Influence of Physical and Chemical Treatments upon the Microsomal Enzymes of Testes related to Androgen Biosynthesis*

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Synopsis

Testicular microsomal fraction (10,000 ~ 105,000 x g precipitates) of adult rats which was responsible for enzymic transformation of pregnenolone into testosterone was inactivated by phospholipases C and D, but not by phospholipase A. After the 10 kilocycles/sec. sonication for 60 mins., most of the microsomal enzyme activities were still found in the 105,000 x g precipitates. The specific activities of the enzymes in the precipitates at 105,000 x g became higher than those of the untreated microsomal particles, as inactive protein was solubilized from the microsomal fraction by this sonication. After the 20 kilocycles/sec. sonication for 5 mins., however, appreciable activities of the testicular enzymes related to testosterone formation were found in the supernatant fluid at 105,000 x g, but were mostly precipitated at 165,000 x g for 5 hrs., indicating that the sonication modified the microsomal structure of testis without serious loss of the enzyme activities related to the androgen biosynthesis. Influences of the phospholipases A and C upon the 20 kilocycles/sec. sonicated microsomal fraction of testes were similar to those of the same enzymes upon the intact microsomes. By treatments of it with organic solvents, surface active reagents, and freezing and thawing procedure, the microsomal enzymes could not be solubilized with retention of the enzyme activities.

From the results obtained by the above physical and chemical treatments against testicular microsomal fraction, the status of the testicular enzymes in the microsomal structure was discussed.

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*** The following trivial names were used in this text. pregnenolone, 3 β -hydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione; 17 α -hydroxyprogesterone, 17 α -hydroxypregn-4-ene-3,20-dione; androstenedione, androst-4-ene-3, 17-dione; testosterone, 17 β -hydroxyandrost-4-en-3-one; NAD, nicotinamide adenine dinucleotide; and NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

The testicular enzyme system which concerned with production of testosterone*** from pregnenolone was concentrated exclusively to the testicular microsomal fraction (10,000 ~ 105,000 x g precipitates) of the interstitial cells, while the soluble fraction of testicular glands (105,000 x g supernatant fluid) contained the 20 α -hydroxysteroid dehydrogenase and the activating principle upon the above mentioned enzyme activities of the microsomes (Shikita and Tamaoki, 1965; Inano and Tamaoki, 1968; Shikita *et al.*, 1967).

On the other hand, enzyme activities associated with the membrane system of liver

and other organs were biochemically studied, and among them, a limited number of enzymes were reported to be solubilized by physical and chemical treatments (Reid, 1967). As to the enzymes related to the steroidogenesis, one constituent of the 21-hydroxylase system which was associated with adrenal microsomes was isolated and purified as cytochrome P-450, which plays the role of activating the molecular oxygen in the course of the hydroxylation (Omura *et al.*, 1965).

In this paper, the enzymes related to testosterone biosynthesis from pregnenolone were studied of the status in the microsomes, by application of several physical and chemical treatments which had been used for solubilization of microsomal enzymes.

Materials and Methods

Radioactive steroid precursors

Progesterone-4-¹⁴C (146.6 μ Ci/mg), androstenedione-4-¹⁴C (71.0 μ Ci/mg) and 17 α -hydroxyprogesterone-7 α -³H (0.025 μ Ci/mg) were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.), and pregnenolone-4-¹⁴C (738.3 μ Ci/mg) and 17 α -hydroxyprogesterone-4-¹⁴C (113.6 μ Ci/mg) were obtained from Radiochemical Centre (Amersham, England). These radioactive steroids were diluted respectively with non-radioactive steroids, and appropriate amounts of substrates were added to the testicular enzyme preparations, so that saturation of the substrates with the used enzyme preparations was insured. Before use, the radiochemical purities of the radioactive steroids were examined by thin layer chromatography.

Phospholipases

The following phospholipase preparations were used in this experiment to modify the phospholipids of the testicular microsomes.

Phospholipase A (Phosphatide acyl-hydrolase): This preparation was prepared from the venom of Russell vipers and was free from the proteolytic enzymes. Shortly before use, the activity was assayed by the method of Habermann and Neumann (1954).

