

## ZONATION OF HEMOPROTEIN P-450 AND CYTOCHROME $b_5$ IN THE ADRENOCORTEX OF MAMMALS

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### INTRODUCTION

Histologically the mammalian adrenocortex consists of three zones: the glomerular, the fascicular, and the reticular. However, the origin and function of these zones are unknown, although there have been many investigations on the subject and theories have been suggested such as cell migration, the transformation theory, and the zonal theory (1). Namely, it has been proposed that each zone has a specific function in biosynthesis and secretion of adrenocortical hormone (2) or, on the contrary, that the zones are the result of these processes during the development of adrenocortical cells (3). To study the function of each zone it seems very useful to investigate the zonation of hemoprotein P-450, an important component of the mixed function oxidase systems for the hydroxylation of steroids (4, 5).

The concentrations of hemoprotein P-450 or cytochrome  $b_5$  per milligram protein of microsomes (6) or mitochondria (7) prepared from homogenates of whole adrenocortex have already been reported. However, the contents of hemoproteins in the different zones of the adrenocortex have not been studied.

### MATERIALS AND METHODS

Pig, beef, and rabbit adrenal glands were obtained from a local slaughter house, chilled on ice, and transported immediately to the laboratory. Fat and connective tissues were carefully removed. The glands were scraped from the capsule, and each gland was cut into 200 sections with a razor at  $-20^{\circ}\text{C}$ . The sections were thin enough to transmit light from a Nippon Kogaku model 3 shadowgraph. Sections were prepared from five glands. The frozen sections of adrenal glands were carefully divided into the three

zones and the medulla under the binocular microscope, the procedure being monitored on the shadowgraph giving 10-fold magnification. Homogenates of the respective zones were subfractionated by the method of Schneider and Hogeboom (8). Though the zones of the adrenocortex were influenced by the age, sex, and species of animal (9), pig or beef adrenocortex generally consisted of the outer zona glomerulosa (one-fifth of the radius), the zona reticularis (one-third of the radius), and the zona fasciculata (the remaining area). These facts were confirmed histologically with a Carl Zeiss Ultraphoto II optical microscope (Carl Zeiss, Inc., New York).

Light absorption microspectra of the sections were measured in a Carl Zeiss UMSP type 1 universal microspectrophotometer. Microspectrophotometry was carried out with a light beam of 5  $\mu$  diameter unless otherwise stated. Adrenal glands were placed on block holders. The glands and holder were maintained at  $-20^{\circ}\text{C}$  and the glands were sectioned at a thickness of 20  $\mu$ . The absorption spectra of the hemoprotein P-450-CO complex in the sections were obtained after incubation of slices in 0.2 M K-phosphate buffer at pH 7.5, saturated with carbon monoxide for 10 min, hemoprotein P-450 in the sections on the slides was reduced by treatment with 1 mg of sodium dithionite per ml of 0.2 M K-phosphate buffer at pH 7.5; thus the reduced hemoprotein P-450-CO complexes were formed. Then sections were immediately placed under anaerobic conditions by covering them with a cover glass sealed at the edges of the preparation by bonding adhesive glue (Cyanobond, Sumitomo Chemical Co. Ltd.) in order to prevent the autoxidation of reduced hemoprotein P-450-CO complex, which is rapidly converted to oxidized hemoprotein P-450 even on brief exposure to a very low concentration of oxygen (10).

The hemoprotein contents of the tissue homogenates and the subfraction were determined with a Cary Model 14 recording spectrophotometer (Cary Instruments, Monrovia, Calif.) at room temperature.

Cytochrome  $b_5$  content was determined by the method of Garfinkel (11). The estimation of microsomal cytochrome  $b_5$  in the difference spectrum of the nicotinamide adenine dinucleotide (NADH) reduced form minus the oxidized form was not affected by the contaminating hemoglobin because hemoglobin is physiologically in the reduced form, and NADH-cytochrome  $b_5$  reductase (Enzyme Commission Number 1.6.2.2) cannot reduce methemoglobin. The content of hemoprotein P-450 was estimated by taking a value of  $115 \text{ mM}^{-1} \text{ cm}^{-1}$  as the molar extinction coefficient between 450 and 500  $m\mu$  in the difference spectrum of the sodium dithionite-reduced CO complex and the oxidized form, to exclude complication of interference from the absorption spectra of the contaminating hemoglobin CO complex.

Protein content was determined by the biuret

reaction, after addition of 1% sodium cholate to the sample to remove turbidity, with crystalline bovine albumin as the standard (12). Increase in absorption in the biuret reaction due to heme in the test samples was avoided by using the reference containing the hemoproteins in 4% NaOH.

