IMMUNOCHEMICAL AND IMMUNOELECTRON MICROSCOPE STUDIES ON LOCALIZATION OF NADPH-CYTOCHROME c REDUCTASE ON RAT LIVER MICROSOMES

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ABSTRACT

By the use of ferritin-conjugated antibody (conjugate) indirect immunoelectron microscopy, NADPH-cytochrome c reductase was localized on rat liver microsomes. Most microsomes in the sections had from 1 to 12 conjugates on their outer surfaces. Among the conjugates, 83% was estimated to bind to NADPH-cytochrome c reductase at a molecular ratio of 1:1, 12% at the ratio of 2:1, and 5% at the ratio of 3 or 4:1. The correlation between immunochemical and morphological data confirmed that most of the NADPH-cytochrome c reductase reacted with the conjugates. Subsequent morphological analyses have revealed that the enzyme is distributed homogeneously on the outer surfaces of microsomes but heterogeneously within microsomes in groups of three to five enzyme molecules.

Liver microsomes contain NADH-linked and NADPH-linked electron transport systems which participate in various oxidative reactions: drug metabolism, desaturation of fatty acids, etc.. Two flavoproteins (NADPH-cytochrome c reductase and NADH-cytochrome b_5 reductase) and two cytochromes (cytochrome P_{450} and cytochrome b_5) are major components of these membrane-bound, electron transport systems. The enzymes interact with one another in the transfer of electrons from NADH or NADPH to the terminal electron acceptors of the reactions. The problem of how these enzyme proteins are arranged in the membrane to accommodate their function has received considerable attention. The distribution of the enzymes among microsomes (intermicrosomal distribution) has been shown immunochemically to be homogeneous (15). Their distribution within microsomes (intramicrosomal distribution) has been suggested by biochemical studies to be as follows: (a) cytochrome b_5 and cytochrome P_{450} may be present as a multicomponent complex with their flavoproteins such that a single molecule of flavoprotein links to a number of the corresponding cytochrome molecules within a complex (1, 5, 18); and (b) the association between flavoprotein and cytochrome is not permanent, so the flavoprotein can move freely among the complexes (5, 18). If that were the case, several complexes should locate close to one another in a microsome. Little has been shown concerning their ultrastructural localization, except that cytochrome b_5 has been localized homogeneously among microsomes. In this report an attempt was made to visualize the inter- and intramicrosomal distributions of NADPH-cytochrome c reductase by the use of ferritin-conjugated antibodies.

MATERIALS AND METHODS

Animals

Male albino rats (Donryu strain) weighing 150-200 g were used. They were fed laboratory chow ad libitum, and fasted for 14-16 h before sacrifice.

Preparation of NADPH-Cytochrome c Reductase and Ferritin

NADPH-cytochrome c reductase was prepared from rat liver according to the procedure of Omura and Takesue (10). Ferritin was prepared from horse spleen according to the procedure of Granick (2, 3), and further purified by recrystallizing eight times.

Assay of NADPH-Cytochrome c Reductase

NADPH-cytochrome c reductase activity of microsomes was assayed by measuring the rate of reduction of horse heart cytochrome c by NADPH according to the procedure of Omura and Takesue (10). The amount of the enzyme was calculated from the rate by using a conversion factor of 20 μmol of cytochrome c reduced per min per mg enzyme, and was expressed as the percentage of total microsomal protein, which was determined by the method of Lowry et al. (6) with bovine plasma albumin as the standard.

Preparation of Rabbit Antiserum Against Rat NADPH-Cytochrome c Reductase and Sheep Antiserum Against the Fab Fragment of Rabbit Immunoglobulin G (IgG)

Each rabbit was given eight – ten intramuscular injections of 5 mg of the enzyme in Freund's complete adjuvant (final vol 3–4 ml). After 3 wk, an intravenous injection of 0.5 ml of the enzyme solution (2–3 mg) was given. For primary immunization, a sheep was given six – eight intramuscular injections of 100 mg purified Fab fragment in Difco Freund's incomplete adjuvant (final vol 10 ml). After 2 wk, the sheep was given another series of six – eight intramuscular injections of 100 mg of purified Fab fragment as described above. The antisera were tested by double diffusion against the purified enzyme and by immunoelectrophoresis against rabbit IgG a week after the last injection. An immunoglobulin G fraction of the antisera was prepared by repeated precipitation with 33% saturated ammonium sulfate, followed by chromatography on a Sephadex G-200.

Iodination of IgG

IgG was labeled with eigher ^{125}I or ^{131}I by the use of chloramine T according to a slight modification of Hunter's procedure (4). The reaction mixture (0.4 ml) contained 0.5 – 1 mCi of the labeled NaI, 0.1 M K phosphate buffer pH 7.4, about 2 mg of IgG, and 2 mg of chloramine T. The reaction was started by the addition of chloramine T and allowed to proceed at 0°C for 15–30 s, after which it was stopped by adding 0.2 ml of 4.8 mg/ml Na metabisulfate, followed by 0.2 ml of 20 mg/ml of KI. The labeled IgG was separated from free ^{125}I or ^{131}I by chromatography on a Sephadex G-50 column which had been previously equilibrated with 0.05 M K phosphate buffer pH 7.4. In most cases the specific radioactivity

obtained was about 10^5 cpm per μg of IgG. Approximately two atoms of I were calculated to bind per molecule of IgG.

Preparation of Ferritin-Conjugated IgG (Conjugate)

IgG was conjugated to ferritin using toluene 2, 4 diisocyanate according to the procedure of Schick and Singer (14). The conjugate was separated from free ferritin and IgG by chromatography on a Bio Gel A 1.5M column, 200–400 mesh (Bio Rad Laboratories, Richmond, Calif.) (9). A conjugate fraction eluted between the void volume and free ferritin was used (see Results).

