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GENERAL REVIEW

STEROIDOGENESIS AND CELL STRUCTURE

BIOCHEMICAL PURSUIT OF SITES OF STEROID BIOSYNTHESIS

BUN-ICHI TAMAOKI

National Institute of Radiological Sciences, Anagawa-4-chome, Chiba-shi 280, Japan

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SUMMARY

Steroidogenesis is discussed at the different levels of the whole body, organ, cell, subcellular components and biomembrane, in order to pursue the sites of production of steroid hormones in the body. From the information on the sites of steroid transformations in a cell, intracellular transport of steroidal precursors, intermediates, and final products are integrated together with the biosynthetic pathways of the steroid hormone.

1. INTRODUCTION

IN THIS review, relationships between steroidogenesis and cellular components are discussed. This information is helpful towards the understanding of the specific cellular function of endocrine organs in relation to steroidogenesis [1, 2]. Up to the present time, steroidogenic sites have been studied at the following levels: (1) whole body, (2) organ tissue, (3) cellular level, (4) subcellular level, (5) specific organella and (6) constituents of organella and finally toward molecular level.

For determination of sites of steroidogenesis, quantitation of the metabolites at the various levels, and more rationally, distribution of the enzyme activities related to steroidogenesis have been investigated. With the development of biochemical endocrinology, enzymological studies related to steroidogenesis have been strongly encouraged. During the past two decades, the pathways of steroid hormone biosynthesis, and the functions and characteristics of the enzymes involved at each step of steroidogenesis and its metabolism have been clarified. Recently, some multi-enzyme systems related to steroid hydroxylation [3, 4] and enzyme reaction mechanism related to the side-chain cleavage of cholesterol and progesterone [5–7] have been studied in relation to steroid biosynthesis.

Steroidogenesis at the whole body level is not described in detail here, as it is well accepted that steroid hormones are produced in the body under certain physiological conditions. The factors which influence the steroidogenesis in the whole body includes species, sex, age, stage of development (fetus, new-born, immature, mature, old and senile, and on and off of breeding season) and physiological conditions, such as pregnancy, estrus cycle, the presence of stress etc.

2. STUDY OF ORGANS AS SITES OF STEROIDOGENESIS

In order to demonstrate that steroid hormones are produced and secreted by a specific organ, the organ is removed and pathological symptoms arising are inves-

tigated. To establish this point more clearly, it is necessary to determine whether or not the symptoms caused by removal of the organ can be recovered by administration of an extract of the organ, a purified preparation of the extract or a steroid hormone itself. This is the special technic developed by endocrinologists.

For example, after removal of the testes, loss of sperm production, and atrophy of accessory sex organs such as seminal vesicle and prostate are observed as the deficient symptom due to orchiectomy. Also following this operation, a fall in the level of androgens and their metabolites in blood and urine is observed. By administration of testosterone to castrated animals, atrophied accessory organs recover, but there is no improvement in the sperm production. From these results, it is concluded that androgens are produced in the testes and secreted from them. Accessory sex organs are regulated exclusively by this specific chemical stimulator, whereas spermatogenesis itself is not restored by administration of the hormone.

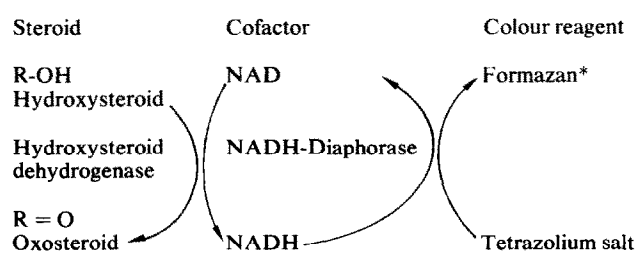
Recently, as another approach to this problem, macroautoradiographic analysis of the distribution of radioactive steroid precursors in the whole body has been used [8]. In such autoradiogram, organs which show specific affinities for the steroid precursors (and their metabolites) are visually demonstrated by the accumulated radioactivity. The involvement of tissues located in this way will subsequently be confirmed by biochemical investigation of the tissue *in vitro*. Furthermore, the concentration of steroids in the venous blood obtained directly from the organ will be found to be significantly higher than in the peripheral blood. From such studies, it may be possible to show that production of a particular steroid hormone is limited to a specific tissue or organ, whose function was represented by the biological actions of the hormones.

3. INTERCELLULAR DISTRIBUTION OF THE ENZYME ACTIVITIES RELATED TO STEROIDOGENESIS IN ENDOCRINE ORGANS

As tissue consists of several different components and cell types, it is necessary to examine which parts of the organ or which cell groups in a specific tissue are responsible for biosynthesis of steroid hormones.

Among the enzymes related to biosynthesis and metabolism of steroid hormones, hydroxysteroid dehydrogenases (hydroxysteroid oxido-reductases) are histochemically demonstrated by chemical process [9] as shown in Fig. 1.

Though the reaction is common among hydroxysteroid dehydrogenases,



*Deposited as insoluble blue dye at the site where enzymic dehydrogenation occurs [9].

Fig. 1. Enzyme reactions applied for histochemical detection of hydroxysteroid dehydrogenases.

specific dehydrogenase activity is histochemically demonstrable by selecting a specific steroid as the substrate for the reaction, taking advantage of substrate specificity of the enzyme. Apart from dehydrogenases, however, other enzyme activities related to steroidogenesis such as those of hydroxylases, lyases, hydrogenases, isomerase etc. are not histochemically detectable by this procedure. In such cases therefore, biochemical methods combined with other approaches must be employed to study the distribution of enzyme activities related to formation of steroid hormones among cells of a particular organ or tissue. As it is difficult to generalise about the distribution throughout all tissue involved in steroid production, individual organs will be discussed separately.

Testis [10]

Histologically the testis consists of several different cells, such as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, spermatozoa, Sertoli cells, and interstitial cells, in addition to cells of blood vessels and basement membranes. According to present information, it is well established that the enzyme activities related to androgen formation are exclusively localized into the testicular interstitial cells:

(1) By local X-irradiation of 1,000 R to testes of rats, spermatogenesis is completely destroyed in 1 month, while interstitial cells and Sertoli cells remain histologically almost unchanged, as shown in Fig. 2. The weight of X-irradiated testes is reduced to almost half of the normal value, while the weight of accessory organs is found to be practically unchanged in comparison with those of normal animals. These results suggest that the cells which produce and secrete enough androgens to maintain the weight of accessory organs at the normal level are resistant to X-irradiation and would most likely be interstitial or Sertoli cells. Biochemically, enzyme activities involved in androgen formation from pregnenolone are also confirmed in X-irradiated testes [11, 12].

When human chorionic gonadotrophin is administered to a rat whose testes have been locally X-irradiated with 1,000 R, histological examination shows specific hypertrophic and functional features of the interstitial cells, but there is no remarkable histological changes in the Sertoli cells. On the other hand, the weight of accessory sex organs is increased by the administered gonadotrophin. Biochemical analysis of enzyme activities of testes reveals that the activities of Δ^5-3β-hydroxysteroid dehydrogenase + $\Delta^5 \rightarrow \Delta^4$-isomerase, 17$\alpha$-hydroxylase, and C_{17}-C_{20} lyase are enhanced by the gonadotrophin, suggesting close relationship between the development of interstitial cells and enhanced androgen production [11, 12].

(2) It is established that the microsomal fraction obtained from testicular homogenates retain all the enzymes related to testosterone formation from pregnenolone [13], as discussed later (Table 1). Specific activities of these enzymes in the microsomal fraction of X-irradiated testes are found to be significantly higher than those of normal testes, suggesting that these enzymes are concentrated in the microsomal fraction of the radio-resistant cells of testes [12].

(3) When animals are immunized with purified hypophyseal luteining hormone (LH), the anti-LH antiserum produced in the bodies inactivates the endogenous LH which is produced and secreted from their pituitary glands. Accordingly, the weight of the testes is remarkably reduced. Histological examination of the testes reveals that not only the cells related to spermatogenesis, but also the inter-

stitial cells are extensively damaged. Thus, contrary to the case of X-irradiated testes, accessory organs are found to be strongly atrophied, in keeping with the reduced enzyme activities involved in androgen production from pregnenolone [14]. To support the specific relationship of LH to the interstitial cells, respiration of such cells dissected from testes by free hand is stimulated *in vitro* by LH but not by FSH[15]. Furthermore, ferritin- and I^{125}-labelled LH administered *in vivo* are located in the testicular interstitial cells exclusively[16, 17].

(4) Among the enzymes related to androgen formation from pregnenolone, Δ^5-3β- and 17β-hydroxysteroid dehydrogenases are histochemically demonstrated in the interstitial cells[9]. As enzymes other than these hydroxysteroid dehydrogenases are localized in the microsomal fraction of X-irradiated testes along with the dehydrogenases, it is suggested that other steroidogenic enzymes are also concentrated in the interstitial cells.

(5) Furthermore, after manual separation under a microscope of the interstitial tissue from seminiferous tubules of rat testicular tissue, progesterone is incubated with the two separate tissue fractions. Then, the interstitial tissue is found to be primarily responsible for androgen production, while the seminiferous tubules show limited production of androgen from progesterone[18]. Recently, it has been reported that cholesterol is converted to androgen exclusively by the interstitial cell fraction, and not at all by the seminiferous tubules[19]. An interstitial cell fraction separated from testicular tissue of guinea pigs by a filtration technic is enriched in the content of 17α-hydroxylase[20].

(6) One month after experimental bilateral cryptorchidism of rats, the weight of the testis is markedly decreased. Histological examination shows that spermatogenesis is completely inactive, and the interstitial cells are rather atrophic. The weight of accessory organs is significantly reduced, but not to the level caused by castration[21].

(7) Histologically confirmed interstitial cell tumors are able to produce androgen from several precursors *in vitro*[22].

From the above facts, it could be concluded that androgens are produced by the interstitial cells of testicular tissue but not by the cells concerned with spermatogenesis in the seminiferous tubules.

Adrenal

In the case of adrenal gland, steroid hormones or corticoids are known to be produced by the cells in the cortex but not the medulla[23]. The adrenal cortex is histologically divided into capsule, zona glomerulosa, zona fasciculata and zona reticularis[24]. Recently, by ultramicrochemical methods, measuring 11β-hydroxylase activity, the enzyme activity which is specific to adrenal cortex in mammals was found to be increased in a border zone between the zona fasciculata and zona reticularis, following stimulation of rats *in vivo* with adrenocorticotrophic hormone. The enzyme activity was distributed almost uniformly over cortical area in unstimulated animals (Fig. 3).

From these results, the 11β-hydroxylating enzyme system is concluded to be stimulated or probably synthesized *de novo* in the cells located around the specific zone of adrenal cortex under the influence of ACTH. In this connection, the content of cytochrome P-450 in bovine or porcine adrenal mitochondria is reported recently, highest in the zona fasciculata, then zona reticularis and zona glomerulosa, but none in the medulla[25].

