± 08	1.09

[a] Nonseparated thymocytes. [b] Data for 3 individual experiments is given. Values listed are the mean cpm of 4 replica tubes plus the standard deviation.

from band B exhibited the greatest mitogenic response. The mitogenic responses of non-separated cells was higher than the responses of bands A, C, D, or the pellet. Thymus cell subpopulations were stimulated with the thymus independent mitogen *E. coli* LPS (10 μg). This data (Table III) shows that cells from band B exhibited the highest degree of stimulation. Cells in the other bands and pellet did not respond to LPS. The cell subpopulation isolated from the lowest density area of the gradient (band A) was rich in large blast-like cells displaying an abundant amount of cytoplasm and large round nuclei. These cells were not stimulated by any of the mitogens but did display the highest basal uptake of tritiated thymidine (Tables, I, III) when incubated without mitogen. Due to the higher level of tritiated thymidine uptake in this cell population it is felt that these cells correspond to the highly mitotic cells of the outer cortext of the intact thymus[11].

There was a significant enrichment of cells in band B responding to mitogenic stimulation when compared to the other bands and unseparated cells. This reactive subpopulation was not, however, morphologically homogenous. It is not clear at the present time which of the morphological cell types respond to the mitogens. Previous investigations have shown that cortisone resistant thymocytes are responsive to PHA and Con A. Therefore, it is probable that the cells isolated from band B correspond to the cortisone resistant immunologically competent cells of the thymus medulla[12]. The morphological heterogeneity of this reactive subpopulation may be due to contamination with nonreacting cells. However, a more plausible explanation would be that this subpopulation contains cells that are in various stages of immunological maturation and thus appear morphologically dissimilar.

Our data (Table III) show a small number of thymus cells isolated from band B that are capable of responding to the thymus independent mitogen *E. coli* LPS. However, unseparated thymus cells exhibited no mitogenic response to LPS, which is in agreement with the data of ANDERSSON et al.[13]. Density gradient separation of thymus cells allowed the isolation of mitogen responsive cells in a single subpopulation. Speculation on the functionality of the thymus cells responding to LPS may lead to the following suppositions. First, the cells may be B cells involved in humoral immunity and correspond to the antibody producing cells described by VITETTA et al.[14]. On the other hand they may represent B cells that are destined to become T cells under the influence of the thymus. A third possibility is that the LPS reactive cells are contaminants from the circulation.

Zusammenfassung. Fraktionen von Thymuslymphozyten der Maus, die im diskontinuierlichen BSA-Dichtegradienten aufgetrennt worden waren, wurden auf ihre Stimulierbarkeit durch die «thymusabhängigen» Mitogene Concanavalin A und Phythämagglutinin sowie durch das «thymusunabhängige» Lipopolysaccharid von *E. coli* untersucht. Eine Lymphozytenfraktion niederer Dichte konnte mit allen Mitogenen stimuliert werden. Dieser Befund wird im Rahmen der Funktion einzelner Subpopulationen der Thymuslymphozyten diskutiert.

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Absence of a Circadian Rhythm of Corticosterone Secretion in Monolayer Cultures of Adult Rat Adrenocortical Cells

We have shown that adult rat adrenocortical cells from the zona fasciculata-reticularis retain their structural and functional differentiation in monolayer culture and secrete corticosterone at an apparently steady overall daily rate when maintained with ACTH[1-3]. The intact adrenal cortex, however, shows a circadian rhythm of responsivity to ACTH in vitro[4], and circadian rhythms of several physiological and biochemical parameters including oxygen consumption and corticosteroid secretion have been reported in organ-cultured adrenal glands[5-7]. It has therefore been suggested[8] that the adrenal cortex possesses intrinsic rhythmicity of function, since rhythms persisted for many days in organ cultures maintained under constant conditions. Because our previous experiments did not exclude the possibility of circadian variations in monolayer cultures, we have examined the secretion of corticosterone by freshly-prepared cultures on a 3 and 6-hourly basis. This is the first time, to our knowl-

edge, that a differentiated function of mammalian endocrine cells has been examined for intrinsic circadian rhythmicity in monolayer culture.

Methods. Tissue culture. Primary monolayer cultures of zona fasciculata–reticularis cells were prepared from the adrenal cortices of 8-week-old (200 g) male Wistar rats maintained on a 12 h light/12 h dark regime. Suspensions of collagenase-hyaluronidase disaggregated cells obtained from the decapsulated cortices of 4 rats killed by cervical dislocation midway through the light period were prepared as described previously[1], and used to set up paired cultures, each of which contained approximately 2×10^6 attached cortical cells. The number of adrenocortical cells in each culture was determined by counting randomly-chosen fields of known area under an inverted phase-contrast microscope at 24 h intervals.

Cultures were maintained at 37.5 °C in 25 cm^2 Falcon tissue culture plastic flasks with 5 ml Dulbecco's Eagle's medium containing 15% fetal calf serum (Gibco) and 100 U/ml penicillin and streptomycin, with 10% CO_2 in air. Porcine ACTH (91.5 U/mg, Sigma) was added to cultures in 50 µl sterile 0.9% (w/v) NaCl solution.