Preparation of Smooth-Surfaced Microsomes

Rat livers were homogenized by 10 strokes of a motor-driven glass homogenizer with a Teflon pestle, in four vol of 30% sucrose solution containing 0.5 mM Mg^{2+}. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 13,000 g for 20 min in a Hitachi RP 40 rotor (Hitachi Ltd., Tokyo, Japan). The supernate was diluted with an equal vol of the same sucrose solution, then 7-ml portions were overlayered onto 2 ml of 45% sucrose cushion. The gradients were centrifuged at 314,000 g for 220 min in a Hitachi RP 65T rotor. The smooth microsomes banded at the interface were taken out with a syringe and diluted to the initial vol of 7 ml with the homogenizing medium, after which the suspension was recentrifuged at 314,000 g for 220 min. Smooth microsomes were thus washed three times and the final pellet was suspended by a mild homogenization in the desired buffer just before use. The microsome pellets were used within 24 h.

Immunoreaction between Microsomes and Antibody or Conjugate, and the Subsequent Washing of Microsomes by Sucrose Density Gradient Centrifugation

The reaction mixture contained 0.1 M K phosphate buffer pH 7.4 and a specified amount of microsomes and rabbit IgG or conjugate (in case of conjugates, the number of conjugate molecules per microsome in the reaction mixture was adjusted to be less than 700). 0.5 – 1.0 ml of reaction mixture was incubated at 25° for 15 min with slow shaking. After incubation, the mixture was layered onto 12 ml of 10 – 30% (wt/vol) sucrose density gradients over 1 ml of 45% sucrose cushion. The gradients were centrifuged at 175,000 g for 60–90 min in a Hitachi RPS 40 rotor. The microsomes banded at the interface between the gradient and cushion were removed with a syringe, and then washed three times (vide supra). The gradients used for washing the microsomes incubated with IgG contained 0.1 M K phosphate buffer pH 7.4, while those for the microsomes incubated with

conjugate contained 0.3 M K phosphate buffer pH 7.4. Such a procedure has been found to be very effective for reducing the extent of nonspecific binding of conjugate to microsomes (Morimoto, Matsuura, Sasaki and Tashiro, unpublished data).

Electron Microscopy

The washed microsomes were fixed for 2 h with 1% OsO_4 solution in 0.1 M K phosphate buffer pH 7.4. These microsomes were washed with the same buffer solution, dehydrated in ethanol, and embedded in Epon 812 in the usual manner. The block was cut on a Porter-Blum MT2 microtome with a diamond knife (Dupont Instruments, Sorvall Operations, Newtown, Conn.). The thin sections (about 500 Å thick) were stained with either a saturated aqueous solution of uranium acetate or lead citrate for 5–10 min, and examined in a Hitachi electron microscope, model HU-12.

RESULTS

Properties of Ferritin-Conjugated IgG

Fig. 1 shows a typical chromatograph resulting from the application of solution (ferritin-conjugated sheep IgG against rabbit normal IgG) to a 2.5 × 37 cm column of Bio Gel A 1.5M (200–400 mesh). As indicated by the arrows, four fractions were eluted. The composition, size, and immuno-
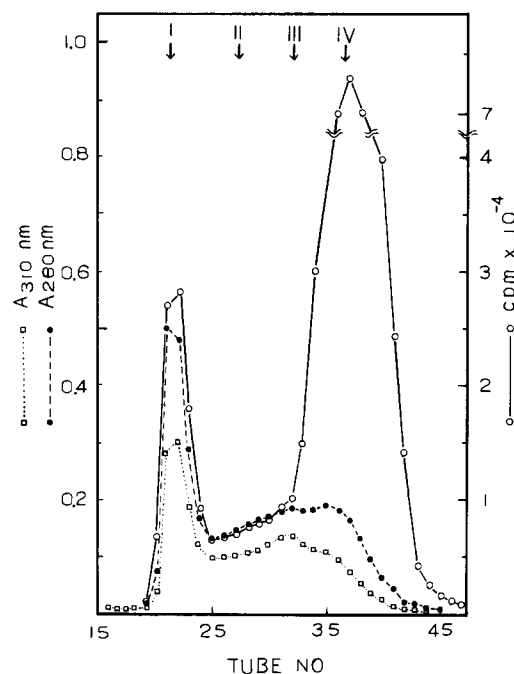
logical properties of each fraction were examined by immunoelectrophoresis in agarose plates and by sucrose density gradient centrifugation. Fraction I contains many polymeric ferritin-conjugated antibodies (about 50%). Fraction II contains mainly monomeric ferritin-conjugated antibodies (about 85%). Fraction III contains free ferritin with some free antibody, and Fraction IV contains free antibody with some free ferritin. The molecular ratios of IgG to ferritin in Fractions I and II were 2.0 and 1.4, respectively. The immunological activity of sheep IgG in Fraction II could not be detected by immunoprecipitation, but could be detected by sucrose density gradient centrifugation as shown in Fig. 2. The antibody activity was estimated to be 30–50% that of unconjugated sheep IgG, as assessed by the amount of labeled normal rabbit IgG bound to the conjugate (when the active sites of an equivalent amount of unconjugated sheep IgG were saturated with an excess amount of normal rabbit IgG).

These results indicate that the conjugates in Fraction II are more homogeneous and consist of a ferritin and an IgG molecule and behave like monovalent antibodies. These properties led us to use the fraction for quantitating immunoreaction products.

