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THE INFLUENCE OF HORMONES ON CELL DIVISION

I. TIME-RESPONSE OF EAR, SEMINAL VESICLE, COAGULATING GLAND AND VENTRAL PROSTATE OF CASTRATE MALE MICE TO A SINGLE INJECTION OF ESTRADIOL BENZOATE

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THE mitogenic actions of hormonal materials have been studied in a variety of tissues belonging to the endocrine system proper or to its "target tissues". The results of many such studies have been tabulated by Eigsti and Dustin [14]. Estrogens and androgens have been demonstrated to be powerful mitotic stimulators of both the stromal tissues and epithelia of such glands as the seminal vesicle and ventral prostate [11, 23]. Bullough and van Oordt [10] have demonstrated that these materials are capable of inducing elevated levels of mitotic activity in the malpighian layer (stratum germinativum) of mouse ear epidermis. Dirschel, Zilliken and Dropp [13], in dose-response studies, have shown that a linear relationship exists between dose level and mitotic activity in mouse seminal vesicle when androgens are employed. Estrogenic materials, however, do not yield simple linear curves in this type of experiment. Experiments have been carried out to determine which hormonal factors may act to inhibit mitotic activity. Green and Ghadially [16] have demonstrated inhibition of mitosis in rodent skin following administration of cortisone. Bullough [5] has presented provocative data derived from *in vitro* studies on the effects of estrogen on mitotic activity of ear epidermis which tends to indicate that this material exerts its influence on cell division by intervention in the glycolytic phases of carbohydrate metabolism. In spite of the research effort which has been expended upon the problem of control of mitotic activity by hormonal materials, no clear picture of the points of hormonal interposition or of the hormonal interrelationships involved has as yet emerged.

Much of the work concerned with the influence of hormones upon cell division has had as its goal the simple demonstration of the fact that a particular hormone is capable of inducing mitotic activity in a given tissue [22]. Data are often presented in relative terms such as the number of mitotic

figures per microscopic field [11] thus making comparison of levels of activity reached by tissues of different cell densities difficult or impossible. Ignorance of the existence of diurnal mitotic rhytms [8] and age factors [7], a lack of appreciation of the proper use of the colchicine technique [6], and absence of control over genetic variability in test animals serve to vitiate much of this work. Burkhart's paper [11] on the early effects of androgenic materials appears to stand alone as a detailed time-response study in this area of investigation. A preliminary study on the time responses of ear and seminal vesicle to estrogenic materials was reported by Allen [1].

The need for time-response data derived from experiments in which intrinsic and extrinsic variability is controlled as closely as possible is apparent. The present paper, the first of a series of time-response studies, deals with the development of mitotic activity in the epithelial cells of ear epidermis, seminal vesicle, coagulating gland and ventral prostate under the influence of a small dose of estradiol benzoate.

MATERIAL AND METHODS

All work was carried out using 90–180 day old, purebred BALB/c Jax castrate male mice. Castration was performed 30 days prior to use of animals for experimental purposes. The experimental stock was housed under conditions of constant 14 hour artificial day length (0700 hours to 2100 hours). A standard laboratory ration and water were provided *ad libitum*. Estradiol benzoate (Scheering) was administered as a single subcutaneous injection of 16 micrograms (100 Rat Units) in .25 cc sesame oil (N.F.). Colchicine (Penick, powdered U.S.P.) was utilized to arrest mitoses and was administered as a single subcutaneous injection of 100 micrograms in .25 cc distilled water 6 hours prior to autopsy time. Solutions of colchicine were routinely made up just prior to use. Autopsy was carried out at 1400 hours. Hormone and

