THE UNIVERSITY OF OKLAHOMA

SCHOOL OF MEDICINE

HORMONAL INFLUENCES ON CATALASE ACTIVITY

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1955

HORMONAL INFLUENCES ON CATALASE ACTIVITY

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ACKNOWLEDGMENT

The writer takes this opportunity to express his appreciation to the many professors and fellow students who have had a part; through instruction, suggestions and criticism, in the work leading to this thesis.

Gratitude is expressed to Doctor Allan J. Stanley who has directed my graduate program and research problem. His instruction and criticism have been invaluable.

A deep sense of indebtedness to various members of the Physiology Department is felt for their encouragement and assistance in numerous respects.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

HORMONAL INFLUENCES ON CATALASE ACTIVITY

CHAPTER I

INTRODUCTION

It is becoming increasingly evident that hormones exert their regulatory effects on tissues and organs by affecting rates of enzymatic processes. Although the effect of hormones on the body, even in minute quantities, is usually marked, the precise chemical functions of the various hormones remained totally obscure until the concept of hormone-enzyme inter-relationships was derived. With this concept as a working hypothesis such progress has been made in the last ten years that it is now possible to give many examples of enzyme systems which are influenced by endocrine substances. Several recent reviews, Dorfman (1952), Lieberman (1953), Porter (1954), Verzar (1952), serve admirably in this manner.

Investigators in this field seem to agree that hormones may exert their influence on enzymes in one of four possible ways: (1) by changes in enzyme concentrations in the tissues, (2) by the hormone functioning as a component of an enzyme system, (3) by the hormone accelerating or inhibiting an

enzyme system, or (4) by direct or indirect effects on accelerators and/or inhibitors of enzyme systems.

With this concept of hormonal regulation of enzyme activity in mind the present study was undertaken in an attempt to determine whether the enzyme catalase is affected by endocrine substances. The physiological role of catalase has not been fully clarified and it was felt that if some of the factors that influence its activity were better known perhaps its physiological function could more readily be understood.

Until recently it was believed that the function of catalase was only a protective one against the toxic effect of hydrogen peroxide by promoting the degradation of hydrogen peroxide to water and oxygen. Keilin and Hartree (1954), however, have shown that catalase may in addition function in coupled oxidations. They observed the oxidation of alcohols to aldehydes in the presence of catalase and hydrogen peroxide slowly generated by chemical reactions or furnished by the action of an enzyme such as xanthine oxidase. Methanol, ethanol, N-propanol, isobutanol, and ethylene glycol were shown to undergo oxidation in this system. From this study Keilin and Hartree concluded that although catalase does promote the decomposition of hydrogen peroxide into water and molecular oxygen, thereby protecting cells from the toxic effects of hydrogen peroxide, the

catalysis of coupled oxidations by means of hydrogen peroxide formed in a primary oxidation is the most important biological property of catalase. Theorell (1951) and Chance (1951) are also of the opinion that the chief physiological function of catalase is its ability to catalyze coupled oxidations.

The mechanism of action of catalase can be more readily understood if it is described in the form of equations. Thus according to the old theory of its action the enzyme (E) combined with the substrate (S), which is hydrogen peroxide, to give an enzyme-substrate complex (ES). This complex then breaks down spontaneously to give the enzyme plus products (P).

$$E + S \longleftrightarrow ES$$
$$ES \longleftrightarrow E + P \qquad \text{(Equation I)}$$

Due largely to the work of Britten Chance (1951), the <u>consecutive reaction theory</u> for catalase is now the generally accepted mechanism for its action.

$$E + S \longleftrightarrow ES$$
$$ES + S \longleftrightarrow E + P \qquad \text{(Equation II)}$$

Here a hydrogen peroxide molecule (S), which is the specific substrate for catalase, reacts with catalase (E) to form the enzyme-substrate complex (ES); an additional molecule of hydrogen peroxide then reacts with the enzyme-substrate complex to give the enzyme plus products (P), which are water

and oxygen in this case. This theory of two consecutive bimolecular reactions (Equation II) is in contrast to the older idea that the activity of catalase could be described by a single bimolecular reaction followed by a spontaneous monomolecular reaction (Equation I). The question then arises as to how the reaction of Equation II can account for the coupled oxidation reactions in which catalase is known to participate. Equation II can be written in a more general form that will allow explanation of both properties of catalase, i.e., the coupled oxidation reaction and the simple decomposition of hydrogen peroxide.

$$E + S \longleftrightarrow ES$$

$$ES + AH_2 \longleftrightarrow E + SH_2 + A \qquad \text{(Equation III)}$$

Here AH_2 represents a hydrogen donor molecule which may be an alcohol. Using ethyl alcohol as an example to demonstrate a coupled oxidation reaction, the equation would be

$$E + H_2O_2 \longleftrightarrow E\text{-}H_2O_2 \qquad \text{(Equation IV)}$$

$$E\text{-}H_2O_2 + C_2H_5OH \longleftrightarrow E + 2\ H_2O + CH_3CH{=}O$$

In the case of the simple decomposition of hydrogen peroxide, a second hydrogen peroxide molecule would serve as the hydrogen donor and react with the catalase-hydrogen peroxide complex in this manner:

$$E + H_2O_2 \longleftrightarrow E\text{-}H_2O_2 \qquad \text{(Equation V)}$$

$$E\text{-}H_2O_2 + H_2O_2 \longleftrightarrow E + 2\ H_2O + O_2$$

From this it is clear that the consecutive reaction theory accounts for both observed actions of catalase, i.e., the decomposition of hydrogen peroxide to water and oxygen and the function of catalase in coupled oxidations.

One further interesting aspect of the coupled oxidation action of catalase arises from the recent work of Tauber (1954). He demonstrated that dilute solutions of crystalline catalase, in the presence of hydrogen peroxide, can oxidize a variety of large molecules by coupled oxidation. Thus, alpha-naphthol and p-phenylenediamine are oxidized to indophenol purple; p-aminobenzoic acid, sulfathiazole, adrenalin, ephedrine sulfate and tyrosine are coupled with catechol by oxidation to form colored compounds. Pyrogallol is oxidized to the insoluble end product purpurogallin and catechol is oxidized to soluble products of different colors. This work suggests another important role of catalase since numerous complex compounds found in nature can be derived from simple phenols and phenol derivatives by oxidative coupling.