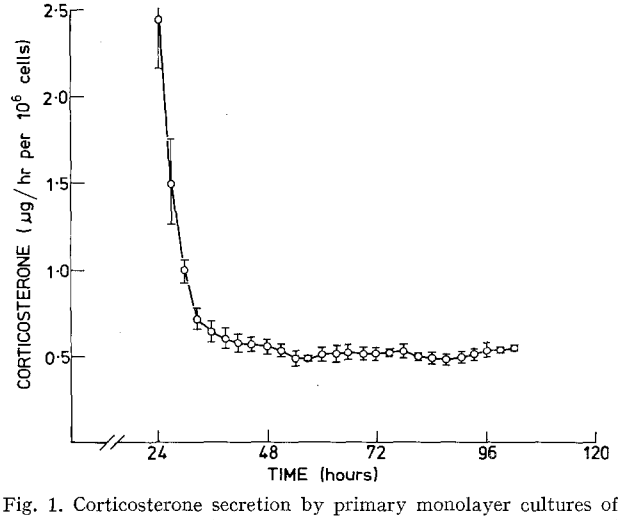

Fig. 1. Corticosterone secretion by primary monolayer cultures of adult rat adrenocortical cells maintained in the presence of 100 mU/ml ACTH. The mean and range of determinations made on 2 cultures set up from the same cell suspension are shown.

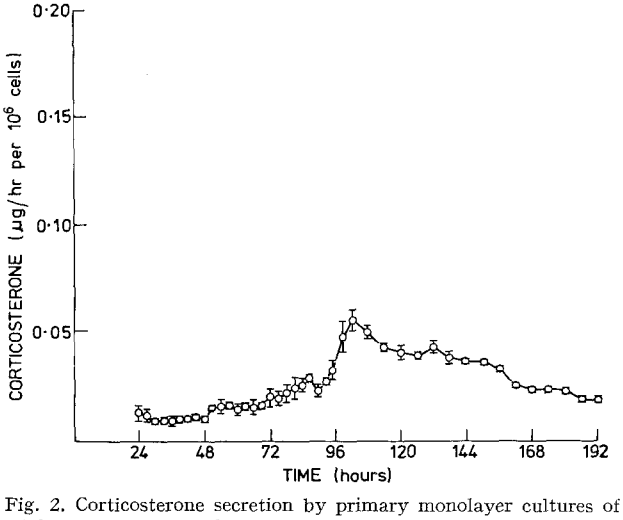

Fig. 2. Corticosterone secretion by primary monolayer cultures of adult rat adrenocortical cells maintained without ACTH. Note that the scales of both abscissa and ordinate differ from Figure 1.

Cultures were allowed to remain undisturbed for 24 h in medium without ACTH during which time the viable cells attached to form the monolayer, they were then rinsed with several changes of fresh medium to remove damaged, unattached cells, and the culture medium was subsequently replaced every 3 or 6 h and stored at −20 °C to await analysis.

Corticosterone assay. The corticosterone output of the cultures was determined using the sulfuric acid-induced fluorescence method[2], determinations being made on dichloromethane extracts of culture medium and corrected for the number of adrenal cells in the culture. To check the specificity of the assay, some daily medium collections from similar cultures were extracted and subjected to Sephadex LH-20 (Pharmacia) column chromatography using the solvent system toluene/dichloromethane/methanol 49:49:2 to separate corticosterone from other fluorogenic steroids.

Results and discussion. The monolayer cultures were maintained on one of two regimes. Stimulated cultures were treated with 100 mU/ml ACTH, fresh hormone being added with each medium change. This concentration of ACTH results in the maximal steroidogenic stimulation of adrenal monolayers[2]. Unstimulated cultures were maintained with complete medium alone, containing less than 0.2 µU/ml ACTH.

A peak level of corticosterone secretion, equivalent to over 50 µg/day per million cells, was achieved immediately on addition of ACTH after 24 h preliminary culture in its absence (Figure 1). This output declined rapidly, however, in spite of the fact that the cultures were treated continually with a maximally-stimulating concentration of ACTH, and within 24 h of commencing stimulation, corticosterone secretion had fallen to a level equivalent to approximately 13 µg/day per million cells and was maintained at this level thereafter.

There was no evidence of circadian variation in the corticosterone output of ACTH-stimulated cultures. The mean hourly outputs of the 6 h quadrants during the 3rd to 5th days of culture inclusive were 0.54 ± 0.012 (SD), 0.53 ± 0.017, 0.52 ± 0.024 and 0.56 ± 0.020 µg per million cells. None of these quadrant outputs was significantly different from the overall mean output of 0.54 ± 0.022 µg/h per million cells during this time. The precision of the assay procedure, expressed as the coefficient of variation, was 6% at the levels of corticosterone measured in the medium from ACTH-stimulated cultures.