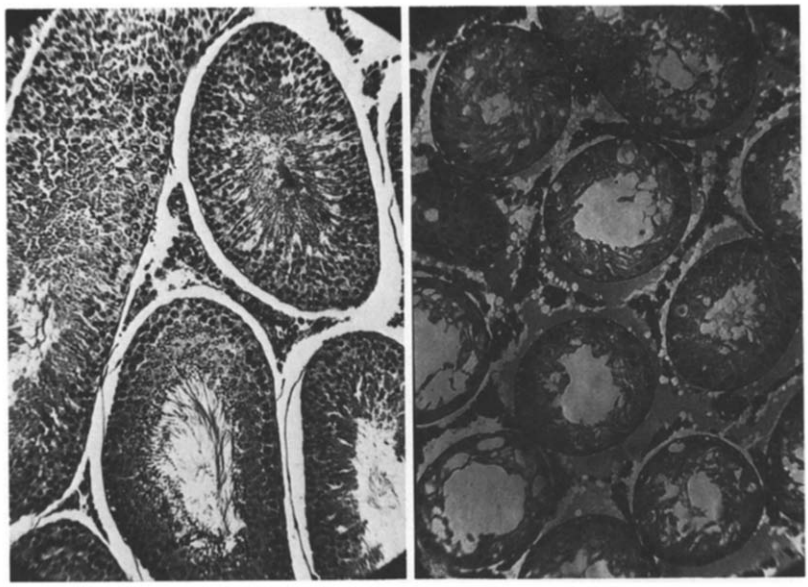


Fig. 2. Photomicrographs of normal testicular tissue (left) and X-irradiated testicular tissue of rat [1,000 R] (right) [× 100].

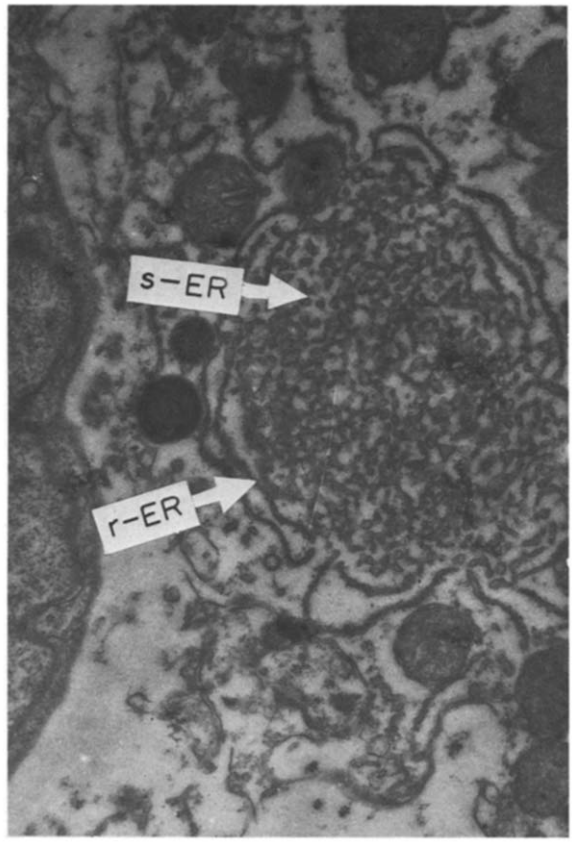


Fig. 5. Electron micrograph of rat testicular interstital cell. (× 60,000). Courtesy of Dr. M. Seki in National Institute of Radiological Science, Chiba-shi, Japan. r-ER and s-ER denotes respectively granular and agranular endoplasmic reticula.

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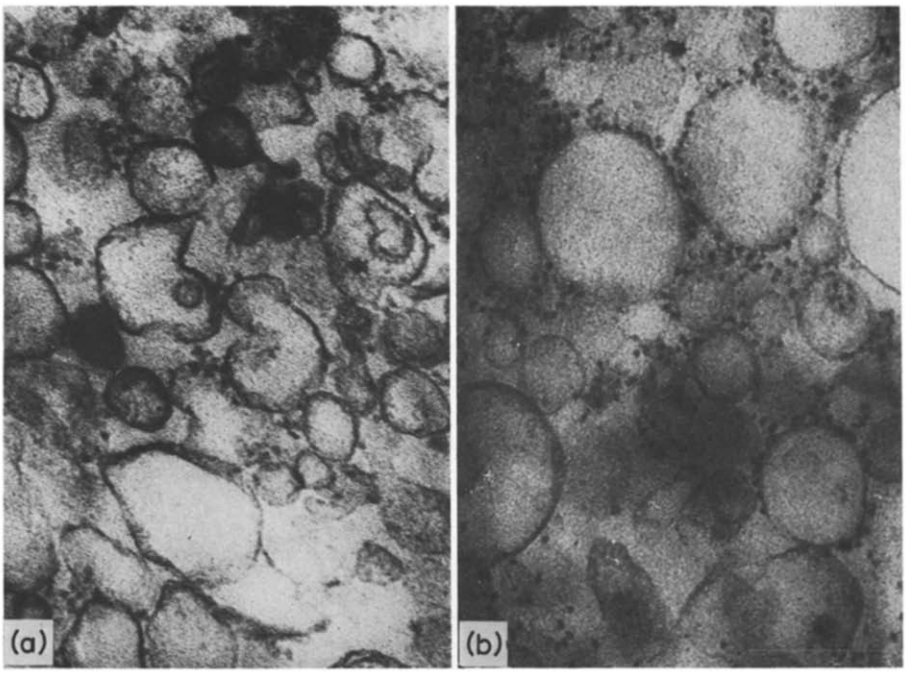


Fig. 8. Electron micrographs of the two different submicrosomal fractions of rat testes (×75,000)[50]. (a) Smooth-surfaced microsomal fraction, (b) Rough-surfaced microsomal fraction.

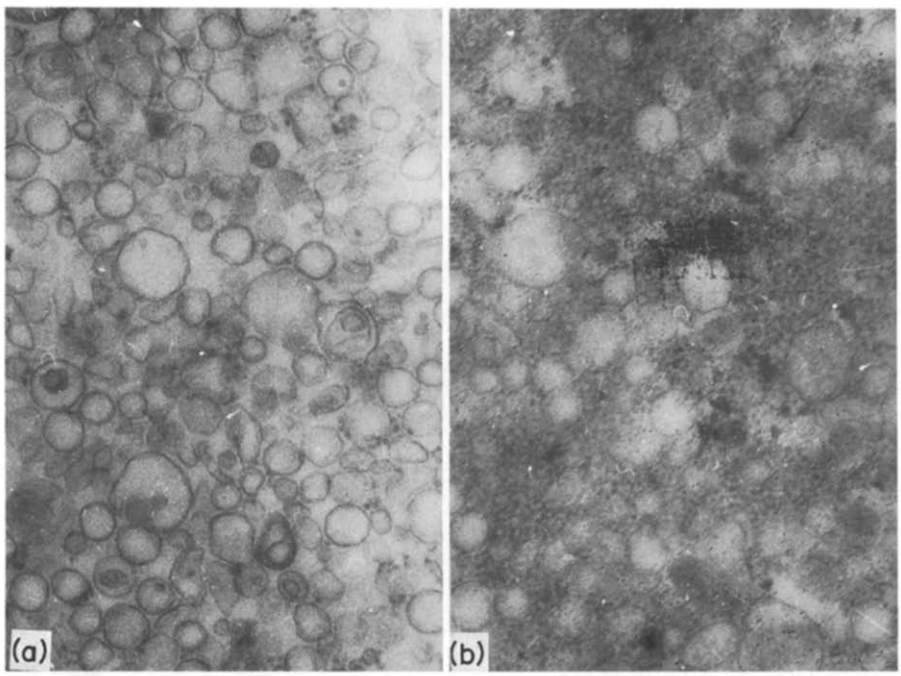


Fig. 9. (a) Electron micrographs of smooth-surfaced microsomal fraction and (b) rough-surfaced microsomal fraction of porcine adrenals (×75,000)[56].

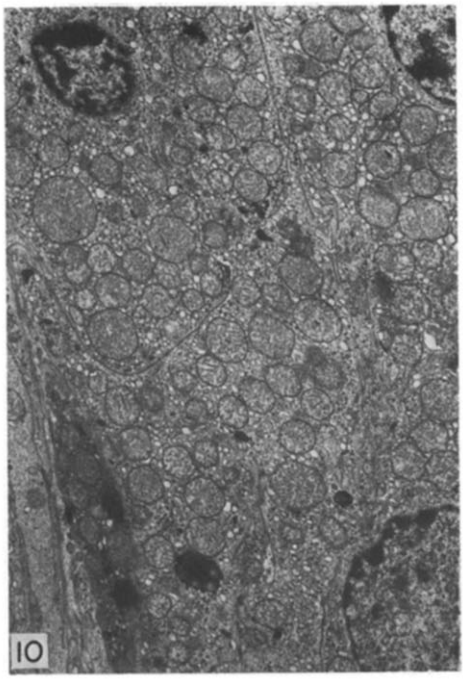


Fig. 10. Electron micrograph of rat adrenocortical cell. (zona fasciculata) (×60,000). Courtesy of Dr. M. Seki in National Institute of Radiological Science, Chiba-shi Japan.

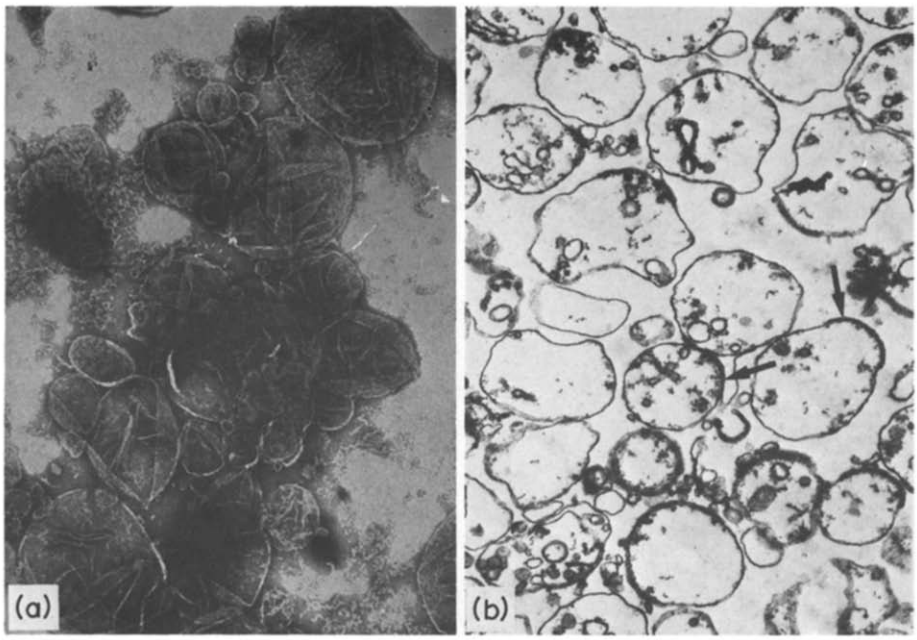


Fig. 11. Electron micrographs of the submitochondrial fractions of bovine adrenal. (a) Negatively stained fraction of purified outer mitochondrial membrane (×35,000). (b) Thin section of inner mitochondrial membrane fraction. The arrows point to fragments of outer membrane still attached to the inner membrane (×18,000). (By courtesy of Dr. Satre M. Biochimie, C.E.N.-G Cedex 85, Faculté de Médecine, 38 Grenoble, France[72])

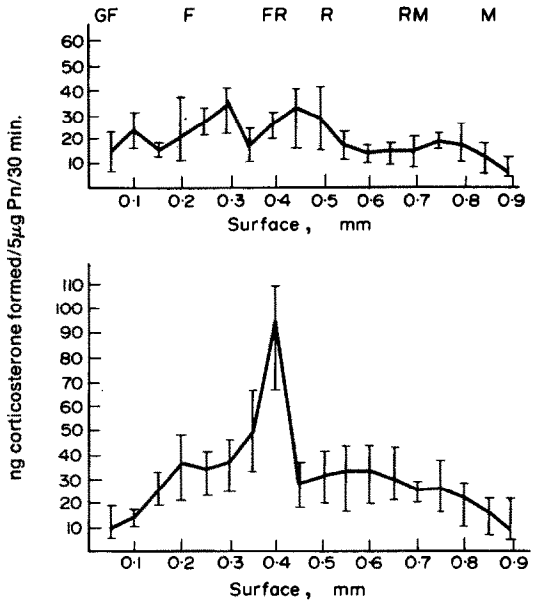


Fig. 3. The quantitative histochemical distribution of 11β-hydroxylase activity based on tissue protein nitrogen (Pn) throughout the normal (upper curve) and ACTH stimulated (lower curve) rat adrenal cortex. Vertical bars represent ranges of activity determined in various regions in the cortex (4 expts). Regions marked G, F, R and M denote respectively zona glomerulosa, zona fasciculata, zona reticularis and medulla; mixed zones are designated by both letters [24].