Investigators in the field of cancer have for many years had an interest in the relationship between catalase activity and malignant growths. In the early years of this century Rosenthal (1912), working with mice, and Brahn (1916), studying cancerous tissues of man, came to the conclusion that liver catalase activity is depressed by malignant growths. It was not, however, until 1941 that active interest in this

subject was revived, largely through the work of Greenstein and his co-workers. Their findings have recently been summarized (Greenstein, 1947) and show the following facts:

1. The decrease of liver catalase is progressive with the growth of the tumor and is most marked in animals bearing rapidly growing tumors.
2. The effect is reversible, i.e., the liver catalase activity returns to normal after excision or spontaneous regression of the tumor. This return to a normal level is rapid, often seen within 24 to 48 hours after removal of the tumor.
3. Of the other liver enzymes studied in tumor-bearing rats, only arginase showed any decrease in activity. The reduction in activity of this enzyme was much less striking than that of catalase.
4. Kidney catalase activity is depressed much less than that of liver; only in mice with spontaneous mammary tumors is it lowered as much as, or more than, liver catalase.
5. The depression of liver catalase is not due simply to the presence of growing tissue, since pregnancy or growing implants of embryonic tissue fail to affect it.

Investigators, assuming that the nutritional state of cancerous animals might be the important factor involved in

the lowering of catalase activity have made numerous attempts to influence the liver catalase of normal animals by diet. Greenstein (1941) found that a 3-day fast had no effect on liver catalase activity of rats. Weil-Malherbe (1948) reported that the catalase activity of normal rat liver was not significantly changed by a high (45% casein) or a low (18% casein) protein diet.

Although it is a well established phenomenon of cancer, the mechanism by which a distant tumor affects liver catalase remains unknown. Of the several hypotheses proposed, the one that seems to have the strongest experimental support suggests that the effect is produced by one or more substances elaborated by the cancer cells and released into the blood stream. Nakahara and Fukuoka (1949) have reported the isolation of a proteose-like material from human cancers which, when injected intraperitoneally into mice, produces a marked diminution in liver catalase. This fraction is water soluble and alcohol insoluble; it could not be isolated from normal tissue, even that adjacent to the tumor, and it had no effect <u>in vitro</u> on homogenates of normal liver. Greenfield and Meister (1951), Adams (1950), and Appleman (1950) have confirmed these findings with experimental tumors and have succeeded in concentrating the "anti-catalase fraction." The preparation of Greenfield and Meister caused a significant

drop in liver catalase activity within 12 hours after it was injected intraperitoneally into mice. At 24 hours after injection of the preparation catalase activity was lowered by more than 50% and after 72 hours the activity was back within normal limits. The depression of liver catalase activity was not accompanied by a significant decrease in liver lactic dehydrogenase, dehydropeptidase, and glutamic-alanine transaminase. The activity of the tumor fractions was not affected by digestion with 6 N HCL for 48 hours.

CHAPTER II

EXPERIMENTAL PROCEDURE

Albino rats of the Holtzman strain were used as experimental animals throughout this study. In order to minimize the variability between groups of animals, each experiment was carefully controlled by using animals of the same age and sex. Determined values of catalase activity of the livers of experimental and untreated animals were compared. Data so derived, as set forth in the various tables to follow, were treated statistically and the probability established.

Catalase activity was measured by the method of Feinstein (1949). This method consists of the use of sodium perborate as the substrate and of the determination of the perborate remaining after catalase action by titration with potassium permanganate.

The livers were removed from the animals under light ether anesthesia and were immediately placed in cold phosphate buffer (M/15; pH 6.8) to remove the excess blood. The tissue was then blotted dry, placed in a glass container and immediately frozen in a deep freeze unit. In earlier experiments

(Troop, 1953, unpublished thesis) we found that freezing of rat liver at -18 degrees C., for periods as long as 12 days had no apparent effect on the catalase activity.

After being frozen for at least one day but never longer than three days, a sample was taken from each lobe of the liver. The samples from any one liver were combined, weighed and finally homogenized in ten times their weight of ice-cold phosphate buffer (M/15; pH 6.8) for one minute in a Waring blendor with a micro-attachment. A one ml. sample of the homogenate was then diluted 1:50 with triple distilled water giving a final dilution of 1:500, and the catalase activity determined.

The substrate, as mentioned above, was 1.5% sodium perborate, tetrahydrated, adjusted to pH 6.8 with concentrated HCl. Eight ml. of substrate and 1.5 ml. of phosphate buffer (M/15; pH 6.8) were placed in a series of four 125 ml. Erlenmeyer flasks. The flasks were covered with watch-glasses and immersed in a bath at 37 degrees C. for exactly 15 minutes after which 0.5 ml. of water was added to the first flask, 0.5 ml. of the tissue homogenate to the next two flasks (giving duplicate determinations for each animal), and 0.5 ml. of water to the fourth flask. After exactly 5 minutes of incubation, 10 ml. of 2 N sulfuric acid were added to each flask to stop the enzyme action. The contents of the flasks

were then titrated with standard potassium permanganate. The titres of the first and fourth flasks, containing perborate and buffer as in the test flasks, but with water replacing the homogenate, were used as base points from which to calculate the perborate destroyed by the enzyme. At various times throughout this study the permanganate reducing capacity of liver tissue itself was determined. For this purpose an additional flask was used containing tissue (1:500 dilution) and buffer but with water replacing perborate. At no time was the liver tissue factor alone great enough to register on the micro-pipette used for titration.

The enzyme activity was expressed as milliequivalents of sodium perborate destroyed per gram, wet weight, of tissue under the conditions specified above.

Cortisone was the first synthetic steroid studied for its effect on liver catalase in the intact rat. Table I presents the data obtained from 24 rats. The animals were divided into four groups, each containing 6 rats. Group 1 was maintained as controls, receiving no treatment. The animals in group 2 were injected with 2 mgm. of cortisone acetate subcutaneously each day for 15 days before being sacrificed and their livers removed and assayed for catalase activity. Those in group 3 received 5 mgm. of cortisone acetate each day for 17 days and the ones in group 4 were given 10 mgm. daily for 15 days.

