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EXPERIMENTAL AUTOIMMUNE ADRENALITIS IN THE RAT

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SUMMARY

A single innoculation of an autologous adrenal extract produced autoimmune adrenalitis in rats. The histological changes were correlated with the plasma corticosterone levels and the capacity of *in vitro* steroid biosynthesis. Peripheral corticosterone values were determined by the competitive protein binding method. The *in vitro* studies were performed using [^{14}C]-progesterone as precursor steroid and incubating with glandular tissue. The immunized female rats showed a significant decrease in plasma circulating corticosterone: 18 $\pm$ 3.6 μg/100 ml to 13.5 $\pm$ 1.4 μg/100 ml ($P < 0.05$).

In vitro studies demonstrated a lower conversion into corticosterone in incubations with the tissue of animals with adrenalitis, possibly due to two facts with a parallel action: (1) Alteration in the 11-betahydroxylase system and in the enzymatic cofactors responsible for the hydroxylation of desoxycorticosterone in C_{11}. Electron microscopy revealed partially destroyed mitochondrial cristae and lipid inclusion bodies, thus confirming this biochemical fact; (2) Slow metabolization of the precursor steroid added to the incubation medium, consequent upon a more accelerated exhaustion of the glandular tissue in immunized animals.

The conclusion arrived at is that immunization provokes tissue lesions in the adrenal glands of female rats discernible both by light and electron microscopy. These lesions can be correlated with the biochemical findings in so far as concerns steroid synthesis.

INTRODUCTION

There exist numerous pathological conditions of unknown origin in which an immunologic mechanism is the suspected physiopathologic agent. The familial incidence in such conditions and the presence of more than one irregularity in the immune system suggest that a genetically determined predisposition may be at play. In many of these patients it has been possible to demonstrate the presence of circulating antibodies[1–4] though doubts still prevail as to their pathogenicity.

In order to determine an equivalent of these human diseases in laboratory animals studies have been carried out with encouraging results. The investigation covering adrenal cortex insufficiency and its relation with autosensitization seem to lead to an attractive though complex field of study. Thus, the production of allergic adrenalitis became possible through the use of adrenal extracts[5–11] which, upon being incorporated to favorable adjuvating substances, originated infiltrative lesions and cellular deterioration within a period of 10–14 days. The animals produce circulating antibodies which can be detected by means of adequate techniques.

The present investigation was aimed at establishing whether the glandular lesion was accompanied by a state of hormonal deficiency, and at the same time whether this deficiency was a consequence of a change in or the blocking of the pathways of steroid synthesis, or of a reduced production of hormones due to the presence of a partially destroyed tissue. The results obtained in animals in which immunization provoked glandular alterations as determined by light and electron microscopy, point to a decrease in the concentration of circulating corticosterone together with a lowered potential capacity in the tissues to bring about hormonal biogenesis, thus indicating a clear relation between glandular tissue damage and hormonal insufficiency.

MATERIALS AND METHODS

Animals used: The young rats used in this investigation were of the Lewis strain and weighed between 150 and 175 g. They were bred in this Institute and were fed on "Forramex". Water was given *ad lib.*

Antigenic material. The glandular tissue was collected from animals sacrificed by exsanguination working in a cool room kept at 4°C. By means of careful dissection the tissue was liberated entirely of fat and connective material. If not used immediately, the organs were preserved at -40°C. The tissue extract was prepared by finely sectioning the glands in a medium of phosphate buffer at pH 7.2, 2 ml of buffer being used per gram of tissue. The tissue was homogenized for 5 min by means of a Teflon homogenizer. The preparation was exposed to ultrasound 20,000 c.p.s., for 3 min using the MSE-Mullard ultrasonic disintegrator. After 1–2 h of additional

extraction, the preparation was centrifuged at 3000 rev./min during 20 min in a refrigerated centrifuge. The supernate consisted in a fine suspension with a protein concentration of approximately 3.5 mg/100 ml; to it was added an equal volume of complete Freund adjuvant and emulsification was carefully undertaken during 30 min at a temperature not exceeding 4°C.

