SOME EFFECTS OF X-IRRADIATION ON THE ADRENAL RESPONSE TO HYPOTHALAMIC STIMULATION IN RATS

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THESIS

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CHAPTER I

Introduction

The concept of stress and the General Adaptation Syndrome (G. A. S.) as proposed by Selye (27) states that any severe alteration of an animal's external or internal environment may elicit compensatory changes on the behalf of the animal. Experimentally, such factors as scalding, immobilization, chemicals, electrical stimulation, radiation, and other stressor agents have been used to initiate adaptative defensive measures.

In general, the essence of this concept is that every stressor by way of afferent nervous impulses or chemical stimulation activates the hypothalamic centers which, in turn, control the activity of the adenohypophysis (anterior pituitary). Stimulation of the hypothalamus, therefore, stimulates the adrenchypophysis to synthesize and secrete the adrenocorticotropic hormone (ACTH). In response to this increase in ACTH, the adrenal glands become overactive, with the resultant increase in the output of abnormal quantities of various steroid hormones. The adrenocorticoids may be classified as being either mineralocorticoids, which influence electrolyte balance in the body, or glucocorticoids, which influence carbohydrate, protein, and lipid metabolism.

In the normal animal the synthesis and secretion of the adrenocorticoids is thought to be dependent upon a neuro-endocrine mechanism. This has been called, collectively, the adrenal-pituitary axis by Bacq and Alexander (3), who outlined various parameters that might indicate activity of this system in an animal under various conditions. These included changes in ACTH content of the anterior pituitary, in circulating eosinophils, in circulating corticosteroids, and changes in adrenal ascorbic acid and cholesterol content. According to Ganong and Hume (8), a discussion of the factors that initiate ACTH secretion in the stressed animal centers around three factors: the circulating level of epinephrine, the circulating level of glucocorticoids, and afferent nervous impulses. Gemzell (9) and others have shown that epinephrine, alone, brings about a decrease in the number of circulating eosinophils, adrenal ascorbic acid and cholesterol depletion, and elevation of the ACTH content of the blood while <u>not</u> affecting the levels of 17-hydroxy steroids in the blood. The concentration of glucocorticoids in the circulation appears to regulate ACTH production via a servo-mechanism, since Peron and Dorfman (25) established that a high level of circulating glucocorticoids inhibited further release of ACTH from the pituitary. Inhibition, indeed, appeared proportional to the general level of steroids circulating in unstressed animals.

That the hypothalamus should be suspected as a mediator of afferent nervous impulses to the pituitary is a logical

deduction; this complex of nuclei surrounds and gives rise to the infundibular stalk of the pituitary from its ventral surface. This anatomical location allows both vessels and fiber tracts going to and from the pituitary to pass through the hypothalamus. The variety of stimuli that result in adrenocorticotropic activity suggests that many pathways contribute to the afferent input of the hypothalamus. Naute (21) concluded that the hypothalamus forms part of two neural circuits, one of which connects with the limbic forebrain structures, the other with a medial zone of the midbrain.

Several lines of evidence support the contention that the hypothalamus does control the activity of the adenohypophysis and hence adrenocorticotropic activity. Slusher (28, 29) and Suzuki and Romanoff (32) have observed in both cats and rats that stimulation of the medial and posterior regions of the hypothalamus resulted in rapid, marked increases in the adrenal steroid output. Mason (17) noted similar plasma 17-hydroxycorticosteroid elevations to follow posterior hypothalamic stimulation. Following acute lesions of the median eminence, McCann and Haberland (20) noted as much as fifty per cent reduction of pituitary ACTH content in rats. Stress-induced eosinopenia in cats was prevented following posterior hypothalamic lesions as reported by Porter (26) and by Anand and Dua (1, 2). Story et al. (31) attempted to determine if other higher centers of the brain were necessary for adrenal cortical secretion of supra-optimal amounts of 17-hydroxycortisteroids due to operative trauma. Stepwise removal of

the brain down to the hypothalamus resulted in no diminution in the maximal adrenal cortical response following the operative procedure. After removal of the hypothalamus there was a persistence of adrenal corticoid secretion up to six hours; the pituitary was left intact. Story concluded that the central nervous system above the hypothalamus was not essential for adrenocorticoid production. This would tend to support the work of Knigge (13), who observed that at certain time intervals corresponding to low plasma corticosteroid levels the pituitary still contained an above-control level of ACTH. From this and other evidence at hand, it would appear that the posterior regions of the hypothalamus exert the more profound effect on the pituitary.

In regard to the effects of ionizing radiation, Selye (27) stated that the penetrating nature of X-rays rendered them eminently capable of evoking the general-adaptation-syndrome. Patt et al. (23) demonstrated that whole-body X-irradiation activates the pituitary-adrenal axis as evidenced by a decrease in the adrenal cholesterol content and compensatory adrenal hypertrophy. Subsequently, by using hypophysectomized animals, Patt et al. (24) showed the adrenal gland to be dependent upon an intact pituitary gland.

Binhammer and Crocker (6) and Wexler and Pencharz (33) noted a marked decrease of adrenal ascorbic acid at one hour post-irradiation with a concomitant sustained adrenal hypertrophy. Oster et al. (22) observed a similar response

accompanied by an ascorbic acid depletion from muscle and plasma. Eechaute et al. (7) reported a threefold rise in the plasma corticosterone concentration of rats subjected to 750r total-body X-irradiation two and one-half hours later.

More recently Hameed and Haley (11), by administering 650r whole-body and body-only irradiation to rats, caused a marked increase in both the plasma and the adrenal gland steroid levels seventy-two hours later, with return to control values on the succeeding day. Hypophysectomized rats showed no change in hormone concentration. Data obtained after pre-treatment with an adrenal cortical inhibitor indicated that the depressing effect of radiation on the adrenal gland was not sufficient to prevent the adrenals from responding to ACTH. Summarily, the authors concluded that X-irradiation acted as a nonspecific stress agent. These data, in general, substantiated those obtained previously by Lott and Gaugl (16), who reported that plasma corticosterone levels change in a relatively short time in irradiated rats.

In attempting to determine if X-irradiation acted as a direct stress agent, Mateyko and Edelman (18) found that localized body irradiation produced changes in pituitary ACTH concentration of the same order of magnitude as whole-body irradiation, thus suggesting a nonspecific effect. Conversely, they observed that ACTH activity in the pituitary gland increased after localized hypophyseal irradiation; herein a direct effect on the gland was suggested. Bacq and Martinovitch (5) suggested

that this activity was stimulated by the hypothalamus. Their contention was based upon the fact that the "first reaction," that occurring during the first twenty-four hours post-stress, was abolished after the production of lesions in the hypothalamus. Additionally, Bacq and Martinovitch reported that the adrenal glands of newborn rats and those of hypophysectomized rats with pituitary grafts in the anterior chamber of the eye did not react in the usual manner to X-irradiation; there was a slight decrease in ascorbic acid but an increase in cholesterol. The implication was that the hypothalamus mediated the response. Bacq and Fischer (4) showed that the initial adrenocorticoid response, that occurred the third hour post-irradiation, could be abolished by pre-treatment of the animals with a barbiturate (Nembutal) and morphine; this pharmacological treatment has been shown to block hypothalamic control of the adenohypophysis.

In the discussion of their results, Lott and Gaugl (16) suggested that the delay in the response observed in head-shielded animals with respect to whole-body irradiation indicated that the pituitary did not yet "know" the system was under stress. On the other hand, evidence that indicates an extreme sensitivity of the central nervous system (CNS) to ionizing radiation has been mounting. Smirnova (30) found that stimulation of the hypothalamus in irradiated rabbits produced a two-phase dilator response in contrast to a pre-irradiation constriction response. Livanov (15) by means of chronically implanted hypothalamic electrodes noted alteration

of the slow waves in the rabbit following total-body irradiation. Lebedinskii and Nakhil'nitskaya (14) reported that 50r whole-body irradiation produced an increased excitability of the autonomic centers in the hypothalamic region. Haley et al. (10) reported that total-body irradiation with 600r altered the rate at which rats will press a bar in order to receive stimulation from electrodes located in the posterior hypothalamus. Monnier and Krupp (19) showed that thresholds for the EEG arousal reaction, evoked by stimulation of the midbrain reticular formation on the postero-ventral hypothalamus, decreased following 400 or 600r and increased after 900r.

Kimeldorf and Hunt (12) stated that

> From the data available, we can conclude that the hypothalamus, midbrain, and hippocampus are particularly radio-sensitive regions of the brain. However, little can be said about the precise sites or mechanisms for the action or irradiation in these areas.

The foregoing evidence strongly implicates the hypothalamus as an essential element in the initiation of the pituitary-adrenal response to a stress agent. The question arises as to exactly where in the hypothalamus is the adrenal-pituitary response to X-irradiation "triggered" or initiated. Moreover, does ionizing radiation act directly on specific centers in the brain or does it act indirectly via the production of some humoral agents? Finally, what role does the hypothalamus play in the radiation-syndrome? The purpose of the present study was to attempt to answer these questions by determining the effects of two stressor agents, X-irradiation and electrical

stimulation applied either singly or together, on the activity of the adrenal-pituitary axis. The parameters measured were changes in plasma corticosterone, in circulating eosinophils, and in adrenal gland weight.

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CHAPTER II

Materials and Methods of Procedure

Healthy, adult, female Sprague-Dawley rats of average weight (192.1 ± 68.5 grams) were used in this study. In this body weight range, the size and weights of the brain were relatively constant.