The 5-ene-3β-hydroxysteroid dehydrogenase in the human adrenal is found concentrated in the outer part of zona fasciculata, while in the case of rat adrenal, this enzyme activity is demonstrated in all zones of cortex [9].

Ovary

Estrogens are recognized to be produced by the follicle, as the amount of secreted estrogen increases with development of follicles in ovarian tissue under the influence of the pituitary follicle stimulating hormone. Ovarian tissue is roughly divided into three parts, namely the corpus luteum, follicular tissue, and follicular fluid. When the enzyme activity of cholesterol side-chain cleavage is examined of its intercellular distribution, it is found concentrated in both corpus luteum and follicular tissue (mostly *theca interna*), and negligibly in the follicular fluid [26]. Similarly, 5-ene-3β-hydroxysteroid dehydrogenase activity is also demonstrated mostly in corpus luteum and *theca interna* of the ovarian tissue by histochemical method [9].

Placenta

The trophoblast becomes active and differentiates into the following types of cells, during the course of embedding in the uterine mucosa; an inner cytotrophoblast with distinct cell boundaries and an outer syncytiotrophoblast with no cell boundaries [27]. The enzyme activities related to steroidogenesis such as 5-ene-3β-hydroxysteroid dehydrogenase, glucose-6-phosphate dehydrogenase, and NADH diaphorase are demonstrated histochemically in the syncytiotropho-

blast[28, 9]. Finally, throughout the above discussion of steroidogenesis at the organ and cellular levels, it is concluded that steroid hormones are produced by specific cells of the specified endocrine organs.

4. INTRACELLULAR DISTRIBUTION OF THE ENZYMES RELATED TO STEROIDOGENESIS

A variety of subcellular structures such as the nucleus, mitochondria, endoplasmic reticula, Gorgi apparatus, lysosomes, other organellae and cellular fluid have been revealed under electron microscope. The *de novo* steroidogenesis from radioactive precursors has been demonstrated *in vitro* with cell-free preparations of steroid-producing organs. This directly suggests that cell structure itself is not obligatory for the steroidogenesis. What then is the site of biosynthesis of steroid hormone among various cellular components as mentioned above?

At the present time, it is difficult to localize, within the cell structure, the enzyme activities related to steroidogenesis with the electron microscope or by histochemical methods. Therefore, it is necessary for biochemical investigation to isolate the cellular constituents individually, after destruction of cell structure by homogenization. For this purpose, a conventional differential centrifugation as outlined in Fig. 4 is employed. To confirm the validity of this procedure, it is essential to examine the isolated fractions and compare them with the native particles observed in the cell with the electron microscope. Thus isolated nuclear and mitochondrial fractions are found to contain nuclei and mitochondria in a

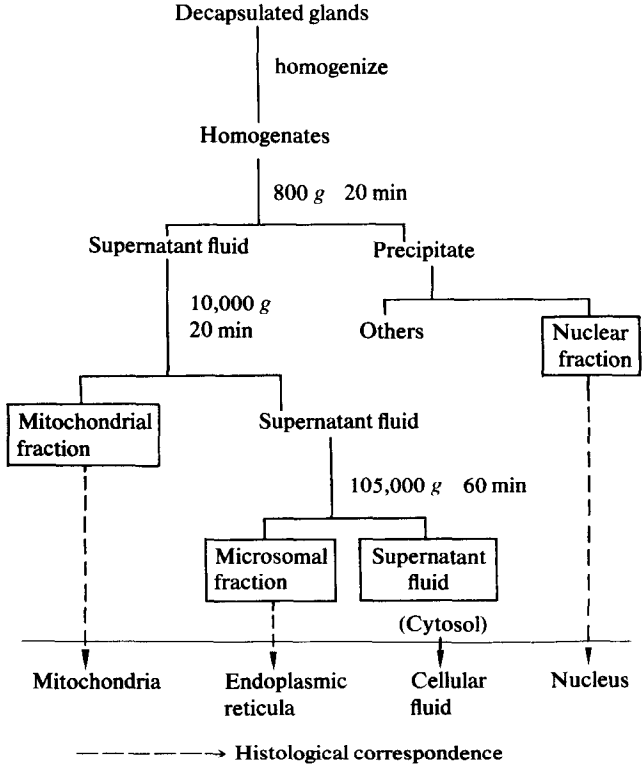


Fig. 4. Procedure of fractionation of cellular components.

state more or less like those observed in the intact cell structure. On the other hand, the structure of endoplasmic reticulum observed in the intact cell structure is broken by homogenization into smaller particles than those of mitochondrial particles. These particles which originate in endoplasmic reticula are referred to as the microsomal fraction. The supernatant fluid remaining after centrifugation at 105,000 *g* contains mainly cellular fluid diluted with isotonic or hypertonic sucrose solution which was used for homogenization. This will also contain certain principles which are solubilized from the particles during the differential centrifugation and other separating procedures. Conventionally, the supernatant fluid is named as cytosol fraction.

Each subcellular fraction is incubated with steroid precursors under identical conditions and the enzyme activities present in the fractions are compared. Generally, the enzyme activities are tentatively expressed as the amount of product formed in a certain incubation time per unit weight of protein[10]. From the results obtained, intracellular distribution of enzyme activities could be determined, considering the following remarks:

(1) Status of enzyme: If an enzyme is located in a certain particle, but is solubilized during homogenization or subsequent separating procedures, the enzyme activities are at least partially found in the soluble fraction. To demonstrate that a certain enzyme is located to a specific membrane structure or particle, it is a fundamental requirement that the enzyme is bound to specific subcellular particles sufficiently tight to ensure against redistribution during the fractionation procedure. In other words, when the enzyme activity is found in a cytosol fraction, it is necessary to pay attention to both possibilities that the enzyme was originally located in the cellular fluid or in certain membrane structures of intact cell but is solubilized from it. On the other hand, a soluble enzyme may become attached to a "particular" fraction, and would be partly washed out from the particle.

(2) Balance sheet: It is necessary to compare the enzyme activity of cell-free homogenates with the sum of individual activities of the enzyme in each subcellular fraction. When there is significant decrease in the sum of individual activities of subcellular fractions, in comparison with that of cell-free homogenates, a recombination study is required to be undertaken by mixing particle fractions and cytosol fraction in possible combinations. In fact, activating "principles" for testicular microsomal enzymes[13] and for the enzymes of adrenal mitochondrial and microsomal fractions[29] are reported. Moreover, as a result of homogenization and centrifugation procedures, some enzyme activity is reversibly or irreversibly lost. For instance, by unconsciously changing osmotic pressure, the mitochondrial structure is easily swollen and ruptured with loss of some active principle(s) into the soluble fraction. Details of this matter are discussed in relation to intramitochondrial distribution of adrenal 11β-hydroxylating system.

(3) It is recommendable to check the agreement of the intracellular distribution of enzyme activity with distribution of other parameters of the enzyme detected by the methods other than enzyme assay. For example, testicular 17α-hydroxylase, and C_{17}-C_{20} lyase which produces 17α-hydroxyprogesterone and transforms it into androstenedione are located in the microsomal fraction. These enzymes most likely involves cytochrome P-450 for their enzyme reactions, because it is known that these enzyme activities are reduced significantly in the

presence of carbon monoxide. As cytochrome P-450 is demonstrated only in the microsomal fraction of rat testes, the intracellular distribution of the enzyme activities as established by the enzyme assays is further supported by the physicochemical determination of its essential component or the P-450 [30].

(4) It is also advisable to examine the relationship between the enzyme activities related to steroidogenesis and the activities of other enzymes known to be specific markers for the organellae.

Testis

When testes of the rat are homogenized and separated into subcellular fractions as described above, all the enzyme activities related to testosterone biosynthesis from pregnenolone are found in the microsomal fraction (Table 1). Intracellular distribution of the same enzymes in other species such as mouse, rabbit, guinea pig and man is similar to the one of rat testes [31].

Table 1. Intracellular distribution of enzymes related to androgen formation from pregnenolone in rat testes

Enzyme	Mitochondrial fraction	Microsomal fraction	Cytosol fraction*
Δ^5-3β-hydroxysteroid dehydrogenase + $\Delta^5 \rightarrow \Delta^4$ isomerase	11·8†	35·8	0·9
17α-Hydroxylase	16·3	37·2	0·4
C_{17}-C_{20} Lyase	4·5	13·6	1·8
17β-Hydroxysteroid dehydrogenase	15·0	43·6	0·6

*Supernatant fluid at 105,000 *g*.
†$\times 10^{-4}$ μmole products/mg protein.

On the other hand, estrogen-producing enzyme system is found in equine testicular tissue [32, 33]. This aromatizing enzyme activity is found mostly in the testicular microsomal fraction [33], being similar to the distribution of the enzymes related to testosterone formation from pregnenolone in the same tissue.

The cholesterol side-chain cleavage enzyme system which consists of 20α-hydroxylase, 22R-hydroxylase and C_{20}-C_{22} lyase is found mostly in the mitochondrial fraction of testicular tissue [34], being similar to its distribution in adrenal and placental tissues. Particularly, since only interstitial cells of the testes can convert cholesterol to androgens [19], mitochondria which have the enzyme activity of cholesterol side-chain cleavage must come from the interstitial cells, but not from seminiferous tubules of testes.

Supernatant fluid obtained by centrifugation at 105,000 *g* is reported to stimulate the activities of testicular 17α-hydroxylase and C_{17}-C_{20} lyase in case of rat [13] and guinea pig [35]. Also the cytosol fraction contains the 20α-hydroxysteroid dehydrogenase which transforms 17α-hydroxyprogesterone to 17α,20α-dihydroxy-4-pregnen-3-one [36]. The role of this dehydrogenase has been discussed in relation to androgen biosynthesis [37]. Finally, intracellular distribution of the enzymes related to androgen biosynthesis is summarized in Table 2.