Immunization. Each rat was given a single intradermal injection of 0.2 ml of the antigenic mixture in the interdigital areas of the foot pads; at the same time they were injected in the dorsal area with 0.5 ml of *Bacillus pertussis* (concentration of 202×10^9 cells/ml) in order to enhance antibody response. The studies were carried out 15 days after immunization. The animals were sacrificed by decapitation without prior anesthesia, and the evaluation was made in the following groups: normal (non-treated) rats; rats used as controls (treated with Freund adjuvant and *B. pertussis*) and immunized rats (treated with Freund adjuvant, *B. pertussis*, and adrenal antigen).

Histological examination. A portion of the glands from the different groups of animals was set aside for histological examination. After embedding the specimens in paraffin, 6 μm sections were obtained and submitted to hematoxylin-eosin stain and PAS reaction.

In the case of electron microscope study the tissues were fixed in glutaraldehyde with 0.1 M, pH 7.25, phosphate buffer. Fixation was carried out with osmium tetraoxide in the same buffer. Subsequently, the tissues were treated with propylene oxide and mounted in Epon 812. The sections were obtained with a Porter-Blum microtome and stained with uracil acetate and lead citrate.

Animal procedure. Fifteen days after immunization the animals were killed by decapitation without anesthesia at 8 am, the adrenals being rapidly removed. The glands derived from 10 normal, 8 control and 12 immunized male rats, and from 10 normal, 8 control and 12 immunized female rats, were cleared of surrounding fat and connective tissue. Adrenals from rats of the same group were then pooled and weighed.

Table 1. Identification of corticosterone and aldosterone

Stage of purification	Solvent	S.A. d.p.m./μg	
		B	Aldo
After addition of the corresponding control steroid	—	1430	750
1st t.l.c.	Benzene 90% Methanol 10%	1610	840
Acetylation			
2nd t.l.c.	Benzene 95% Methanol 5%	1560	730
Hydrolysis			
3rd t.l.c.	Benzene 4% Ethyl acetate 1%	1500	—

B = Corticosterone.
Aldo = Aldosterone.

Table 2. Identification of 11-desoxycorticosterone

Stage of purification	Solvent	S.A. d.p.m./μg
After addition of DOC acetate	—	1950
1st t.l.c.	Benzene 96% Methanol 4%	1900
2nd. t.l.c.	Benzene 98% Methanol 2%	1860
Hydrolysis		
3rd t.l.c.	Benzene 93% Methanol 7%	1850

Blood from each rat was submitted individually to plasma corticosterone determination which was performed in accordance with the competitive protein binding method described by Murphy[12] with some modifications[13].

Incubation. The glands were sectioned in slices and preincubated during 30 min in Krebs–Ringer-bicarbonate buffer at pH 7.4 to which had been added 100 mg % glucose. At the end of this period the glandular tissue was divided in aliquots in bottles containing 5 ml of the buffer solution to which was added 0.5 μCi [^{14}C]-progesterone (S.A. 37 mCi/mmol.) The incubations were carried out in a water bath at 37°C under constant agitation during 30, 60 and 90 min in an atmosphere containing 95% oxygen and 5% carbon dioxide. At the end of each period the reaction was stopped by adding cold dichloromethane to the incubation tubes, the metabolic products being then rapidly extracted.

Chromatographic separation of steroids. Thin-layer and paper chromatography were used for the separation of the various steroids. Detection of the different radioactive fractions obtained in the chromatographic separation was accomplished by means of a Packard Radiochromatogram Scanner, model 7201[14–17].

Isotope dilution analysis. The isotope dilution procedure used to identify most of the radioactive metabolites consisted of elution of the radioactive peak from a paper chromatogram with methanol, addition of about 50 μg of carrier steroid, determination of specific activity of this solution, t.l.c. on silica gel of the free steroid and its acetate (if formed) with determination of specific activity following each step. Amounts of the steroid were determined by ultraviolet spectrophotometry (Beckman D. B.) at about 240 nm against a methanol blank. Allen's correction (18) was employed (Tables 1, 2 and 3).

