A Histochemical Study of 3(β)-Sterol Dehydrogenase in the Adrenal Cortex of the Developing Mouse[1]

JOHN M. ALLEN
Department of Zoology, The University of Michigan, Ann Arbor, Michigan

The function of the X-zone of the adrenal cortex of the mouse has long been problematic. Howard ('39) suggested that this zone might function in androgen secretion under certain circumstances. This contention, however, is by no means certain (Jones, '57). Attempts to characterize this zone enzymatically have done little to clarify its functional status. Allen ('58 and '59) found that both aliesterase and DPN-diaphorase reached maximum activity levels in the X-zone about the 14th day of age. After this time the activities of these enzymes declined. The results suggested emergence of some functional activity prior to the 14th day of age which might be associated with either lipid or oxidative metabolism. No inference relating to secretory activity could be drawn from these studies.

Recent development of a histochemical method for the localization of 3(β)-sterol dehydrogenase (Wattenberg, '58) has made possible the visualization of enzymatic activity associated with certain phases of steroidogenesis. Application of this method to the study of the X-zone might be expected to yield information relating to the capacity of this area for the formation of intermediates in the synthesis of particular steroid hormones. The present paper deals with histochemically determined 3(β)-sterol dehydrogenase activity in the X-zone and permanent cortex of the adrenal gland of developing mice.

METHODS

All work was carried out on adrenal glands from BALB/c Jax mice. Newborn and older animals were dated in reference to the time of birth as determined by animal inspection at 0800 hours. 3(β)-sterol dehydrogenase was determined histochemically by a modification of the method proposed by Wattenberg ('58). This substrate solution contained the following reactants: 0.01 M Δ^5-androstene-3(β)-ol-17-one (dehydroepiandrosterone) (Sigma) in acetone, 1.5 cm^3; diphosphopyridine nucleotide (DPN) (Sigma), 10 mgm; 0.1 M phosphate buffer pH 7.5, 8 cm^3; 0.03 M potassium cyanide pH 7.5, 5 cm^3; nitro blue tetrazolium (Dajac), 3.5 mg or blue tetrazolium (General Biochemicals), 7 mg; and water to make 30 cm^3. This simplified substrate mixture gave results equivalent to or superior to those obtained with the substrate mixture originally proposed by Wattenberg ('58). Glands for study were sectioned at 12 μ in a microtome crystotat maintained at −20°C. Prior to sectioning these glands were thrust into the interior of small cubes of liver and were quick frozen by placing them in a test tube immersed in a slush of dry ice and acetone. The use of liver tissue as a surrounding made the sectioning of very small glands a simple matter. Sections were mounted on glass slides with no adhesive. Chemical fixatives were not employed. Before immersion in the histochemical substrate solution these sections were removed from the cryostat, rapidly dried in an air stream, and defatted in acetone at room temperature for 30 seconds. The acetone extraction improved the sharpness of the reaction and did not affect its intensity or localization. Sections were incubated at 37°C for 30 minutes when substrate solutions containing nitro blue tetrazolium were used or for one hour when substrate solutions containing blue tetrazolium were used. During this incubation a stream of nitro-

[1] This work has been supported by funds from a U. S. Public Health Service grant C-2751(C-3), The Horace H. Rackham School of Graduate Studies of The University of Michigan, and the Cancer Research Institute of The University of Michigan.

gen was used to agitate the solution. Histochemical analysis was carried out on a minimum of 10 animals equally divided between the sexes for each age period studied.

Although prior study of DPN-diaphorase activity in the developing adrenal gland had been undertaken (Allen, '59) it seemed pertinent to re-examine this activity under conditions in which $DPNH_2$ was supplied as such rather than generated by the action of alcohol dehydrogenase upon ethanol (Cascarano and Zweifach, '59). The substrate solution for the histochemical determination of DPN-diaphorase under these conditions contained: $DPNH_2$, (Sigma) 10 mg; 0.1 M phosphate buffer pH 7.5, 8 cm^3; 0.03 M potassium cyanide pH 7.5, 5 cm^3, nitro blue tetrazolium (Dajac), 3.5 mg or blue tetrazolium (General Biochemicals) 7.0 mg; and water to make 30 cm^3. Sections for use with this substrate solution were treated exactly as for the demonstration of 3(β)-sterol dehydrogenase. Optimum incubation time when nitro blue tetrazolium was used was 4 minutes at 37°C or 8 minutes at 37°C when blue tetrazolium was employed.