The 11β-hydroxylation of testosterone has been found in the testicular tissue

Table 2. Distribution of enzyme activities related to androgen production among the cellular components

Cellular component	Residing enzyme activity
Mitochondrial fraction	Cholesterol-20α- and 22R-hydroxylases C_{20}–C_{22} Lyase
Microsomal fraction	Δ^5-3β-Hydroxysteroid dehydrogenase $\Delta^5 \rightarrow \Delta^4$ Isomerase 17α-Hydroxylase C_{17}–C_{20} Lyase 17β-Hydroxysteroid dehydrogenase
Cytosol fraction	20α-Hydroxysteroid dehydrogenase Activating principle for the microsomal enzymes

of a teleost (rainbow trout, *Salmo gairdneri*) [38]. Its further metabolite, 11-oxotestosterone shows androgenic activity in a fresh-water teleost (*Orizias latipes*) 10–17 times as much as that of testosterone itself [39, 40]. As these 11-oxygenated steroids are also detected in the blood, these could be regarded as active hormones specifically in these teleosts. 11β-Hydroxylase activity was found to be localized into the fish testicular mitochondrial fraction, while 17α-hydroxylase, C_{17}–C_{20} lyase and 17β-hydroxysteroid dehydrogenase are located in the microsomal fraction [41]. In this connection, it will be remembered that the 11β-hydroxylase which is regarded as specific for adrenocortical cells in cases of mammalians is also located exclusively in its mitochondrial fraction [23].

Adrenal

In the adrenal mitochondrial fraction, cholesterol side-chain cleaving enzyme system, 11β-hydroxylase and 18-hydroxylase are found, while 17α- and 21-hydroxylases and Δ^5-3β-hydroxysteroid dehydrogenase with $\Delta^5 \rightarrow \Delta^4$ isomerase are found mostly in the microsomal fraction [23]. In cytosol fraction, activating principles to 11β- and 18-hydroxylases in the mitochondrial fraction are reported [29, 42]. The above results are summarized in Table 3. Cytochrome P-450 is found in the mitochondrial and microsomal fractions of the adrenal tissue, in agreement with the above intracellular distribution of adrenal hydroxylases and lyase [43].

Placenta

In placental mitochondrial fraction, cholesterol side-chain cleavage enzyme and also cytochrome P-450 which is to be involved in the above enzyme system are found [44]. On the other hand, the aromatizing enzyme system is significantly concentrated in the microsomal fraction, but P-450 is detected in this fraction less than that in the mitochondrial fraction [45]. Δ^5-3β-Hydroxysteroid dehydrogenase and the isomerase activities are found in all subcellular fractions, but the greatest activities of these are concentrated in mitochondrial and microsomal fractions [46].

Table 3. Intracellular distribution of the adrenal enzyme activities related to corticoidogenesis

Cellular component	Residing enzyme activity
Mitochondrial fraction	Cholesterol-20α-Hydroxylase Cholesterol-22R-hydroxylase C_{20}–C_{22} Lyase 11β-Hydroxylase 18-Hydroxylase
Microsomal fraction	Δ^5-3β-Hydroxysteroid dehydrogenase $\Delta^5 \rightarrow \Delta^4$ Isomerase 17α-Hydroxylase 21-Hydroxylase
Cytosol fraction	Activating principle for 11β- and 18-hydroxylase

5. INTRAMICROSOMAL DISTRIBUTION OF THE ENZYMES RELATED TO STEROIDOGENESIS

Examination of endoplasmic reticula in the cell structure under electron microscope shows two distinctly different types of membrane structure, as follows (see Fig. 5):

(1) *Granular endoplasmic reticula*. As shown in Figs. 5 and 6, the outer surface of the membranes of granular reticulum bears large number of adherent ribosomes. Protein biosynthesis in translation stage is proposed as its major function. The

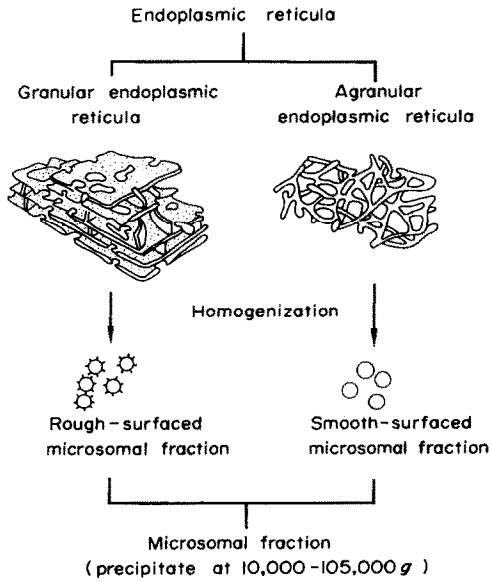


Fig. 6. Relationship between the two types of endoplasmic reticula and microsomal fractions.

attached ribosomal particles are regarded therefore as a site of protein biosynthesis[47].

(2) *Agranular endoplasmic reticula.* This organella consists of interconnected tubular structures, as shown in Figs. 5 and 6, and the outer surface of the membranes of this type of reticulum bears no ribosomes[47]. Function of this membrane is suspected to be related to lipid synthesis in general.

The rough-surfaced microsomal fraction is derived by homogenization from granular endoplasmic reticula, and smooth-surfaced microsomal fraction comes from agranular endoplasmic reticula (Fig. 6). As the whole gland is homogenized and then the microsomal fraction is isolated, the microsomal fraction contains of (1) particles derived from both granular and agranular endoplasmic reticula and (2) particles derived from different types of cells in the same gland (the latter problem is to be discussed later in this review).

Recently, it became possible to separate the microsomal fraction into rough-surfaced and smooth-surfaced microsomal fractions by sucrose density gradient centrifugation, by different affinities of two microsomes for cesium ions and accordingly their different specific gravities.

Procedure for submicrosomal fractionation

As shown in Fig. 7, on top of hypertonic sucrose solution (1·30 M) which contains CsCl 15 mM, the microsomal suspension in 0·25 or 0·33 M sucrose solution which also contains CsCl 15 mM is gently layered. Thus prepared tubes

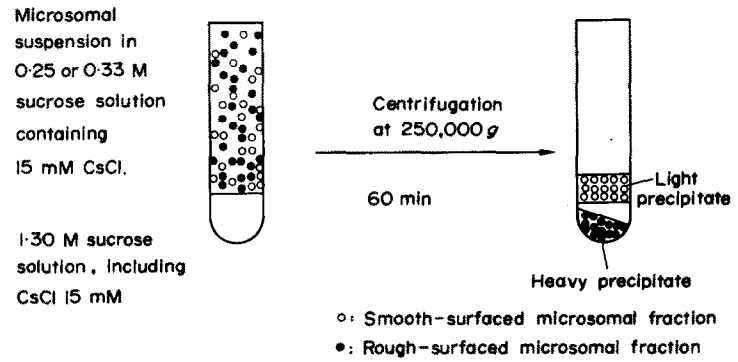


Fig. 7. Procedure of separation of microsomal fraction into rough-surfaced and smooth-surfaced submicrosomal fractions

are centrifuged at 250,000 *g* for 1 h. After centrifugation, heavy precipitates are observed at the bottom of the tubes, while light precipitate is found at the boundary area of the two different sucrose solutions. According to the electron-microscopic observation, the light precipitate consists mostly of smooth-surfaced membrane structure, while heavy precipitate is a mixture of rough-surfaced membrane and free ribosomes. Based on the morphological observation of the microsomal subfractions, the former fraction is designated as smooth-surfaced microsomal fraction, and the latter is designated as rough-surfaced microsomal fraction. The above procedure is essentially similar to the method by Dallner *et al.*[48], which was applied to the subfractionation of hepatic microsomal fraction. Recently, by application of the zonal centrifugation technic, a more detailed spectrum of distribution of microsomal particles was obtained[49].

Chemical analysis and enzyme assay of the two microsomal subfractions

When microsomal subfractions are subjected to chemical analysis of protein and RNA, relatively high content of RNA per unit weight of protein is detected in the rough-surfaced microsomal fraction, in agreement with an abundancy of ribosomal particles (RNA-protein) on the surface of membrane structure, and free ribosomes observed in this fraction under electron microscope.

Enzyme activities related to steroidogenesis are examined for both microsomal subfractions and the results obtained by enzyme assay are described individually for each organ. Again in this kind of study, it is essential that enzymes to be examined are bound to either granular or agranular endoplasmic reticula and their fragments (microsomes) tight enough not to be displaced in these separation procedures.

Testis [50]

Electron microscopic examination of the microsomal subfractions. As shown in Fig. 8, the fraction obtained as a heavy precipitate consists mainly of a rough-surfaced membrane structure with ribosomal particles on the outer surface. The other fraction obtained as a light precipitate consists of smooth-surfaced membrane structures without ribosomal particles on its surface.

Protein, RNA and cytochrome P-450 contents of microsomal subfractions. Contents of RNA and P-450 per unit weight of protein in the microsomal and its two subfractions are listed in Table 4. RNA content is higher in the rough-surfaced microsomal fraction than in the smooth-surfaced one, whereas the cytochrome P-450 is detectable only in the smooth-surfaced microsomes between the two subfractions.

Table 4. Contents of RNA and cytochrome P-450 in subcellular fractions of rat testes [50]

Fraction	RNA/protein*	P-450/g protein/ml†
Microsomal fraction	0·19	4
Smooth-surfaced microsomes	0·14	11
Rough-surfaced microsomes	0·42	0
Mitochondrial fraction	Not measured	0
Cytosol	Not measured	0

*Protein and RNA were determined by the copper-Folin and orcinol reactions.

†Expressed as difference of the optical densities at 450 and 500 mμ in the carbon monoxide difference spectra.

Distribution of testicular enzyme activities related to testosterone formation from pregnenolone between two microsomal subfractions. The combined activities of two subfractions are found to be significantly lower than the activity of the initial microsomal fraction (Table 5). Nevertheless a major portion of the remaining enzyme activities is found in the smooth-surfaced microsomal fraction.

Table 5. Intramicrosomal distribution of the enzyme activities related to androgen biosynthesis, and influence of heated supernatant fluid at 105,000 *g* upon the enzyme activities [50]

Fraction*	Enzyme activities			
	5-ene-3β-hydroxysteroid dehydrogenase†	17α-hydroxylase	C_{17}–C_{20} lyase	17β-hydroxysteroid dehydrogenase
	Specific enzyme activities (μg products/mg protein)			
Ms	1·10	0·65	0·46	0·34
s-Ms	4·18	1·67	1·14	0·94
r-Ms	2·00	0·56	0·59	0·37
s-Ms + h-Sup	6·36	4·09	2·14	1·14
s-Ms + h-Sup	4·33	1·72	1·33	0·63
	Total enzyme activities per pair of testes (μg/testes)			
Ms	24·2	14·4	10·1	7·6
s-Ms	11·7	4·7	3·2	2·7
r-Ms	3·0	0·8	0·9	0·6
s-Ms + h-Sup	17·8	57·3	30·0	15·9
r-Ms + h-Sup	6·5	2·6	2·0	0·9

*Ms, s-Ms, r-Ms and h-Sup denote respectively microsomal fraction, smooth- and rough-surfaced microsomal fraction and heated cytosol.

†Coupled with $\Delta^5 \rightarrow \Delta^4$ isomerase.