The fact that initial peak levels of corticosterone secretion in ACTH-stimulated cultures were approximately 4 times the final steady levels (Figure 1) tends to confirm our previous calculations[2] that the continuous steroid output of the confluent adrenal monolayers is lower than the maximum output of an equivalent number of cells in vivo.

Initial levels of corticosterone recovered in the medium from unstimulated adrenal cultures were very low, equivalent to less than 0.2 µg/day per million cells (Figure 2). The output of corticosterone then rose as the

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extent of further metabolism to 11-dehydrocorticosterone (21-hydroxypregn-4-ene-3, 11, 20-trione)[9] slowly declined, before finally falling to low levels again after the 4th day of culture. Analysis of the quadrant outputs of corticosterone in unstimulated cultures failed to reveal any evidence of significant circadian variation from a steady rise and fall, such fluctuations as were measured falling essentially within the limits of experimental error, the coefficient of variation of the assay procedure being 10% at these low levels of corticosterone.

We have demonstrated previously[2] that the predominant fluorogenic steroid secreted by adrenal monolayers maintained without ACTH for several weeks is 20α-dihydroprogesterone (20α-hydroxypregn-4-ene-3-one), owing principally to the decline in 21-hydroxylase activity that takes place in the absence of ACTH[3]. During the initial phase of culture examined here, however, the fluorogenic steroid secreted by both ACTH-stimulated and unstimulated cultures was accounted for as corticosterone when medium extracts were subjected to Sephadex LH-20 column chromatography.

The present experiments have therefore failed to reveal any significant circadian variation in the corticosterone output of either stimulated or unstimulated adrenal monolayer cultures. This finding is in marked contrast to results obtained with organ-cultured hamster[5,6] and lemming[7] adrenal glands, where circadian rhythms of corticosteroid secretion often exceeded 40% of the mean output in amplitude and persisted for over a week in ACTH-stimulated and unstimulated cultures. The present results do not exclude the possibility of unsynchronized rhythmicity in the individual cells in the non-proliferating confluent monolayers, but there was no evidence of any rhythm of diminishing amplitude, such as might be expected to result from a gradual desynchronization of individual cellular rhythms.

The absence of rhythmicity in monolayer cultures and its apparent persistence in organ cultures suggests that organized tissue structure is important for its maintenance in vitro, although a species difference between adrenal rhythms in the rat and in the hamster and lemming can not be ruled out. The significance of endogenous adrenal rhythmicity in vivo, however, is doubtful. It has been shown that deafferentation of the medial basal hypothalamus in the rat results in both a high pituitary ACTH content[10] and high steady levels of corticosterone secretion[11], similar results being obtained with suprachiasmatic lesions[12]. These experiments indicate that variations in corticotrophin releasing factor synthesis[13] resulting in variations in ACTH secretion[14] are responsible for at least the greater part of the circadian variations in steroid secretion by the rat adrenal cortex in vivo. Such a mechanism is consistent with our failure to detect circadian variations in the steroid output of monolayer-cultured rat adrenal cells. Thus the significance and origin of the endogenous rhythms of steroid secretion in organ-cultured adrenals remain to be determined.

Résumé. La stéroïdogénèse par les cellules surrénaliennes du rat adulte (zones fasciculaire et réticulaire), cultivées en couche monocellulaire a été examinée dans le but de trouver les rythmes circadiens, semblables à ceux qui ont été observés dans la sécrétion de corticosteroïdes des glandes surrénales en culture organotypique. On n'a pas observé de rythmes de sécrétion, ni dans les cultures stimulées par ACTH ni dans les cultures non stimulées, ce qui indiquerait que si le cortex surrénalien possède en effet une fonction rythmique intrinsèque, cela doit dépendre de la structure histotypique du tissu glandulaire.

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Certain Features of Hypophyseal Intermediate Lobe Materials as Seen in Tissue Culture[1]

In an attempt to purify, characterize and determine the function of bovine hypophyseal intermediate lobe materials i.e., intermediate lobe tissue and intraglandular colloid, it was learned that materials characterized at one point in time may, within 24 to 48 h, show different characteristics. Since these materials are so highly active, and if meaningful data are to be secured from ongoing chromatographic and immunological studies, it was felt that some time relationship be established between the breakdown of intermediate lobe tissue, the ultimate formation of intraglandular colloid[2], and the elaboration of various substances during the breakdown period. Tissue cultures were utilized to secure this information and this communication, in part, is based on these findings.

Previous investigations[2–7], established an activity period for intermediate lobe materials, from 1 to 3 days, based on the response of cells of mesodermal origin, in various organs, to an injection of these materials. Mesodermal cells are induced to differentiate and proliferate along red blood cell lines.

Gel filtration studies of intermediate lobe materials demonstrate the existance of 4 separate substances in fractions of intermediate lobe materials, and for convenience are labeled WB_1–WB_4. All of these fractions activate cells of mesodermal origin to various degrees. At this time it has not been determined whether these fractions are breakdown products of a specific intermediate lobe cell or a combination of products from profilerating intermediate lobe cells. Nevertheless, tissue culture techniques have added some basic knowledge to this study.

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