TABLE I

LIVER CATALASE ACTIVITY AFTER VARIOUS DOSES OF CORTISONE

Group	Treatment	Number of Animals	Adrenal Weight	Change in Body Wt.	Activity*	Range
1	CONTROLS	6	21.6 mg	-	798	(760-874)
2	CORTISONE (2mg/day; 15 days)	6	18.8	-42 gm	588**	(500-678)
3	CORTISONE (5mg/day; 17 days)	6	14.5	-100	468 **	(431-509)
4	CORTISONE (10mg/day; 15 days)	6	16.1	-121	397**	(364-434)

* milliequivalents of sodium perborate destroyed per gram of tissue.
** significant at .1% level of probability.

This table was reproduced from Masters Thesis: The Effect of Cortisone on Rat Liver Catalase Activity. R. C. Troop, University of Oklahoma, School of Medicine, 1953.

The catalase activity of the liver was found to decrease progressively as the dosage of cortisone was increased. Doses of 2 mgm. gave a 26% depression, 5 mgm. a 41% depression, and finally the 10 mgm. dosage gave a 50% depression. The increase in dosage also caused a decrease in both adrenal weight and body weight.

After observing this marked response to cortisone, it was decided to study the effect of adrenocorticotropic hormone (ACTH) on this enzyme system. Two experimental groups of animals were used (Table II). One group was given 2 mgm. of ACTH[1] per day, subcutaneously, for 12 days and the other group was injected with 5 mgm. of ACTH daily for 14 days. The results were negative in that neither of the treated groups showed a significant difference from the control group. It must be pointed out, however, that data derived from the group receiving 5 mgm. of ACTH each day were on the borderline of statistical significance, indicating that a series of animals receiving higher doses of ACTH might yield data which would be significantly different. As will be shown later, this experiment has now been repeated with larger groups of animals and higher doses of ACTH, but at no time have we observed a depression of catalase activity with ACTH. This

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[1]Armour's ACTH, lyophilized, Standard LA-1-A. This preparation was used throughout this work except where otherwise indicated.

TABLE II

LIVER CATALASE ACTIVITY FOLLOWING ACTH TREATMENT

Treatment	Number of Animals	Adrenal Weight	Change in Body Wt.	Activity	Range
CONTROLS	6	21.6 mg	-	798	(760-874)
ACTH (2mg/day; 12 days)	6	26.5	-11 gm	728*	(613-844)
ACTH (5mg/day; 14 days)	4	31.0	-29	712**	(629-769)

* not significant at 5% level of probability.
** significance doubtful.

This table was reproduced from Masters Thesis: The Effect of Cortisone on Rat Liver Catalase Activity. R. C. Troop, University of Oklahoma, School of Medicine, 1953.

interesting difference between the action of cortisone and ACTH will be discussed later.

The influence of ACTH on liver catalase activity was studied further in four additional groups of rats (Table III). The animals in groups 1 and 2 were females; those in group two received lyophilized ACTH (Armour's) in saline at the rate of 2 mg. daily for 6 days. The liver catalase activity did not differ significantly from that of the untreated female controls. The animals in groups 3 and 4 were males. Group three was maintained as controls and group 4 was given ACTH in a gel preparation[1]. Six units (1 unit equivalent to one mgm.) were injected subcutaneously daily for six days. Here again there was no difference in catalase activity between treated and control animals, indicating that ACTH does not depress the liver catalase activity in the rat. The enlargement of the adrenals in the treated animals in groups 2 and 4 indicated that a systemic response was elicited from the ACTH.

Comparison of groups 1 (female controls) and 3 (male controls) of Table III gives a definite indication of a sex difference in liver catalase activity; the males having much the higher value. Because of the possible importance of a sex difference in the hormonal control of catalase activity,

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[1]Courtesy of Dr. S. L. Steelman, Armour Laboratories, Research Department, Chicago, Ill. (Long-Acting HP ACTHAR Gel).

TABLE III

LIVER CATALASE ACTIVITY IN MALE AND FEMALE RATS FOLLOWING ACTH TREATMENT

Group	Treatment	Number of Animals	Adrenal Weight	Change in Body Wt.	Activity	Range
1	CONTROLS (females)	6	26.9 mg	-	441	(339-497)
2	ACTH (females, 2 mg/day; 6 days)	5	32.6	-	465*	(418-499)
3	CONTROLS (males)	8	18.7	+50 gm	967	(850-1077)
4	ACTH-gel (males, 6 units/ day; 10 days)	8	22.5	+29	957*	(818-1076)

* not significant at 5% level of probability.

this point was investigated further. Fifteen untreated rats of the same age were used. Eight of the animals were males and seven of them were females. Table IV shows that the liver catalase activity of the male rat is approximately 25% greater than the corresponding activity of the female.

TABLE IV

SEX DIFFERENCE IN LIVER CATALASE ACTIVITY

Sex	Number of Animals	Activity	Range
MALES	8	967	(850-1077)
FEMALES	7	752	(644-834)

A sex difference in catalase activity of the livers of animals has been noted by other investigators. Adams (1950) found the liver catalase activity in the male mouse to be from 20 to 30 percent higher than in the female mouse. Schultze and Kuiken (1941) noted a greater liver catalase activity in male rats than in female rats but their experiments were not controlled in a manner that will allow a quantitative value to be given for the difference.

With this sex difference in view, it was of immediate interest to determine whether catalase activity could be quantitatively affected by appropriate sex hormone administration. To this end a number of female rats were given testosterone as shown in Table V. The animals in groups 1 and 2 were mature females; the ones in group 2 received

TABLE V

LIVER CATALASE ACTIVITY IN FEMALE RATS AFTER TESTOSTERONE TREATMENT

Group	Age	Treatment	Number Animals	Adrenal Weight	Change in Body Wt.	Activity	Range
1	mature	CONTROLS	5	-	+15 gm	490	(449-518)
2	mature	TESTOSTERONE (5mg/day;10 days, subcutaneously)	6	-	-7	580*	(511-630)
3	166 d.	CONTROLS	7	27.2 mg	+7	636	(516-742)
4	166 d.	TESTOSTERONE (2 ca. 20mg pellets subcutaneously for 12 days)	8	22.1	+11	727**	(683-826)
5	79 d.	CONTROLS	7	28.9	+36	752	(644-834)
6	79 d.	TESTOSTERONE (5mg/day; 11 days, intramuscularly)	8	19.9	+27	717***	(649-828)

* significant at 1% level of probability.
** significant at 5% level of probability.
*** not significant at 5% level of probability.

testosterone[1], 5 mgm. daily subcutaneously for 10 days, after which the livers were removed and assayed for catalase activity. The treated animals had a significantly higher liver catalase activity than the controls. The significance of the difference between the means of these two groups was at the 1% level of probability.