RESULTS

3(β)-sterol dehydrogenase

Day of birth (fig. 1). Substrate solutions employing nitro blue tetrazolium or blue tetrazolium yielded very low levels of acticity in the zona fasciculata. Some cells in the zona fasciculata had relatively high activity as compared to their neighbors. When blue tetrazolium was employed as an electron acceptor the cells of the zona glomerulosa showed no activity. These same cells, however, showed levels of activity equal to those seen in the zona fasciculata when nitro blue tetrazolium was used. Sections from male and female animals showed equivalent patterns and levels of activity. At this time (and at all others investigated) deposits of reduced tetrazolium were confined to the general cytoplasm of reactive epithelial cells. Connective tissue cells were negative.

Seven days of age (fig. 2). Sections incubated in substrate solutions containing either nitro blue tetrazolium or blue tetrazolium showed an increase in activity of the cells of the zona fasciculata. Sections incubated in nitro blue tetrazolium solutions showed low levels of activity in the zona glomerulosa which were equal to those seen on the day of birth and which were lower than levels prevailing in the zona fasciculata at 7 days of age. Sections incubated in blue tetrazolium showed no reaction in the zona glomerulosa. In a few fortuitous sections it was possible to see that two or three layers of cells lying next to and intermingled with the cells of the medullary tissue gave no reaction with either electron acceptor. These cells probably represent the "interlocking zone" of Waring ('35). No sex difference in enzyme activity was noted.

Fourteen days of age (fig. 3). Sections incubated in substrate solutions containing blue tetrazolium showed levels of activity in the zona fasciculata which were equivalent to those seen at 7 days of age. The cells of the zona glomerulosa and of the X-zone were negative.

Sections incubated in substrate solutions containing nitro blue tetrazolium showed levels of activity in the zona fasciculata equivalent to those noted at 7 days of age. Activity in the zona glomerulosa showed no change over that noted at the day of birth and at 7 days of age. The X-zone of most glands examined showed no reaction. However, occasional glands from females at this age gave a moderately positive reaction in a band of cells situated in the innermost region of the X-zone, i.e., in that portion of the X-zone just adjacent to the adrenal medullary tissue. Except for this occasionally present active area in the X-zone of female animals no sex difference was noted.

Twenty-one days of age (figs. 4 and 5). Sections incubated in substrate solutions containing blue tetrazolium showed slight increases in the activity of the enzyme in the cells of the zona fasciculata. As at previous ages the cells of the zona glomerulosa and the X-zone were negative. No sex difference was present in enzyme activity in any of the cortical zones.

Sections incubated in solutions containing nitro blue tetrazolium showed moderate increases in the activity of the enzyme in the zona fasciculata over that seen at 14 days of age. The activity of the zona glomerulosa was equivalent to that seen

at previous ages. When present, the X-zone of all males examined was negative. The X-zone of all females examined, however, showed the presence of moderate activity in an inner band of cells situated adjacent to the medullary tissue. No sex difference in the activity of any other cortical region was noted.

Steps were taken to determine the time of development of the inner active region of the X-zone in females. A number of animals were examined at 13, 14, 15, and 16 days of age for the presence or the absence of this activity. These results are summarized in table 1. The data show that this activity was never observed in male animals (which at these times still possessed a clearly discernible X-zone). It was, however, a constant feature of the zone in the female of 16 days of age but was only occasionally noted at 13 or 14 days of age.

Twenty-eight days of age (figs. 6 and 7). No divergence from the situation prevailing at 21 days of age was noted.

Sixty and ninety days of age (figs. 8 and 9). Patterns and levels of activity were equivalent to those noted at 21 and 28 days of age.