Table 3. Identification of progesterone

Stage of purification	Solvent	S.A. d.p.m./μg
After addition of progesterone	—	8410
1st t.l.c.	Benzene 90% Methanol 10%	8300
2nd t.l.c.	Benzene 4% Ethyl acetate 1%	8310

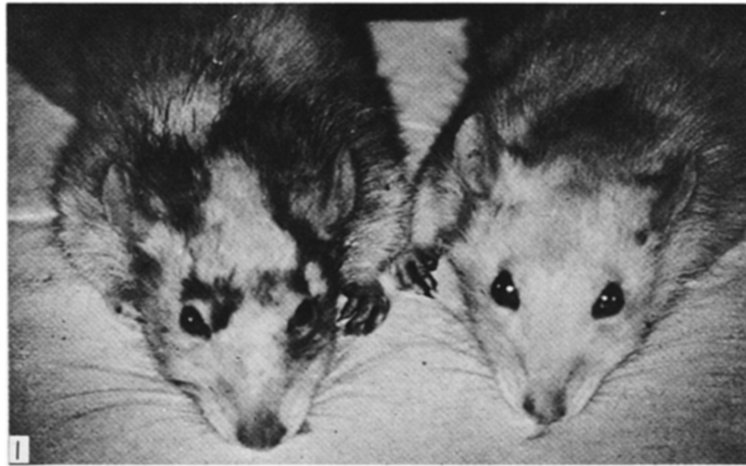

Fig. 1. (Left) Female rat sensitized with adrenal extract and complete Freund adjuvant. (Right) Non-sensitized female rat used as control.

RESULTS

Throughout the experiments the sensitized animals were watched closely in order to detect clinical changes revelatory of a possible adrenal failure. Upon comparing with non-injected animals few variations were found as to weight, appetite or behaviour. A few of them, however, exhibited some degree of apathy and hair of a darker color on the head, accompanied by alopecic areas over the neck (Fig. 1).

In view of the fact that previous studies[11] had revealed that the more significant glandular lesions manifested themselves 15 days after the injection of antigenic material, it was decided to sacrifice the different groups of animals at the end of this period. The peripheral corticoid values in non-injected (normal) female rats amounted to 18.6 ± 3.6 μg/100 ml as against 13.5 ± 1.4 μg/100 ml in immunized animals ($p < 0.05$). The female rats injected only with Freund adjuvant and *B. pertussis* did not reveal significant variations with respect to the normals: 18.9 ± 5.2 μg/100 ml (Fig. 2).

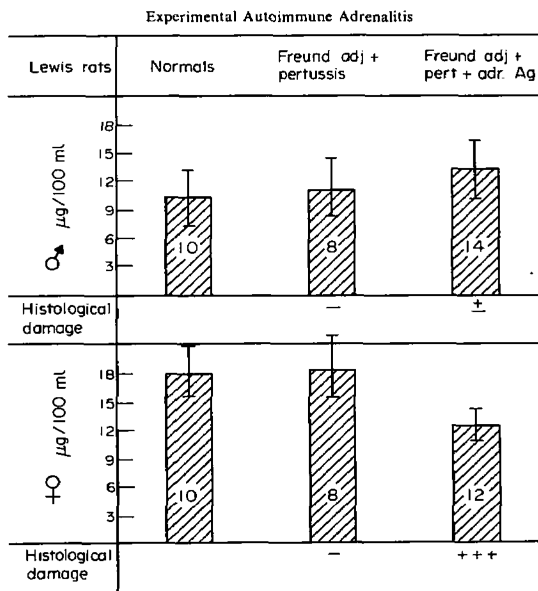

Fig. 2. Peripheral corticosterone in female and male rats (Mean ± S.D.). Numbers inside bars indicate animals used in each group.

The decrease in peripheral corticosterone in the immunized female rats was accompanied by a histological picture characterized by mononuclear cell infiltrates, mainly composed of lymphocytes and plasma cells. These cells were grouped together in foci of different sizes localized throughout the adrenal cortex, excepting the glomerular area (Figs. 3 and 4).

In the fasciculata and reticularis zones some of the adrenal cells had lost their typical stranded pattern; others revealed anomalies such as eosinophilia and vacuolation of the cytoplasm.

Electron microscopy also revealed infiltrates around the cortical cells (Fig. 5) and inside them as well on account of the rupture of the membrane (Fig. 6). This lymphocytic penetration was accompanied by evident changes in the cellular architecture (Fig. 7). Moreover, the electron microscope study revealed organelle lesions and serious cellular damage.

In some of the sections swollen mitochondria were present; they lacked a clear-cut membrane and showed alterations in the cristae which were entirely absent at times. Lipid inclusion was observed within the mitochondria (Fig. 8). It was also possible to detect the presence of lysosomes in cells without affecting the mitochondrial structure. Some mitochondria in scarcely affected cells presented inclusions in the form of large droplets (Fig. 9).