The animals in groups 3 and 4 were also mature females. The rats in group 4 received implants of two 20 mgm. testosterone pellets[2] subcutaneously 12 days before the liver was excised for assay of its catalase activity. It was calculated that approximately 0.6 mgm. of the implanted material was absorbed per day. Here again the testosterone treated animals had greater liver catalase activity than did their controls. The difference is not so great as in groups 1 and 2 but it is statistically acceptable at the 5% level of probability.

Finally, the immature female rats in groups 5 and 6 were studied. The animals in group 6 were injected intramuscularly with 5 mgm. of testosterone[3] daily for a period of 11 days. The difference in the liver catalase activity of these two groups was not statistically significant. This immediately raises a question as to why testosterone failed to raise the catalase activity in this group (group 6) as it

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[1]Repositol Testosterone, Pitman-Moore Co.

[2]Oreton-F Pellets, Courtesy of Dr. E. Henderson, Schering Corporation, Bloomfield, New Jersey.

[3]Oreton-F, Aqueous Suspension of testosterone U.S.P. Courtesy of Dr. E. Henderson, Schering Corporation.

did in groups 2 and 4. Possible reasons for this difference will be discussed later.

Since testosterone promotes an increase in catalase activity when administered to the mature female rat, it was felt that castration of the male animal might reduce this enzyme activity. Accordingly a group of 8 male rats was castrated and the catalase activity of their livers was compared with that of the livers of 7 control animals. Table VI shows the results of this procedure. The operated animals were castrated via the abdominal approach ten days before they and their controls were sacrificed and analyzed for enzyme activity. Although the average catalase value for the castrated group was slightly higher than for the control group the difference did not stand when the data were statistically analyzed.

Effect of Hydrocortisone on Liver Catalase Activity

When hydrocortisone became available to us, its influence on catalase activity was tested. This compound, unlike cortisone, is considered to be a true secretory product of the adrenal cortex (Hechter and Pincus, 1954). Hydrocortisone[1] was administered by stomach tube at the rate of 5 mgm. per day for 10 days. The recent work of Hyde (1954) has shown that

[1]Hydrocortone Acetate, saline suspension, Sharp and Dohme Courtesy Dr. Elmer Alpert, Merck and Co., Rahway, New Jersey.

TABLE VI

EFFECT OF CASTRATION ON LIVER CATALASE ACTIVITY IN MALE RATS

Condition of Animals	Number of Animals	Change in Body Wt.	Adrenal Weight	Activity	Range
CONTROLS	7	+12 gm	20.2 mg	712	(602-813)
CASTRATED	8	-23	20.7	655*	(576-755)

* not significant at 5% level of probability.

hydrocortisone is almost completely absorbed from the gastrointestinal tract when given in this manner. That good absorption occurred in this experiment (Table VII) is indicated by the sharp reduction in adrenal weight of the animals given hydrocortisone. The catalase activity was depressed 32% in the treated animals making it apparent that hydrocortisone acts similarly to cortisone with respect to catalase activity.

TABLE VII

LIVER CATALASE ACTIVITY AFTER HYDROCORTISONE TREATMENT

Treatment	Number of Animals	Adrenal Weight	Change in Body Wt.	Activity	Range
CONTROLS	8	18.1 mg	+50 gm	967	(850-1077)
HYDROCORTISONE (5mg/day; 10 days)	8	11.6	-2	654*	(441-865)

* significant at 1% level of probability.

Effect of Adrenalectomy on Liver Catalase Activity

It might be inferred that since cortical hormones cause a depression of catalase activity, adrenalectomy would promote an increase in this enzyme. To test this assumption the work in Table VIII was carried out. These animals were males, 77 days old.

The operated group was adrenalectomized 12 days before autopsy and maintained on dry sodium chloride ad lib. There was no difference in catalase activity of the two groups.

TABLE VIII

EFFECT OF ADRENALECTOMY ON LIVER CATALASE ACTIVITY

Condition of Animals	Number of Animals	Change in Body Wt.	Activity	Range
CONTROLS	8	+50 gm	967	(850-1077)
ADRENALECTOMIZED	6	+31	948	(896-1058)

Effect of Hypophysectomy and Growth Hormone on Catalase Activity

In 1951 Gaebler and Mathies studied liver catalase activity in hypophysectomized rats. They found a marked increase in catalase activity in female rats after removal of the pituitary. Small doses of growth hormone did not lower the high catalase activity of these operated animals but large doses reduced the elevated levels toward normal. Similar large doses produced small decreases of catalase activity in normal animals.

Our work along these same lines is essentially in agreement with the findings of Gaebler and Mathies (1951). This study was done on three groups of male rats of the same age. Group 1 (Table IX) was maintained as controls, groups 2 and 3 were hypophysectomized when they were 55 days old. Group 2 received no further treatment but group 3 received 0.4 mgm. of Armour's growth hormone beginning 30 days after operation and continuing through the 107th day postoperatively;

from then until autopsy, which was 170 days after hypophysectomy, these animals received 0.8 mgm. daily of growth hormone.

TABLE IX

EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE ON LIVER CATALASE ACTIVITY IN MALE RATS

Group	Treatment	Number Animals	Adrenal Weight	Activity	Range
1	CONTROLS	3	21.9 mg	755	(646-847)
2	HYPOPHYSECTOMIZED	5	5.6	871	(772-971)
3	HYPOPHYSECTOMIZED plus GROWTH-HORMONE	4	8.1	849	(707-949)

Although it was felt that the groups in this series were too small to allow statistical analysis, it appears that hypophysectomy promoted an increase of catalase activity while replacement therapy with growth hormone, in the small doses used, did not cause an appreciable change.

Mechanism of Catalase Depression by Cortisone and Hydrocortisone

Throughout this part of the study an explanation of the depressing effect of cortisone and hydrocortisone on catalase was being sought. Two lines of evidence persuaded us that the availability of iron for the synthesis of catalase, which by the work of Sumner and Dounce (1937) has been shown

to contain iron in its molecule, might be an important cog in the mechanism. First, Fukuoka and Nakahara (1951) have reported that the depression of catalase by the fraction that they isolated from tumor tissue can be neutralized by the injection of iron salts. It appears, from this observation, that the tumor material might be producing its effects on liver catalase by combining with iron and in some way removing it from the circulation, thus limiting its availability for the synthesis of catalase.