Electrical stimulation of the hypothalamus was accomplished by means of a single, permanently implanted, monopolar electrode. Placement of the electrode was affected by means of a Horsley-Clark stereotaxic apparatus (Figure 1) manufactured by the Kopf Instrument Company (Tejunga, California). DeGroot stereotaxic coordinates (2) were used to ascertain the exact position of the electrode. These coordinates were 4.5 - 5.0 mm. anterior from the external auditory meatus, 0.5 mm. lateral from the longitudinal cranial suture, and a vertical deviation of 9.0 mm. from the surface of the skull. Using these coordinates, the tip of the electrode, assuming no deflection upon entering the skull, came to rest in the posterior nuclei of the hypothalamus. These coordinates were used for each animal tested. The exact location of the electrode was confirmed by X-rays and histological examination of stained brain sections made at autopsy.

The electrodes used were #00 stainless insect pins (Clay-Adams Company, New York). The head of each pin was clipped

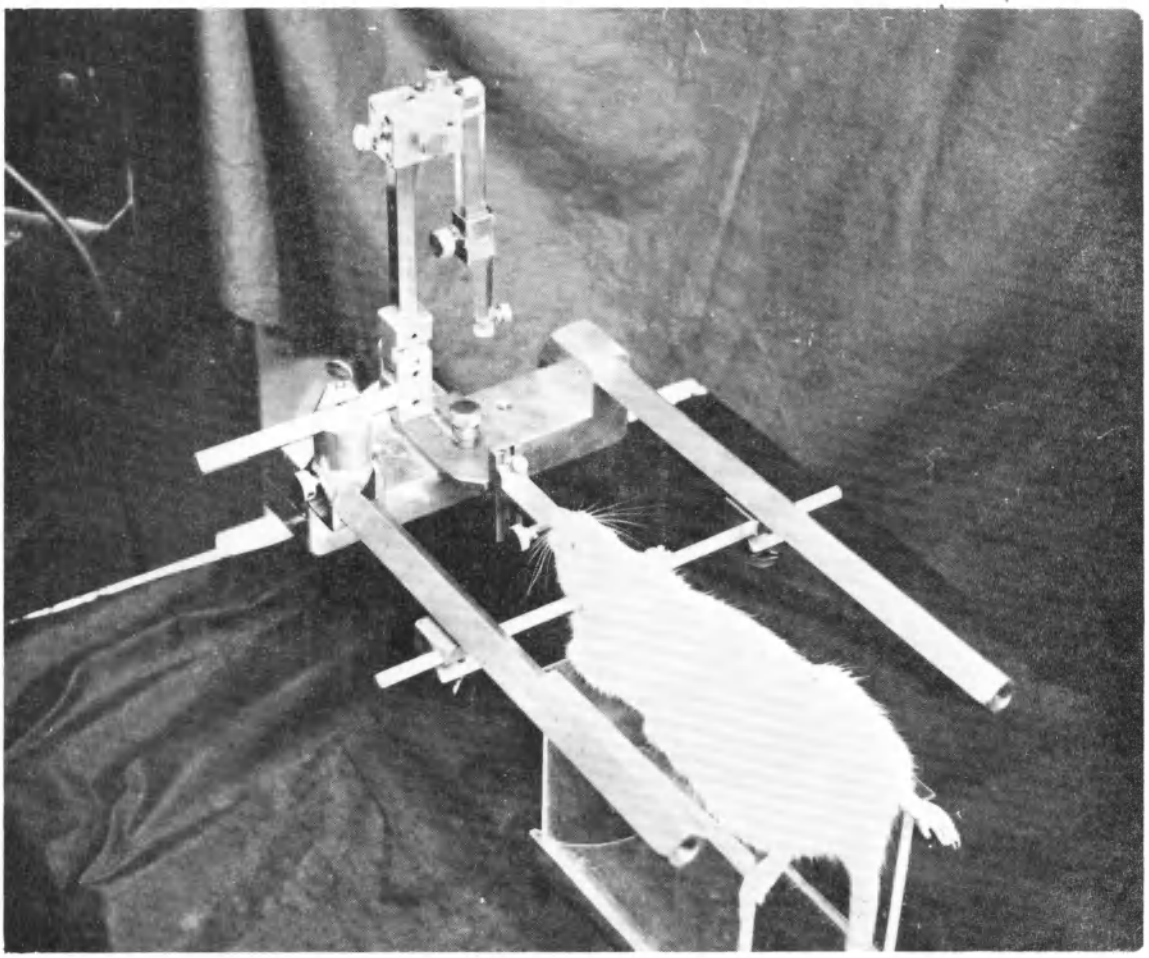

Fig. 1 -- Stereotaxic instrument

off uniformly. The electrode was insulated by means of a specially prepared liquid insulator (Epoxylite Corporation, El Monte, California). The electrode was dipped into the insulator and drawn out slowly, keeping it in contact with the wall of the vessel to assure that no bubbles formed along its length. The insulated pin was then allowed to air-dry for approximately one hour, at which time a second coat was applied in an identical manner. The electrode was next placed in a drying oven overnight at a temperature of approximately 80° C. The diameter of the electrode, after this step, was 0.4 mm. Prior to implantation the insulation was scraped from the distal end by the use of a dissecting microscope. This step resulted in exposing approximately 0.5 mm. of the electrode tip. Sterilization prior to implantation was accomplished by 1:750 Zepharin chloride for one hour.

In all of the experiments, implantation and testing was performed in groups of three or four animals. The operative procedure was as follows: The animal to be tested was anesthesized with sodium Nembutal, intra-peritoneally (33 mg./kg. body weight) and mounted in the stereotaxic unit (Figure 1). The head and neck of the animal were then shaved and a midline incision in the skull was made. The connective tissue overlying the calvarium was scraped aside and the resultant bleeding stopped by means of an electro-cautery. The tragus of the ear was next cut to allow the ear bar of the stereotaxic instrument easier access into the external auditory meatus. The animal was fixed in the Horsley-Clark instrument

by placing the ear bars into the auditory canal with the incisor bar placed behind the upper incisor teeth. The head of the animal was centered by clamping the ear bars in place, and fastening the nose tightly by the nose clamp. If properly positioned, the animal was unable to move its head even if it happened to awaken during the operative procedure.

Two small holes were then drilled in the skull by means of a hand drill. These were placed along an anterior-posterior line, one in the frontal and the other in the parietal bone on the side contra-lateral to the electrode placement. Into these holes two steel jeweler's screws were securely set; these served to anchor the protective cap which was subsequently affixed to the skull. The electrode carrier was next positioned above the site of entry into the skull; this site was carefully marked and a burr hole 1.5 mm. in diameter was made by means of a dental drill. Caution was necessary at this point to insure that the drill did not inadvertently, in passing through the skull, damage the cortex. The electrode carrier was then carefully repositioned to insure that the electrode was centered in the burr hole. It was then lowered to the desired depth and carefully detached from the electrode carrier. To insure that the electrode remained stationary, a protective cap was constructed about the electrode from Nuweld dental acrylic (L. D. Caulk Company, Milford, Delaware). The two steel screws were also covered by the acrylic to aid in immobilizing the electrode. The cap was built up to a height of approximately

.8 centimeter. Once the acrylic was dry, crystalline Sulfathiazole was sprinkled into the wound to prevent possible bacterial infection and the incision was closed.

The animals were then returned to the communal cages for a post-operative period of at least one week to allow for complete recovery and to regain any lost weight. The animals were maintained both pre- and post-operatively on Purina laboratory Chow and tap water ad libitum.

The day prior to testing, the animals were taken to the room in which the testing was to be performed and weighed. Each animal was placed in an individual covered cage; this was done to allow for more complete adaptation to temperature, light, noise, and the like, since any one of these factors could serve to activate the adrenal-pituitary axis. This procedure was completed some fifteen - eighteen hours prior to testing. Constant lighting was maintained in this room; the temperature was relatively constant at 24 $\pm$ 2° C.

On the day of testing, the animals were anesthesized early in the morning, and care was taken to insure that testing was initiated at the same time of day. This was done to compensate for the diurnal variation of the adrenal cortical steroids. The animals were allowed to remain quiet in their cages for a period of one hour, since Beigelman and Slusher (1) have shown that with Nembutal anesthesia there was an initial depression in the concentration of the steroid production, followed during the first hour by a subsequent return to control or "resting"

levels. At the end of this interval, the animals were individually removed from their cages and placed dorsal side up on a restraining board to await experimentation.

In this study, three physiological parameters were monitored: the plasma corticosterone, the absolute eosinophil count, and the weights of decapsulated adrenal glands. Tail blood samples (1 - 1.2 ml.) were collected for the subsequent pre-testing period corticosterone analyses and eosinophil count determinations.

Givner's and Rochefort's (4) method for determining plasma corticosterone content was utilized in this study. It may be outlined as follows: Following centrifugation of the blood sample, one part (.5 ml.) plasma was withdrawn and added to one part de-ionized water and six parts two, two, four-trimethylpentane (iso-octane). This mixture was mechanically shaken for one minute and centrifuged for three minutes. The iso-octane layer was aspirated; one-half milliliter of the aqueous layer was transferred to another tube containing six parts de-ionized water and fifteen parts chloroform. The aqueous layer was washed with Spectrograde chloroform by shaking for one minute prior to centrifugation for three minutes. The aqueous layer was aspirated and to the chloroform was added one part 0.1 N sodium hydroxide; the mixture was shaken for one minute and centrifuged at 2000 rpm for three minutes. The basic aqueous layer was aspirated and ten parts (5 ml.) of the remaining chloroform layer was transferred

to another tube containing two parts (1 ml.) of a solution made up of seven parts thirty-six N sulfuric acid and three parts absolute ethanol. The mixture was gently shaken by hand for fifteen seconds, centrifuged for three minutes, and allowed to sit for a period of thirty minutes from the time of addition of the acidified ethanol solution. This period of time was carefully controlled in all determinations to allow the development of uniform fluorescence. At the end of the thirty minute period, the chloroform layer was aspirated, and the remaining acidic layer transferred to quartz cuvettes. Readings were initiated within five minutes.