Contrary to the findings in female rats, in the males no differences were observed in the values of circulating corticosterone: normal rats, 10.6 ± 3.9 μg/100 ml; controls, 10.8 ± 5.0 μg/100 ml, and immunized rats,

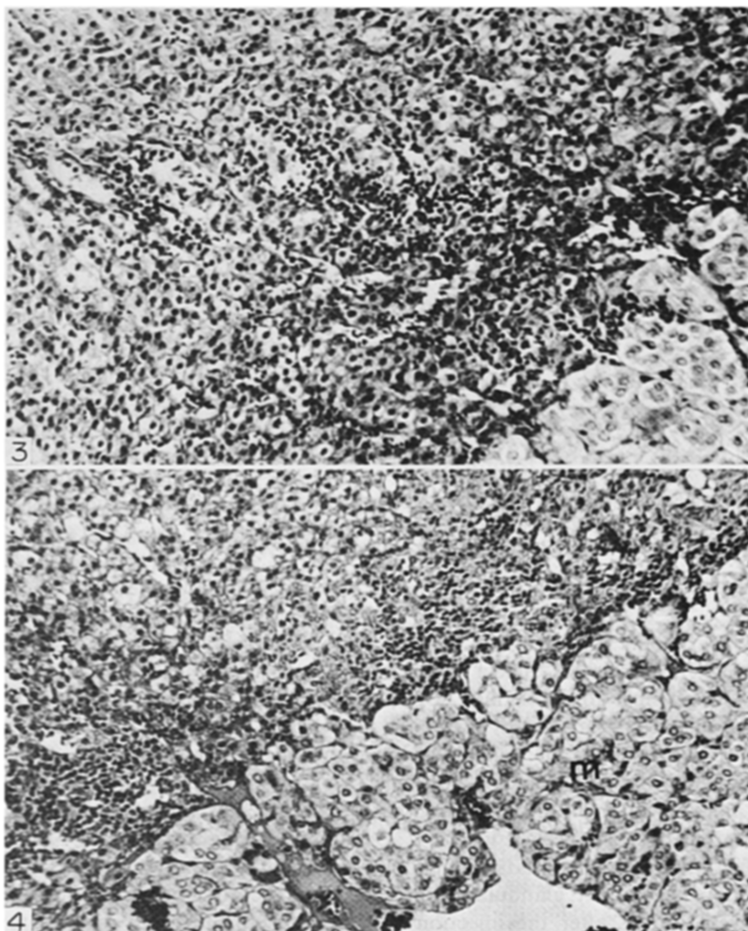

Fig. 3. Adrenal cortex of female rat which had received an injection of adrenal extract in complete Freund adjuvant. There are foci of cellular infiltration in the fasciculata and reticularis areas (H & E × 120).

Fig. 4. Adrenal cortex of female rat which had received an injection of adrenal extract in complete Freund adjuvant. The greatest degree of infiltration, in which lymphocytes predominate, is observed in the reticularis area. The medullary zone (m) is not affected (H & E × 120).

13.7 ± 3.8 μg/100 ml (Fig. 2). Accordingly, the histological picture did not reveal the previously described structural and infiltrative changes.

Metabolism of [^{14}C]-Progesterone. The homogenates of control and immunized rats were incubated with [^{14}C]-progesterone during 30, 60 and 90 min. In Fig. 12 can be appreciated the percentage of progesterone metabolized by both groups, both in female and in male rats. As will be seen, while no differences exist in the male group in the proportions of metabolized precursor steroid in the different incubation times, nor in the synthesis of the steroids studied, in the case of female rats the progesterone added to the medium undergoes a process of transformation which is slower in the immunized animals (Fig. 10). This fact is quite remarkable at 60 and 90 min of incubation times. At the same time the control and immunized female rats behave in a different manner as regards the production of metabolites. Thus, the quantity of synthesized corticosterone (Fig. 11) is significantly lower in animals with adrenalitis than in the controls after 30 and 60 min incubation. The equalized percentages at 90 min could be due to a relatively greater transformation of corticosterone to other metabolites, as suggested by the increased transformation to aldosterone (Fig. 12). In accordance with this finding more desoxycorticosterone is recovered from the glands of the treated rats than from those of the control rats after completion of the first period of incubation; at the same time there is less conversion to corticosterone (Fig. 13).