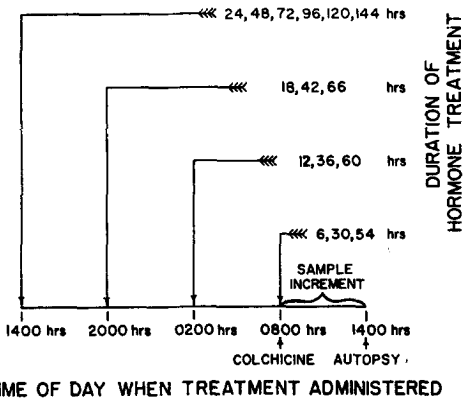

Fig. 1. Plan of experimental time relations employed to allow harvesting of mitoses during a fixed time period. Horizontal axis indicates time of day when treatments were given, arrows show time of administration of hormone for a given duration of treatment (right vertical axis).

colchicine administration were adjusted to this autopsy time as indicated in Fig. 1. Six hour samples of mitotic activity were thus made available from 0 hours to 72 hours following hormone treatment and further 6 hour samples covered the periods following hormone treatment of 90–96 hours, 114–120 hours, and 138–144 hours. Castrate controls received identical treatment save for omission of estradiol benzoate. This experimental procedure serves to fix all experimental variables. Animals were killed by cervical dislocation and subjected to immediate autopsy. Seminal vesicle, coagulating gland, ventral prostate, and samples of pinna were dissected out, fixed in Bouin's fluid, subjected to standard histological processing procedures, serially sectioned at 8 microns, stained in Harris haematoxylin and eosin and mounted. For analysis of mitotic acitivity resting nuclei versus colchicine arrested methaphases of the acinar epithelial cells of ventral prostate, coagulating gland and seminal vesicle and the epithelial cells of the malpighian layer of pinna were differentially counted. Three thousand non-dividing cells were observed in each tissue in each animal and 5 animals constituted a typical experimental group. Counting was carried out at 440 diameters magnification using an ocular counting grid. To prevent prejudicing of counts, slides were randomized and counted as unknowns. Raw counts were converted to per mille mitotic indices, the per mille indices multiplied by 100 and converted to logarithms. From these figures standard errors of the means and 95 per cent confidence limits were calculated using standard statistical methods. Zero values were corrected for discontinuity by dividing the number of observed cells into 250.

RESULTS

Comparison of mitotic indices exhibited by the four investigated tissues in animals with intact testes as opposed to castrate control animals (Table I) indicates that all tissues show a decrease in mitotic activity following castration. Using *t*-tests this decrease is shown to be significant in all cases ($P = < .02$).

The response of ear epidermis to hormone injection in castrate males is presented graphically in Fig. 2. Inspection of this figure indicates that ear epidermis undergoes a pronounced drop in mitotic activity during the 6 hour period (0–6 hours) immediately following administration of estradiol benzoate. The *t*-test indicates this drop to be significant ($P = < .02$). A significant increase in mitotic activity over castrate levels is noted in the period 6–12 hours following hormone administration ($P = < .05$). Peak activity is reached about 24 hours after estrogen treatment. Any lag period preceding commencement of mitotic activity in this tissue is obscured by the above mentioned depression following estrogenization. Inspection of the "plateau" portion of the curve shows a series of regular fluctuations to be present which have an apparent period of 24 hours. Analysis of variance indicated that these points form a heterogeneous series ($P = < .001$ between 12–72 hours) thus suggest-

TABLE I

Mitotic activity at six hour intervals following the injection of 16 micrograms estradiol benzoate into castrate male mice. For each tissue mean mitotic indices as log (per mille mitotic index × 100) followed by standard error values are given. The number of donor mice for tissues examined follows in brackets.