Fluorometric determination of corticosterone in the resultant extract was carried out with a Model 110 Turner Fluorometer (Turner Associates, Incorporated, Palo Alto, California). The primary filter was a Corning No. 47-B; the secondary filter was a Corning No. 2A-12. The light source was a General Electric near ultra-violet bulb No. 4-W. A micro-cuvette adapter (Turner Cat. # 110-865) was also necessary to hold the small cuvettes. The foregoing filters and U-V bulb were suggested specifically for corticosterone determinations by the Turner Company.

Absolute eosinophil counts were made, using the direct-chamber counting method of Pilot (5). A standard white cell blood pipette was filled with blood to the one mark and diluted to the eleven mark with Pilot's stain; the pipette was shaken for thirty seconds on a pipette shaker and allowed to stand

for a minimum of fifteen minutes prior to counting. After discarding the staining fluid in the stem of the pipette, the field of a standard hemacytometer was filled with the blood sample and allowed to stand for at least three minutes to permit the cells to settle onto the counting grid; each field was filled with the fluid from a separate pipette. All nine chambers of each grid were counted and the values of each grid of the hemacytometer averaged. The average number of cells per cubic millimeter was reported. The eosinophils appeared as large ruby-red cells that were easily distinguishable. Pilot's stain consists of one part ten per cent sodium carbonate, fifty parts propylene glycol, and ten parts of phloxine and forty parts distilled water. The sodium carbonate served to lyse all leucocytes except eosinophils, while the propylene glycol rendered the red blood cells invisible, and the phloxine stained the eosinophils a ruby-red color.

Electrical stimulation was accomplished with a Grass S4-B stimulator delivering 3.0 volts at a pulse frequency of twenty-five per second. The pulse duration and pulse delay was one millisecond. This pattern was previously shown by Porter (6) to elicit an eosinophilic response in rats. The stimulus pattern was delivered in alternating periods of five seconds stimulation followed by five seconds of rest for a period of five minutes. To stimulate, one lead was connected to the implanted monopolar electrode while the other lead was attached to one ear. The ear, therefore, served as an indifferent electrode.

Unfiltered X-irradiation was delivered from a General Electric Beryllium window X-ray unit, 120 KVP, 5 ma. The target distance was fifty cm. thus giving a calculated air dose of 188r per minute. Whole-body irradiation was administered for a total of 5.3 minutes, giving a cumulative dose of 996r. This dose has been established as an LD_{100} dose (3). The LD_{100} dose was chosen in order to insure maximum effects in regard to the alarm reaction.

Following electrical stimulation and/or X-irradiation the animals were returned to their respective cages. They were allowed to rest quietly until the completion of their particular test interval. Care was taken to prevent noise and excessive traffic in the room in which the animals were housed.

At intervals of three, six, twelve, and twenty-four hours following the test period the animals were sacrificed individually, as quickly as possible, with a Harvard guillotine (Harvard Apparatus Company, Dover, Massachusetts). The blood collected was used for both the corticosterone determination and eosinophil count. The adrenal glands were removed and decapsulated to minimize weighing errors due to adherent fat, and weighed on a Mettler balance. Prior to removal, a coagulating current was passed through the implanted electrode in the brains of the test animals to aid in identifying the electrode site. The brains were then removed and placed in buffered formalin for fixation prior to histological sectioning on a frozen microtome. The tissues were stained with thionin.

The experiments were divided into the following series:

1. Sham-irradiated and unstimulated
2. Stimulated and sham-irradiated
3. Irradiated and unstimulated
4. Irradiated and stimulated
5. ACTH injected, unstimulated and sham-irradiated
6. ACTH injected, stimulated and sham-irradiated
7. ACTH injected, irradiated and unstimulated

All animals received whole-body X-irradiation. The adrenocorticotropic hormone (ACTH) was obtained from the Nutritional Biochemical Company, Cleveland, Ohio. The ACTH was injected (15 unit per 100 grams body weight) into the lateral tail veins.

In preliminary runs, an attempt was made to implant a venous cannula into a given animal so that blood sampling could be done with as little disturbance to the animal as possible. Moreover, with such a technique, it was hoped that the blood could be taken at the various time intervals from the same animal and analyzed accordingly. Due to the inability of the implanted cannulas to remain open, however, the procedure had to be abandoned. The animals, therefore, were divided into several groups (four animals per group); each was sacrificed at a given time interval following the test period; id est, at three, six, twelve, and twenty-four hours. Moreover, due to the failure to obtain more animals from animal suppliers during the month of May and June, a

series of experiments involving the effects of simultaneously applying ACTH, stimulation, and X-irradiation had to be postponed for future studies.

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CHAPTER III

Results

The data reported here were obtained from 100 animals. This does not include the number that were required to learn the stereotaxic procedure and to establish the present experimental format. The data are presented as curves containing mean values. In every curve, the pre-stimulus value was obtained from determinations made ten minutes prior to the test period. The tables present summaries of the data including the standard error of the mean (SEM) for each group of animals in a given series. Figure 2 shows the position of a typically implanted electrode in a lateral, radiographic view of the rat skull. Figure 3 shows the locus of the electrode tip as indicated by the lesion made by an externally applied electrical current. The tip can be seen located in the posterior-median area of the hypothalamus.

Figure 4 and Table 1 contain data showing the effects of X-irradiation on the corticosterone response to hypothalamic stimulation. It was apparent that stimulation alone, X-irradiation alone, as well as applying irradiation and stimulation simultaneously, resulted in an increase in corticosterone levels at the third hour and sixth hour. In the stimulated group, return of the corticoid concentration toward control levels occurred at the twenty-fourth hour. In the simultaneously stimulated