DISCUSSION

The injection of adrenal extracts results in damage to adrenal gland tissue in rats. The adrenalitis thus

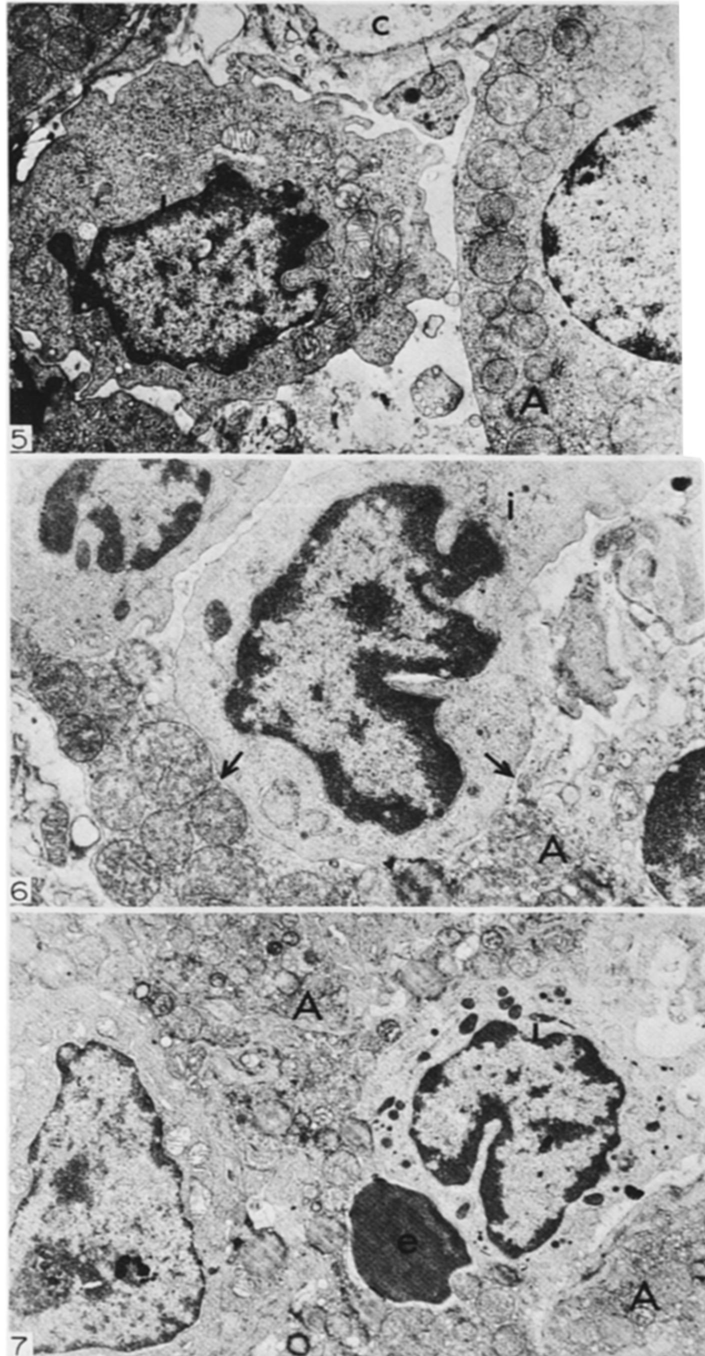

Fig. 5. Fasciculata zone of adrenal gland corresponding to female rat sensitized with adrenal antigen in complete Freund adjuvant. Infiltrating cell (I) among several well-preserved adrenal cells (A). (C) capillary (× 19720).

Fig. 6. Internal fasciculata zone of adrenal gland of rat immunized with adrenal antigen and complete Freund adjuvant. Both the cytoplasm and organelles reveal a good state of preservation (N). The membrane cannot be observed at the site of lymphocyte penetration (arrow).

Fig. 7. Reticularis zone of adrenal gland of female rat sensitized with adrenal antigen in complete Freund adjuvant. The adrenal gland (A) is irregularly shaped and reduced in size. (l) lymphocyte; (e) erythrocyte. (× 12550).