Secondly, Hamilton, Gubler, Ashenbrucker, Cartwright and Wintrobe (1951) have shown that adreno-cortical extract or cortisone (4-8 mgm.) produces acute hypoferremia in rats. The hypothesis that cortisone may be depressing catalase by virtue of its hypoferremic action was tested by giving iron with cortisone to determine whether any reversal or lessening of the depression would result. Table X shows the findings of this procedure. The group of animals receiving both cortisone and iron were given ferrous sulfate[1] intramuscularly in sufficient quantity to equal 1 mgm. of iron daily. This amount of ferrous sulfate was dissolved in 0.2 cc. of distilled water for injection. The site of injection was alternated between the two heavy thigh muscles of the rats.

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[1]Baker's Reagent grade ferrous sulfate. All animals in this study were fed Purina Dog Chow which contains approximately 32 mgm. of iron per 100 grams of feed.

TABLE X

EFFECT OF IRON AND CORTISONE, COMBINED, ON LIVER CATALASE

Treatment	Number of Animals	Adrenal Weight	Change in Body Wt.	Activity	Range
CONTROLS	6	20.5 mg	0	923	(852-1014)
CORTISONE (5mg/day; 10 days)	6	15.0	-7 gm	501	(400-616)
CORTISONE (5mg/day; 10 days) plus IRON (1mg/day; 10 days)	6	18.9	-57	435	(390-495)

The data in Table X confirm the marked depression of catalase by cortisone but there is no indication that the intramuscular administration of iron salts prevents or lessens this process, rather the indication is that the iron injections produced a small additional depression.

Prevention of Cortisone-Induced Adrenal Atrophy

During the attempt to determine whether or not plasma iron plays a role in the reduction of liver catalase activity caused by cortisone acetate, an interesting and unexpected influence on the adrenal glands was noted. As indicated in Table X, the administration of 5 mgm. of cortisone acetate per day produced a very significant reduction in the weight of the adrenal glands. This reduction was expected and is thought to be due to suppression of ACTH output by the

anterior pituitary gland. When a similar group of animals (group 3, Table X) was given the same amount of cortisone and in addition 1 mgm. of iron intramuscularly, a striking reduction of the atrophy occurred. This observation was followed up with a number of experiments designed to clarify the mechanism through which the iron was acting to prevent the cortisone-induced adrenal atrophy. The data in Table XI confirm the original observation, in that cortisone (group 2) reduced the adrenal weight and the addition of iron to the regimen (group 3) prevented the cortisone-induced adrenal atrophy. In fact, it is seen that when the adrenal weight is expressed on a body weight basis, to off-set the greater loss of weight in the animals receiving both cortisone and iron, the iron actually caused an adrenal hypertrophy even during cortisone administration. It is further noted (group 4) that when iron was given alone, a marked increase in adrenal weight resulted.

Further, it was of interest to establish whether or not iron given orally would produce the same effect on the adrenal glands as when given intramuscularly. Table XII shows the results of this experiment. In this instance the iron was given by stomach tube and it is seen that the results are, in general, the same as when iron was given intramuscularly. Thus cortisone caused the usual atrophy; iron given concurrently with the cortisone prevented the atrophy, and

TABLE XI

INFLUENCE OF IRON SALTS ON CORTISONE-INDUCED ADRENAL ATROPHY

Group	Number Animals	Treatment	Body Weight		Adrenal Weight	
			Initial	Final	Absolute	Relative*
1	6	DISTILLED H_2O (.25 cc/day; 10 days I.M.)	301 gm	305 gm	20.9 mg	13.7 mg
2	6	CORTISONE (3 mg/day; 10 days)	308	261	13.9	10.8
3	7	CORTISONE (3 mg/day; 10 days) plus IRON ($FeSO_4$) (1 mg/day; 10 days I.M.)	305	239	19.6	16.5
4	7	IRON ($FeSO_4$) (1 mg/day; 10 days I.M.)	311	286	27.0	19.3

* mgm of adrenal tissue per 100 gms of body weight.

TABLE XII

INFLUENCE OF IRON SALTS ADMINISTERED BY STOMACH INTUBATION ON CORTISONE-INDUCED ADRENAL ATROPHY

Group	Number Animals	Treatment	Body Weight		Adrenal Weight	
			Initial	Final	Absolute	Relative*
1	6	CONTROLS	327 gm	320 gm	24.9 mg	15.6 mg
2	5	CORTISONE (5 mg/day; 10 days)	337	275	16.1	12.0
3	6	CORTISONE (5 mg/day; 10 days) plus IRON ($FeSO_4$) (2 mg/day; 10 days in stomach)	340	262	20.5	15.9
4	4	IRON ($FeSO_4$) (2 mg/day; 10 days in stomach)	361	331	28.5	17.5

* mgm of adrenal tissue per 100 gms of body weight.

iron given by itself produced adrenal hypertrophy. It must be mentioned here that the values were more erratic in this experiment than when the iron was given in the muscle, that is, the adrenal weights in groups 3 and 4 were spread over a wider range. This may be a reflection of the variability in iron absorption by the gut of the individual animals.

Up to this point, the iron compound used was ferrous sulfate but because this substance often produced a tissue reaction at the site of injection a milder iron salt was sought. On the basis of the work of Reznikoff and Goebel (1937), ferrous gluconate[1] was chosen and used throughout the remainder of this investigation. It was found to produce very little or no irritation at the site of injection and no apparent discomfort to the animals following injection. The effects of this compound were studied in four groups of animals, as indicated in Table XIII. Here the same pattern is seen, i.e., ferrous gluconate tends to maintain the adrenal weight during cortisone administration and when ferrous gluconate is given alone adrenal hypertrophy results.

In view of the fact that iron causes adrenal enlargement to about the same extent as ACTH, it was of interest to determine whether iron would potentiate the action of ACTH. In Table XIV it is seen that iron given concurrently with

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[1]Courtesy of Dr. A. Scribner, Winthrop-Stearns Inc., New York City, New York.

TABLE XIII

INFLUENCE OF IRON SALTS ON CORTISONE-INDUCED ADRENAL ATROPHY

Group	Number Animals	Treatment	Body Weight		Adrenal Weight	
			Initial	Final	Absolute	Relative*
1	4	DISTILLED H_2O (.2 cc/day; 10 days I.M.)	197 gm	203 gm	20.1 mg	19.8 mg
2	4	CORTISONE (3 mg/day; 10 days)	192	162	14.6	17.9
3	5	CORTISONE (3 mg/day; 10 days) plus IRON (gluconate) (1 mg/day; 10 days I.M.)	187	141	17.9	25.7
4	4	IRON (gluconate) (1 mg/day; 10 days I.M.)	192	180	23.4	26.2

* mgm of adrenal tissue per 100 gms of body weight.