Immunochemical Analyses

An indirect procedure was used for visualizing the ultrastructural localization of NADPH-cytochrome c reductase on microsomes. This procedure involves two successive immunoreactions: microsomes vs. rabbit IgG against rat NADPH-cytochrome c reductase (the first-step reaction), and microsome-rabbit IgG complexes vs. ferritin-conjugated sheep IgG against rabbit IgG (the second-step reaction). Three kinds of control experiments were designed to evaluate the specificity of the immunoreaction: control 1 is for the first-step reaction, in which microsomes are incubated with the same amount of nonsensitized rabbit IgG as in the experiment, followed by a second step with the same amount of ferritin-conjugated sheep IgG as in the experiment. Control 2 is for the second-step reaction, in which the ferritin-conjugated sheep IgG was replaced by the same amount of ferritin-conjugated sheep normal IgG. Control 3 is a blocking control for a second-step reaction, in which microsome-rabbit IgG complexes were preincubated with a large amount of sheep IgG against rabbit IgG.
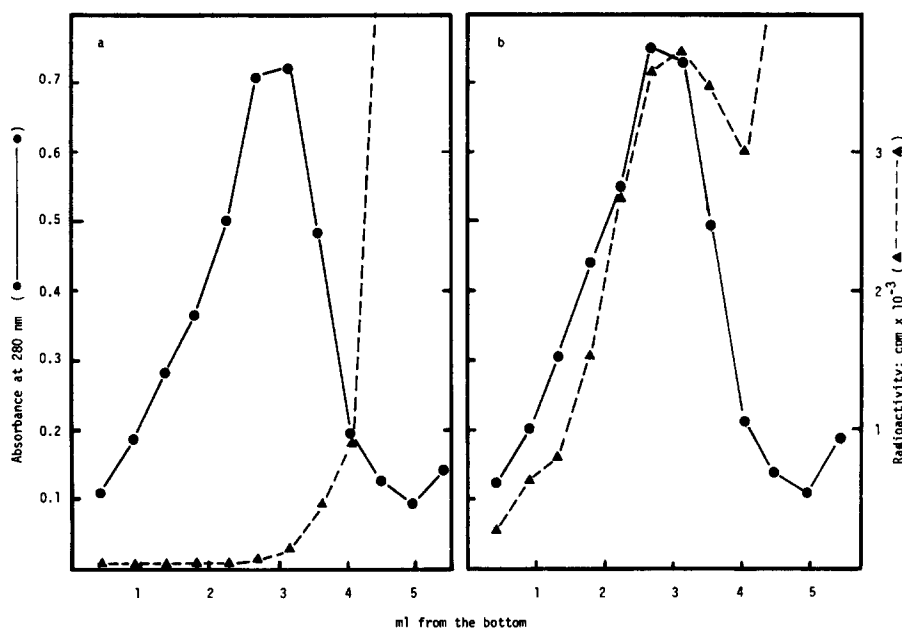
THE FIRST-STEP REACTION: The ex-

FIGURE 1 A Bio Gel A 1.5 M chromatograph of ferritin-conjugated ^{131}I-labeled IgG.

FIGURE 2 Sedimentation profiles of the immunoreaction mixture of ferritin-conjugated sheep IgG against normal rabbit IgG and ^{131}I-labeled normal sheep IgG(a) or ^{131}I-labeled normal rabbit IgG(b). A portion from the reaction mixture was layered onto 5 ml of 10 − 60% (wt/vol) sucrose density gradients containing 0.1 M K phosphate buffer, pH 7.4. These gradients were centrifuged at 150,000 g for 4 h in the cold, using a Hitachi RPS 65T rotor.

tent of the immunoreaction was examined as follows. A fixed amount of microsomes was incubated at 25°C for 15 min with various amounts of ^{131}I-labeled normal rabbit IgG as the control or ^{131}I-labeled rabbit IgG against rat NADPH-cytochrome c reductase. After incubation, the microsomes were washed three times with 0.1 M K phosphate buffer pH 7.4 as described in Materials and Methods. Portions from the microsome samples were counted in a well-type scintillation counter.

The results are shown in Fig. 3. On this graph, molecules of IgG bound per molecule of NADPH-cytochrome c reductase (vertical axis) are plotted against molecules of IgG added per molecule of the enzyme (horizontal axis). In the control, the amount of IgG bound increased slightly in a linear fashion with the addition of IgG, reaching a plateau at a ratio of 1,600, at which figure the number of molecules of IgG bound per molecule of NADPH-cytochrome c reductase was 0.6. In the experiment, the amount of IgG bound increased much more, also linearly, and plateaued at a ratio of 2,500 with five molecules of IgG bound per molecule of the enzyme. The enzyme activity of
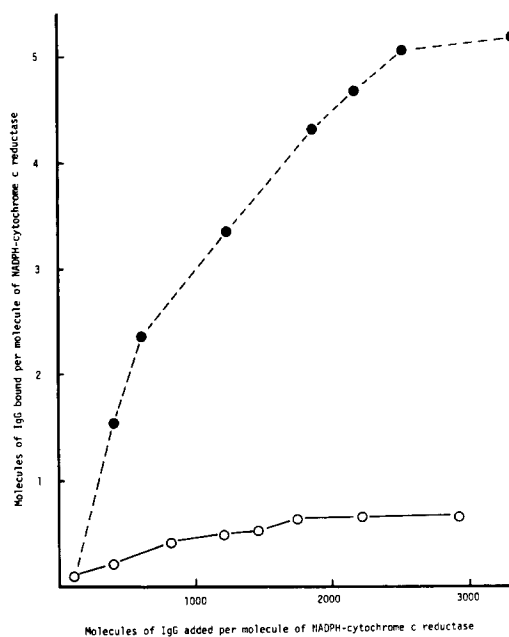

FIGURE 3 Saturation of microsomes with ^{131}I-labeled normal rabbit IgG (O—O) or ^{131}I-labeled rabbit IgG against rat NADPH-cytochrome c reductase (●- - -●).

microsomes incubated with rabbit IgG at a molecular ratio of 2,500:1 was measured. As Fig. 4 shows, the control microsomes reduced cytochrome c at the same rate as untreated microsomes, while the experimental microsomes functioned at one-tenth the rate of the control. The enzyme activity was not inhibited further by increasing the molecular ratio in the reaction mixture. Thus, both experiments indicate that antigenic determinants were saturated with rabbit antienzyme IgG at the molecular ratio of about 2,500.

THE SECOND-STEP REACTION: Our IgG fraction prepared from sheep antirabbit IgG serum contained about 5% of specific antibody. This antibody loses its activity by 50–70% when conjugated to ferritin. About 2% of ferritin-conjugated antirabbit IgG sheep IgG is, therefore, considered responsible for the subsequent immunoreaction. Considering this antibody activity and the extent of nonspecific binding of the conjugates, we determined the molecular ratio of the conjugate to
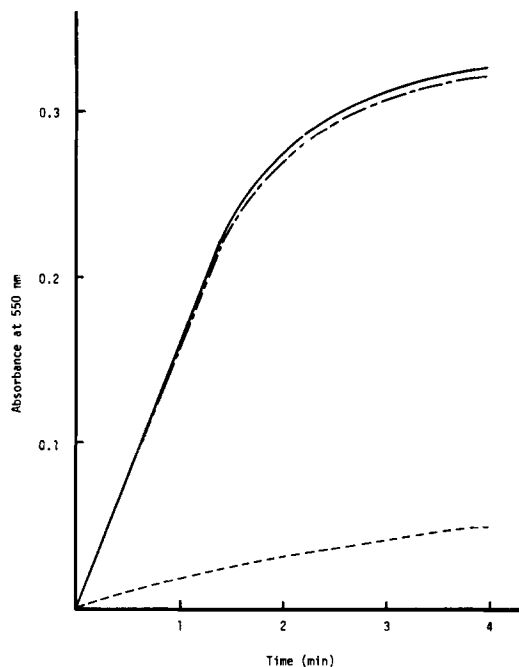