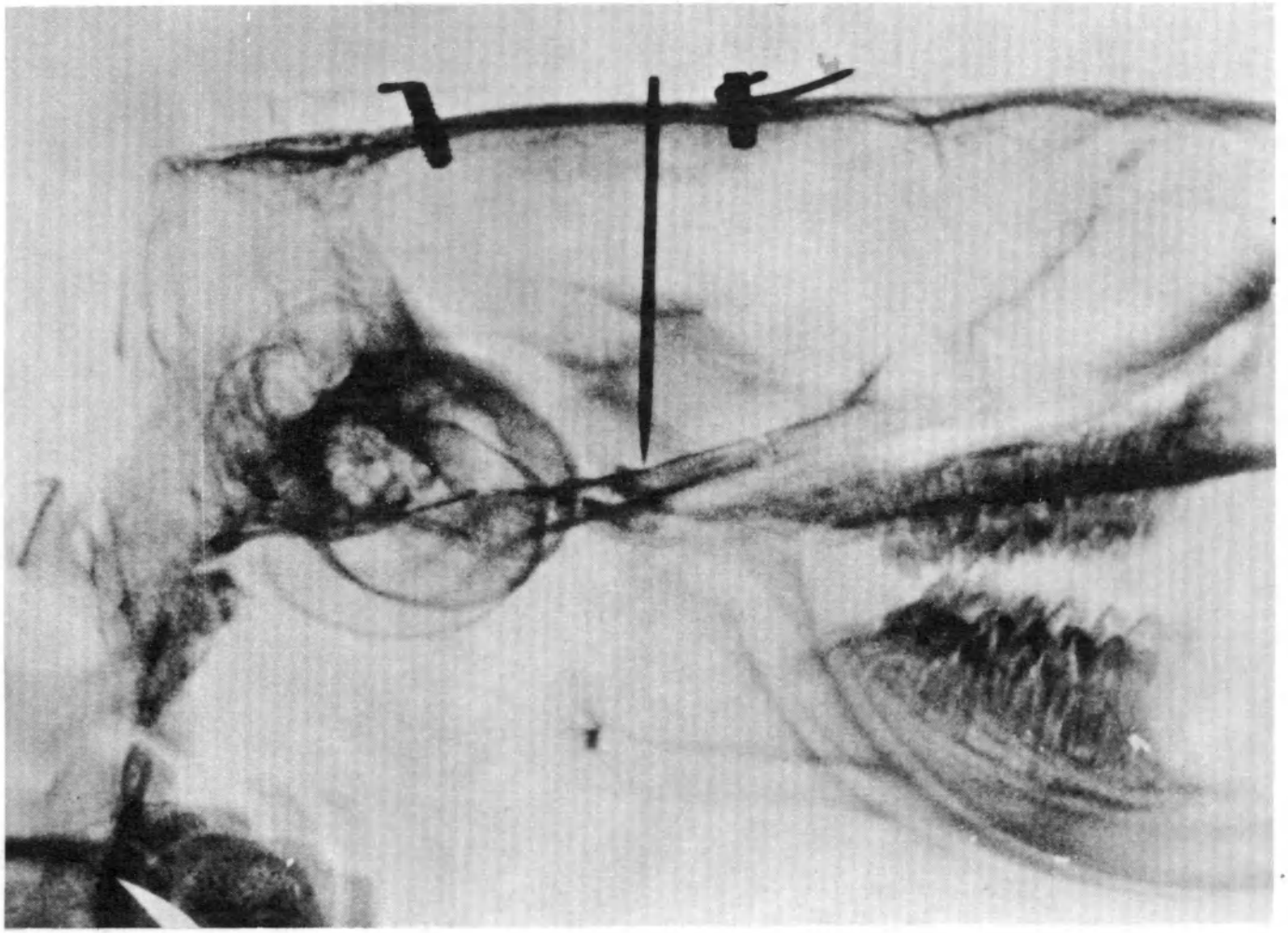

Fig. 2 -- X-ray of rat skull showing electrode placement

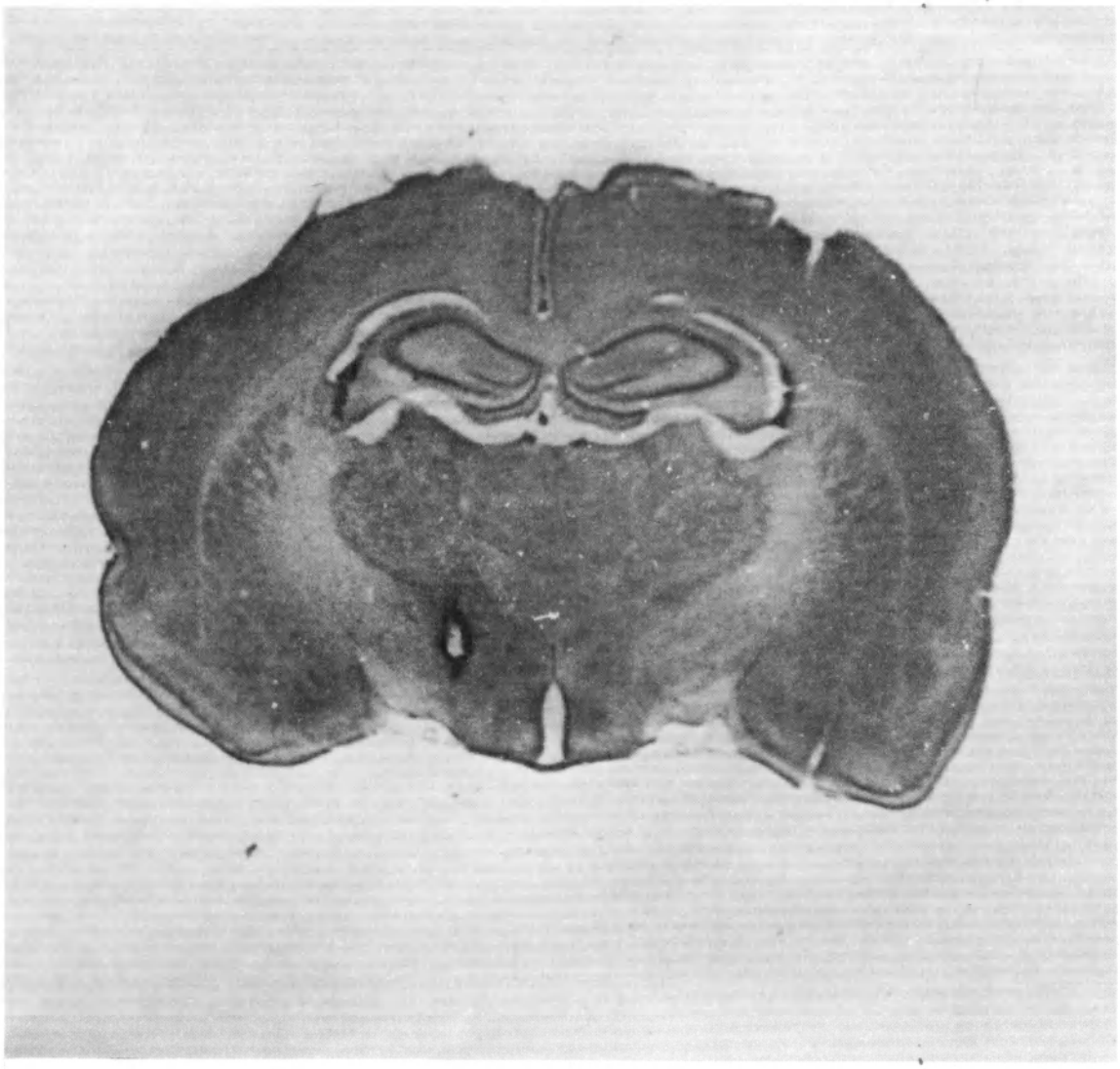

Fig. 3 -- Histological section of rat brain showing site of stimulation.

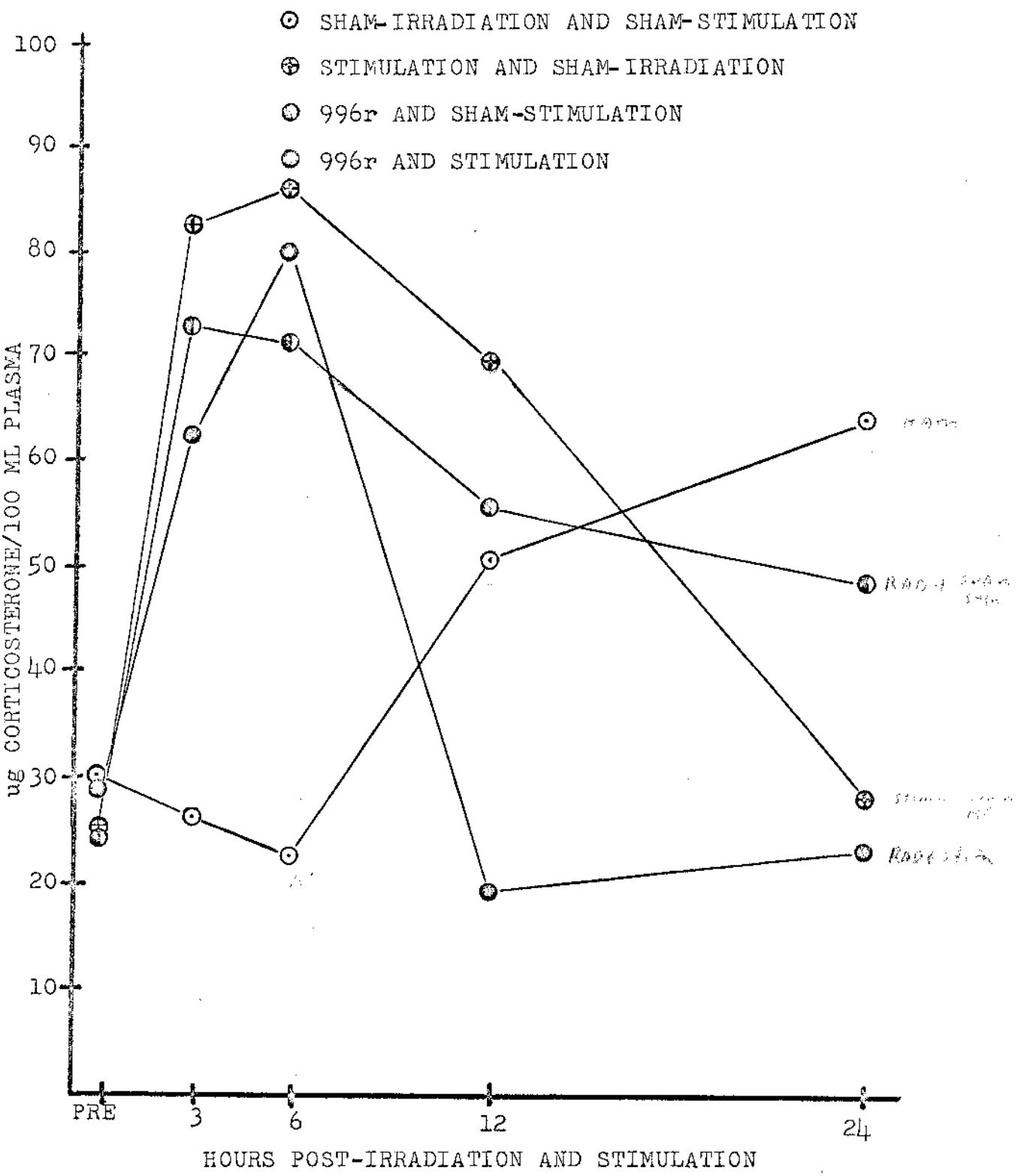

Fig. 4 -- A summary of the effects of X-irradiation and hypothalamic stimulation on the corticosterone level in rats.