TABLE XIV

AUGMENTATION OF ACTH WITH IRON SALTS

Group	Number Animals	Treatment	Body Weight		Adrenal Weight	
			Initial	Final	Absolute	Relative*
1	4	CONTROLS	288 gm	304 gm	19.3 mg	12.8 mg
2	6	ACTH (2 mg/day; 10 days)	289	298	21.6	14.5
3	6	ACTH (2 mg/day; 10 days) plus IRON (gluconate) (1 mg/day; 10 days, I.M.)	288	257	24.4	19.0

* mgm of adrenal tissue per 100 gm of body weight.

ACTH (group 3) produces a greater enlargement of the gland than ACTH given alone (group 2). This difference is seen when the adrenal weights are expressed either as absolute values, i.e., mean weight for a single adrenal gland or as relative weights, i.e., mgm. of adrenal tissue per 100 grams of body weight. The increase in the relative weight in group 3 is somewhat out of proportion to the increase in absolute adrenal weight of this group. This is due to the loss of body weight suffered by the animals in group 3. These data then show that ferrous gluconate given intramuscularly in sufficient quantity to supply the equivalent of 1 mgm. of iron (8.3 mgm. of ferrous gluconate) will potentiate the "adrenal enlarging action" of ACTH.

Influence of Cortisone on Catalase Activity In Vitro

Having established that cortisone will inhibit catalase in vivo, it was decided to determine whether or not this inhibition would also occur in a homogenous in vitro system. This procedure should give some insight into the mechanism of inhibition in that if inhibition could be demonstrated in vitro it could be assumed that the steroid was probably combining in some fashion with the catalase molecule and thus inhibiting its normal activity. The binding of certain steroids to proteins has been demonstrated (Samuels, 1954). This problem was approached in the following way: crystalline

catalase[1] was obtained and preliminary experiments were carried out to determine what concentration of the enzyme would be the most convenient for the assay procedure. The crystalline catalase was placed in cold buffer, 1 mgm. per cc., and the solution was centrifuged to remove insoluble material. A sample of the supernatant was then further diluted to give a final concentration of 0.5 mgm. of catalase per ml. of solution. One cc. samples of this solution were placed in two tubes, one of which contained 1 mgm. of cortisone acetate in 0.4 cc. of saline. The other tube containing 0.4 cc. saline alone served as control. This mixture was incubated at 37 degrees C. for 30 minutes. Samples of 0.5 cc. were then taken from each tube and assayed in the usual manner for catalase activity. This procedure was repeated with quantities of cortisone acetate ranging up to 25 mgm. In no case was there a difference between the activity of the cortisone-catalase mixture and the control catalase solution indicating that in a homogenous cell free system such as this, cortisone does not inhibit catalase as it does in the intact animal. This is taken to mean that cortisone does not combine directly with the catalase molecule, at least in the absence of the intact cell. However, this observation in no way rules out the possibility that cortisone could be shown to inhibit catalase activity in surviving tissue in vitro.

[1]Delta Chemical Company, New York, N.Y.

CHAPTER III

DISCUSSION

This study and similar studies of other investigators indicate that the hormonal regulation of catalase activity is an extremely complex mechanism and that our knowledge of the subject is probably still insufficient to allow a precise description to be made. Nonetheless, it is interesting to speculate whether or not the various hormonal factors studied have a common basis for their effect on liver catalase activity. One might assume that the adrenal corticoids are the primary hormonal entities influencing the action of this enzyme, since the corticoids produce the greatest effect seen to date. On this assumption it follows that the increased activity seen after hypophysectomy might be due to a reduction of endogenous corticoid production. Another line of evidence that fits this hypothesis is the work of Begg, Dickinson and White (1953). They demonstrated a 20% reduction of liver catalase activity in male rats treated with the synthetic estrogen diethylstilbestrol. It is known that estrogen causes stimulation of the adrenal cortex via an increased release of corticotropin by the anterior pituitary (Ingle, 1953)

In this indirect way the corticoids may also be causing the catalase depression seen in the estrogen treated animal. The lower catalase activity in females as compared to that of males might be explained in a similar manner and here it is well to note that the adrenal glands of the female rat are always larger than those of the male. Just as it is known that estrogens promote adrenal function, it is equally well known that testosterone causes a pronounced decrease in adrenal weight and presumably in adrenal function (Selye and Stone, 1950). One might interpret this to be part of the mechanism responsible for a higher catalase activity in males than in females.

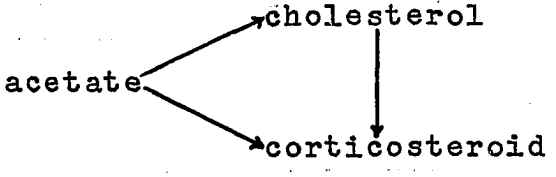
The observation, in our studies, that testosterone administered to mature female rats gives an indication of increased catalase activity can likewise be explained on this basis. Thus, when testosterone, which causes a decrease in adrenal function, is given to the mature female rat the output of adrenocortical hormones is depressed resulting in an elevation of catalase activity. The fact that our data indicate only a slight increase of activity takes on fuller meaning when it is considered that the mature female animals receiving testosterone were simultaneously producing estrogen which tends to increase adrenal function and thereby lower catalase activity.

While the above discussion holds for the mature female rat, it was observed in Table V (p. 18) that although testosterone promoted an increase in catalase activity in the mature female (groups 2 and 4), similar treatment of immature animals (group 6) produced no change in enzyme activity. It therefore appears that the immature female (producing no estrogen) responds to testosterone administration with regard to catalase activity in a different manner than does the mature animal.

Pursuing the hypothesis that the corticoids are the primary factor in catalase regulation, it might be supposed that castration in the male would decrease the catalase activity. There was some indication that this did occur. It is interesting to note, in conjunction with the slight difference between the catalase activity of these groups (Table VI, p. 21) that the adrenal weights were unchanged.