Phospholipase C (Phosphatidylcholine cholinephosphohydrolase): The enzyme preparations used were prepared by the fractional precipitation with ammonium sulfate from the filtrate of the media in

which the following bacteria were cultured (Macfarlane and Knight, 1941; Molnar, 1962). In this paper, the phospholipase which originated in *Bacillus cereus* was denoted as C₁ and the one obtained from *Clostridium welchii* was designated as C₂. Phospholipase D (Phosphatidylcholine phosphatidohydrolase): The preparation was extracted and purified from the cabbage leaves (Matsumoto, *et al.*, 1967).

Tissue preparation

Male rats of the Wistar strain (about 2 month-old), bred in this Institute were used for this experiment. Immediately after decapitation of the animals, the testicular glands were removed and reserved in the chilled 0.25 *M* sucrose solution. The glands were decapsulated and then homogenized in twice their weight of ice-cold 0.25 *M* sucrose solution with a loose-fitting Teflon-glass homogenizer. The homogenates were then centrifuged at 800 $\times$ g for 20 mins.

The precipitates containing unbroken cells, cell wall, nuclei, and other large particles, *etc.* were discarded. The supernatant fluid obtained at 10,000 $\times$ g was centrifuged at 10,000 $\times$ g once more for 20 mins. to remove the mitochondrial contaminants, and then, the supernatant fluid was centrifuged at 105,000 $\times$ g for 60 mins. The precipitates were suspended in 50 times their weight of 0.25 *M* sucrose solution, sedimented again at 105,000 $\times$ g, and was used as the microsomal fraction. According to electron microscopic examination, the microsomal fraction thus obtained was found as the mixture of rough and smooth-surfaced microsomes, but not contaminated with its mitochondrion (Inano and Tamaoki, unpublished findings).

Incubation

The radioactive precursors were transferred to the incubation flasks to which two drops of propylene glycol per each flask and NADPH or NAD as cofactor were then added. Thereafter, testicular microsomal preparations which had been treated by several physical and chemical procedures were added to the flask mentioned above. Each mixture was incubated at 37.5° for 60 mins. with continuous shaking, under the atmosphere of the O₂-CO₂ (95:5) mixture.

Extraction and isolation of the metabolic products

Immediately after the incubation of the steroid substrates with the testicular microsomal preparation, 15 ml of methylene dichloride was added to each incubation flask, and the mixture was shaken vigorously to arrest any further enzyme reaction. After centrifugation of it at 400 $\times$ g for 10 mins., the

lower layer was removed by a pipette and reserved. The upper layer which contained denatured protein precipitate was reextracted twice more with 15 ml of methylene dichloride, each time. The pooled extract was dehydrated with anhydrous sodium sulfate and evaporated to dryness under the reduced pressure at room temperature. Then, thus obtained steroid fraction was subjected to the analysis of silica-gel thin layer chromatography for separation of the metabolites, developed by the benzene-acetone (18:2) system, and other systems, as previously reported (Shikita and Tamaoki, 1965).

Quantitation of the steroid metabolites

The steroid fractions were separated and purified by repeated thin-layer chromatographies with and without chemical derivations, after extraction from the scraped silica-gel powder on the spots which were detected autoradiographically. An aliquot of the extract was transferred to a counting vial, and the organic solvents were evaporated with a stream of nitrogen. Then, 11 ml of the liquid scintillator which consisted of PPO or 2,5-diphenyloxazole 0.4% and POPOP or 1,4-di-[2-(5-phenyloxazolyl)]-benzene 0.01% in the redistilled toluene was added to the vial, and then, the radioactivity of the ^{14}C or ^3H involved in the sample was measured by a liquid scintillation spectrometer (Nuclear Chicago, System 725, Des Plaines, Ill., U.S.A.). The efficiency of counting radiocarbon was 74%, and, for the double isotope tracer experiment, the efficiencies of simultaneous counting ^{14}C and ^3H were respectively 57% and 32%.

Expression of the enzyme activities related to androgen biosynthesis

As all the enzymes related to testosterone formation from pregnenolone or, Δ^5 -3 β -hydroxysteroid dehydrogenase with Δ^5 - Δ^4 isomerase, 17 α -hydroxylase, C_{17} - C_{20} lyase and 17 β -hydroxysteroid dehydrogenase were located together in the testicular microsomal fraction and could not be separated from each other at the present, the testicular enzyme activities were expressed tentatively as follows; Δ^5 -3 β -hydroxysteroid dehydrogenase together with the isomerase was expressed as the sum of Δ^4 -3-oxosteroids and their derivatives originating in the pregnenolone added as the substrate. 17 α -Hydroxylase activity was expressed as the sum of 17 α -hydroxyprogesterone, androstenedione, testosterone and their derivatives, transformed from the progesterone which was used as the substrate. The C_{17} - C_{20} lyase activity was designated as the sum of androstenedione, testosterone and other C-19 steroids derived by the side-chain cleavage of

17 α -hydroxyprogesterone.