FIGURE 4 NADPH-cytochrome c reductase activity of microsomes incubated with rabbit IgG at a molecular ratio of IgG to NADPH-cytochrome c reductase of 2,500:1. (———), Unincubated microsomes; (— · —), Microsomes incubated with normal rabbit IgG; (- - - -), Microsomes incubated with rabbit IgG against rat NADPH-cytochrome c reductase.

NADPH-cytochrome c reductase in the reaction mixture to be about 50:1, so as to produce a molecular ratio of active conjugate to enzyme of 1:1.

Microsome-rabbit IgG complexes, i.e., the products in the first-step reaction, were incubated at 25°C for 15 min with ferritin-conjugated [125]-labeled sheep IgG. The complexes were then washed three times with 0.3 M K phosphate buffer pH 7.4, and counted. The results were computed in terms of molecules of conjugate bound per molecule of enzyme. Thus, we obtained 0.12 – 0.14 for the control and 0.8 – 1.1 for the experiment, the difference being statistically significant. The samples were then examined by electron microscopy.

Morphological Analyses

IDENTIFICATION OF THE IMMUNO-REACTION PRODUCTS: As seen in Fig. 5, ferritin molecules were clearly identifiable and different from ribosomes. About 27% of microsome sections in the control have from one to three ferritin molecules (see Table I), and about 80% in the experiment have from one to twelve (see Table II). These ferritin molecules were present on the outer surface. Thus, the experimental image demonstrates a clear difference from the control with respect to the number of positive sections as well as the number of ferritin-conjugated antibodies bound.

Although the presence of groups of ferritin molecules may indeed be due to the properties of the antigen, i.e., clusters of NADPH-cytochrome c reductase (group form) or multiple antigenic determinants on a single molecule (amplified form), this type of labeling could also result from the presence of polymeric ferritin-conjugated antibodies contaminating the conjugate fraction. A linear grouping of conjugates separated from each other by 50 Å (which length corresponds to the core-core distance or thickness of two coat protein layers of ferritin molecules, see Fig. 6, indicated by arrows) may result from polymeric ferritin-conjugated antibodies. Linear arrays were also observed, but rarely, inside the microsomal vesicle away from the membrane (Fig. 6, indicated by an arrowhead), although their significance cannot be explained.

Since amplified and group forms are indistinguishable from one another in the electron micrographs, in order to examine the molecular ratio of conjugate to NADPH-cytochrome c reductase all conjugates lying on an arc 250 Å long or inside a
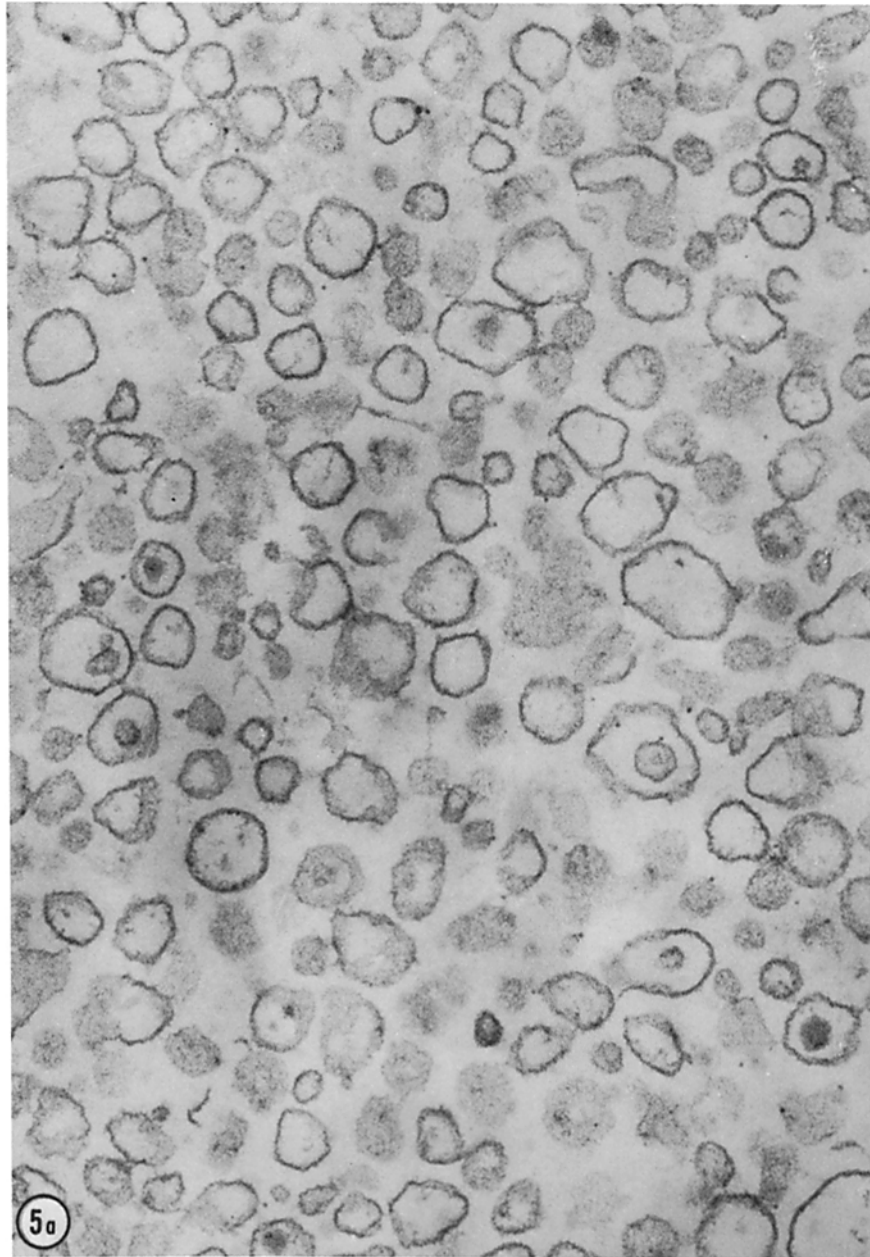
FIGURE 5 a General view of control microsomes. Most microsome sections are not lageled with conjugates. × 82,500.