The 17α-hydroxylase and C_{17}-C_{20} lyase in particular are found predominantly in the smooth-surfaced microsomal fraction in accordance with the distribution of the cytochrome P-450, which is most likely involved in the hydroxylation and the side-chain cleavage [30]. Specific enzyme activity in the smooth-surfaced fraction is three times as much as that of the rough-surfaced fraction. By adding heated supernatant fluid (cytosol fraction), the apparent enzyme activities of the microsomal subfractions are increased, and the sum of the enzyme activities in subfractions activated by the heated cytosol fraction approaches the level of that of intact microsomal fraction.

At least, one of the reasons why the enzyme activities related to testosterone biosynthesis of two subfractions are significantly decreased after the gradient centrifugation is that one or more components essential for enzyme reactions, but not the cytochrome P-450 itself, is partially solubilized by treatment of the microsomal fraction with the hypertonic sucrose solution. This solubilized principle(s) is partly replaced with the heated 105,000 *g* supernatant fluid. Previous similar studies of enzymic distribution among the microsomal subfractions did not give clear-cut results [51], probably because of the reduction of the enzyme activities by treatment with the hypertonic sucrose solution as explained above.

After incubation of androstenedione with rat testicular microsomes, 7α-hydroxyandrostenedione and 7α-hydroxytestosterone were identified as metabolites, in addition to testosterone [52]. The 7α-hydroxylase activity involved in the above conversion was localized in the smooth-surfaced microsomal fraction of the interstitial cells [53].

As already mentioned, the equine testicular microsomal fraction retains aromatizing enzyme system, including 19-hydroxylase and "aromatase", and is regarded as the site of production of estrogenic steroids found in the stallion's urine[33]. Recently, the microsomal fraction obtained from equine testicular homogenates has been separated into the rough- and the smooth-surfaced microsomal fractions by a method similar to that used for rat testicular microsomal fraction. Then, 19-hydroxylase, which converts androstenedione to 19-hydroxyandrostenedione, and aromatase activities are found in the smooth surfaced microsomal fraction more than in the other subfraction[54].

Adrenal[55, 56]

Electron-microscopic observation of rat and porcine microsomal subfractions. According to observation of subfractions in the electron microscope, the smooth-surfaced fraction consists mainly of smooth-surfaced membranes, and the other heavy fraction consists of a mixture of many free ribosomes with a limited amount of membrane structure (Fig. 9). For the sake of convenience, however, the latter fraction is named as "rough-surfaced microsomal fraction" in spite of its morphological appearance.

Distribution of RNA and the cytochrome P-450 between the microsomal subfractions. The rough-surfaced microsomal fraction contains about 2–7 times as much RNA per unit weight of protein as the smooth-surfaced microsomal fraction (Table 6). This finding is in agreement with the presence of many free

Table 6. Contents of RNA and cytochrome P-450 in submicrosomal fractions of porcine and rat adrenal glands[56]

Fraction	RNA/protein*		P-450/g protein/ml†	
	Pig	Rat	Pig	Rat
Microsomal fraction	0·39	0·16	79	39
Smooth-surfaced microsomes	0·24	0·06	191	88
Rough-surfaced microsomes	0·50	0·42	0	0

*See note of Table 4.

†Expressed as the difference of the optical densities at 450 and 500 mμ in the carbon monoxide difference spectra.

ribosomes in the rough-surfaced fraction. On the other hand, cytochrome P-450 is concentrated in the mitochondrial and microsomal fractions, but not in the cytosol fraction. The P-450 is located mainly in the smooth-surfaced microsomal fraction between the subfractions (Table 6).

Intermicrosomal distribution of the enzyme activities related to corticoidogenesis. The metabolites of progesterone produced by smooth-surfaced microsomal fraction of porcine adrenal are identified as 11-deoxycorticosterone, 17α-hydroxyprogesterone and 11-deoxycortisol. When pregnenolone is incubated with the same preparation, progesterone is identified as a metabolite, in addition to the above three metabolites. From the quantitative analysis of the enzyme

products formed by the two microsomal subfractions, it is concluded that Δ^5-3β-hydroxysteroid dehydrogenase, the isomerase, 17α-hydroxylase and 21-hydroxylase are concentrated in the smooth-surfaced microsomal fraction in case of porcine adrenal. A similar distribution is found in the rat adrenal, from which however, the 17α- hydroxylase is missing (Table 7).

Table 7. Intermicrosomal distribution of the enzyme activities related to corticoidogenesis in porcine and rat adrenal glands [56]

Fraction*	Δ^5-3β-hydroxysteroid dehydrogenase + $\Delta^5 \rightarrow \Delta^4$ isomerase		21-hydroxylase		17α-hydroxylase
	Specific enzyme activities (nmoles products/mg protein)				
	pig	rat	pig	rat	pig
Ms	73	67	49	62	26
s-Ms	159	635	86	401	49
r-Ms	30	16	3	4	5
s-Ms/r-Ms	5·3	39·7	29·7	100·2	9·8
	Enzyme activities per g adrenal (nmoles products/g tissue)				
Ms	333	2308	214	2170	133
s-Ms	145	1937	72	1217	45
r-Ms	5	28	0·3	5	1
s-Ms/r-Ms	29·0	69·2	26·0	243·4	45·0

*See note of Table 5.

The distributions of testicular and adrenal enzyme activities related to steroidogenesis between smooth-surfaced and rough-surfaced microsomal fractions are in agreement with histological findings that in these steroid-producing cells of interstitial or adrenocoritical tissue, agranular endoplasmic reticula are abundant and well-developed. Thus one concludes that agranular endoplasmic reticula of these cells are a site of steroidogenesis in these cells. [57–59].

Placenta [60]

The same procedure of subfractionation of the microsomal fraction as described above is applied for the preparation obtained from human term placentae. Chemical analysis of the two microsomal subfractions reveals that the content of RNA relative to protein in the rough-surfaced microsomes is significantly higher than in the smooth-surfaced microsomes (Table 8). This is in agreement with the electron-microscopic observation on the distribution of ribosomal particles between the two subfractions. Thereafter, distribution of the enzyme activities related directly to estrogen biosynthesis, namely 19-hydroxylase and aromatase are examined in the two subfractions. As shown in Table 9, in contrast with the intermicrosomal distribution of the enzyme activities found in testicular or adrenal tissue, these enzyme activities related to estrogen formation are almost equally distributed between the two microsomal subfractions. Electron-microscopic observation of these two subfractions indicated that they showed distinct characteristics due to granular and agranular endoplasmic reticula [60], as already mentioned in cases of testicular and adrenal microsomal subfractions. Immuno-

Table 8. RNA and protein contents in the smooth- and rough-surfaced submicrosomal fractions of human term placenta[60]

Fraction	RNA μg/ml	Protein μg/ml	RNA/Protein
s-Ms	20·5	720	0·028
r-Ms	88·0	500	0·176
t-Ms	14·5	380	0·038

Table 9. Intermicrosomal distribution of enzyme specific activities related to estrogen synthesis in human term placenta[60]

Substrate, (nmole)	Enzyme	Enzyme specific activity nmole product/mg progein		
		s-Ms*	r-Ms*	t-Ms*
Androstenedione (17·5)	19-OHase	0·15	0·21	0·25
	Aromatase	0·11	0·14	0·18
Testosterone (17·4)	19-OHase	0·05	0·08	0·12
	Aromatase	0·03	0·03	0·08
Dehydroepiandrosterone (17·4)	19-OHase	0·07	0·18	0·14
	Aromatase	0·05	0·15	0·12

*s-, r-, and t-Ms denote respectively smooth-surfaced, rough-surfaced and total microsomal fraction.

chemical investigation of human chorionic gonadotrophin in the ultrastructure of placental cells shows that the cisternae of the granular endoplasmic reticula of the syncytiotrophoblast react strongly against HCG[61].

Corpus luteum

Ovarian tissue obtained from rats treated with human chorionic gonadotrophin and pregnant mare serum gonadotrophin to produce superovulation is found to be rich in corpora lutea. As these corpora lutea are not readily separated from other ovarian tissue, whole ovarian tissue is homogenized and the microsomal fraction is isolated from the homogenates. After separation of the microsomal fraction into the two different subfractions by the procedure described above, each subfraction is examined with the electron-microscope, and activity of Δ^5-$^3\beta$-hydroxysteroid dehydrogenase, the isomerase to pregnenolone is measured. The enzyme activity is found in the rough-surfaced microsomal fraction rather than in the smooth-surfaced one[62].

Discussion on the distribution of enzyme activities within microsomal fractions. As described above, certain enzyme activities related to steroidogenesis are found in the smooth-surfaced microsomal fraction of testicular and adrenocortical cells. On the other hand, in cases of placenta and ovarian tissue with corpora lutea, these enzyme activities are distributed almost evenly between the two microsomal subfractions. Therefore, it is not possible to generalize about the localization of steroid-producing sites in the endocrine cells. But it is concluded that the intermicrosomal distribution of the enzyme activities related to steroidogenesis depends on organ specificities.

Agranular endoplasmic reticulum is mainly composed of granular cisternae and coarse tubules, but as development proceeds, the attached polyribosomes become more widely spaced and patches of smooth-surfaced reticulum appear [63]. Recently, the biogenesis of hepatic endoplasmic reticula, particularly their proteins has been extensively investigated by Omura *et al.* [64–66]. As the marker proteins, NADPH cytochrome *c* reductase and cytochrome b_5 were employed. After administration of radioactive leucine to the animal, incorporation of the amino acid into the hepatic proteins in the microsomal subfractions was measured in the time course. As a result, cytochrome *c* reductase is firstly synthesized on the surface of granular endoplasmic reticulum and then these proteins are transferred to agranular endoplasmic reticula. Proteins which are transferred to agranular endoplasmic reticula seem to attach to the membranes loosely at an early stage, and then become gradually absorbed or connected to the membrane structure tightly as a component of endoplasmic reticulum. Finally, distribution of cytochrome *c* reductase seems to be evenly distributed between two types of reticula.

The final pattern of intermicrosomal distribution of the hepatic cytochrome *c* reductase and cytochrome b_5 is very similar to those of placenta and ovary loaded with corpora lutea. These organs are known as having relatively high activity of protein biosynthesis and turnover [67]. Therefore, being similar to the fate of the cytochrome *c* reductase in liver, the enzymes related to steroid production in placenta and corpus luteum are actively synthesized by ribosomes on the surface of granular endoplasmic reticula and transferred to agranular endoplasmic reticula. The proteins including the enzymes related to steroid biosynthesis are metabolized in agranular endoplasmic reticulum with similar turnover rate of protein to the rate of biosynthesis in the granular endoplasmic reticulum, and so a certain size of the protein pool is maintained.

On the other hand, testicular interstitial cells and adrenocortical cells seem to have a fairly low rate of protein turnover. Enzyme proteins related to steroidogenesis in testicular and adrenal glands are synthesized by ribosomes on the surface of granular endoplasmic reticula fairly slowly. Thus synthesized protein is then transferred to agranular endoplasmic reticula, and is deposited into the well-developed endoplasmic reticula, as a large enzyme pool. Therefore, due to the small amount of the enzyme synthesized on the surface of granular endoplasmic reticula at the moment of sacrifice, low activity of the enzyme is detected in the rough-surfaced microsomal fraction, while high enzyme activity is detected in the smooth-surfaced microsomes of testicular and adrenal tissues. This is probably due to significantly accumulated enzymes to agranular membrane structure.