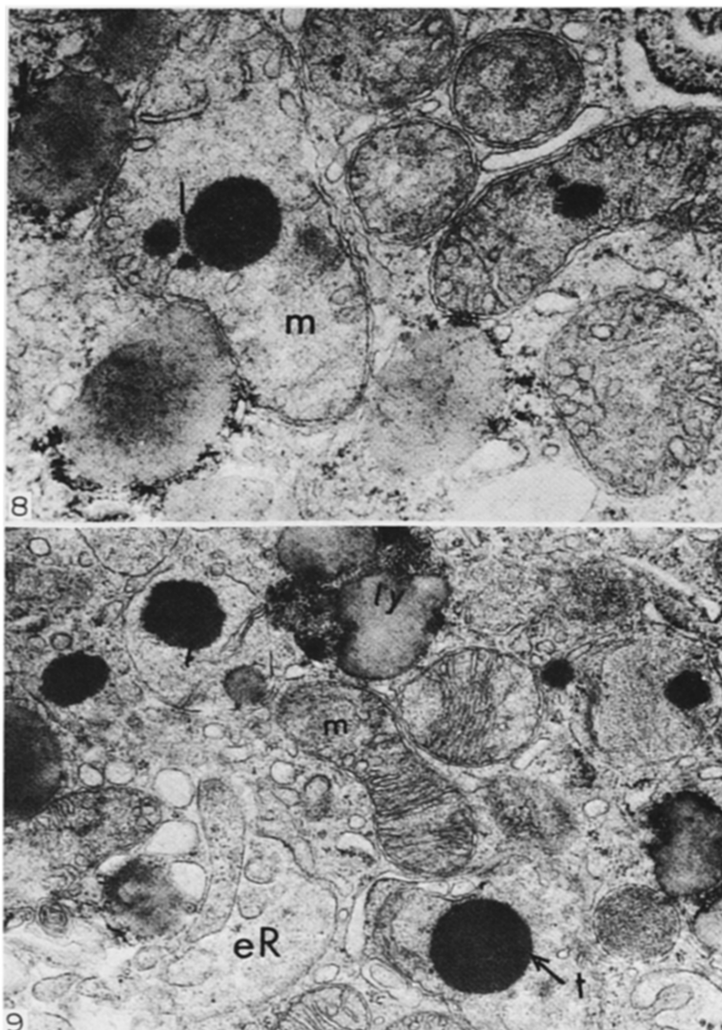

Fig. 8. Ultrastructural view of cortical cell of rat sensitized with adrenal antigen in complete Freund adjuvant. Swollen and deformed mitochondria (m) accompanied by loss of hyaline structure, ruptured membranes and lipid inclusion bodies (l). (× 19660).

Fig. 9. Ultrastructural view of cortical cell of rat sensitized with adrenal antigen in complete Freund adjuvant. Mitochondria (m) with normal crystalline structure. Evidently deteriorated endoplasmic reticulum (eR); lipids (l) in mitochondria. Lysosomes (ly); inclusion body in mitochondria, shaped like a large droplet (t). (× 19660).

induced is greater in the female animals. The males, on the other hand, do not usually reveal histological changes even at ultrastructural level.

The lesions commence to become detectable towards the tenth day of injection of the antigenic material, their maximum severity being apparent by the 15th day. A single immunization is sufficient to produce the glandular lesions which are not rendered worse by the administration of subsequent injections.

The histological picture of experimental adrenalitis in rats is similar to that observed in other experimental autoimmune conditions[19, 20]. The cellular lesions and the infiltration of mononuclear cells predominate in the fasciculata and reticularis areas of the adrenal cortex.

Rats with adrenalitis exhibit mild clinical abnormalities; in only some of those under chronic treatment a reduced activity was observed as well as hair pigmentation and alopecic patches. This reduced clinical repercussion is also observed in other animals with induced autoimmune disease[21, 24].

The parameters investigated in order to endeavor to correlate the histological findings with possible biochemical changes in hormonal synthesis were the evaluation of peripheral corticosterone and the *in vitro* studies of glandular tissue.