DPN-diaphorase

Patterns of diaphorase activity were equivalent to those previously described (Allen, '59). Substitution of $DPNH_2$ for DPN, alcohol and alcohol dehydrogenase resulted in somewhat higher levels of activity in all areas. Likewise the substitution of nitro blue tetrazolium for blue tetrazolium resulted in appreciably greater dye deposition in all areas of the adrenal cortex. The important point is that there is little possibility that the activity of DPN-diaphorase is a limiting factor in the study of 3(β)-sterol dehydrogenase in the developing adrenal gland of the mouse. At all stages investigated and in all areas of the cortex dye deposition due to the activity of DPN-diaphorase was many times greater than dye deposition due to the action of the 3(β)-sterol dehydrogenase-DPN-diaphorase system.

Tests for specificity and quantitative determinations

Specificity. Sections incubated in substrate solutions from which DPN had been omitted failed to give any reaction. Likewise sections incubated in substrate solutions from which dehydroepiandrosterone and acetone had been omitted failed to react. Sections from which the steroid had been omitted but which included acetone also failed to react. When tested on sections derived from 90-day old females and 21-day old females it was found that the substitution of Δ^4-androstene-3, 17-dione (androstenedione) or Δ^4-androstene-17(β)-ol-3-one (testosterone) or estratriene-3-ol-17-one (estrone) or estratriene-3, 17-diol (estradiol) for dehydroepiandrosterone yielded no reaction. Δ^4-pregnene-3(β)-ol-20-one (pregnenolone) yielded a reaction comparable in distribution and intensity to that obtained with dehydroepiandrosterone. From these studies it is concluded that the histochemical reactions obtained with dehydroepiandrosterone represented true enzymatically catalyzed oxidations of the substrate and were not artifactual localizations of the "nothing dehydrogenase" type recently described by Zimmermann and Pearse ('59). Further it appears that the activity of the enzyme studied is restricted to the 3(β)-ol grouping, for negative reactions were obtained with the 17

TABLE 1

Time of appearance of 3(β)-sterol dehydrogenase activity in the X-zone[1]

Age	Males		Females	
	Present	Absent	Present	Absent
days				
13	0	4	1	7
14	0	14	5	5
15	0	3	10	1
16	0	14	9	0

[1] Total number of animals investigated at a particular age is the horizontal sum of the appropriate column.

(β)-ol grouping of testosterone and estradiol.

Quantitation. Serious attempts to determine the activity of 3(β)-sterol dehydrogenase by quantitative methods met with failure. Using the method proposed by *Talalay and Dobson* ('53) it was found that complex formation between DPN and cyanide (Colowick, Kaplan and Ciotti, '51) interfered with optical density measurements of DPN reduction at alkaline pH values. Systems from which cyanide was omitted failed to show any reduction of DPN between pH 7.0 and pH 9.0. Due to the small amount of adrenal tissue available for analysis even partial purification of homogenates was out of the question. Attempts to measure 3(β)-sterol dehydrogenase activity through the use of a coupled reaction system involving dehydroepiandrosterone, DPN and dichlorophenolindophenol (Allen, unpublished data) as the electron acceptor likewise met with failure. In this case it was found that an unknown reducing material was present in adrenal homogenates which resulted in a rapid and non-enzymatic reduction of dichlorophenolindophenol. This reducing material did not interefere with determinations of DPN-diaphorase in developing adrenal glands (Allen, '59) because of the high rate of enzyme catalyzed reduction relative to non-enzymatic reduction. Finally, attempts to utilize the method proposed by Beyer and Samuels ('56) met with failure due to the inadequate size of tissue samples for analysis.

DISCUSSION

The significance of these results depend upon the role of 3(β)-sterol dehydrogenase in steroid synthesis. An implication in the formation of estrogens is unlikely due to the inability of the enzyme to oxidize the hydroxyl group of ring A of either estrone or estradiol. A similar implication applies to the failure of the enzyme to oxidize the 17(β)-ol group of estradiol. However, a role in the biogenesis of corticosteroids and androgens is likely (Dorfman, '55). Thus 3(β)-sterol dehydrogenase is apparently involved in the formation of progesterone from pregnenolone by oxidation of the 3(β)-ol group of the latter compound. Progesterone is an intermediate in the biosynthesis of cortisol and corticosterone and may be an intermediate in the formation of aldosterone. The enzyme is also involved in the production of the adrenal androgen, Δ^4-androstene-11 (β)-ol-3, 17-dione, by virtue of its capacity to oxidize the 3(β)-ol group of dehydroepiandrosterone. Thus the presence of 3(β)-sterol dehydrogenase may be indicative of either corticosteroid or androgen formation.