17 β -Hydroxysteroid dehydrogenase activity was expressed as the yield of the testosterone derived from the administered androstenedione as the substrate. The activities of the enzymes defined above were expressed in the ratios (in %) of the products derived by the enzymes to the administered substrates in the tables of this report.

Results and Discussion

Effect of phospholipases upon the microsomal enzyme activities of testes.

1. Phospholipase A

The microsomal fraction of rat testes was adjusted at pH 8.6 with 0.5 M Tris-HCl (pH 9.0) and then the phospholipase A was added (final concentration, 0.1 % w/v). Then, the mixture was incubated at 4° for 16 hrs. (Imai and Sato, 1960; Isselbacher *et al.*, 1962). After the incubation, the mixture was then adjusted at pH 7.4 and an aliquot was reserved for examination of the influence of this lipase upon the microsomal enzyme activities, while the other aliquot was centrifuged at 105,000 $\times$ g for 60 mins., and the precipitates and the supernatant fluid were respectively assayed of the enzyme activities involved in each fraction.

When the untreated microsomal fraction of rat testes was recentrifuged at 105,000 $\times$ g, about one third of total Δ^5 -3 β -hydroxysteroid dehydrogenase activity was found in the supernatant fluid. After treatment with the venomous lipase preparation, total enzyme activity of the lipase-treated mixture was reduced to about 75 % of the sum of the activities found in both the supernatant fluid and the precipitates of the untreated preparation. When the phospholipase-treated microsomal fraction was centrifuged at 105,000 $\times$ g, the activities in the supernatant fluid and the precipitates were respectively comparable with the corresponding activities of the untreated microsomal fractions.

When 17 β -hydroxysteroid dehydrogenase activity was examined of the above mentioned two fractions, the similar tendency to the case

of Δ^5 - 3β -hydroxysteroid dehydrogenase was observed (Table 1).

2. Phospholipases C and D.

The phospholipases C_1 and D were respectively incubated at pH 7.0 with the testicular microsomes, and then the testicular enzyme activities were examined of the supernatant fluid and the precipitates of the treated and untreated microsomal preparations, res-

pectively.

As shown in Table 2, all the enzyme activities related to testosterone formation from pregnenolone were reduced remarkably by the phospholipase C preparation originated in *Bacillus cereus*, while, the activities in the supernatant fluid were negligibly small. Also, after incubation of the testicular microsomal fraction with phospholipase D, the testicular enzyme activities were not significantly detected in the supernatant fluid at $105,000 \times g$, being consistent with the case of phospholipase C_1 .

It is known that phospholipase A removes the fatty acid from the 2-position of phospholipid, which is a component of the cytomembrane, whereas phospholipase C and D release choline and diglyceride respectively from the phospholipid. (Ansell and Hawthorne, 1964). The microsomal enzyme activities related to testosterone production were inhibited by phospholipases C and D, but not significantly by the phospholipase A preparation. The similar tendency as stated above was reported also of adenosine triphosphatase of cerebral microsomal fraction, which was partly solubilized by some surface active reagents (Swanson *et al.*, 1964).

Table 1. Effect of phospholipase A upon the microsomal enzyme activities of rat testes

Preparation	Δ^5 - 3β -hydroxysteroid dehydrogenase*		17 β -hydroxysteroid dehydrogenase*	
	Sup**	Ppt**	Sup**	Ppt**
Untreated microsomal fraction	21.5	60.0	7.5	51.0
Phospholipase A treated fraction	27.5	56.9	5.3	74.8

* Enzyme activity was expressed as the ratio of products in % to the administered substrate (5 μg per flask).