Sample category	Ear	Seminal vesicle	Coagulating gland	Ventral prostate
Normal	2.954 ± .031 (5)	1.620 ± .222 (5)	1.899 ± .062 (5)	2.220 ± .049 (5)
Castrate	2.644 ± .073 (4)	1.032 ± .119 (5)	1.038 ± .154 (4)	1.056 ± .023 (4)
0–6 hrs.	2.377 ± .070 (4)	1.027 ± .156 (4)	.898 ± .006 (5)	
6–12	2.902 ± .045 (5)	1.388 ± .281 (5)	.896 ± .029 (5)	
12–18	3.014 ± .075 (5)	1.390 ± .233 (5)	1.149 ± .151 (5)	
18–24	3.085 ± .013 (5)	1.846 ± .158 (5)	1.143 ± .143 (5)	1.151 ± .148 (5)
24–30	2.973 ± .034 (4)	2.607 ± .029 (5)	3.326 ± .078 (5)	
30–36	2.943 ± .117 (5)	3.110 ± .080 (5)	3.218 ± .119 (5)	
36–42	3.004 ± .084 (5)	3.043 ± .036 (5)	3.578 ± .048 (5)	
42–48	3.097 ± .060 (5)	3.019 ± .049 (5)	3.183 ± .029 (5)	1.279 ± .148 (5)
48–54	2.820 ± .063 (5)	3.269 ± .025 (5)	3.283 ± .038 (5)	
54–60	2.809 ± .023 (5)	2.941 ± .076 (5)	3.112 ± .069 (5)	
60–66	2.908 ± .062 (5)	3.100 ± .075 (5)	3.177 ± .062 (5)	
66–72	2.974 ± .039 (5)	3.188 ± .019 (5)	3.012 ± .048 (5)	1.635 ± .072 (5)
90–96	2.715 ± .064 (5)	2.708 ± .081 (5)	2.580 ± .018 (5)	
114–120	2.650 ± .025 (5)	2.596 ± .048 (5)	2.486 ± .064 (5)	
138–144	2.635 ± .052 (5)	2.595 ± .049 (5)	2.554 ± .088 (5)	1.312 ± .206 (3)

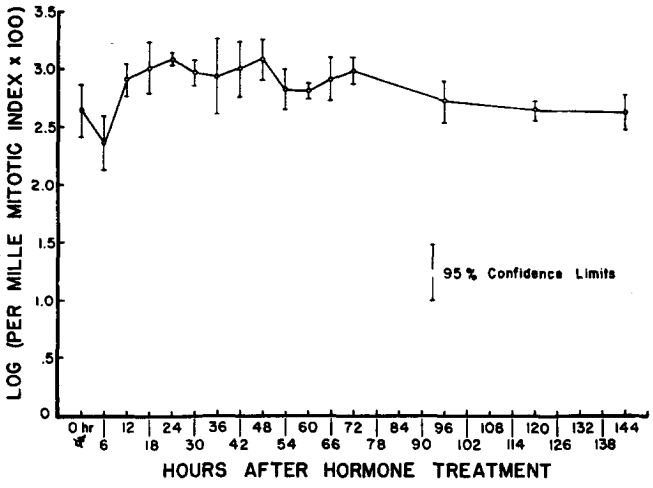

Fig. 2. Time-response of ear malphigian layer epithelial cells to a single injection of 16 micrograms of estradiol benzoate. Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X-6$) to X hours.

ing the fluctuation to be real. Mitotic activity has returned to castrate levels 96 hours following administration of hormone.

Data obtained for seminal vesicle are represented graphically in Fig. 3. A significant increase in mitotic rate above castrate levels is noted during the period 18–24 hours following hormone administration ($P = < .001$). Peak activity is reached about 36 hours following hormone administration. The "plateau" portion of the curve presents a series of regular fluctuations which have an apparent period of 18 hours. Analysis of variance indicated that