The presence of 3(β)-sterol dehydrogenase in the permanent cortex of the adrenal gland is not surprising. In this location it is most likely involved in corticosteroid formation. The behavior of the enzyme during development indicates that by the time of birth the adrenal cortex of the mouse has a capacity for the production of corticoids. The activity of the enzyme increases slowly until levels of activity comparable with those noted in the adult gland are reached by the 21st day of age. Aliesterase (Allen, '58) and DPN-diaphorase (Allen, '59) also reached adult levels of activity by the 21st day of age. These results point to the acquisition of adult levels of function by the adrenal cortex of the mouse by the end of the third week of life. Similar conclusions have been drawn by Moog ('53, '54) from studies of a different nature.

The behavior of 3(β)-sterol dehydrogenase in the X-zone is by no means as clear cut. Activity was never observed in the male but in the female moderate levels emerged in the inner portions of the zone between the 13th and the 16th day after birth. This activity, in the female, remained into adult life. The identification of an enzyme in the X-zone which may be implicated in steroidogenesis is of significance. Up to the present time the general concensus of opinion regarding the X-zone has been that neither androgen nor corticoid production takes place in this tissue. Allen ('59) mentioned failure to find 3(β)-sterol dehydrogenase in the X-zone when using blue tetrazolium as an electron acceptor. The use of nitro blue tetrazolium, a far more sensitive electron acceptor, has invalidated this observation. It appears that the X-zone, by virture of its content of 3(β)-sterol dehydrogenase, must now be viewed as a potential steroid hormone producer. The decision as to whether it

may produce androgens or corticoids, both, or neither is impossible on the basis of this work.

The course of development of 3(β)-sterol dehydrogenase activity in the X-zone differs markedly from that of either aliesterase and DPN-diaphorase. Both aliesterase (Allen, '58) and DPN-diaphorase (Allen, '59) reached their maximum expression in the X-zone by the 14th day of age and then declined in activity. The behavior of both enzymes was identical in males and females. The association of 3(β)-sterol dehydrogenase activity with the X-zone of the female but not with that of the male suggests the factor(s) responsible for the maintenance of the zone in females may also be involved in the development of the activity of the enzyme. Experiments carried out by Jones ('57) indicate that the factor responsible for the maintenance of the X-zone is probably pituitary luteinizing hormone (LH). Androgen production by the testis of the developing male suppresses production of LH and leads to the atrophy of the zone. This hypothesis accounts for the presence of 3(β)-sterol dehydrogenase in the female X-zone from the 14th day onward. It fails, however, to account for the similarities of behavior of aliesterase and DPN-diaphorase. It is possible that other humoral factors regulate the development of aliesterase and DPN-diaphorase in this tissue or that the early development (prior to 14 days of age) of these enzymes is not under endocrine control. In this latter case the activity of aliesterase and DPN-diaphorase might reflect metabolic changes associated with the early growth of the zone (Allen, '59).

SUMMARY

The activity of 3(β)-sterol dehydrogenase in the X-zone and permanent cortex of the mouse adrenal gland during development was investigated using histochemical methods. The enzyme was absent from the X-zone of the male. In the female, however, the inner portions of the zone gave a moderate reaction by the 16th day of age. This activity remained into adult life. In the permanent cortex, low levels of activity were present in the zona glomerulosa and the zona fasciculata at the time of birth. The activity of the zona fasciculata gradually increased during the first 21 days after birth. By the end of this time levels of activity were comparable with those observed in the zona fasciculata of 90-day old males and females. There was no increase in the activity of the enzyme in the cells of the zona glomerulosa over that observed on the day of birth. No sex difference in the activity of the zona glomerulosa or the zona fasciculata was noted. The possible role of 3(β)-sterol dehydrogenase in the biosynthesis of adrenal cortical hormones and androgens is discussed. It is concluded that the presence of 3(β)-sterol dehydrogenase in the cells of the X-zone may be indicative of a capability for steroid hormone formation.

LITERATURE CITED

Allen, J. 1958 A chemical and histochemical study of aliesterase in the adrenal gland of the developing mouse. Anat. Rec., *132:* 195–208.