On administration of gonadotrophin, remarkable development of agranular endoplasmic reticula is observed, indicating the active synthesis of the membrane structure [68]. In this regard, ICSH (LH) stimulates the phospholipid [69] and protein [70] synthesis in the isolated interstitial cells. It seems probable that a part of the phospholipid and protein thus synthesized would be utilized for the biosynthesis of the smooth-surfaced endoplasmic reticula.

The other possible explanation of this discrepancy of the intermicrosomal distributions of the enzymes among several steroid-producing organs could be proposed as follows: Enzyme proteins are synthesized on the free ribosomes, instead of the surface of granular endoplasmic reticula. Then, protein synthesized in this way is incorporated into both types of endoplasmic reticula in different

ratios, depending upon the organ specificities. Probably, both speculative proposals would in part be used to expalin the whole story of this phenomenon.

As the intermicrosomal distribution of the aromatizing enzyme system was different for human placenta[60] and equine testes [54], it is suggested that the nature of the enzyme itself is independent of its intermicrosomal distribution, but the distribution is related to the rates of biosynthesis and turnover of protein in the cell and also the mode of intracellular transfer of the protein between the organellae *in vivo*.

6. INTRAMITOCHONDRIAL DISTRIBUTION OF ENZYMES RELATED TO STEROID BIOSYNTHESIS

Abundant mitochondrial particles are found in adrenocortical cells, and these particles are characterized by their round shape and by tubular or vesicular cristae (Fig. 10). As stated above in the intracellular distribution of the enzymes related to corticoidogenesis, 11β-hydroxylase and cholesterol side-chain cleaving enzyme system (cholesterol 20α- and 22R-hydroxylases and C_{20}–C_{22} lyase) are found to be located in the mitochondrial fraction. Morphologically mitochondria are divided into three parts, namely outer membrane, inner membrane and matrix fractions. In order to study sites of steroidogenesis within mitochondria, e.g. pregnenolone synthesis from cholesterol and 11β-hydroxylation, the following procedures are employed: Firstly, mitochondria are ruptured by osmotic shock [71–73], and sonication[74]. Then mitochondrial fragments are separated into outer membrane, inner membrane fractions and matrix fraction by density gradient centrifugation. Each fraction is examined—(1) with electron microscope (2) by marker enzyme assay—to establish the nature of the isolated mitochondrial components. Outer membrane and inner membrane fractions are found to be fairly homogeneous and distinctly different from each other under the electron microscope[72] (Fig. 11). Assay of the enzymes in hepatic mitochondrial components shows that the outer membrane fraction contains a high concentration of monoamine oxidase, amytal-insensitive NADH-cytochrome *c* reductase and cytochrome b_5, while the inner membrane fraction contains NADPH diaphorase, which is regarded as specific for the inner membrane. The supernatant fluid obtained at 17,000 *g* contains a high concentration of malate dehydrogenase, which is probably derived from the matrix[71].

It is already established that an electron-transfer enzyme system is involved in steroid 11β-hydroxylation and cholesterol side-chain cleavage, starting from NADPH[75], shown in Fig. 12. After destruction of mitochondrial structure,

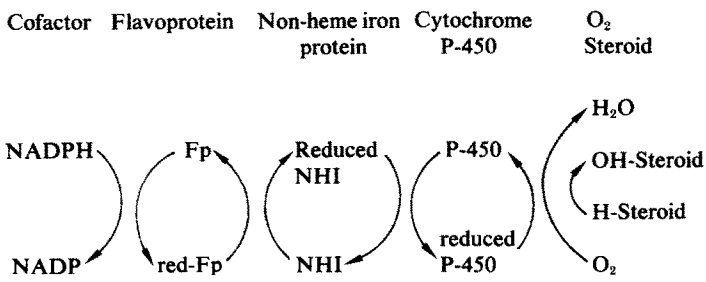


Fig. 12. Enzyme reaction mechanism involved 11β-hydroxylation[75].

each mitochondrial subfractions lacks significant enzyme activities related to the above reactions. However, when non-heme iron protein (adrenodoxin) is added to each subfraction, significantly enhanced activities of 11β-hydroxylase and cholesterol side-chain cleaving enzymes are observed in the inner membrane fraction, but not in the other subfractions (Table 10). In agreement with the distribu-

Table 10. Intramitochondrial distribution of the enzyme activities related to corticoidogenesis and cytochrome P-450 in rat adrenal [71]

Fraction	Enzyme		
	Cytochrome P-450*	11β-Hydroxylase†	Cholesterol side-chain cleaving enzyme‡
Matrix	0	0	0
Outer membrane	0·2	0	0·08
Inner membrane‡	1·4	1·3	1·13

*mμmoles/mg protein.

†The enzyme activities are assayed after addition of adrenal non-heme iron protein and expressed as mμmoles of product/mg protein.

‡+ matrix.

tion of the enzyme activities, the cytochrome P-450 which is the site of molecular oxygen activation and plays the final role of steroid hydroxylation and cleavage (Fig. 12) is also detectable only in the inner membrane structure of adrenal mitochondria [71, 72].

7. DISCUSSION

Cholesterol which is an obligatory intermediate in steroid hormone biosynthesis is available to steroid-producing cells through the following ways: (1) direct transport from serum as cholesterol itself, (2) after enzymic hydrolysis of cholesterol ester deposited in the cell (3) biosynthesis within the cell from precursors by joint action of endoplasmic reticula and cytosol.

Cholesterol administered *in vivo* is mostly deposited as lipid droplets, which are found in the fine structure of adreno-cortical cells [76], and also on the surface of adrenal mitochondria [77]. In this connection, ultrastructure of adrenal cortex has been thoroughly investigated, in relation to the corticoidogenesis and role of lipid droplets [78].

Based on the findings of the various distributions of enzymes described above, the following scheme is proposed; Cholesterol in the cell reaches the inner membrane of the mitochondria of the adrenal cortex cell (probably, the same holds good for cells of other hormone-producing tissue), where the side-chain of the sterol is cleaved. As a result, pregnenolone is synthesized on the inner membrane. Pregnenolone thus synthesized is passed through the outer membrane of the mitochondria and reaches the surface of agranular endoplasmic reticula, where pregnenolone was converted first to progesterone by Δ^5-3β-hydroxysteroid dehydrogenase, and by $\Delta^5 \rightarrow \Delta^4$ isomerase and then 21-hydroxylated,

or hydroxylated at the C-17 and then C-21 positions, forming 11-deoxycorticosterone and 11-deoxycortisol. The 11-deoxycorticoids are again transferred to the mitochondria, particularly to its inner membrane, and there these are 11 β-hydroxylated. Probably, 18-hydroxylation would occur in the same locality. Then, corticosterone, 18-hydroxy-11-deoxycorticosterone, cortisol, and probably aldosterone are finally biosynthesized within the adrenocortical cell and secreted as hormones from the cells into circulation (Fig. 13).

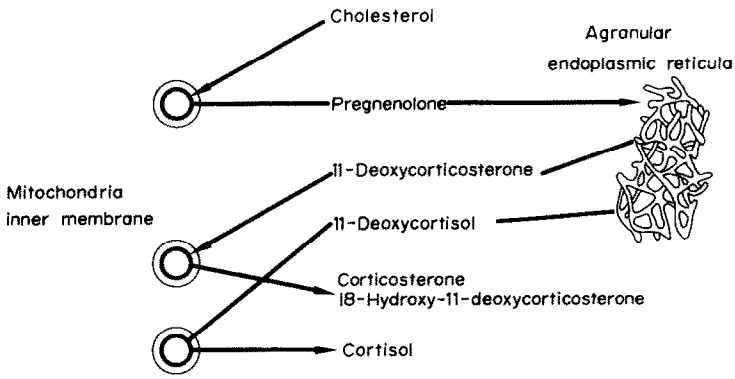


Fig. 13. Sites of corticoidogenesis in adrenocortical cells.

In the case of testis, cholesterol is also transformed into pregnenolone by its mitochondria, most likely by inner membrane enzymes. Pregnenolone is then converted to testosterone by Δ^5-3β-hydroxysteroid dehydrogenase $+\Delta^5 \rightarrow \Delta^4$ isomerase, 17α-hydroxylase, C_{17}-C_{20} lyase and 17β-hydroxysteroid dehydrogenase, all of which are located in agranular endoplasmic reticula of the interstitial cells (Fig. 14). In the case of equine testicular tissue, however, estrone is

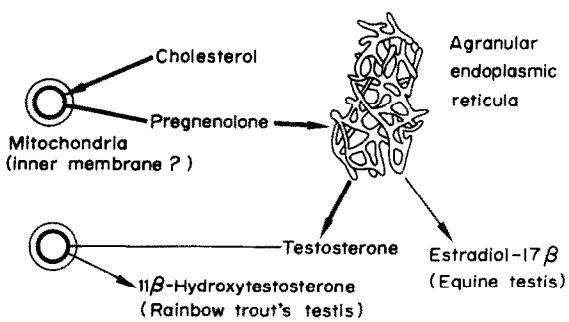


Fig. 14. Sites of steroidogenesis in testicular interstitial cells.

synthesized from androstenedione by the smooth-surfaced microsomal fraction. Furthermore, testosterone is 11β-hydroxylated by testicular mitochondrial fraction of a teleost, the rainbow trout. The two latter cases present an important problem of biological significance regarding the enzyme systems specific to these species.

The biosynthetic sites of estrogens in placenta have not been fully examined, and detailed pathway and mechanism of aromatization are under active investiga-

tion[79, 80]. Cholesterol is also transformed by the mitochondrial enzymes to pregnenolone which is then converted to C-19 steroids. The C-19 steroids produced such as androstenedione, dehydroepiandrosterone and testosterone are converted to estrogens by both granular and agranular endoplasmic reticula of placental cells (Fig. 15).

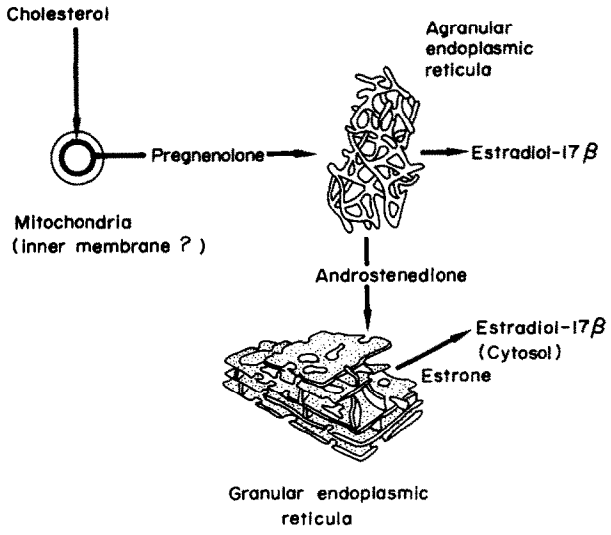
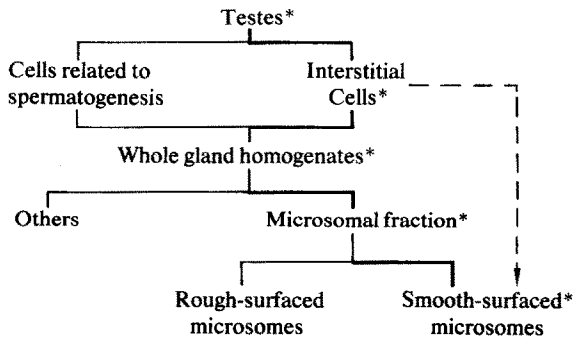


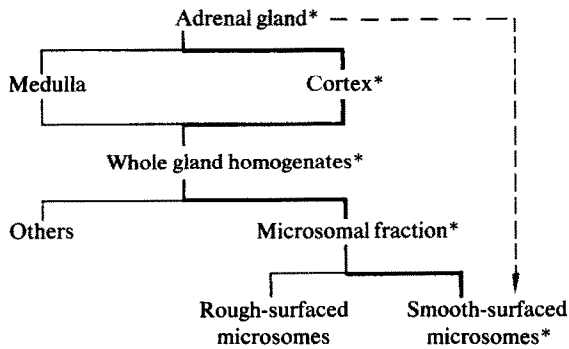
Fig. 15. Sites of estrogen biosynthesis in placental cells.