circle of diameter 250 Å were tentatively assumed to bind to one antigen molecule. This value was calculated from the angle formed between two Fab arms of antigen-antibody complex which was most frequently present in solution (16). Among 2,660 conjugates examined in the experimental speci-men, 2,200 conjugates (83%) bound to antigen at a molecular ratio of 1:1, 308 (12%) at a ratio of 2:1, and 152 (5%) at a ratio of 3 or 4:1. The results clearly indicate that about 90% of NADPH-cyto-chrome c reductase molecules reacted with the conjugate sample bound to a single conjugate.
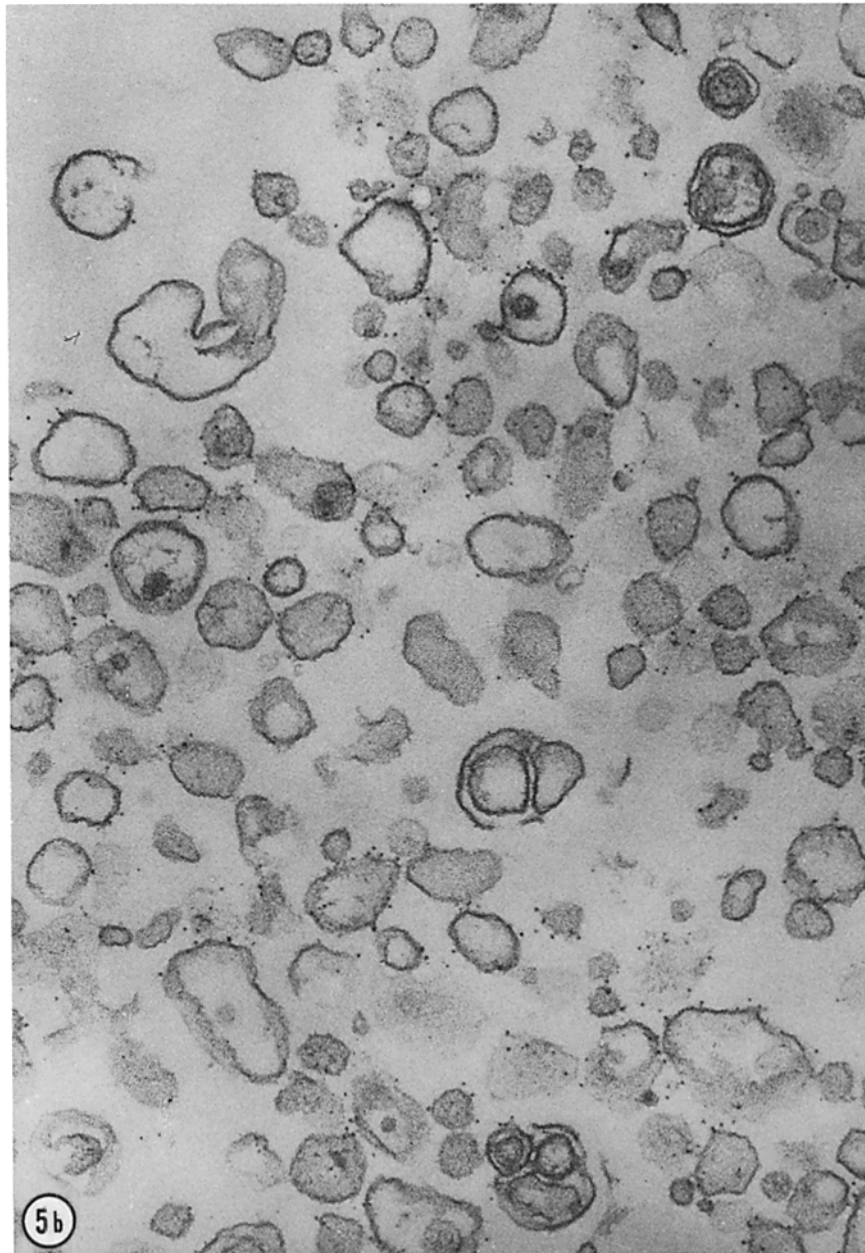
FIGURE 5 *b* General view of experimental microsomes. Most conjugates are present on or near the surface of microsome sections. In contrast to the control microsomes, most sections are labeled with conjugates. × 82,500.

CORRELATION BETWEEN SIZE OF MICROSOME SECTION AND NUMBER OF CONJUGATES BOUND: Microsome sections appeared in various forms as seen in Fig. 5 *a, b:* (*a*) Circular forms with distinct outlines, of which the thickness is comparatively homogeneous (cross section). The length of this line is proportional to the lateral area of the microsome section. (*b*) Circular forms with distinct outlines, part of which were replaced by an electron-dense mass, where the

TABLE I

Correlation between the Size of Microsome Sections and the Number of Conjugates Bound on Them in the Control Specimens

Circumference of microsome section	No. of sections with 0-4 or more conjugates				
	0	1	2	3	4 or more
Å					
501–1,000					
1,001–1,500	10	1	1		
1,501–2,000	23	4	4		
2,001–2,500	28	8	1		
2,501–3,000	34	9		2	
3,001–3,500	42	12	2	2	
3,501–4,000	39	6	7		
4,001–4,500	15	3	1	1	
4,501–5,000	10	4	1	1	
5,001–5,500		2	2		
5,501–	1		1		

TABLE II

Correlation between the Size of Microsome Sections and the Number of Conjugates Bound on Them in the Experimental Specimens

Circumference of microsome section	No. of sections having 0-12 conjugates								
	0	1	2	3	4	5	6	7	8-12
Å									
501–1,000		1	1						
1,001–1,500	3	5	5		2				
1,501–2,000	8	5	5	3		1			
2,001–2,500	7	1	3	5	6	2			
2,501–3,000	13	2	12	18	9	1	2		
3,001–3,500	5	1	2	5	4	3	2		1
3,501–4,000		1	1	9	3	9	1	3	
4,001–4,500	5	1	1	6	6	1	1	1	
4,501–5,000	4	1		2	2	3	5		1
5,001–5,500	1	1		4	3	1	1	1	
5,501–	5		1	1	1	1	6	1	4

microsome may have been tangentially sectioned. (c) Electron-dense mass with no distinct contour (tangential section). Form (a) was exclusively used for the subsequent morphological analyses.