——— 1959 A chemical and histochemical study of DPN-diaphorase activity in the adrenal cortex of the developing mouse. Ibid., *134:* 385–395.

Beyer, K., and L. Samuels 1956 Distribution of steroid-3(β)-ol dehydrogenase in cellular structures of the adrenal gland. J. Biol. Chem., *219:* 69–76.

Cascarano, J., and B. Zweifach 1959 Factors influencing the histochemical demonstration of coenzyme-dependent dehydrogenases and diaphorases. J. Biophys. Biochem. Cytol., *5:* 309–318.

Colowick, S., N. Kaplan and M. Ciotti 1951 The reaction of pyridine nucleotide with cyanide and its analytical use. J. Biol. Chem., *191:* 447–457.

Dorfman, R. 1955 Steroid hormone metabolism. In: The Hormones, vol. 3, ed. G. Pincus and K. Thimann. Academic Press Inc., New York, pp. 589–664.

Howard, E. 1939 Effects of castration on the seminal vesicles as influenced by age, considered in relation to the degree of development of the adrenal X-zone. Am. J. Anat., *65:* 105–149.

Jones, I. 1957 The Adrenal Cortex. Cambridge University Press, Cambridge.

Moog, F. 1953 The functional differentiation of the small intestine. III. The influence of the pituitary-adrenal system on the differentiation of phosphatase in the duodenum of the suckling mouse. J. Exp. Zool., *124:* 329–346.

Moog, F., D. Bennett and C. Dean 1954 Growth and cytochemistry of the adrenal gland of the mouse from birth to maturity. Anat. Rec., *120:* 873–892.

Talalay, P., and M. Dobson 1953 Purification and properties of a β-hydroxysteroid dehydrogenase. J. Biol. Chem., 205: 823–838.

Wattenberg, L. 1958 Microscopic histochemical demonstration of steroid-3-beta-ol dehydrogenase in tissue sections. J. Histochem. Cytochem., 6: 225–232.

Waring, H. 1935 The development of the adrenal gland of the mouse. Quart. J. Micr. Sci., 78: 329–366.

Zimmermann, H., and A. Pearse 1959 Limitations in the histochemical demonstration of pyridine nucleotide-linked dehydrogenases ("Nothing Dehydrogenase"). J. Histochem. Cytochem., 7: 271–275.

PLATE 1

EXPLANATION OF FIGURES

All figures represent a final magnification of 140 times. Processing and printing of all photographs was carried out under identical conditions.

All figures show the distribution of 3(β)-sterol dehydrogenase activity when nitro blue tetrazolium was used as the electron acceptor and incubation was carried out at 37°C for 30 minutes.

1 Day of birth. Low levels of activity are present in the cells of the zona glomerulosa and the zona fasciculata. The adrenal medulla is unreactive (lower half of figure).

2 Seven day male. Activity in the zona fasciculata has increased over that seen on the day of birth (fig. 1) but activity in the zona glomerulosa remains unchanged.

3 Fourteen day female. Activity in the zona fasciculata is equivalent to that noted at 7 days of age (fig. 2). Activity in the zona glomerulosa remains unchanged. The X-zone shows low levels of activity in its inner portions (bottom of figure).

4 Twenty-one day male. Activity in the zona fasciculata is slightly increased over that noted previously (figs. 1, 2, and 3). Activity in the zona glomerulosa remains unchanged. Activity is absent in the X-zone (bottom of figure).