TABLE I

THE EFFECTS OF X-IRRADIATION ON THE ADRENAL RESPONSE TO POSTERIOR HYPOTHALAMIC STIMULATION IN RATS

Test Groups	Hours Post-Stimulation and/or Irradiation							
	Pre-stimulus	3	% Change	6	% Change	12	% Change	24	% Change
Eosinophils (cells/mm^3)									
Sham-irradiated + sham-stimulated	122 ± 58*	42 ± 10	- 66**	42 ± 2	- 66	64 ± 10	- 48	77 ± 35	- 37
Stimulated + sham-irradiated	67 ± 32	44 ± 22	- 34	20 ± 13	- 70	21 ± 4	- 70	76 ± 32	+ 13
996r + sham-stimulated	87 ± 41	29 ± 7	- 67	15 ± 12	- 83	18 ± 10	- 79	11 ± 6	- 87
996r + stimulated	114 ± 30	39 ± 11	- 65	20 ± 13	- 82	13 ± 3	- 89	13 ± 3	- 89
Corticosterone (ug/100 ml plasma)									
Sham-irradiated + sham-stimulated	30 ± 12	26 ± 7	- 13	23 ± 5	- 23	51 ± 9	+ 70	63 ± 10	+110
Stimulated + sham-irradiated	25 ± 8	83 ± 4	+232	86 ± 1	+244	69 ± 4	+ 76	27 ± 6	+ 8
996r + sham-stimulated	24 ± 11	73 ± 4	+204	71 ± 9	+196	66 ± 9	+175	47 ± 6	+ 96
996r + stimulated	29 ± 5	63 ± 3	+117	80 ± 17	+141	19 ± 4	- 34	23 ± 6	- 21

*Standard error of mean for four animals
**Per cent change from pre-stimulus value

and irradiated group, however, a return to pre-test levels occurred at the twelfth hour. No additive effect of X-irradiation and stimulation was noted. It was interesting to note that the steroid level in the irradiated group failed to return to pre-testing levels even by the twenty-fourth hour.

Figure 5 and Table 1 indicate a similar eosinophil response in all three test groups of animals, at least during the third, sixth, and twelfth hour post-test period. Indeed, all four groups of animals showed an immediate drop in eosinophil count, id est, at the third hour. The eosinophil count in the control group, however, began to return to pre-test values at the twelfth hour. In general, it was clear that all three experimental conditions brought about similar states of eosinopenia in the animals. There was no evidence of a differential response in the test animals with the exception noted in the animals that were stimulated only. They exhibited a recovery in eosinophil count at the twenty-fourth hour post-testing. No recovery was present in any of the X-irradiated groups.

The data in Figure 6 and Table 2 indicate that X-irradiation at 996r resulted in a slight but sustained increase in adrenal weights, whereas electrical stimulation alone brought about a slight but sustained decrease in adrenal weights. Animals that received both 996r and stimulation exhibited a slight decrease in adrenal weights followed by a recovery by the sixth hour.

Figure 7 and Table 3 contain a summary of the data concerning corticosterone responses following the application of

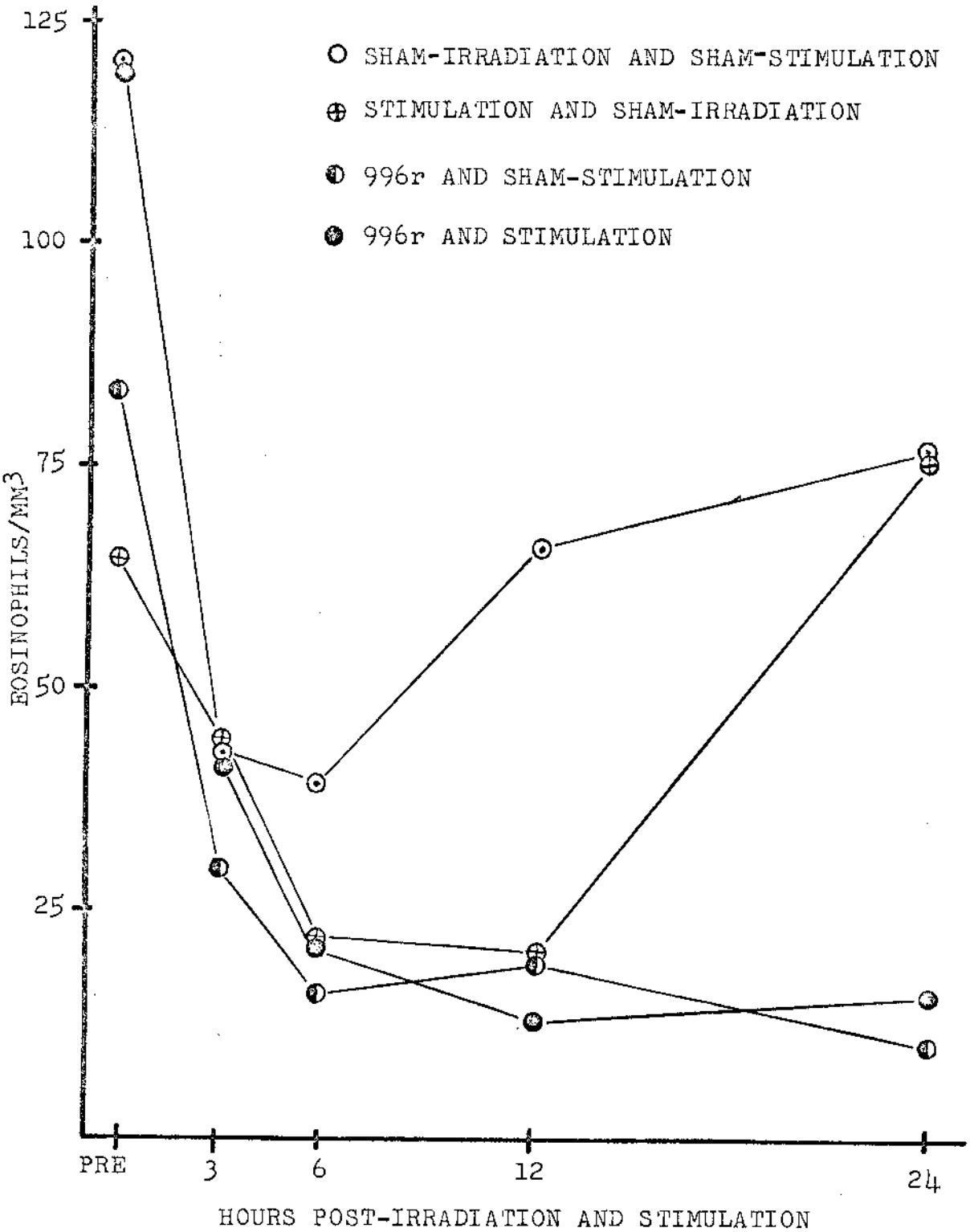

Fig. 5 -- A summary of the effects of X-irradiation and hypothalamic stimulation on the eosinophil count in rats.

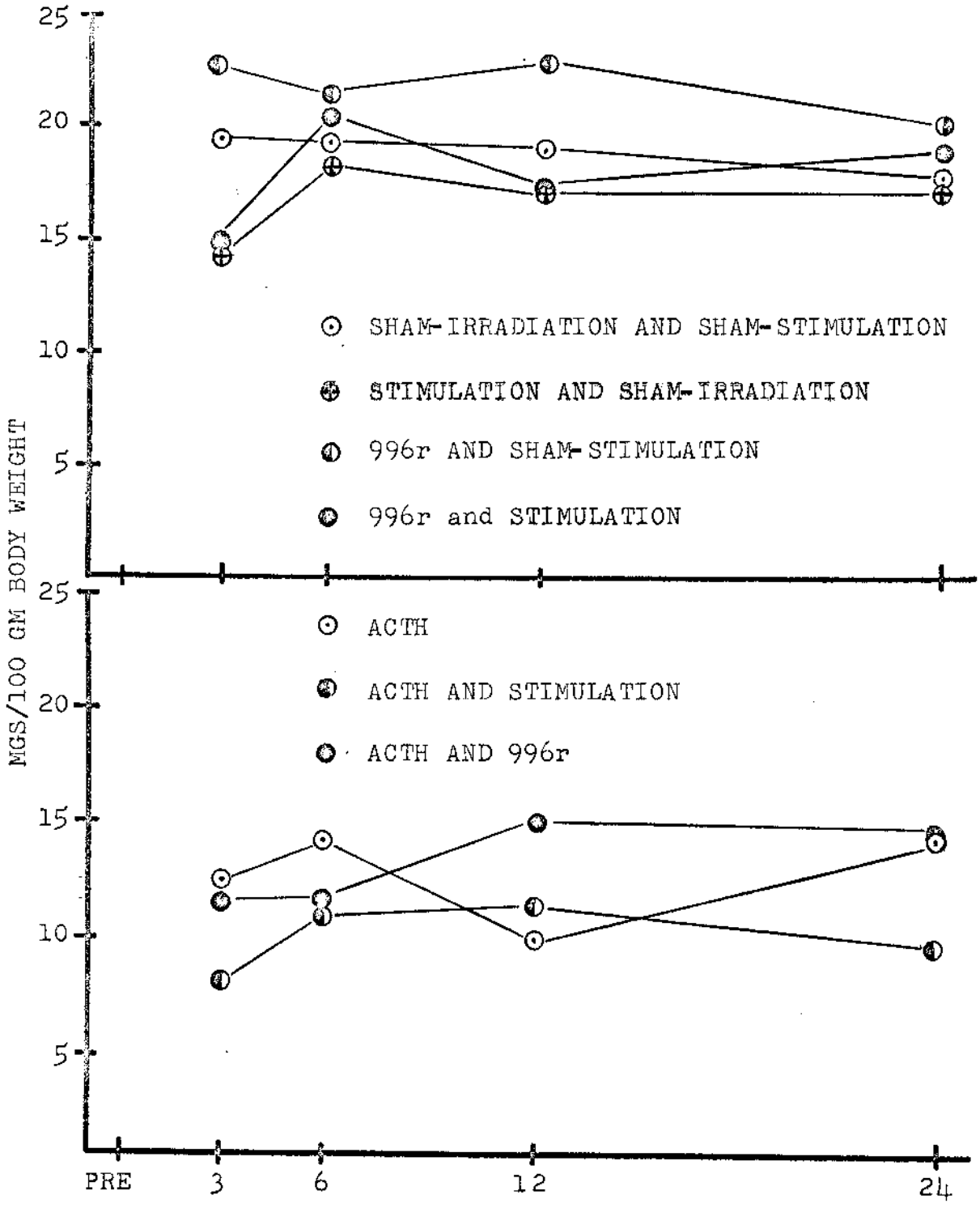

Fig. 6 -- A summary of the effects of ACTH, hypothalamic stimulation, and irradiation on the adrenal gland weight in rats.