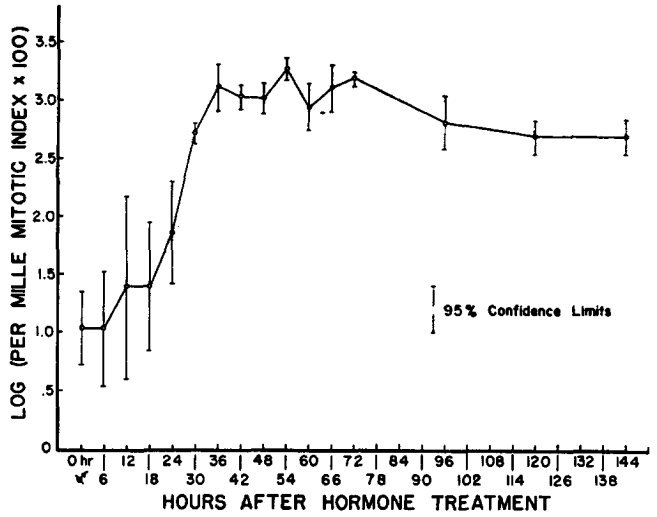

Fig. 3. Time-response of seminal vesicle epithelial cells to a single injection of 16 micrograms of estradiol benzoate. Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X - 6$) to X hours.

these points form a heterogenous series ($P = < .01$) thus suggesting the presence of real periodicity in the data. Mitotic activity is depressed after 72 hours but is still significantly greater (by inspection) than castrate levels 144 hours following hormone administration.

Mitotic activity exhibited by coagulating gland is presented graphically in Fig. 4. The first significant increase in mitotic activity over castrate levels occurs during the period 24–30 hours following administration of estradiol benzoate ($P = < .001$). Peak activity appears to be reached at about 42 hours. Inspection of the "plateau" portion of the curve indicates periodic fluctuations at 12 hour intervals. Analysis of variance indicate these points to form a heterogenous series ($P = < .01$) and suggests the fluctuations to be real. Mitotic activity is depressed 96 hours following hormone injection but is still significantly higher than castrate levels (by inspection) 144 hours after treatment.

Ventral prostate response is represented graphically in Fig. 5. Samples were taken only for the periods 18–24, 42–48, 66–72 and 138–144 hours following hormone treatment. It will be noted that only slight increases in mitotic activity are exhibited by the tissue following estrogen administration. Only during the period 66–72 hours after hormone treatment is an increase in mitotic activity above castrate levels noted ($P = < .001$).

Fig. 5 also shows data for all tissues examined plotted on the same set of coordinates. Examination of the data presented in this fashion makes evident

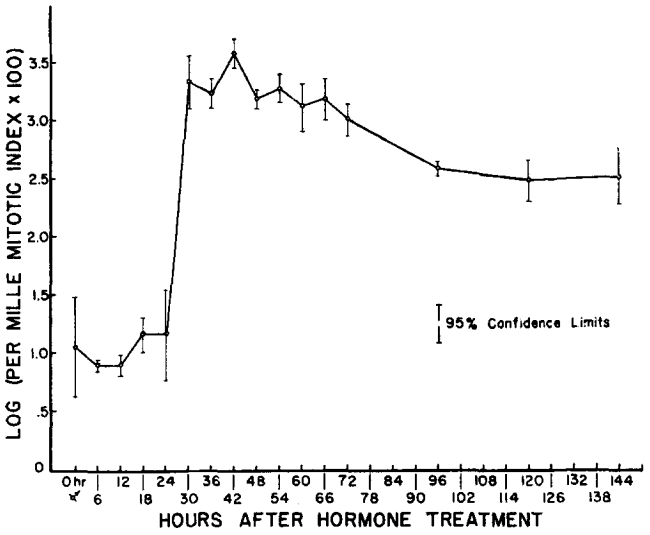

Fig. 4. Time-response of coagulating gland epithelial cells to a single injection of 16 micrograms of estradiol benzoate. Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X-6$) to X hours.