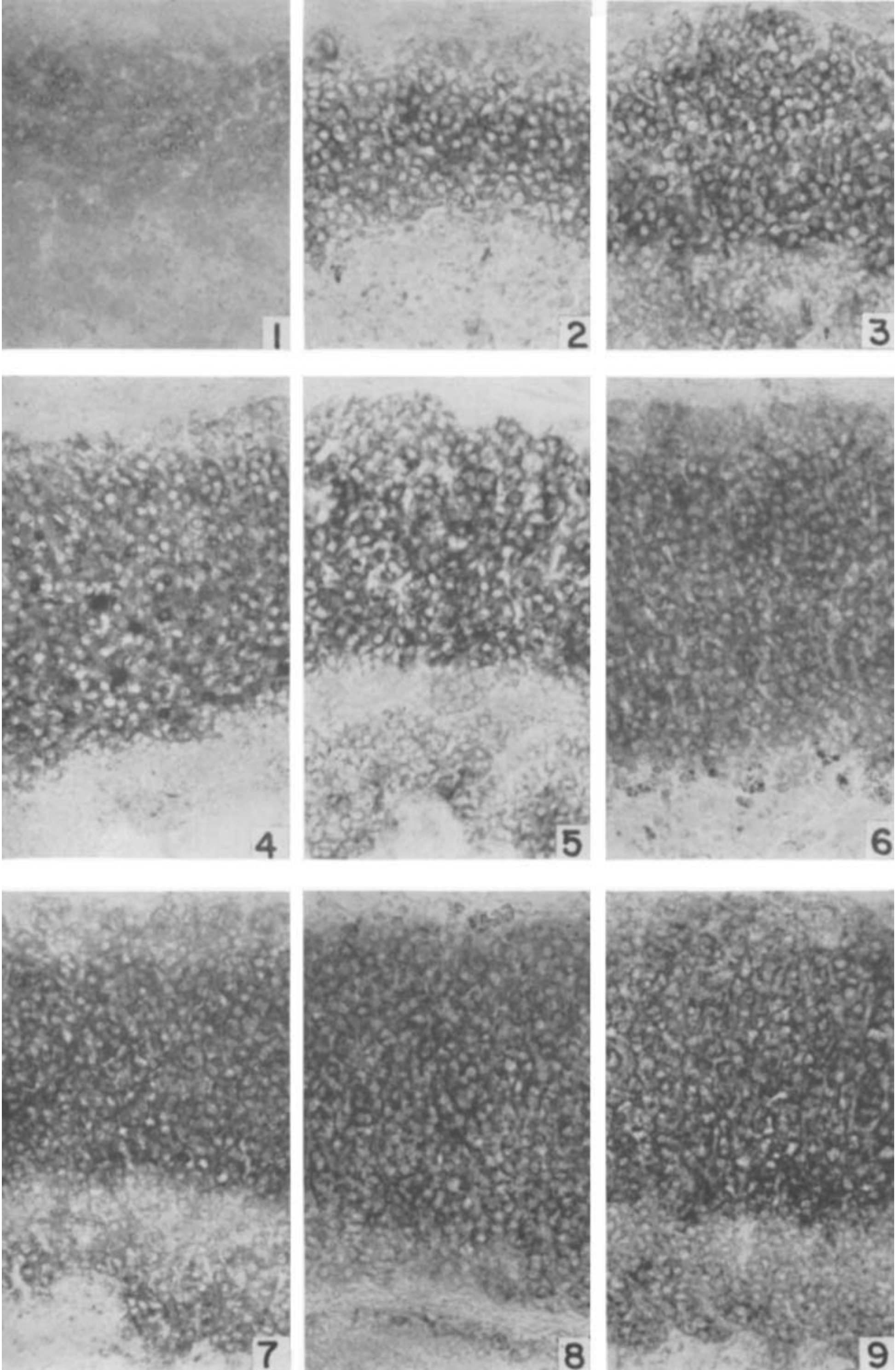
5 Twenty-one day female. Activity in the zona glomerulosa and the zona fasciculata are as in the 21-day old male (fig. 4). The innermost cells of the X-zone (bottom of figure) show moderate activity.

6 Twenty-eight day male. Activity in the zona glomerulosa and the zona fasciculata remains unchanged over that seen at 21 days of age (figs. 4 and 5). The X-zone has largely disappeared.

7 Twenty-eight day female. Activity in the zona glomerulosa and the zona fasciculata remains as at 21 days of age (figs. 4 and 5). Moderate activity is present in the inner portions of the X-zone (bottom of figure).

8 Sixty day male. Activity in the zona glomerulosa and the zona fasciculata is equivalent to that seen at 28 days of age (figs. 6 and 7). The X-zone has disappeared.

9 Sixty day female. Activity in the zona glomerulosa and the zona fasciculata is equal to that seen at 28 days of age (figs. 6 and 7). The well developed X-zone (bottom of figure) shows moderate levels of activity in its inner areas.


1
2
3
4
5
6
7
8
9