**Sup and Ppt denote respectively the supernatant fluid and the precipitates obtained at $105,000 \times g$.

Table 2. Influence of phospholipases C_1 and D upon the microsomal enzyme activities of rat testes

Preparation	Enzyme Activities*							
	Δ^5 -3 β -OH DHG		17 α -OHase		the Lyase		17 β -OH DHG	
	Sup**	Ppt**	Sup	Ppt	Sup	Ppt	Sup	Ppt
Control***	65.9		39.0		63.3		62.0	
Phospholipase C_1 treated fraction	5.2	3.7	1.8	5.1	4.4	11.3	1.9	24.1
Phospholipase D treated fraction	2.9	44.7	1.7	19.9	3.4	32.6	2.4	58.8

* Enzyme activities were expressed as the ratios of the products in % to the administered substrate (10 μg per flask).

Δ^5 -3 β -OH DHG, 17 α -OHase, the lyase and 17 β -OH DHG were respectively the abbreviation of the Δ^5 -3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase, the C_{17} - C_{20} lyase and 17 β -hydroxysteroid dehydrogenase.

** same as the note of Table 1.

*** Total activity of the untreated microsomal fraction.

From the results obtained from the experiments with the phospholipases, it is suggested that, as an essential part, the enzyme activities related to testosterone synthesis from pregnenolone required the microsomal phospholipoidal component which was susceptible to phospholipases C and D.

Although there was no qualitative difference between the influences of phospholipases C and D, Δ^5 -3 β - and 17 β -hydroxysteroid dehydrogenase activities of testicular microsomes were not so much inhibited by the phospholipase D as the lyase and 17 α -hydroxylase activities, both of which required molecular oxygen and NADPH (Inano *et al.*, 1967; Nakano *et al.*, 1967). Probably, the dehydrogenases group and the group of oxygen-requiring enzymes would occupy different positions in the microsomal particles, particularly in the connection to microsomal phospholipid layer.

Effect of sonication upon the microsomal enzyme activities

1. 10 kilocycle/sec. sonication

The microsomal fraction of rat testis was suspended in the Tris-HCl buffered solution (pH 7.4), and was sonicated with a 10 kilocycle/sec. (hereafter, abbreviated as KC) sonicator (Tominaga Co. Ltd., Tokyo) for 0, 30, and 60 mins. Then, the resultant preparations were respectively centrifuged at 105,000 $\times$ g for 60 mins., and the supernatant fluid and the precipitates of each preparation were simultaneously incubated with progesterone-4- 14 C and 17 α -hydroxyprogesterone-7 α - 3 H. When the 17 α -hydroxylase and the C₁₇-C₂₀ lyase activities as previously defined were examined, very limited activities of the enzymes were found in the supernatant fluid. The specific activities of the enzymes in the particles (105,000 $\times$ g precipitate) were relatively higher than the untreated microsomal fraction, as shown in Figure 1, probably because the protein which was devoid of the enzyme activities was solubilized from the microsomes

Fig. 1. Influence of 10 KC sonication upon the specific activities of the enzymes and protein content in the 105,000 $\times$ g precipitates.
 ○ protein content in the 105,000 $\times$ g precipitates
 ● specific activity of 17 α -hydroxylase
 ▲ specific activity of the C₁₇-C₂₀ lyase

by this sonication, whereas the enzyme activities themselves were not influenced by the sonication (Fig. 1).

2. 20 KC sonication

Testicular microsomal fraction was treated with a 20 KC sonicator (Umeda Electric Co. Ltd., Tokyo) for 5 mins. with cooling. Being consistent with the previously obtained results with the 10 KC sonication, the specific activities of the enzymes in the 105,000 $\times$ g precipitates after the 20 KC sonication become higher than the corresponding activities of the untreated microsomal fraction. Moreover, as indicated in Table 3, appreciable activities of the enzymes were found in the supernatant fluid obtained at 105,000 $\times$ g after 20 KC sonication, and the specific activi-

Table 3. Influence of sonication of 20 KC upon testicular microsomal enzyme activities

Preparation	3 β -OH DHG		Enzyme activities*				17 β -OH DHG	
	Sup**	Ppt**	17 α -OHase Sup	Ppt	the Lyase Sup	Ppt	Sup	Ppt
Control	4.4	61.9	3.4	65.8	4.9	56.9	5.7	76.3
Sonicated preparation	53.8	48.6	20.1	33.1	22.1	21.1	57.5	46.5
Sep. at 165,000 $\times$ g***	18.4***	26.3***	4.3***	9.8***	7.5***	13.2***	13.1***	41.7***
Recombination****	36.8		12.1		14.0		52.2	

* Same as the note of Table 2, but the amount of the substrates per flask was 2 μ g.