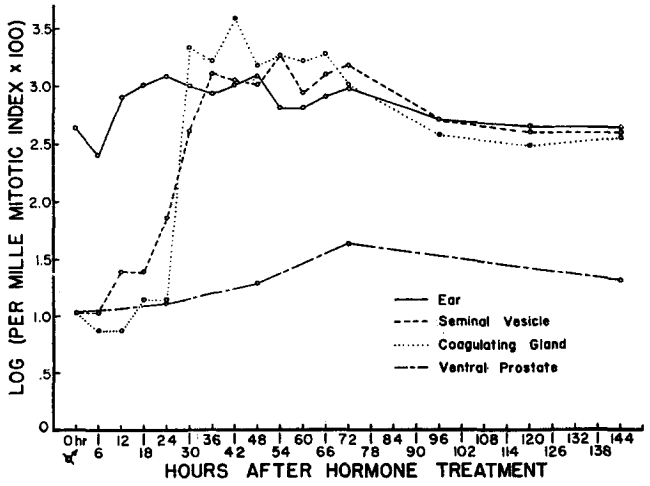

Fig. 5. Time-response of the epithelial cells of malpighian layer of ear, acinar epithelial cells of seminal vesicle, coagulating gland and ventral prostate to a single injection of 16 micrograms of estradiol benzoate. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X-6$) to X hours.

several further points. (*a*) All four tissues show a lag period before commencement of elevated mitotic activity occurs. The lag period for ear is 6 hours, for seminal vesicle 18 hours, for coagulating gland 24 hours and for ventral prostate at least 48 hours. (*b*) The rates at which the tissues reach the "plateau" portions of their curves differ. The rate, expressed as the average per cent of increase above castrate levels per hour, for ear epidermis is 22 per cent/hour, for seminal vesicle 17 per cent/hour and for coagulating gland 131 per cent/hour. (*c*) Although curves for ear, seminal vesicle and coagulating gland reach apparently comparable levels during the period 36–72 hours following hormone treatment, comparison of the mean of means indicates that the average level reached by seminal vesicle is greater than that reached by ear epidermis ($P = < .05$) and that coagulating gland reaches higher average levels than does seminal vesicle ($P = < .01$). (*d*) In terms of relative levels of activity, seminal vesicle and coagulating gland are far more active in their response to estrogen than is ear epidermis.

DISCUSSION

Bullough and van Oordt [10] found that castration lowers mitotic activity in ear epidermis of male mice and that estrogen treatment of castrate males raises this activity. The results reported here confirm and quantify these observations and extend them to the epithelia of seminal vesicle, coagulating gland and ventral prostate of castrate male mice.

The depression of mitotic activity observed in ear epidermis immediately following administration of estradiol benzoate may be due to the action of hormones of adrenal origin released in response to the injected estrogen. Gemzell [15] has demonstrated an increase in the formation and liberation of adrenocorticotrophic hormone in rats following administration of estradiol benzoate, and Green and Ghadially [16] have demonstrated inhibition of epidermal mitosis by cortisone. Inhibition of mitotic activity under the influence of adrenal fraction(s), such as cortisone, may account in part for the lag periods present in the responses of seminal vesicle and coagulating gland to estrogen.

The pronounced difference in time of response to hormonal treatment between ear and seminal vesicle and coagulating gland may be closely linked with the hormone sensitive system(s) through which these materials exert their effect upon cell division. A possible point of hormonal intervention in mitotic activity may be via an effect upon metabolic activity. From a study of the *in vitro* actions of oestrone on ear epidermis Bullough [5] suggests

that this material may effect mitotic activity through action on some metabolic step between extracellular glucose and intracellular pyruvate. The hexokinase reaction was strongly favored as the estrogen sensitive rate limiting step. Estrogen has been shown to increase the activity of this enzyme in uterus [26] but biochemical observations on this effect in other target tissues are lacking. *In vitro* studies on the mitogenic action of insulin on ear epidermis [4] indicate a similar influence on the hexokinase system. Studies on the effct of insulin on mitosis in ventral prostate would be of interest in view of the low levels of activity obtained under the conditons of estrogen administration reported here.

A second possible point of hormonal intervention in mitotic activity might involve reactions concerned with the oxidative metabolism of pyruvate. These pathways are, in general, stimulated by androgenic materials and inhibited by estrogens [20]. Bullough and Johnson [9] observed that cell division in ear epidermis is dependent upon the oxidation of pyruvate. The high levels of mitotic activity seen in seminal vesicle and coagulating gland following estrogenization tend to discount reactions involving the oxidation of pyruvate as rate limiting under these circumstances. O'Connor's suggestion [18] that control of cell division may depend upon a balance between the glycolytic and oxidative phases of carbohydrate metabolism is of merit in light of the above discussion.