In this connection, the intercellular distribution of the enzymes is correlated to the intracellular distribution and then to distribution within the organellae, in order to establish that steroid hormones are synthesized by specific organella of specified cells of steroid-producing organs. For example, the microsomal fraction obtained from the homogenates of whole tissue is derived from endoplasmic reticula of steroid-producing cells as well as non-steroidogenic cells. But, as already stated in the section on intercellular distribution, androgen production from pregnenolone is mainly concerned with the testicular interstitial cells. Therefore, the enzyme activities related to testosterone formation from pregnenolone are found within the interstitial cells and then in the microsomal fraction. Furthermore, they are located in the smooth-surfaced microsomal fraction. Therefore, as indicated by the broken line in Fig. 16, the enzyme activities found in the smooth-surfaced microsomal fraction could be concluded as having originated in the agranular endoplasmic reticula of the testicular interstitial cells. A similar account is applicable to the adrenal microsomal enzymes such as Δ^5-3β-hydroxysteroid dehydrogenase + the isomerase, 17α- and 21-hydroxylases which are related to corticoidogenesis. As explained in Fig. 17, these enzyme activities found in the smooth-surfaced microsomal fraction are deduced to be derived from the agranular endoplasmic reticula of adrenal cortex. This is however indirect evidence and, in future, functions of the organella of a single cell type or homogeneous cell group should be examined in the same manner, when a technique for this purpose becomes available, possibly involving use of cell cultures. Culture of a specified cell *in vitro* is a promising method, but, once the cell is cultured *in vitro*, some



*Positive enzyme activities related to androgen biosynthesis from pregnenolone

Fig. 16. Correlation between intercellular distribution and intracellular distribution of the enzyme activities related to androgen formation from pregnenolone.



*Positive enzyme activities of Δ^5-3β-hydroxysteroid dehydrogenase, 21- and 17α-hydroxylases.

Fig. 17. Relationship between intercellular and intracellular distributions of adrenal Δ^5-3β-hydroxysteroid dehydrogenase + $\Delta^5 \rightarrow \Delta^4$ isomerase, 21-hydroxylase and 17α-hydroxylase.

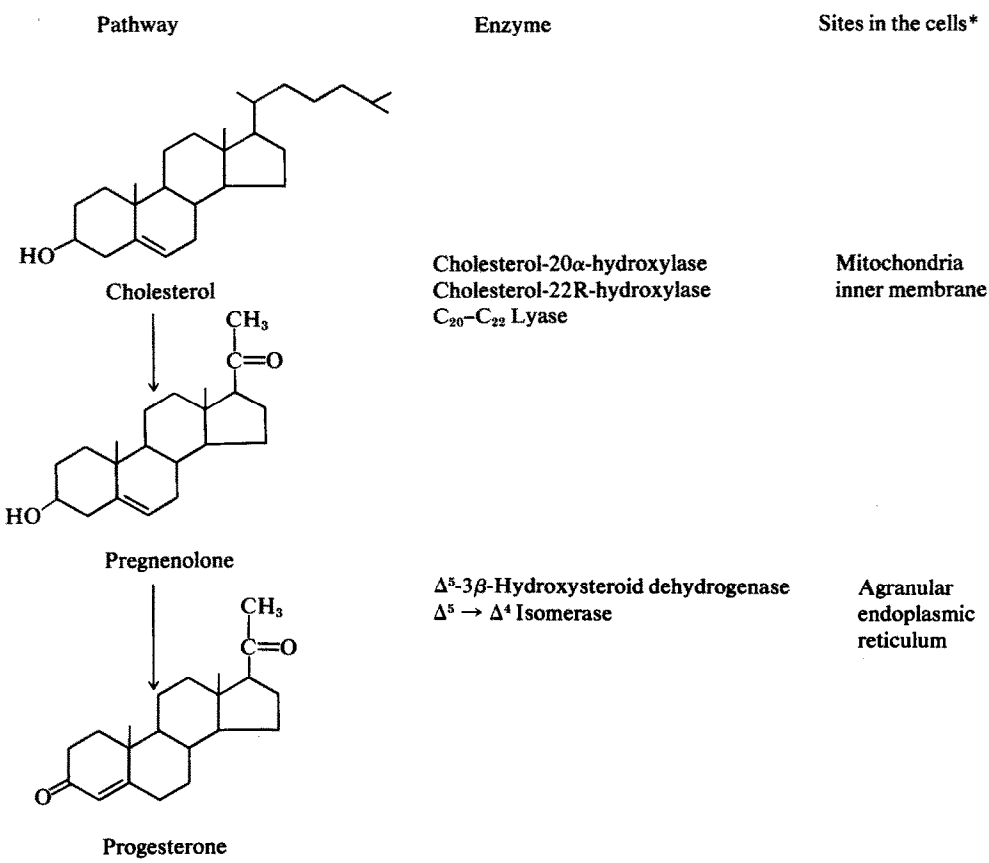
steroidogenic enzyme activity which had been maintained *in vivo* is reported to disappear [81].

Owing to the fact that the enzymes or their components are localized to specific organella and are tightly bound to the membrane structure, it becomes possible to clarify and correlate biosynthetic pathways of steroid hormones, intracellular transport of steroid precursors and hormones, and their biosynthetic sites.

At the same time, binding makes it difficult to solubilize enzyme protein from membrane structure without loss of their activities. Testicular microsomal enzyme protein is particularly tightly bound to its microsomal membrane structure. By sonication at 10 kilocycles, a significant part of the microsomal protein (other than the enzymes) is solubilized, but enzyme proteins themselves remain a part of the microsomal fraction. These enzymes are not solubilized in active form by treatment with several kinds of phospholipases, detergents, organic solvents or by freezing and thawing [82].

Lately, however, one of the testicular microsomal enzymes, 17β-hydroxysteroid dehydrogenase has been solubilized for the first time in this laboratory, and then purified by fractional precipitation with ammonium sulfate, ultra-filtration, and Sephadex, Bio-Gel, and other column chromatographic procedures. By these methods, the enzyme is purified about 1,800 times on the basis of specific activities, calculated from the microsomal fraction. The final preparation of the enzyme is devoid of the other microsomal enzyme activities in the testis [82]. Further purification and characterization of the dehydrogenase are in progress.

Thus, almost all the enzymology related to steroidogenesis makes use of enzyme-carrying bio-membrane as the enzyme sources, as these are not yet solubilized. In comparison with other soluble enzyme systems, enzyme kinetics, quantitative relationship among enzyme, cofactor, substrate, and incubation time, optimal temperature and optimal pH are reasonably studied in this microsomal state. From a different point of view, enzymological knowledge based on bio-membrane actively offers a better opportunity for understanding the function of organella in the cell. In future, when enzymes on bio-membranes are



*Adrenocortical cells, testicular interstitial cells, placental syncytiotrophoblast, corpus lutea and follicular theca interna cells.

Fig. 18. Sites of steroidogenesis and related enzymes on the pathways of progesterone synthesis.

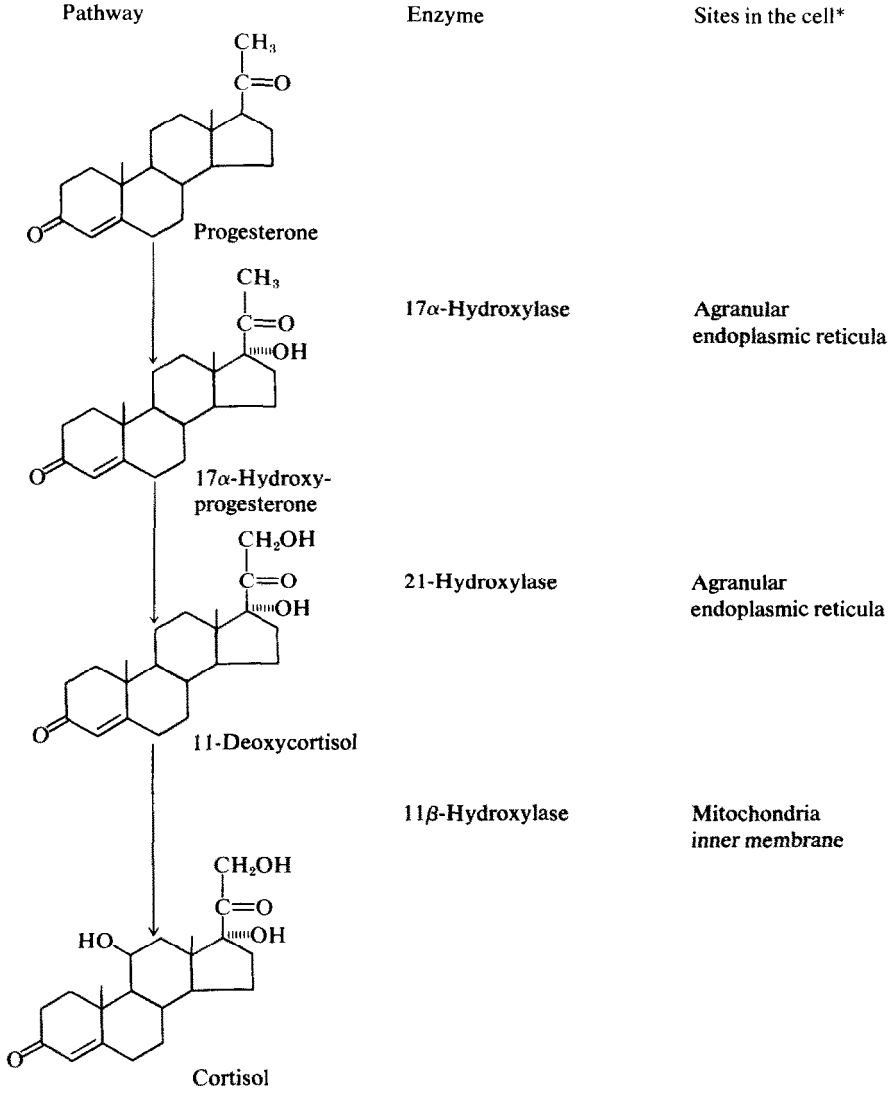


Fig. 19. Sites of steroidogenesis and related enzymes on the pathway of cortisol biosynthesis.

solubilized, enzymology on the basis of solubilized enzymes and their protein chemistry will be explored. This will be the final step towards understanding steroidogenesis at the molecular level.