** Sup and Ppt respectively denotes the supernatant fluid and the precipitate at 105,000 $\times$ g, except the figures marked with ***.

*** The supernatant fluid obtained at 105,000 $\times$ g was centrifuged at 165,000 $\times$ g, and the enzyme activities in the supernatant fluid and the precipitates were assayed.

**** The activities of the fraction recombined of the supernatant fluid and the precipitate obtained at 165,000 $\times$ g as stated above.

ties of the enzymes were relatively lower than the specific activities of the precipitate obtained simultaneously. This was presumably caused, due to the dilution of the enzyme activities with the solubilized microsomal protein which had no enzyme activity related to the production of testosterone.

In order to examine the status of the enzyme activities in the supernatant fluid obtained at 105,000 $\times$ g furthermore, it was centrifuged at 165,000 $\times$ g for 5 hrs., and then the distribution of the enzyme activities were examined of both the supernatant fluid and the precipitates thus obtained.

After the centrifugation, total enzyme activities of the recombined mixture was found to be remarkably reduced in comparison with the activities of the supernatant fluid at 105,000 $\times$ g. The remaining activities were found in the both fractions, and under the condition of centrifugation at 165,000 $\times$ g, more activities found in the precipitates than in the supernatant fluid. The recombination of the precipitates with the supernatant fluid obtained at 165,000 $\times$ g showed no significant increase to the sum of the individual activities of the two fractions.

By 10 and 20 KC sonications, some part

of protein which was constituent of the microsomal particles and was devoid of the steroidogenic activity was solubilized from the microsomal particles, judging from the increased protein concentration but no enzyme activity in the supernatant fluid, and also from the increased specific activities of the enzymes in the precipitates after the sonications.

The active principles remained in the precipitates at 105,000 $\times$ g after 10 KC sonication, and it is suggested that the particles which contained the enzyme activities, still after the sonication, had the similar physical feature to the intact microsomal fraction during the sedimentation, even though the inert protein was solubilized from the particles by this treatment.

On the other hand, by 20 KC sonication, physical characteristics of a part of the microsomal fraction were changed, or, in other words, a part of the enzymically active particles which had been sonicated was no more precipitable at 105,000 $\times$ g. Furthermore, by centrifugation at 165,000 $\times$ g, however, major part of the Δ^5 -3 β -hydroxysteroid dehydrogenase and the other three enzymic activities were found in the precipitates.

At this stage, total enzyme activities of

the recombined fraction of the resultant supernatant fluid and precipitates which were obtained at centrifugation at $165,000 \times g$ were remarkably diminished, in comparison with the corresponding activity before the centrifugation. This suggests that, during this treatment, the enzyme activities which had been solubilized were inactivated, or at least, the essential component(s) of the enzyme systems were inhibited irreversibly.

The Δ^5 - 3β -hydroxysteroid dehydrogenase and the isomerase which were found in the microsomal fraction of bovine corpus luteum (Cheatum and Warren, 1966) and adrenal gland (Cheatum *et al.*, 1967) were detected in the supernatant fluid at $105,000 \times g$ after sonication, but still, could not be considered as being completely solubilized, because the enzyme activities were precipitated by further centrifugation at $186,000 \times g$.

Therefore, from the results obtained with sonication, it could be concluded that testicular microsomal fraction consists of the following three parts, namely; 1. protein which could be solubilized by 10 KC sonication and had no activity of steroidogenesis, 2. the other part of protein which was not solubilized at 10 KC but solubilized at 20 KC. This fraction did not contain any appreciable enzyme activities, or if any, easily inactivated. 3. the particles which remained in the insoluble state after 10 and 20 KC sonication, and contained all the enzyme activities, more or less reduced by sonication and the following centrifugation.

Influence of the phospholipases upon the sonicated microsomal enzymes of testis

As inactive protein was solubilized, and physical properties of the microsomal particles were changed by 20 KC sonication, the microsomal fraction which had been treated by sonication was subjected again to phospholipases A and C, in order to examine whether or not the sonicated microsomal fraction showed different attitudes to these phospholipases, in comparison with case of intact microsomal fraction.