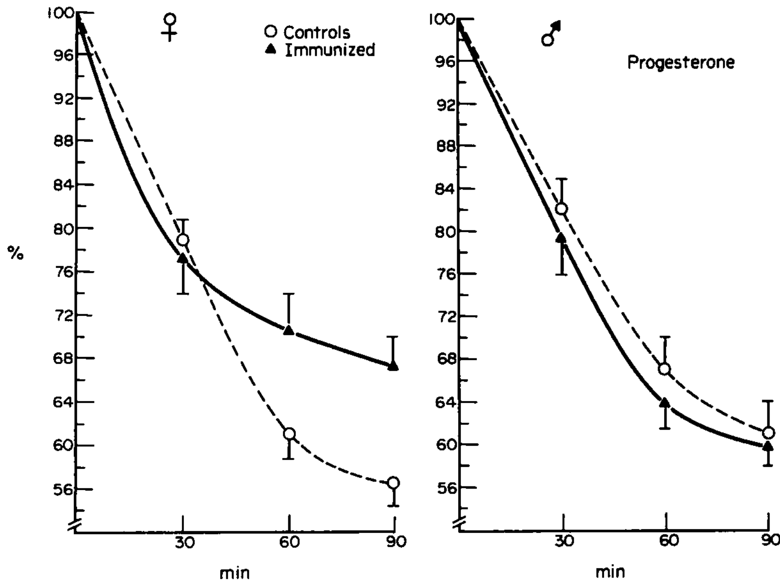

Fig. 10. Non-metabolized [^{14}C]-progesterone after incubation during 30, 60 and 90 min with homogenates obtained from the adrenals of control and immunized rats. (Mean ± S.D. from triplicates of pooled adrenals).

The values of circulating corticosterone were significantly lower in the immunized female animals by the 15th day of the injection of the antigenic material. The males, whether treated or untreated, exhibited similar values of corticosterone in peripheral blood, thus confirming the normalcy of the histological picture revealed by microscopy.

The *in vitro* studies of glandular tissue incubated with [^{14}C]-progesterone during 30, 60 and 90 min pointed to a dissimilar utilization of the precursor steroid in female as compared with male animals. In the latter, metabolization of the added substrate as well as its bioconversion into the different metabolites of the hormonal synthesis, did not show any difference between the injected rats when compared with the controls. On the contrary, in the group of female rats the corticosterone synthesis was lower in the immunized animals after the 30 and 60 min incubation periods. The drop registered in the controls at the end of 90 min would indicate its transformation into other cortical steroids to which corticosterone serves as precursor. The ascending curve of aldosterone through the different incubation periods would constitute an example of the foregoing. In both groups of female rats, metabolization of the progesterone added to the medium showed a similar utilization during the first 30 min of the study; however, in the subsequent periods a delay was observed in the incubation with the glands of immunized animals. This finding could be suggestive of a more rapid exhaus-

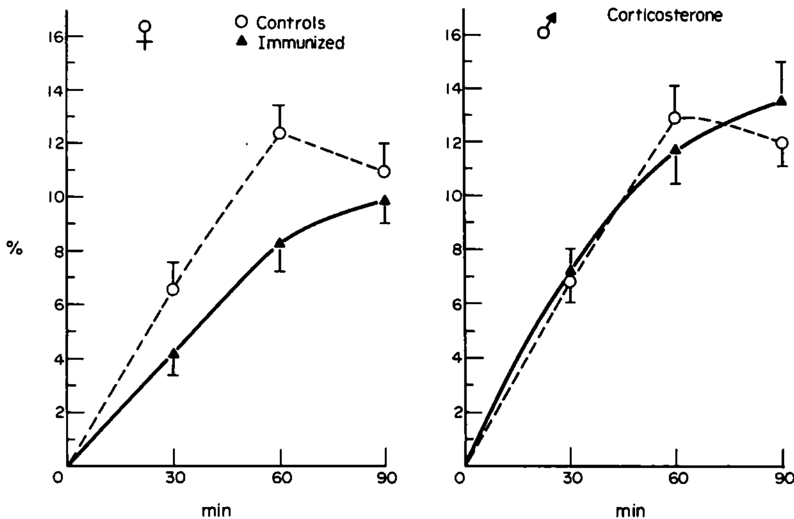

Fig. 11. [^{14}C]-corticosterone synthesized from [^{14}C]-progesterone after incubation during 30, 60 and 90 min with homogenates obtained from the adrenals of control and immunized rats.