The circumferences of microsome sections and the number of conjugates bound on them were measured in magnified electron micrographs of the control and experimental specimens. The results are arranged in Table I (control) and Table II (experimental) to demonstrate the correlation between the size of microsome sections and the number of conjugates bound. In the control, 27% of the microsome sections bound from one to three

conjugates, while in the experiment 79% bound from 1 to 12 conjugates approximately in proportion to their size. Statistical analysis with the null hypothesis at a significant level of 1% confirmed the presence of a positive correlation between the section size and number of conjugates bound in the experiment. This correlation was not found in the control.

CORRELATION BETWEEN IMMUNO-CHEMICAL AND MORPHOLOGICAL DATA, AND THE RELATIVE NUMBER OF NADPH-CYTOCHROME C REDUCTASE MOLECULES INVOLVED IN THE IMMUNOREACTION: Immunoreaction products measured by morphological methods were computed in terms of the number of conjugates bound per microsome, which was then compared with that obtained by immunochemical methods. For this computation, the content of NADPH-cytochrome c reductase and the thickness of microsomal membrane were measured. The average enzyme content of 15 preparations was $0.5 \pm 0.1\%$ of microsomal protein. The average thickness of the outer electron-dense layer measured in 13 highly magnified electron micrographs ($\times 360,000$) was 20.5 ± 4.7 Å, which was the same as the thickness of the inner electron-dense layer. The average thickness of the clear area between the two dense layers was 17.0 ± 5.0 Å. The mol wt of the enzyme and the density of protein have been reported to be 90,000 daltons (13) and 1.27 g/cm³ (11), respectively. Thus, the number of the enzyme molecules per microsome, the amount of membrane protein per microsome, and the surface area per microsome were calculated to be $7.22, 2.16 \times 10^{-16}$ g, and 4.52×10^6 Å², respectively [assuming that (a) microsomes are spherical vesicles with a diameter of 1,200 Å (17) and (b) the microsomal membrane protein is localized to both surfaces of the membrane with a double layer of lipid at the center, and is visualized as an electron-dense layer in the micrographs]. The number of microsomes was then calculated from the biochemical data by dividing the amount of microsomal protein by 2.16×10^{-16} g or from the morphological data by dividing the lateral areas of microsome sections by 4.52×10^6 Å².

Since it is difficult to determine precisely the thickness of the sections used in electron microscopy, sections with an interference color of grayish tone were preferentially employed. Although these sections are considered to have a wide range of thickness below 600 Å, a section thickness of 500 Å was assumed here. The number of conjugates
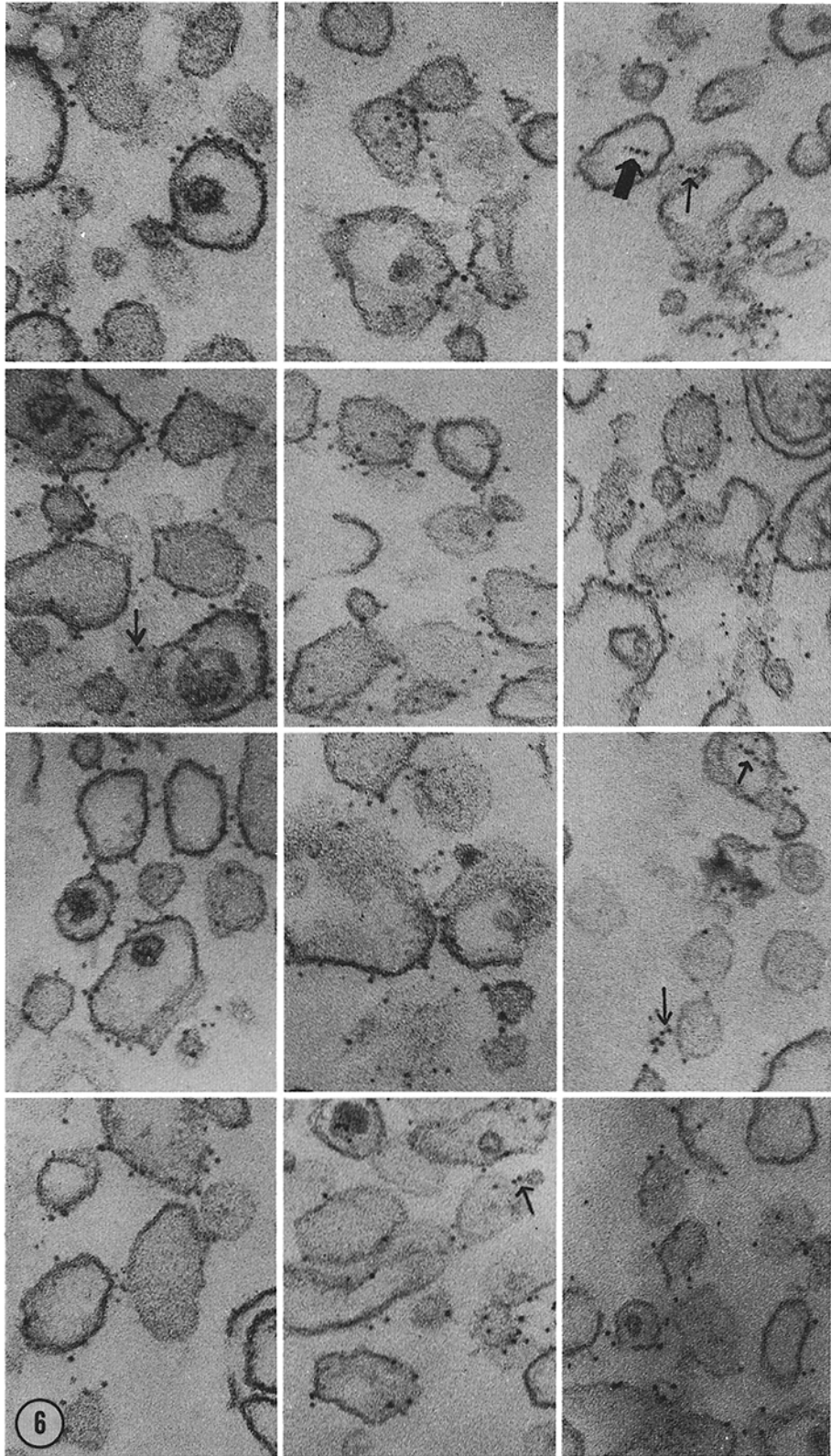
FIGURE 6 Galleries of the experimental microsomes. Most mcrosome sections are labeled with conjugates, which are heterogeneously distributed in each section. Possible images of polymeric ferritin-conjugated antibodies are indicated by an arrow or an arrowhead. × 100,000.