TABLE II

THE EFFECTS OF X-IRRADIATION ON ADRENAL WEIGHT CHANGES FOLLOWING HYPOTHALAMIC STIMULATION IN RATS

Test Groups	Hours Post-Stimulation and/or Irradiation			
	3	6	12	24
Sham-irradiated + sham-stimulated	* ** 19.1 ± 0.8	19.2 ± 0.1	19.1 ± 1.4	18.6 ± 2.1
Stimulated	14.0 ± 2.4	18.2 ± 4.7	17.1 ± 1.0	17.5 ± 2.4
Irradiated	22.2 ± 2.8	21.3 ± 2.9	23.5 ± 3.9	21.1 ± 2.4
Stimulated + irradiated	14.9 ± 2.4	20.6 ± 1.7	17.2 ± 2.1	19.7 ± 1.1

*Mean weight in mg paired adrenal wt/100 gm body weight
**Standard error of mean

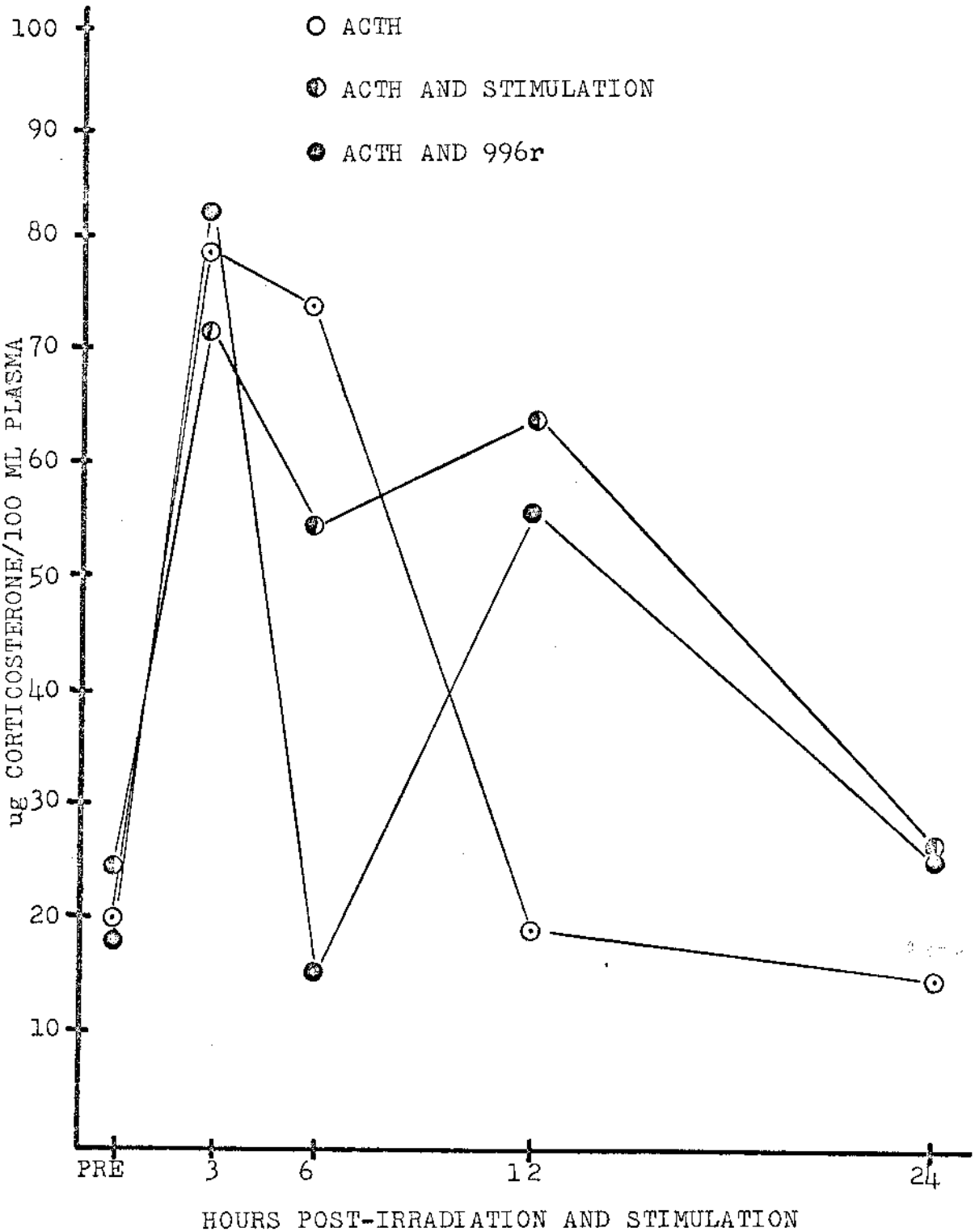

Fig. 7 -- A summary of the effects of ACTH, hypothalamic stimulation, and X-irradiation on corticosterone level in rats.

TABLE III

THE EFFECTS OF ACTH ON THE ADRENAL RESPONSE TO POSTERIOR HYPOTHALAMIC STIMULATION AND X-IRRADIATION IN RATS

Test Groups	Hours Post-Stimulation and/or Irradiation							
	Pre-stimulus	3	% Change	6	% Change	12	% Change	24	% Change
Eosinophils (cells/mm^3)	*		**						
ACTH	101 ± 32	26 ± 7	- 74	44 ± 11	- 56	58 ± 25	- 43	30 ± 14	- 70
ACTH + 996r	75 ± 29	39 ± 11	- 48	18 ± 5	- 76	15 ± 8	- 80	18 ± 12	- 76
ACTH + stimulated	116 ± 26	48 ± 18	- 59	39 ± 7	- 66	27 ± 14	- 77	65 ± 10	- 76
Corticosterone (ug/100 ml plasma)									
ACTH	20 ± 5	78 ± 10	+290	73 ± 3	+265	19 ± 2	- 5	15 ± 6	- 25
ACTH + 996r	19 ± 4	82 ± 2	+332	15 ± 2	- 21	56 ± 2	+195	25 ± 6	+ 32
ACTH + stimulated	24 ± 3	72 ± 6	+200	54 ± 3	+125	63 ± 5	+163	27 ± 2	+ 13

*Standard error of mean for four animals
**Per cent change from pre-stimulus value

ACTH alone, ACTH and hypothalamic stimulation, and finally, ACTH and whole-body X-irradiation applied simultaneously. It was evident from this data that the ACTH either in combination with stimulation or with irradiation produced an immediate (third hour post-testing) response similar to that induced by ACTH alone. A return to pre-test levels occurred by the twelfth hour in the animals receiving ACTH alone. In the animals that received both ACTH and stimulation, a return to pre-test levels occurred at the twenty-fourth hour post-testing. Interestingly enough, the animals receiving ACTH and 996r whole-body X-irradiation exhibited a return to pre-test levels of steroid at the sixth hour followed by a secondary rise at the twelfth hour before returning to pre-test levels at the twenty-fourth hour post-testing. This biphasic response in steroid levels occurred in all of the animals tested.

As shown in Figure 8 and Table 3, the eosinophil response at all three experimental conditions was relatively similar, id est, an immediate and sustained eosinopenia. The eosinopenia noted in the animals receiving ACTH and X-irradiation appeared to be more severe than in animals receiving ACTH alone or in combination with hypothalamic stimulation. Indeed, there was evidence of a recovery in the rats receiving electrical stimulation and ACTH. Figure 6 and Table 4 contain data that indicate that changes were relatively similar. The increase in weights shown in animals receiving X-irradiation and ACTH at the twelfth and twenty-fourth hour may not be statistically significant.