1. Influence of phospholipase A upon the microsomal enzymes sonicated with 20 KC

The microsomal suspension in Tris-HCl buffered solution was sonicated with 20 KC for 2 min., and then the phospholipase A solution was added to the sonicate which was adjusted at pH 8.6 by 0.5 M Tris-HCl buffer (pH 9.0). Then the mixture was incubated at 4° for 16 hrs. After the incubation, the mixture was adjusted at pH 7.4 by 0.1 N HCl, then centrifuged at $105,000 \times g$ for 60 mins.

As shown in Table 4, the supernatant fluid at $105,000 \times g$ of the sonicated and then phospholipase A-treated preparation had somewhat increased activities of these two hydroxysteroid dehydrogenases in comparison with these of the supernatant fluid of the untreated microsomal fraction, while the 17α -hydroxylase and the lyase activities in the supernatant fluid were not significantly increased by

Table 4. Influence of phospholipase A upon the sonicated microsomal preparation of rat testes

Preparation	Enzyme activities*							
	3β -OH DHG		17α -OHase		the Lyase		17β -OH DHG	
	Sup**	Ppt	Sup	Ppt	Sup	Ppt	Sup	Ppt
Control	3.1	71.5	2.9	78.5	5.6	48.3	3.0	64.9
Sonicated and Phospholipase A-treated fraction	30.1	48.1	7.7	31.3	6.4	15.7	11.3	42.0

* The amount of the substrates per flask was 5 μ g., and expressed same as mentioned in the note of Table 2.

** The supernatant fluid (Sup) and the precipitates (Ppt) at $105,000 \times g$.

these combined treatments. Furthermore, recombination of the supernatant fluid and the precipitates proved no significant increase due to the interaction between the two fractions.

These results were found principally consistent with the results obtained by incubation of the sonicated preparation with the phospholipase A at 37° for 10 mins.

The supernatant fluid at 105,000 × g in which the 3β- and 17β-hydroxysteroid dehydrogenase activities were significantly increased by the procedures was further centrifuged at 165,000 × g for 5 hrs. By the centrifugation, major part of the two dehydrogenase activities were found in the precipitates. Specific activities of the enzymes were found higher in the 165,000 × g precipitates than the supernatant fluid (Table 5).

2. Influence of phospholipase C upon the sonicated microsomal enzyme activities

Testicular microsomal suspension was sonicated in the same manner as above described, and an aliquot of the sonicate was centrifuged at 105,000 × g for 60 mins., while the other aliquot was incubated with phospholipase C₂ preparation at 37° for 30 mins. After incubation with the lipase, the incubation mixture was centrifuged at 105,000 × g for 60 mins. Then, the sonicated preparation without treatment with phospholipase C₂, and

Table 5. Further studies on the effect of sonication and phospholipase A treatment upon testicular hydroxysteroid dehydrogenases

	Enzyme activities*			
	3β-OH DHG Sup**	17β-OH DHG Ppt**	Sup	Ppt
Control	5.5	62.7	4.3	38.9
Sonicated and phospholipase-A treated fraction	72.0	75.0	12.1	40.8
Sep. at***				
165,000 × g	7.8*** (2.7)	29.9*** (8.7)	3.8*** (0.7)	36.2*** (4.5)

* Expressed as the note of Table 2. (Substrate per flask, 5 μg).

** at 105,000 × g except the figures marked with ***.

***The supernatant fluid at 105,000 × g was centrifuged at 165,000 × g for 5 hrs. The figures in parenthesis indicate the specific activities of the enzymes (μg of product/mg protein × 10²).

the sonicated and then phospholipase C-treated preparations were respectively examined of the enzyme activities related to androstenedione synthesis from pregnenolone, after centrifugation at 105,000 × g.

As shown in Table 6, the enzyme activities in the supernatant fluid of the sonicate were significantly enhanced in comparison with the corresponding activities of the unsonicated microsomal preparation, being con-

Table 6. The combined effects of sonication and phospholipase C₂ upon testicular microsomal enzyme activities

Preparation	Enzyme activities*					
	3β-OH DHG Sup**	17α-OHase Ppt**	Sup	Ppt	the Lyase Sup	Ppt
Control	3.3	79.2	2.7	75.0	4.5	86.5
Sonicated 20 KC fraction	52.9 (14.7)***	74.8 (26.7)	27.5 (7.6)	74.4 (26.6)	23.7 (6.6)	73.6 (26.3)
Sonicated and then Phospholipase C ₂ -treated fraction	4.4 (8.8)	6.0 (1.8)	1.6 (3.2)	5.2 (1.5)	4.8 (9.6)	8.4 (2.5)

* Expressed same as the note of Table 2, (Substrate per flask, 1 μg).