bound per microsome was thus calculated to be 1.13 for the control and 7.54 for the experiment, using the morphological data shown in Tables I and II. These values paralleled those obtained by radioimmunoassay in the second-step reaction, indicating that about 15% of the conjugates present on the experimental microsomes result from nonspecific binding.

INTRAMICROSOMAL DISTRIBUTION OF NADPH-CYTOCHROME C REDUCTASE: Conjugates were found on most microsome sections, but did not appear to be present homogeneously on the outer surface (Fig. 6). Because the correlation between immunochemical and morphological data suggested that most of the enzyme molecules reacted with conjugates at a molecular ratio of 1:1, the intramicrosomal distribution of the enzyme was examined in the following way.

A microsome vesicle with a diameter of 1,200 Å contains, on the average, 7.22 molecules of NADPH-cytochrome c reductase. If these enzyme molecules are present homogeneously on the outer surface of the microsome membrane, one enzyme molecule will be present in every 6.3×10^5 Å^2 (which corresponds to a circumference of 1,260 Å in cross section, since the thickness of the thin section was assumed to be 500 Å). This area is tentatively defined as the "unit area," which is introduced in the subsequent morphological analysis.

Among 660 cross sections examined in 20 electron micrographs at a magnification of 100,000, 19.8% of the sections bound no conjugates, 79.8% bound from one to eight conjugates, and 0.3% bound more than nine conjugates. Microsome sections having one to eight conjugates were classified into eight classes (classes 1-8) according to the number of conjugates. Sections in each class were further classified in the following way according to the number of "unit areas" in which the conjugates were distributed. For example, class 4 microsome sections which bind four conjugates were classified into types 1, 2, 3, and 4, depending upon the number of unit areas from 1 to 4 which the conjugates are found. Similarly, microsome sections in classes 5, 6, 7 and 8 were classified into types 1-5, 1-6, 1-7, and 1-8, respectively. The distribution of these types in each class is presented as the relative frequency, and the results are arranged in a tabular form so as to allow one to visualize the correlation between the class number and the type distribution (Table III). The underlying principle is that, since the number of conjugates bound per microsome section was

found to be proportional to the size of the microsome section, the mode will be proportional to the class number if the conjugates are distributed homogeneously.

The results (Fig. 7) show that the mode increased in a stepwise fashion from 1-2 at class 4 and from 2-3 at class 7. Types 5-8 were not observed in classes 5-8, nor type 1 in classes 7 and 8. These results indicate that the enzyme is not

TABLE III

Relative Frequency of Various Types in each Class of Microsome Section (see Text)

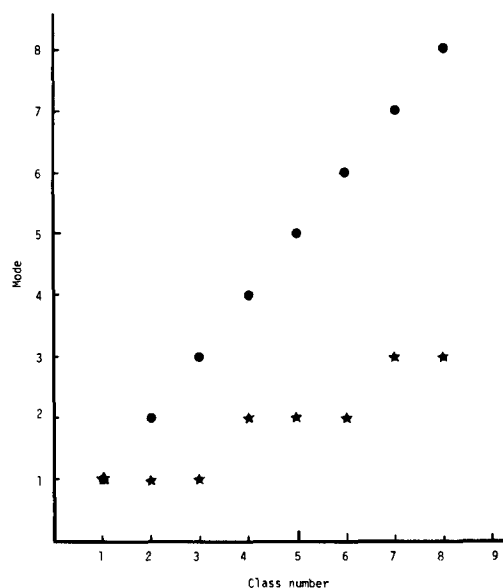| Class	Relative frequency of types 1-8				
	1	2	3	4	5-8
	%			%	
1	100				
2	88	12			
3	71	27	2		
4	44	53	3		
5	27	55	18		
6	5	84	11		
7		45	55		
8		37	50	13	

FIGURE 7 Variation of mode as a function of class number. The mode is expressed as type number, and the class number corresponds to the number of conjugates bound to the microsome section. Dots (●) lying on a sloping straight line indicate the relation between mode and class number when conjugates are homogeneously distributed in each microsome vesicle. The results are plotted as stars (★).

distributed homogeneously but heterogeneously as shown by the grouping of ferritin-conjugated IgG molecules on each microsomal vesicle.

DISCUSSION

Conjugate Counts

The correlation between immunochemical and morphological data confirmed that ferritin molecules on the outer surface of microsome vesicles were the immunoreaction products between NADPH-cytochrome c reductase and ferritin-conjugated sheep IgG. The conjugate-enzyme (antigen) relationship was then examined to clarify what percentage of the conjugates bound to antigen at a molecular ratio of 1:1. It was found that 83% of the conjugates bound at the above ratio, 12% at a ratio of 2:1, and 5% at a ratio of the 3 or 4:1. These results indicate that a value equal to 90% of the number of conjugates corresponds to the number of the enzyme molecules bound. It can be concluded, therefore, that at least 80% of the enzyme molecules reacted with the conjugates under the present experimental conditions, because the number of conjugates bound per microsome and the number of enzyme molecules per microsome were estimated to be 6.41 (experimental-control) and 7.22, respectively.