If the above hypothesis of hormonal control of catalase activity is to hold it must also explain the apparent paradox that whereas exogenous cortisone and hydrocortisone cause a marked depression of liver catalase activity in the rat, administration of ACTH or adrenalectomy produce no observable change. This apparent inconsistency might be resolved by a consideration of the biochemical processes in adrenocortical secretion and the role that ACTH is thought to play in corticosteroidogenesis.

The well-established view that adrenal cholesterol is a potential precursor of corticosteroid hormones plus the recognition that acetate is synthesized to cholesterol in the adrenal gland (Srere, Chaikoff and Dauben, 1948) have led to the extensive study of these two substances as corticosteroid precursors. For a time it was thought that acetate was converted to cholesterol as an obligatory intermediary (Hechter, Solomon, Zaffaroni and Pincus, 1953), which in turn was converted through a series of intermediates to hydrocortisone and corticosterone. The recent studies of Stone and Hechter (1954) now make it seem more likely that acetate is converted through alternate pathways, only one of which involves cholesterol.



With the knowledge that cholesterol is a major precursor and that hydrocortisone and corticosterone are the major end products of corticosteroid biosynthesis, Hechter, Pincus and their co-workers (see Hechter and Pincus, 1954) were able to formulate a reaction sequence for corticosteroid biosynthesis and to substantiate it by perfusion studies of the adrenal cortex. Figure 1 illustrates their scheme. The available evidence strongly indicates that ACTH enters into this sequence between cholesterol and pregnenolone; that is, ACTH

action stimulates the degradation of the cholesterol side chain.

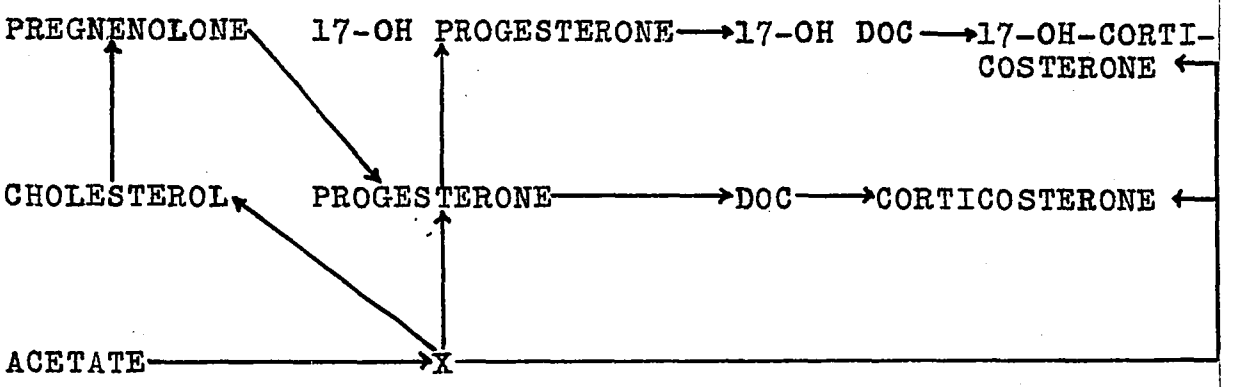


FIGURE 1

REACTION SEQUENCE OF CORTICOSTEROID BIOSYNTHESIS
(after Hechter and Pincus)

The postulated alternative pathway of acetate conversion to corticoid, which does not involve acetate conversion to cholesterol, is not significantly increased by ACTH. The suggestion from these experiments is that corticoid synthesis, in the absence of ACTH, may proceed primarily from acetate through the noncholesterol pathway. When ACTH is present, the cholesterol pathway is utilized as well.

As already mentioned, in all species studied one or two corticosteroids are regularly found in highest concentration in the adrenocortical secretion: hydrocortisone and corticosterone. However, the relative proportions of these compounds are markedly different in various species but the

ratio is fairly constant within a species (Bush, 1953).

The characteristic ratios of the two major components of the adrenalcortical secretion (hydrocortisone: corticosterone) are apparently influenced by a number of factors. Thus, while acute ACTH treatment does not appreciably alter the characteristic ratio, chronic treatment profoundly modifies the corticosteroid pattern (Kass, 1954). Kass feels that in addition to its acute direct action in corticosteroidogenesis, ACTH alters the pattern of corticosteroid formed by influencing the synthesis of enzymes involved in corticosteroidogenesis.

Although cortisone and hydrocortisone depress catalase activity, neither ACTH nor adrenalectomy influence this enzyme. This apparent inconsistency may, perhaps, be resolved in the light of the foregoing discussion. It is clear that the genesis of the adrenocortical secretion is a very complex and labile mechanism and that the ratio of the active corticoids may well play a significant role in the biochemical response to this secretion. It is felt then that the best explanation at the present time for the lack of an effect with ACTH on catalase activity lies in the concept of relative proportions of the corticoids. This explanation is admittedly speculative, and other theories will have to be considered as new evidence is found. This concept of hormonal balance as an important factor in the influence of hormones on enzymes is

not without precedence. The hormonal regulation of the enzyme hexokinase is a classical example. Insulin on the one hand and growth hormone and 11-oxycorticosteroids on the other have a competitive action on this enzyme, the latter inhibiting the reaction catalyzed by hexokinase and the former overcoming this inhibition. Evidence is accumulating to indicate that the control of hexokinase activity is primarily dependent on the ratio of pituitary-adrenal to insulin activity, rather than on the absolute amounts of either hormone. In the absence of all three, carbohydrate utilization (as an index of hexokinase activity) may be almost normal (Engel, 1954). This normalcy of enzyme function in the absence of the competitive forces may be the reason our experiments failed to show any change in catalase activity after adrenalectomy.

One other point that fits the concept that the adrenocortical secretion may be the principal hormonal factor in catalase regulation is the observation by Begg (1951) that together with the decreased liver catalase level in cancerous animals there is an enlargement of the adrenal glands with loss of ascorbic acid and cholesterol.

Other investigators have studied hormonal influences on catalase activity. Begg (1953) using the rat as experimental animal showed that while cortisone in doses of 1-3 mgm. per day will depress the liver catalase activity, adrenalectomy

has no effect on this liver enzyme. These observations are in agreement with our earlier findings. There appears to be a species difference in catalase activity between the rat and mouse. Adrenalectomy in the mouse results in a depression in liver catalase activity, (Adams, 1952). Cortisone will restore the normal level. Adams further reports that the catalase activity in females is lower than in males and that the level in females can be elevated to that of the male by testosterone administration. Castration of adult male mice also produces a depression of enzyme activity that can be normalized by testosterone injections. There is precedent for opposite effects in enzyme concentration in different species after the administration of the same hormone. Testosterone causes quite different effects on kidney alkaline phosphatase in the rat and mouse, (Kochakian, 1947).