Furthermore, the status of the enzyme protein in the membrane structure should be examined in more detail not only from the physico-chemical nature of bio-membrane, but also from the viewpoint of function as biochemically characteristic membranes. On this point, although there are many possible combinations of substrate with the enzymes present together in the bio-membrane, the sequential order of enzyme reactions for steroid hormone biosynthesis is fixed and accord-

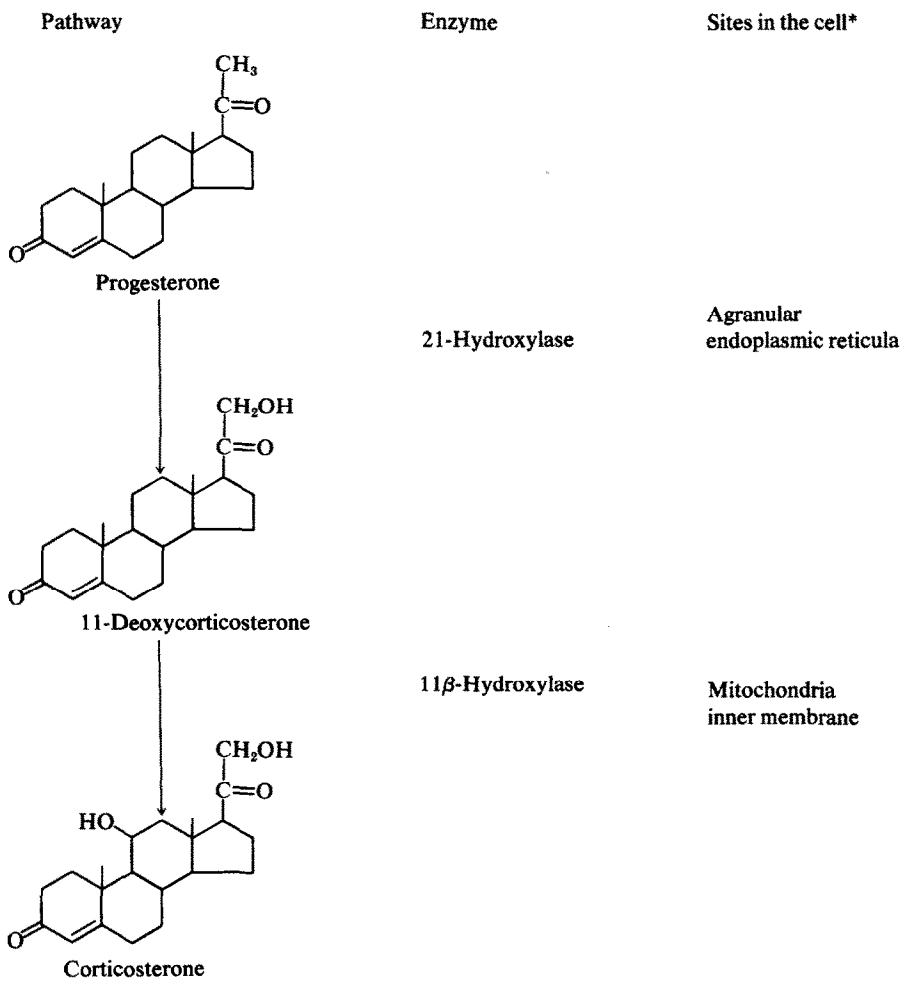


Fig. 20. Sites of steroidogenesis and related enzymes on the pathway of corticosterone synthesis.

ingly the biosynthetic pathway settled. This is explained only partially by substrate preference of the different enzymes which coexisted in the same membrane. For instance, in the case of rat testes, pregnenolone is converted exclusively to progesterone by Δ^5-3β-hydroxysteroid dehydrogenase + the isomerase, but never 17α-hydroxylated even in the presence of excess NADPH and molecular oxygen that is favourable for the 17α-hydroxylase but not for the dehydrogenase [83]. Once progesterone is produced, it is sequentially hydroxylated at its 17α-position. This means that the order of the dehydrogenation and then the hydroxylation has been fixed in this organ of this species, and can not be changed by altering incubation conditions such as cofactors or incubation atmosphere. As it is known that 7α-hydroxyandrostenedione inhibited Δ^5-3β-hydroxysteroid

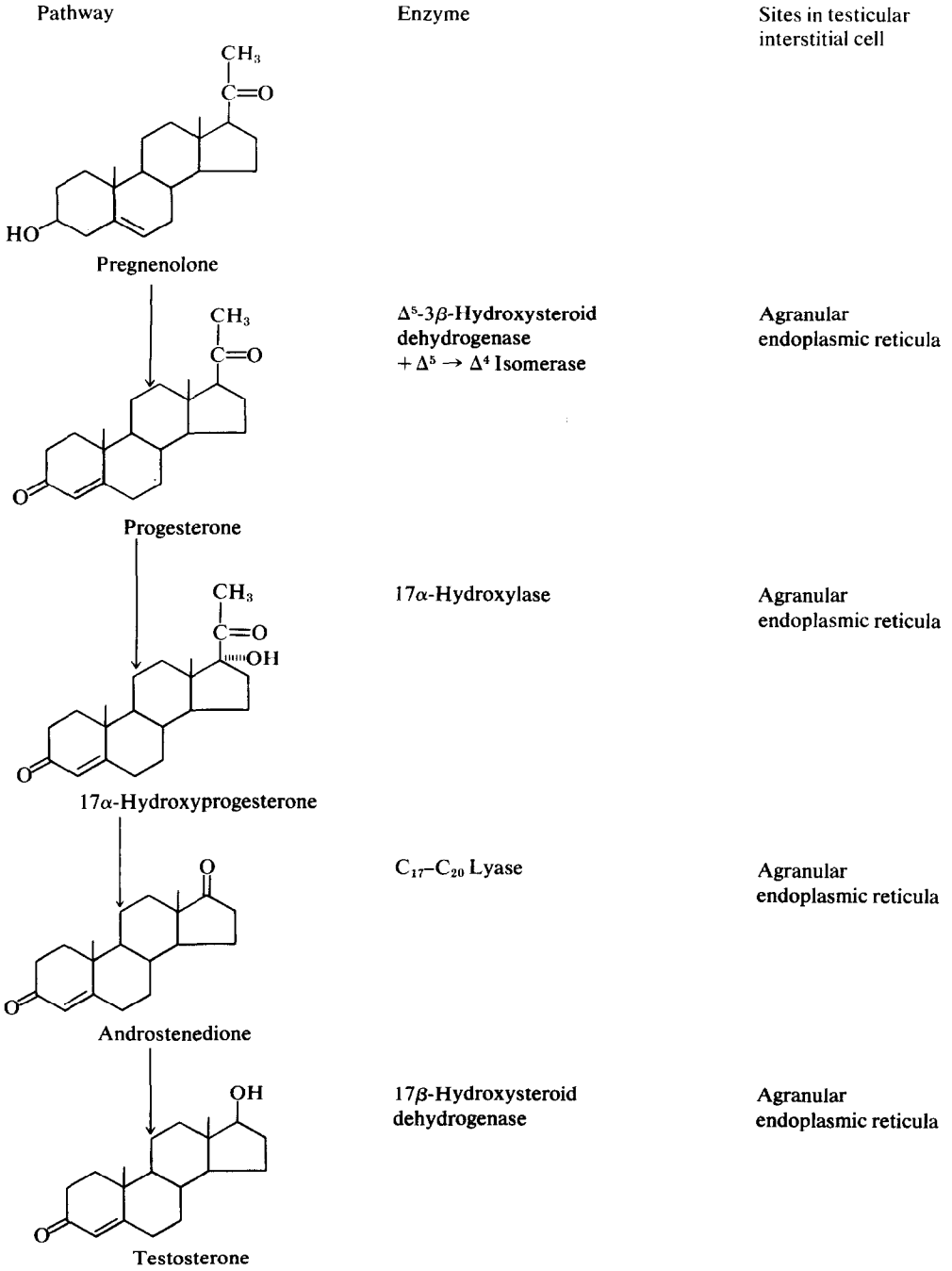
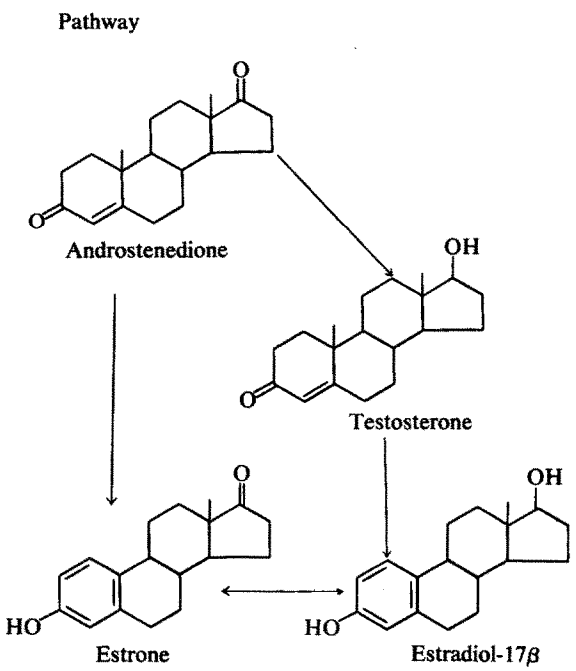


Fig. 21. Sites of androgen biosynthesis and their related enzymes on the pathway via Δ^4-3-oxo route.



*Placental cell of syncytiotrophoblast.
†In case of equine testis, agranular endoplasmic reticula.

Fig. 22. Sites of steroidogenesis and related enzymes on the pathway of estrogen biosynthesis.

dehydrogenase + the isomerase competitively, but not the 17α-hydroxylase or C_{17}–C_{20} lyase [84], pregnenolone is incubated with rat testicular microsomal fraction in the presence of the 7α-hydroxyandrostenedione, in order to shift Δ^4-pathway to Δ^5-pathway by this inhibitor. As a result, however, pregnenolone is neither 17α-hydroxylated nor converted to further Δ^5-metabolites [83]. In the course of testosterone biosynthesis from C^{14}-progesterone, radioactivity could not be efficiently trapped by non-radioactive 17α-hydroxyprogesterone added to the incubation medium in significantly large amounts compared to the substrate [85].

From these facts, it is speculated that enzyme proteins are not scattered at random over the membraneous plane of endoplasmic reticula, but these enzymes which are mutually correlated in steroid biosynthetic pathways are located together in an organized group. The manner of organization in the enzyme groups is genetically determined [83]. When the precursor reaches a group of these enzymes, multiple enzyme reactions are sequentially performed until the final product is formed without excretion of several intermediates from this enzyme group. Thus, free exchange of *de novo* synthesized intermediate with trapping steroids in the cellular fluid or outside of the membrane is very unlikely.

As stated above, a certain steroidal intermediate is obliged to transfer from one organella to the other in the course of steroid hormone biosynthesis. It is likely that the intracellular transport of steroid precursor from cell wall to cytoplasm,

to endoplasmic reticula and to mitochondria would be mediated by steroid so-called "transfer receptors". After biosynthesis of hormones, steroids are secreted from the steroid-producing cells to circulate through blood vessels, and to lymphatic system etc. [86, 87].

There are still several important problems to be solved. For instance, what role, if any, have other organellae such as lysosomes, Golgi apparatus, etc. in steroid biosynthesis and secretion. On this point, cytochrome P-450 was recently detected in Golgi complex of liver [88].

Finally, on the basis of present knowledge of steroidogenesis, the relationship among biosynthetic pathways of steroid hormones, the related enzymes and sites of steroid transformation in the specified cells of steroid-producing organs are summarized in Figs. 18–22.

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