## RESULTS AND DISCUSSION

Table I shows the contents of hemoprotein P-450 in homogenates of the zona glomerulosa, zona fasciculata, and zona reticularis of pig and beef adrenocortexes. The values for these homogenates mainly reflect the mixed contributions from these hemoprotein P-450's of the microsomes and mitochondria, since the microsomes and mitochondria of adrenocortexes contain most of the hemoprotein P-450. The zona fasciculata contained more hemoprotein P-450 than the zona glomerulosa or zona reticularis. The contents of hemoprotein P-450 in these zones were also carefully measured with the microspectrophotometer. The value for the hemoprotein P-450 content measured with the microspectrophotometer depends on the density of the cell cluster, the cell nucleus, and the thickness of the section. However, similar qualitative results for the hemoprotein P-450 contents were obtained by careful, repeated investigations of different regions of the zones in different samples. The results of microspectrophotometry of beef adrenal glands are illustrated in Fig. 1 (parts labeled G, F, R, M). Fig. 2 is replotted as the difference spectra of the reduced hemoprotein P-450-CO complex minus the oxidized hemoprotein P-450, and is based on the oxidized and reduced absorption curves of Fig. 1. This figure shows that the peak at 450  $m\mu$  due to the hemoprotein P-450-CO complex is higher in the zona fasciculata than in the other zones, because the CO complexes of the

TABLE I  
*Contents of Hemoprotein P-450 in the Homogenates of Different Zones of Ox or Male Pig Adrenocortex*

Zones	Animals	
	Oxen	Male pigs
Zona glomerulosa	0.05	0.04
Zona fasciculata	0.12	0.08
Zona reticularis	0.07	0.06

The content is expressed in  $m\mu$ moles per mg protein. Values show averages of five determinations of the hemoprotein content of different samples. The standard errors were within 10% of the means.

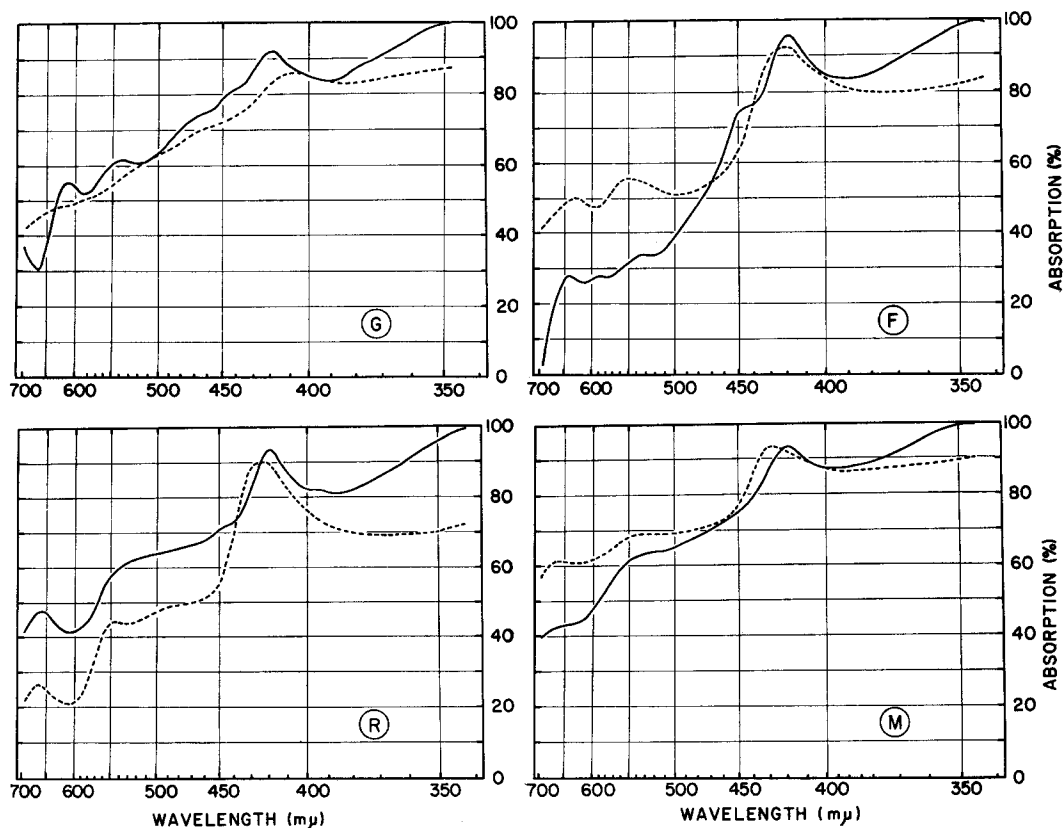


FIGURE 1 Absorption spectra of various zones (zona glomerulosa, zona fasciculata, and zona reticularis) in section of ox adrenocortex and adrenomedulla. The spectra show the light absorptions with a beam of  $5\mu$  diameter seen with a microspectrophotometer. The thickness of the sections of adrenal glands was  $20\mu$ . ----, oxidized form; —, sodium dithionite reduced form + carbon monoxide. G, zona glomerulosa; F, zona fasciculata; R, zona reticularis, M, adrenomedulla.