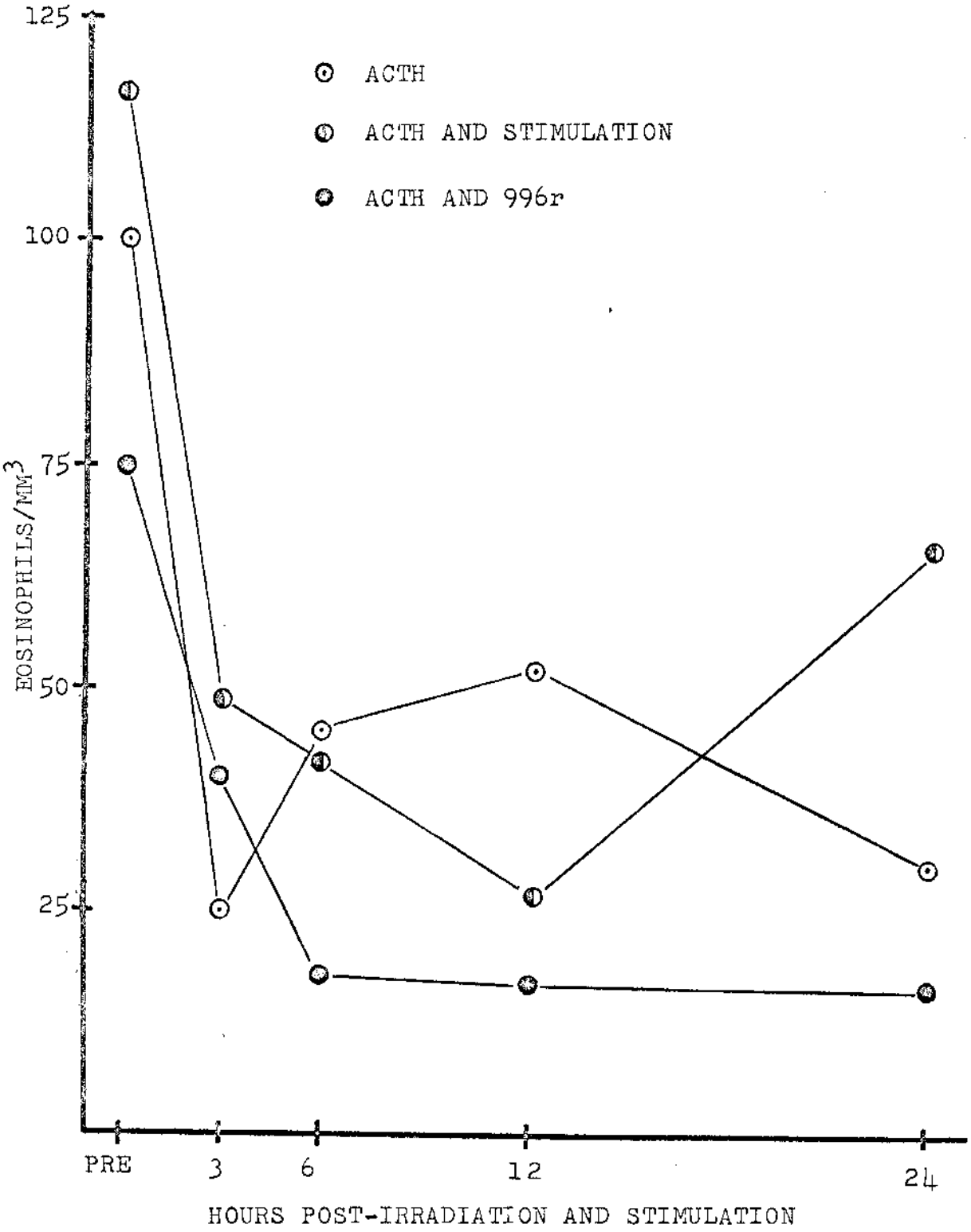

Fig. 8 -- A summary of the effects of ACTH, hypothalamic stimulation, and X-irradiation on the eosinophil count in rats.

TABLE IV

THE EFFECTS OF ACTH ON ADRENAL WEIGHT CHANGES IN RATS FOLLOWING X-IRRADIATION AND HYPOTHALAMIC STIMULATION

Test Groups	Hours Post-Stimulation and/or Irradiation			
	3	6	12	24
	* **			
ACTH	12.6 ± 2.5	14.3 ± 0.6	10.0 ± 0.2	13.8 ± 0.9
ACTH + stimulated	8.7 ± 0.3	10.8 ± 0.4	10.2 ± 1.2	9.6 ± 0.4
ACTH + irradiated	11.6 ± 0.2	11.4 ± I.0	14.9 ± 2.1	14.1 ± 2.0

*Mean weight in mg paired adrenal wt/100 gm body weight
**Standard error of mean

In general, the data indicate an inverse relationship between corticosterone levels and eosinophil count, particularly at the third and sixth hour post-testing. This relationship was quite clear in the sham-irradiated and electrically stimulated group. In this series, a distinct recovery in the eosinophil count was noted at the twenty-fourth hour post-stimulation; at this time the corticoid level had returned to pre-test levels. This inverse relationship between the corticoid level and eosinophil count was also striking in the X-irradiated animals throughout the entire time sequence. In those animals that were X-irradiated and stimulated, the inverse relationship was clear at the third and sixth hour; however, at the twelfth hour post-testing, a profound drop in corticoid occurred. At this point it was expected that there would occur a recovery of the eosinophil count. This event was not observed. The inverse relationship was also clear cut in those animals receiving ACTH alone, although recovery in the eosinophils was not as great as was expected; indeed, another drop in count was noted at the twenty-fourth hour post-injection. The inverse relationship, however, was clearly defined in those animals receiving ACTH and electrical stimulation. Again, a slight recovery in eosinophil count was noted at the twenty-fourth hour post-testing and at the same time in which there was a marked drop in corticoid levels. One of the more striking observations was found in the rats that received ACTH and 996r X-irradiation. The eosinophil

response in this group was similar to the other test group in that there was a marked and sustained eosinopenia. The corticosterone response, however, was distinctly biphasic in character, with "spiking" occurring at the third hour and twelfth hour while dropping at the sixth and twenty-fourth hour. This observation was wholly unexpected.

CHAPTER IV

Discussion

The experimental procedures used in this study and the problems involved with each were numerous. Since not all stereotaxic instruments were exactly alike, it followed that the published stereotaxic atlases derived from various animals using a certain kind of apparatus were also dissimilar. This meant that corrections in the coordinates had to be made to make sure that the implanted electrode was properly placed. Insulating the electrodes without the presence of bubbles along the pins was another obstacle. Making and molding the acrylic material used in the protective cap was no easy task. Bleeding of the cranial tissue had to be controlled by pressure and electro-cautery to prevent undue effects in the animals. Givner and Rochefort's fluorometric method (11) is comparatively new and is basically a modification of the older method of Guillemain (13). Numerous "check" runs had to be made to validate the use of the newer method in determining corticosterone in relatively small (less than one milliliter) samples of plasma. In this regard, the fluorometric technique for the determination of corticosterone possesses some inherent handicaps. The specificity of the methods in use depends upon the removal of neutral lipids by extraction with a compound such as iso-octane and phenolic compounds such as estrogens with a base.

Chloroform will extract fat-soluble compounds other than corticosterone. The extraction procedure of Guillemin et al. (14) was used in the initial stages of this investigation and found to be, in the hands of this investigator, highly erratic. The method of Givner and Rochefort proved to be more reliable since the substitution of the ethanol-sulfuric acid mixture for thirty N sulfuric acid yielded a more stable fluorescence of the corticosterone solution. Preliminary trials using this method yielded recoveries in excess of eighty-seven per cent corticosterone; from this it was concluded that the method would yield valid results. The overall mean pre-stimulus corticosterone value was 24.4 ± 6.8 ug corticosterone/100 ml. plasma, which agreed favorably with the "resting" values reported by other workers: 19.4 ug as reported by Moncloa et al. (21), and 15.2 ug reported by Guillemin, et al. (14), while Zenker and Bernstein (30) obtained a value of 33.3 ug/ml. plasma.

Another point should be raised at this time concerning the histological examination of the brain tissues at autopsy. The position of the electrode was established on the basis of graphs found in DeGroot (7). Most of the electrode tips were estimated to be in the posterior-median regions of the hypothalamus. In three animals, however, the tips were estimated to be in the anterior-medial portion of the hypothalamus. One must keep in mind that the distances involved here ranged between 0.5 to 1.0 millimeter. Considering the possibility of

deflection of the electrode upon being placed, coupled with the overall size of the lesions made prior to autopsy, the exact position of the electrodes could only be estimated. The data indicate, however, that since similar responses were observed in all of the animals in a given series, one could assume that the electrodes were in similar functional loci. This may be an important factor since some workers have shown that electrical stimulation and/or lesions in various portions of the hypothalamus produce different effects in regard to corticoid output, adrenal ascorbic acid content, and eosinophil response. Indeed, the hypothalamus has been rigorously investigated in order to determine whether or not some semblance of functional zonation exists. McCann and Haberland (23) observed the abolishment of adrenal ascorbic acid depletion which normally occurred one to four hours following unilateral adrenalectomy with median eminence lesions. Porter (25) prevented the stress-induced eosinopenia with posterior hypothalamic lesions while anterior lesions showed no response. Destruction of the mamillary and tuber cinereum regions yielded varying degrees of response. Slusher (26, 27) and Suzuki and Romanoff (28), on the other hand, reported elevated 17-hydroxycorticoid levels immediately following posterior lesions. Mason (20), by stimulating an area in the anterior medial hypothalamus of monkeys, the periventicular area, observed marked 17-hydroxycorticoid output of magnitude similar to that brought about by an intravenous injection of

ACTH shown to produce a maximal rate of rise. From this one could conclude that electrical stimulation of the posterior-median regions may bring about marked elevations of adrenal cortical hormones. The present data, in regard to the corticosterone response to electrical stimulation of the hypothalamus, do support those of Slusher (26, 27), Suzuki and Romanoff (28), and D'Angelo et al. (6), who used rats in their studies.

In regard to the eosinophil response, the largest eosinopenia following stimulation, as described by Anand and Dua (1), was elicited from the antero-medial regions of the hypothalamus; eosinopenias resulted from lesions in the more posterior areas of the complex but were not of the magnitude of those seen following anterior stimulation. The data presented here coincided with those reported in an earlier paper (2).

In assessing the role of the adrenal glands in stress, one must consider the statements of Bacq and Alexander (4). They contend that the adrenal response was not necessarily proportional to the concentration of ACTH and therefore to corticoid levels in the blood. D'Angelo et al. (6), moreover, presented evidence that adrenal activation could be done with the mere presence of an electrode in the hypothalamus; therefore, following stimulation the presence of the electrode may serve to potentiate or prolong the effects of excitation. This may account for the failure of the corticosterone levels to return to pre-testing levels in most of the animals tested before the twenty-fourth hour post-stimulation.