A third point at which hormonal materials may control mitotic activity is through an effect upon the alkaline phosphatases. Chèvremont and Firket [12] have reviewed evidence which implicates a relation between phosphatase activity and mitotic activity. The action of this enzyme in cell division may be due to (*a*) facilitation of transmembrane glucose transport [21] or (*b*) to its involvement in the synthesis of DNA [12]. Castration depresses the activity of epithelial alkaline phosphatase in rat ventral prostate while estrogen administration restores values to near normal [2]. Since the mitotic response of rat ventral prostate to estrogen [11] is similar to that observed in the mouse, these results indicate that, in this tissue, alkaline phosphatase is not a limiting factor to mitotic activity.

Roberts and Szego [19] followed respiration in rat uterus after intravenous administration of estradiol and found sharply elevated rates of oxygen utilization in this tissue 20 hours following hormone administration. This increase in respiration corresponds with the beginning of cellular proliferation in this tissue. The similarity of this lag period to that observed in seminal vesicle and coagulating gland before onset of increased mitotic activity is of interest.

The disparity in lag phases between seminal vesicle and coagulating gland

on the one hand, and ear epidermis on the other, may be explained on the basis of different sensitivities to hormone lack in the two types of tissue. Estrogen, during the 18–24 hours following its administration, initiates the development of metabolic systems to levels compatible with the energy requirements of mitosis in seminal vesicle and coagulating gland. In ear epidermis the metabolic systems are not so absolutely hormone dependent and added estrogen serves only to increase the already high levels of activity still further.

Voutilainen [25] has reviewed the literature dealing with the existence of a 24-hour mitotic rhythm in plant and in normal and malignant animal tissues. He concluded that such a rhythm is a generally observed phenomenon. Bullough [8] found rhythmic activity in the tissues of the mouse to be dependent upon such factors as food intake and body activity. In several tissues he showed similar patterns of diurnal rhythms. Blumenfield [3], to the contrary, found a lack of correspondence between various diurnal rhytms observed in several tissues. If all tissues show a similar 24-hour rhythm of mitotic activity, the "plateau" portions of the curves obtained in the present work would be straight lines or would show random fluctuations since they were sampled at 1400 hours. This is not the case. It must be assumed that these tissues (seminal vesicle, coagulating gland and ear) exhibit rhythms of activity in addition to a regular 24-hour cycle. Two factors may be implicated in this cyclic behavior. (*a*) Exogenously administered estrogen may have variable effects depending upon the condition of the cells upon which it acts. If this be the case, exaggeration of cycles might be expected. (*b*) If the pituitary-adrenal axis is compared to a closed-cycle feed-back system [17, 24], it might be expected that exogenously added estrogen may set periodic oscillations in the liberation of adrenal hormones, which would be reflected as regular fluctuations in the mitotic activity of such tissues as ear and seminal vesicle. The fact that the oscillations observed appear characteristic for each tissue suggests the presence of inherent cellular factors.

SUMMARY

The development of mitotic activity following the administration of a small dose of estradiol benzoate has been followed in the epithelia of ear, seminal vesicle, and coagulating gland of the castrate male mouse. Intrinsic and extrinsic variability have been closely controlled. All tissues respond to estrogen by increased mitotic activity. Seminal vesicle and coagulating gland reach the highest absolute values; ear is intermediate in response; and ventral

prostate responds weakly. Ear epidermis responds significantly within 6 hours after hormone administration; seminal vesicle and coagulating gland respond significantly about 24 hours following treatment; and significantly elevated activity is not noted in ventral prostate until 72 hours after hormone treatment. Periodic fluctuations are noted in the mitotic activity curves which suggest the presence of tissue specific rhythms in addition to the previously demonstrated 24-hour mitotic cyle. These results are discussed in terms of the hormonal effects upon the metabolic systems which may be involved in mitotic activity.

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