** at 105,000 × g for 60 mins.

***Figures in the parenthesis indicate the specific activities, (μg of products/mg. protein × 10²).

sistent with the result previously described.

By incubation of the sonicated fraction treated with the phospholipase C₂, however, the remarkable reduction of the enzyme activities was observed both in the precipitates and the supernatant fluid. The specific activities of the precipitate of the sonicate without lipase treatment were remarkably higher than the corresponding activities of the precipitates obtained from the sonicated and then the lipase-treated fraction.

The enzyme activities of the sonicated microsomes related to androstenedione formation from pregnenolone were also strongly destroyed by phospholipase C₂, but not so much by phospholipase A, and this result is consistent with the result obtained by the phospholipases upon the intact microsomal fractions.

Therefore, the inactive protein which was solubilized by the sonication and the enzyme protein seem to locate independently each other in the microsomal particle. In other words, phospholipase C and probably D worked directly and more or less selectively upon the microsomal phospholipid closely connected to enzyme protein, without any interaction of the protein which was devoid of the enzyme activity and solubilized by the sonication.

There was no significant increase by recombination of the precipitates and the supernatant fluid at 105,000 × g, suggesting that these two fractions separated by the centrifugation were not mutually complementary.

Effects of other treatments upon the microsomal enzyme activities of testis

Organic solvents

1. Acetone

The microsomal suspension obtained from rat testes was cooled on ice and then cold acetone (−10°) was added drop by drop into the microsomal suspension with constant stirring. The resultant precipitates were centrifuged off at 400 × g, and the acetone in the

supernatant fluid was evaporated off under the reduced pressure. When the acetone dried powder, acetone soluble fraction, and the recombined mixture of the above two fractions were assayed of the enzyme activities related to testicular steroidogenesis, all these three preparations were found as devoid of the enzyme activities examined.

2. n-Butanol

n-Butanol was added into the testicular microsomal suspension, according to the similar procedure stated in the above case. Then, the mixture was stirred at 0° for 30 mins., and then centrifuged at 400 × g. Then, the water layer was dialyzed against 0.01M Tris-HCl buffered sucrose solution at pH 7.4, in order to remove the organic solvent, but the portion in the cellophane bag had no enzyme activities related to the androgen biosynthesis.

Detergents

When the sodium lauryl sulfate, as an ionic detergent (0.03 %), Triton X-100 (2 %) and sodium deoxycholate (1 % in glycylglycine solution) as non-ionic detergents were added to the testicular microsomal fraction, turbidity of the preparation was remarkably reduced, but the enzyme activities related to the androgen production were destroyed by these detergents.

Freezing and thawing

The testicular microsomal fraction which was suspended in the deionized water was frozen in the liquid nitrogen and then thawed in water bath. This procedure was repeated upto 15 times, and after this treatment, no enzyme activity was observed in the supernatant fluid at 105,000 × g.

Attempts to remove the lipoidal part from the microsomal fraction with organic solvents such as acetone and *n*-butanol, and then to solubilize the enzyme activities related to testosterone formation were not successful. Being consistent with the previous findings by

the usage of phospholipases C and D, this result suggests that intact phospholipid which was connected to the enzyme protein seems to be essential for display of the enzyme activities.

Furthermore, it was proved by turbidimetry that, the detergents were found to be able to solubilize the microsomal fraction, but, by these chemical detergents, the enzyme activities were completely inactivated. Microsomal enzyme system related to hydroxylation of 3,4-benzpyrene in the liver became not precipitable by treatment of a non-ionic detergent or Triton N-101, but was precipitated by further centrifugation at the same gravitational field for prolonged time (Silverman and Talalay, 1967).

By repeated procedure of freezing and thawing, some part of microsomal protein which was devoid of the enzyme activity was solubilized, but the enzyme activities remained in the form of particle.

Individual enzymic characteristics of testicular microsomes had been studied in the state of particles (Lynn and Brown, 1958; Inano *et al.*, 1967, Nakano *et al.*, 1967), and as the testicular enzymes such as 17 α -hydroxylase, the lyase and 17 β -hydroxysteroid-dehydrogenase were inhibited by *p*-chloromercuribenzoate, these microsomal enzymes retained SH-group in the molecules which were related closely to the enzyme activities.

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