Prevention of Cortisone-Induced Adrenal Atrophy

It is felt that two possible explanations can be given for the prevention of cortisone-induced adrenal atrophy by iron salts. First, it is known that any type of stress produces an increase in weight of the adrenals, and it is believed that the weight response in such conditions is an aspect of the adaptation of the adrenals to the increased requirement for cortical hormones by the body (Selye, 1937) (Ingle, 1938, 1939); in other words, the gain in adrenal

weight, under conditions of stress, is considered to be due to an hypertrophy of the cortex with a corresponding increase in adrenal cortical hormone production, and consequently an increased resistance towards the damaging agent. In view of this concept it might be argued that the intramuscular administration of iron exerts the observed effect on adrenal weight simply by acting as a non-specific stress because this procedure does evoke some discomfort in the animals. We do not feel, however, that this accounts for the total degree of adrenal hypertrophy that is seen. It has been shown by many authors that the enlargement of the adrenal cortex in animals under stress can be prevented (Ingle, 1938) (Selye, 1940). It may even become atrophied (Selye and Dosne, 1942) when the animals under stress are treated with sufficiently high doses of adrenal cortical hormones. Since the animals in this study were receiving relatively high doses of cortisone (3-5 mgm.) it is very unlikely that the mild stress associated with the injection of the iron was responsible for the adrenal hypertrophy.

Second, it may be that iron plays a heretofore unsuspected role in the function of the adrenal cortex; perhaps as an integral part of some enzyme system of this gland. There is no direct evidence that this is the case, but two studies point indirectly to this possibility:

1. Hamilton, et al, (1951) have established that the

adrenal cortex plays an essential role in the maintenance of the normal homeostasis of the plasma iron level. Adrenalectomy resulted in a decrease in the level of the plasma iron of rats. This decrease was prevented by administration of small doses of adrenocortical extract or cortisone but not desoxycorticosterone. Larger doses of adrenocortical extract, cortisone or ACTH produced acute hypoferremia in intact rats.

2. Keston, (1954) using an <u>in vitro</u> system containing ascorbic acid, ferrous sulfate, and steroid in an atmosphere of oxygen have shown that this system will promote the introduction of hydroxyl groups into adrenal steroids. The possibility therefore exists that ferrous ions are a necessary component of the enzyme systems that bring about the physiological production of the hydroxycorticoids.

It is felt that the indications from these morphological studies of the adrenal glands are sufficiently suggestive of an important role of iron in adrenal metabolism that tests should now be conducted to assess the <u>functional</u> capacity of the adrenals of animals treated with iron.

Although cortisone has proven to be useful in a variety of clinical conditions, it exerts certain hormonal effects which do not contribute to its therapeutic value and which,

indeed, it would be desirable to avoid if possible. Among these is adrenal atrophy. Much work has been done recently to determine whether one or more of these unwanted effects could be counteracted without diminishing the desired actions of the hormone; the usefulness of cortisone would then be enhanced. The growth-inhibition and other toxic manifestations of cortisone or ACTH have been in part antagonized by a variety of means such as liver therapy (Ershoff, 1951), aureomycin (Meites, 1952), potassium (Whitney and Bennett, 1952) and fat (Kinsell, Olson, Boling, Partridge and Margen, 1951). The adrenal atrophy which occurs when cortisone is given has been prevented by steroids such as methylandrostenediol and testosterone propionate (Winter, Hollings and Stebbins, 1953) (Gaunt, Tuthill, Antonchak and Leathem, 1953).

In the face of these studies, a question of paramount interest concerns the functional capacity of the adrenal gland maintained with iron compounds in the presence of treatment with cortisone. If the functional capacity of the adrenal gland, in this condition, does remain normal the therapeutic applicability of this finding would be overshadowed only by the possibility that iron plays a regulating role in adrenal cortical metabolism.

CHAPTER IV

SUMMARY

1. The influence of numerous hormonal factors on the liver catalase activity of the rat has been studied. This was accomplished either by administering the active material to the animals or by surgical removal of certain endocrine glands. The hormonal control of catalase activity is regarded as one facet of the larger concept that hormones may be exerting their influence in the living organism by regulating the activity of enzymes.

2. Cortisone acetate, administered in amounts ranging from 2 to 10 mgm. daily, was found to produce a marked reduction of liver catalase activity.

3. Contrary to expectations, adrenocorticotropic hormone did not alter the liver catalase activity, even in doses of 6 mgm. per day.

4. A sex difference in liver catalase activity was noted. The activity in the male was approximately 25% greater than the corresponding activity of the female. This finding confirms the work of Schultze and Kuiken (1941) which also demonstrated a higher catalase activity in the male rat than

in the female rat.

5. Testosterone promoted an increase of catalase activity in mature female rats but not in immature females. Possible reasons for this difference in response to testosterone were discussed.

6. Castration of male rats did not alter the catalase activity to a degree that was statistically significant. However, a slight depression of the enzyme activity was indicated.

7. Young adrenalectomized male rats, maintained for 12 days on sodium chloride, did not show a liver catalase activity different from the activity of normal control animals.

8. Hydrocortisone, administered by intragastric intubation, in quantities of 5 mgm. daily produced a decrease in liver catalase activity comparable to the decrease seen after similar quantities of cortisone.

9. The hypothesis that cortisone depresses catalase activity by virtue of its hypoferremic action could not be supported by experimental findings.

10. Cortisone did not suppress catalase activity in an <u>in vitro</u> system.

11. A tentative hypothesis of hormonal control of catalase activity is proposed. This hypothesis takes the findings of this and similar studies into consideration. It is based on the concept that the adrenocortical secretions

are the principal hormonal factors in catalase regulation. The changes in enzymatic activity seen after other endocrine changes are, according to this hypothesis, due to simultaneous quantitative or qualitative changes in adrenocortical secretion.

12. It has been demonstrated in this study that the adrenal atrophy that occurs in rats treated with cortisone can be prevented by the simultaneous administration of iron salts either intramuscularly or orally. It has been further demonstrated that iron salts will potentiate the "adrenal-enlarging action" of adrenocorticotropic hormone.

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