Adrenal hypertrophy and atrophy was one of the earliest used indices of adrenal activity. Ganong and Hume (10) and McCann (23) showed prevention of compensatory adrenal hypertrophy one month following unilateral adrenalectomy in animals with hypothalamic lesions, whereas Patt et al. (24) showed unilateral adrenal hypertrophy to follow six hours after exposure to X-irradiation. The use of unilateral adrenalectomy for long-term studies would appear to be a more valid parameter but involves laparotomy, which is a major stress in itself and, therefore, is unsatisfactory for short-term studies such as this. Accessory adrenal tissues have been described (12); additionally adrenal corticoids of gonadal origin are known to exist. In view of this it was difficult to rationalize that differences of adrenal weight during the first hours following stress indicate specific adrenocorticotropic activity. Differences in weighing techniques could lead to large sources of error due primarily to adherent fat; decapsulation was imperative for meaningful comparisons. The present study indicates that adrenal weight changes may be inadequate criteria for assaying activity of the adrenal pituitary axis.

In comparing the eosinophil responses that were obtained by X-irradiation alone with those effects of electrically stimulated animals, the data indicate that the effects of the latter stress agent appeared to be slightly smaller in amplitude, and more reversible. The irradiated animals exhibited a slightly greater, sustained, and irreversible response. This

would indicate two separate mechanisms involved in bringing about the observed responses. Both agents appear to produce Selye's "first reaction" as described by Bacq and Alexander (4). Electrical stimulation of the hypothalamus brought about a more pronounced initial reaction while X-irradiation promoted higher sustained values.

In regard to the effects of whole-body X-irradiation on corticosterone levels, the data were in good agreement with those of Lott and Gaugl (19), Hameed and Haley (16), Haley (15), Eechaute et al. (8), French (9), and Binhammer and Crocker (5). The sharpest difference in the data occurred in those animals that received both 996r and electrical stimulation. These animals exhibited the initial increase in corticoid levels, as was observed in the other test groups. However, this response was relatively short-lived and returned to pre-testing levels by the twelfth hour, unlike the other groups. Since X-irradiation alone showed a delayed return to control levels while the stimulated groups returned to control levels at the twenty-fourth hour, one was tempted to conclude that X-irradiation in some manner decreased the recovery time in the stimulated animals.

The inverse relationship between the corticosterone levels and circulating eosinophils in both the stimulated and the X-irradiated animals was in fair agreement with that of French (9), who noted the plasma 17-hydroxycorticosteroid concentration to reach maximal proportions at four to eight hours following

800r radiation and to return to normal twelve hours post-irradiation. He also noted that the initial rise was similar in nature to the response elicited with maximal doses of ACTH. Moreover, he found that the eosinophil response was, in general, the reciprocal of the steroid changes: id est, following irradiation the eosinophil count decreased as the steroid concentration increased

A plausible explanation for the inverse relationship between ACTH or corticoid titer and eosinophil levels may be found in an excellent monograph on the eosinophil leucocyte by Archer (3). He claimed that in stressed animals, the high corticoid levels tend to decrease the number of circulating eosinophils due to the antagonism between corticoids and histamine. According to Archer, there exists a chemotactic attraction of histamine for eosinophils; for example, a considerable local accumulation of eosinophils follows injection of histamine into tissue, often as soon as one-half hour. Intravenous injection of histamine, however, is often followed within five minutes by a reduction in the numbers of eosinophils in the circulating blood. The eosinophils tend to chemically inactivate histamine in some manner. Corticoids and ACTH, on the other hand, produce a reduction in blood histamine which in turn brings about a reduction of eosinophils from the circulating blood. Additionally, Harris, Jacobsohn, and Kahlson (17) have shown that the median eminence contains unusually high quantities of histamine. It may be conceivable that electrical stimulation could alter the

integrity of this tissue to allow release of sudden large quantities of histamine which would further reduce the number of eosinophils, or allow for the prolonged release of histamine. It has been well established that free histamine in tissues may account at least in part for some of the clinical symptoms of stress resulting from permeability changes. The foregoing proposals appear to explain the changes that were seen in corticoid and eosinophil levels during the first six hours post-stress period. They do not explain, however, why recovery in the eosinophil count did not occur when the corticoid concentration returned to resting levels. Perhaps other factors controlling histamine release and/or metabolism were still operative. Indeed, one must remember that following lethal doses of ionizing radiation, such mammals as rats begin to show general radiation symptoms fairly early, id est, within twenty-four hours; therefore, the criteria used for the earlier acute phases in radiation injury may not be applicable in accounting for the more delayed and general symptomology of the radiation syndrome. It was tempting to propose that the earlier adrenal responses observed in the stimulated and/or irradiated animals involved specific radio-sensitive targets while the latter responses were due to a more generalized and indirect effects: for example, the action of humoral agents.

In those cases in which the corticoid levels returned in a relatively short time to resting levels, Knigge's explanation (18) may be valid. He claimed that the hypothalamus, in

mediating afferent impulses, "paces" the pituitary in order that quantities of ACTH that might be deleterious to the adrenal glands are not secreted too quickly. Such an inhibitory response observed in this study following initial high concentrations of corticosterone may have been a beneficial one to protect the adrenals. Along this same line, one must not overlook the possibility of a depletion of available corticoid precursors. Moreover, one must consider the presence of various reported factors as corticotropin-releasing agents, histamine-binding factors, and corticoid-releasing factors discussed by Venning in her recent review on the adrenal cortex (29).

Finally, Lott and Gaugl (19) found that exposure to a sub-lethal dose (230r) of whole-body irradiation produced an initial rise of plasma corticosterone titer at three hours that approached control levels at twelve hours and dropped below the controls at twenty-four hours. A lethal dose (780r) of total-body irradiation elicited a similar response; however, the duration of the initial effect was sustained out to twelve hours post-irradiation. Head-shielding studies, using 230r, produced a decrease in corticosterone levels six hours following exposure, while at 780r a slight increase occurred at three hours. The authors concluded that this delay seemed to indicate a need for experiments extending the present study to include head and body-shielding in the format.

In general, the data presented here indicate that X-irradiation acts as a non-specific stressor agent, due to the fact that the initial (three to six hours post-test) adrenal responses in all three experimental conditions was relatively similar in magnitude and duration. On the other hand, the fact that no recovery in the eosinophils' count was observed in any of the X-irradiated group, coupled with the speed at which the responses were noted (within three hours), indicates a more direct, specific action. The data also enforce the electroencephalographic findings of Monnier and Krupp (22) that indicate a possible radiation target to be in the hypothalamus. Such a target may act as a "trigger" for the release of ACTH by the adenohypophysis.

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CHAPTER V

Summary

The present study concerned the effects of X-irradiation upon the adrenal response to hypothalamic stimulation in rats. Adrenal cortical responsiveness was studied by monitoring the plasma corticosterone levels fluorimetrically; the circulating eosinophil level determined by the direct chamber counting method; and changes in adrenal weight. The stress agents utilized were 996r whole-body X-irradiation and posterior hypothalamic stimulation. The test animals were subjected to electrical stimulation, X-irradiation, and the combination of the two. To judge similarity of response due to an ACTH mechanism, other animals were subjected to ACTH alone, electrical stimulation combined with ACTH, and irradiation combined with ACTH. Blood samples were taken immediately before stimulation or X-irradiation, and at intervals of three, six, twelve, and twenty-four hours post-testing.

The most significant findings were (1) Plasma corticosterone levels increased significantly with the first three hours following all of the experimental conditions. Irradiation combined with stimulation brought about a maximal corticosterone response (increase) in six hours, and returned to control levels at the twelfth hour. Electrical stimulation alone brought about the greatest increase in corticoid

concentration six hours post-stimulation but returned to control level at the twenty-fourth hour. The animals that received irradiation alone never regained the corticoid control level. (2) Eosinopenias resulted from application of all of the above stressors. Irradiation produced more irreversible changes in the eosinophil response than did electrical stimulation. No significant additive effect of combined stimulation and X-irradiation was observed. (3) Animals receiving ACTH in combination with X-irradiation exhibited a distinct biphasic corticosterone response; however, no such response was observed in the eosinophil count. Instead, a slightly more severe and irreversible eosinopenia was noted in this group of animals. The eosinopenia observed in the ACTH injected-electrically stimulated group showed reversibility at the twenty-fourth hour. (4) The data indicate that changes in adrenal weights are inadequate indices to denote activity of the adrenal-pituitary axis. (5) There was a distinct inverse relationship between the corticosterone response and the eosinophil response within the first six hours in all the experiments. At the twelfth and twenty-fourth hour post-testing, however, the inverse relationship was not apparent indicating a secondary effect on the adrenal responses. The foregoing inverse relationship may be explained on the basis of free histamine release or histamine binding mechanisms. (6) The fact that X-irradiation alone and electrical stimulation alone brought about similar corticoid and eosinophil response in the first six hours indicated that a

common "target" or mechanism was affected; however, the later responses indicate that secondary and different mechanisms may be involved. The first response resembled the "alarm reaction" of Selye's GAS concept. Moreover, due to the speed of the two responses and assuming both to be the result of adrenal-pituitary axis activity, one might conclude that at least one target or radio-sensitive area to be located in the hypothalamic area. Such an area would then be in position to "trigger" the release of ACTH and thus to initiate the general stress response.

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