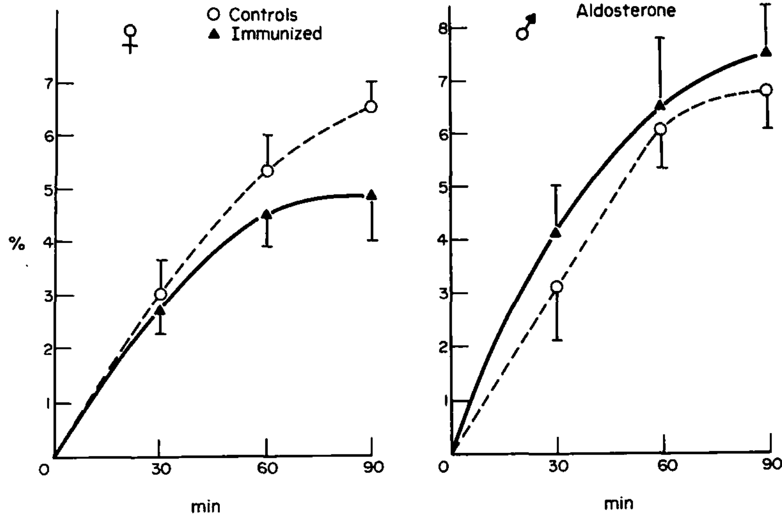

Fig. 12. [^{14}C]-aldostenone synthesized from [^{14}C]-progesterone after incubation during 30, 60 and 90 min with homogenates obtained from the adrenals of control and immunized rats.

tion of the damaged tissue as a consequence of its cellular changes which, at a molecular level, would be manifested by less availability of the cofactors indispensable for hormonal biosynthesis[25].

The lower conversion of desoxycorticosterone (DOC) into corticosterone which is observed at the end of 30 min in the animals receiving adrenal antigen would suggest that the 11-betahydroxilase mitochondrial system and its enzymatic cofactors are at least partially affected in them. Together with this biochemical finding, the ultrastructural image clearly shows the alteration of some of the mitochondrial cristae and the presence of lipid inclusion bodies in their interior. The subsequent drop of DOC at 60 and 90 min would be the consequence of two facts manifesting themselves in a simultaneous manner: the transformation into other intermediaries of the steroid biogenesis, and a lower net synthesis of DOC as a consequence of the drop in progesterone metabolization.

The different parameters evaluated in the present study permit the conclusion that immunization with the adrenal antigen provokes in female rats lesions whose severity can be confirmed microscopically. These lesions are accompanied by a lower potential capacity of the tissues to develop hormonal biosynthesis and a lower concentration of peripheral corticosterone.

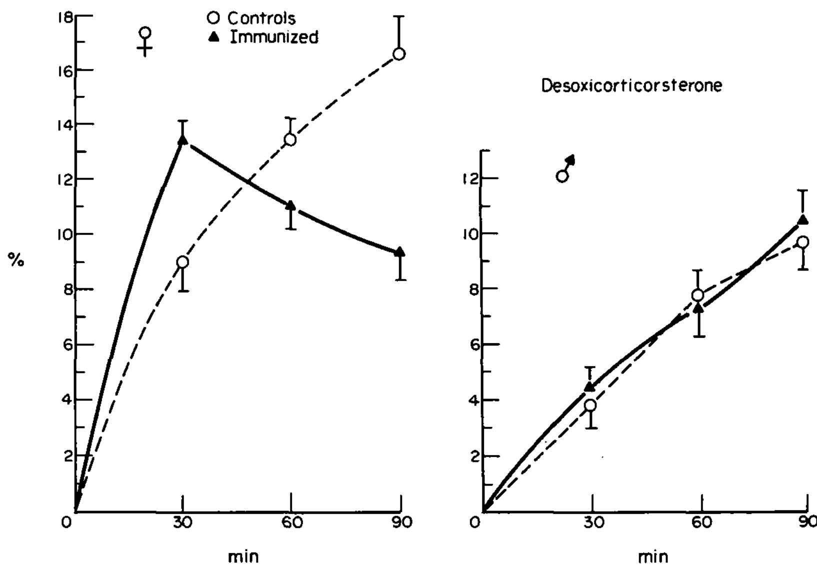

Fig. 13. [^{14}C]-11-desoxycorticosterone synthesized from [^{14}C]-progesterone after incubation during 30, 60 and 90 min with homogenates obtained from the adrenals of control and immunized rats.

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