In our counting, the maximum distance between two ferritin molecules bound to a single antigen in solution was assumed to be 250 Å, and all conjugates lying on an arc 250 Å long or inside a circle of diameter 250 Å were considered to be bound to one antigen. Thus, the "amplified form" was distinguished from the "group forms" only by the geometrical arrangement. However, the following group forms were also counted as "amplified forms," because they are indistinguishable from the "amplified form": (a) Conjugates bound to grouped antigens which formed a cluster or which had a small domain, and (b) conjugates which were present tangentially at a distance of more than 250 Å, but which appeared to be present within 250 Å in cross section. Therefore, it could be considered that more than 83% of the conjugates bound to the enzyme at a molecular ratio of 1:1.

Distribution of NADPH-Cytochrome c Reductase

INTRAMICROSOMAL DISTRIBUTION: The localization of NADPH-cytochrome c reductase to the outer or inner surface of the microsome membrane cannot be elucidated by the present methods, because the ferritin molecule cannot penetrate the membrane and enter the cavity. The present results, however, afford morphological evidence that most of the enzyme is present on the outer surface. In the second-step reaction, at least 80% of the enzyme in microsomes (of which the activity was inhibited by 90%) reacted with ferritin-conjugated antibodies, all conjugates being observed on the outer surface.

In our laboratory the technique developed by Nicolson and Singer (7-9) for two-dimensional visualization of cell surface antigens was not applicable to microsomal membranes. Therefore, a morphometric approach was attempted in order to examine the intramicrosomal distribution.

The results of this analysis (Table III) are seen in Fig. 7, with the mode (vertical axis) plotted against the class number (horizontal axis). Contrary to the homogeneous distribution shown by a series of dots all lying on a sloping straight line in the figure, a distinct stepwise increase in the mode is observed from 1-2 at class 4 and from 2-3 at class 7. Such a regular stepwise increase could be interpreted as follows: (a) This enzyme is distributed not homogeneously but in groups of molecules in each microsome vesicle. (b) Each group consists of three enzyme molecules. (c) The groups may be present homogeneously on microsome vesicles.

Thus, the number of the group constituents is estimated to be three, but if the following points are considered the number will be within a range of three to five. (a) With the present methods, most microsome vesicles are cut into two or three parts and appear as cross or tangential sections in the electron micrographs. A group of enzyme molecules on the vesicle is often cut, and part of the group will be observed as a whole group in the micrographs. (b) Types 1 and 2 in class 4 as well as types 2 and 3 in class 7 did not show much difference in the relative frequency (see Table III). (c) Among microsome sections having six or more conjugates, only 1.1% of "unit areas" contained six conjugates and no "unit areas" contained more than 6. (d) Types 5-8 were not observed in classes 5-8, nor was type 1 in classes 7 and 8.

A consideration of the intragroup arrangement of enzyme molecules is also important for understanding how the arrangement contributes to function. Examination of this feature in greater detail is not possible by current ferritin-conjugated IgG immunoelectron microscope techniques, because the ferritin molecules of the immunoreaction prod-

ucts appear at a distance from the corresponding antigen molecules. In this study, ferritin molecules present in a circumference of 250 Å were considered to be bound to one antigen, but three to five antigens were grouped in one unit area, which indicated that the enzyme molecules are present separately in the group at a finite distance from one another.

INTERMICROSOMAL DISTRIBUTION: As seen in Table II, about 21% of the total microsome sections did not react with ferritin-conjugated IgG. Such negative sections were found in each size, but were slightly more frequent in the smaller sections. Since the enzyme was shown to be present in groups of molecules, some negative sections could be expected. In addition, smooth endoplasmic reticulum is the major, but not the sole, constituent of the smooth microsome fraction thus prepared. Nonmicrosomal membranes such as plasma membrane fragments and Golgi vesicles are considered to be 10% of the total fraction (17). These membranes do not contain NADPH-cytochrome c reductase and, in most cases, are distinguishable from microsomal vesicles in electron micrographs. The negative sections observed in the experimental specimens are, therefore, partly due to the nonmicrosomal membranes which contaminate the specimens.

Thus, the present studies have demonstrated that (a) the intermicrosomal distribution of NADPH-cytochrome c reductase is homogeneous, (b) the intramicrosomal distribution is heterogeneous and the enzyme is present in groups of molecules on the outer surface of the microsomal vesicle, (c) each group consists of three to five enzyme molecules more than 250 Å apart, and (d) each microsome contains about 1.5 groups, on an average.

Franklin and Estabrook (1) have suggested the existence in rat liver microsomes of an electron transport system linking a single molecule of NADPH-cytochrome c reductase to a number of molecules of cytochrome P_{450} within a multicomponent complex. Yang (18) has recently proposed a nonpermanent association between the two enzymes. It could be considered that these enzymes may be present in groups similar to the assembly of cytochrome b_5 and NADH-cytochrome b_5 reductase (5). The present results support that possibility.

It could be argued that the grouping may be formed artificially by lateral diffusion of membrane protein under the present experimental conditions, because the immunoreaction was carried out at 25°C with divalent antibodies, although the conjugates used here are considered to be monovalent. To examine the above possibility, the immunoreaction was attempted at 4° and 25°C with and without glutaraldehyde fixation. However, in addition to the decrease in antigenicity with this modification, the extent of nonspecific binding could not be reduced to a level of one conjugate molecule per microsome. Similar studies are underway with cytochrome P_{450} and cytochrome b_5 as target proteins which are present in higher concentration than NADPH-cytochrome c reductase. The present results have shown that adjacent ferritin molecules in a group keep a distance of more than 250 Å from one another, suggesting that the enzyme molecules may not be grouped artificially by the action of divalent antibodies. If that were the case, the distance between molecules should be less.

Cytochrome b_5 (12, 15, unpublished data) and NADH-cytochrome b_5 reductase (15) were found to be present in all microsomal vesicles. As these enzymes are present in groups of 50 molecules (cytochrome b_5) and five molecules (NADH-cytochrome b_5 reductase) (5), respectively, they account for an average of 1.5 groups per microsome, as does NADPH-cytochrome c reductase. It is very interesting to note that the microsomal enzymes participating in electron transport may be present in groups, and that they are bound in a concentration of 1.5 groups per microsome. Studies on the intramicrosomal arrangement of these enzymes on smooth as well as rough microsomes are in progress.

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