TABLE II  
Contents of Hemoprotein P-450 and Cytochrome  $b_5$  of Microsomes or Mitochondria in the  
Different Zones of Ox or Male Pig Adrenocortex

Zones	Adrenocortex					
	Ox			Male pig		
	Mitochondria	Microsomes		Mitochondria	Microsomes	
	P-450	P-450	Cyt $b_5$	P-450	P-450	Cyt $b_5$
Zona glomerulosa	0.57	0.70	0.19	0.72	0.94	0.14
Zona fasciculata	1.00	1.50	0.27	0.96	1.98	0.26
Zona reticularis	0.70	0.80	0.32	0.89	1.05	0.36

The content of hemoprotein P-450 or cytochrome  $b_5$  is expressed in  $\mu$ moles per mg protein of microsomes or mitochondria of adrenocortex. Values show averages of five determinations of the hemoprotein contents of different samples. The standard errors were within 10% of the means.

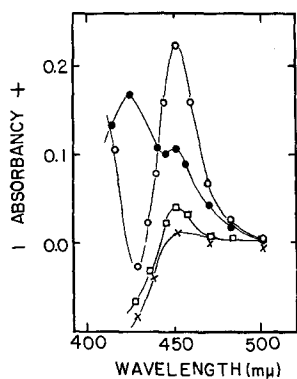


FIGURE 2 Difference spectra of the reduced hemoprotein P-450-CO complex and oxidized hemoprotein P-450 of sections of beef adrenocortex and the medulla. The difference spectra were replotted as absorbancy calculated from the absorption curves of Fig. 1 (G, F, R, M). The correction was made for the turbidity by subtracting or adding the change in absorbance at 500  $m\mu$  as base standard, although the correction remained insufficient for the base line slope. ●—●, zona glomerulosa; ○—○, zona fasciculata; □—□, zona reticularis; ×—×, adrenal medulla.

other known hemoproteins such as cytochrome  $a_3$  and hemoglobin never have absorption peaks at 450  $m\mu$  (13). The decrease in the visible absorption on reduction of the zona fasciculata suggests the existence in these regions of pigments, with absorption which disappear on reduction. These facts may be due to the high concentration of adrenodoxin. The homogenates of the respective zones of beef adrenal glands were subfractionated and the microsomes and mitochondria were isolated. A high concentration of hemoprotein P-450 was found with the microsomes of the zona fasciculata so that these microsomes may be more developed than those in other zones.

The concentrations of mitochondrial hemoprotein P-450 in the various zones less different than those of microsomal hemoprotein P-450 in the various zones.

The content of microsomal cytochrome  $b_5$  increased toward the center of the adrenocortex. The zona reticularis and the adrenal medulla were carefully sectioned to exclude, as far as possible, mutual contamination of the cytochromes. The concentrations of cytochrome  $b_5$  and hemoprotein

P-450 in the microsomes and mitochondria of these zones are summarized in Table II. Similar results were obtained for the zones of pig adrenal glands. Although it was difficult to divide rabbit adrenal glands into zones, the same trend was also observed in them by the microspectrophotometer.

The substrate-induced spectral changes could be observed not only with substrates for hydroxylation, but also with anilines and anisoles, which are not hydroxylated or demethylated in adrenocortical microsomes and mitochondria (14, 15). Thus the spectral changes induced by the substrate may correspond to the content of hemoprotein P-450 in the various zones rather than to the hydroxylation and demethylation activities.

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#### REFERENCES

1. BACHMANN, R. 1954. Die Nebenniere. In Handbuch der mikroskopischen Anatomie des Menschen. Springer-Verlag KG., Berlin. 6:50.
2. DEANE, H. W., J. H. SHAW, and R. O. GREEP. 1948. *Endocrinology*. 43:133.
3. SAYERS, G. 1950. *Physiol. Rev.* 30:241.
4. RYAN, K. J., and L. L. ENGEL. 1957. *J. Biol. Chem.* 225:103.
5. ESTABROOK, R. W., D. Y. COOPER, and O. ROSENTHAL. 1963. *Biochem. Z.* 338:741.
6. ICHIKAWA, Y., and T. YAMANO. 1965. *Biochem. Biophys. Res. Commun.* 20:263.
7. HARDING, B. W., and D. H. NELSON. 1966. *J. Biol. Chem.* 241:2212.
8. SCHNEIDER, W. C., and G. H. HOGEBOOM. 1950. *J. Biol. Chem.* 183:123.
9. ITO, T. 1952. *Folia Anat. Jap.* 24:269.
10. ICHIKAWA, Y., B. HAGIHARA, and T. YAMANO. 1967. *Arch. Biochem. Biophys.* 120:204.
11. GARFINKEL, D. 1958. *Arch. Biochem. Biophys.* 77:493.
12. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. *J. Biol. Chem.* 177:751.
13. WILLIAMS, R. J. P. 1961. *Haematin Enzymes*. Pergamon Press Inc., New York. 1:41.
14. ICHIKAWA, Y., T. YAMANO, and H. FUJISHIMA. 1969. *Biochim. Biophys. Acta.* 171:32.
15. OMURA, T., E. SANDERS, R. W. ESTABROOK, D. Y. COOPER, and O. ROSENTHAL. 1966. *Arch. Biochem. Biophys.* 117:660.