Localization of Peroxidase and Other Microsomal Enzymes in Thyroid Cells*

Toichiro Hosoya,† Shigeru Matsukawa, and Yukifumi Nagai

ABSTRACT: With the aim of elucidating the site of peroxidase in thyroid follicles, a study was made of enzyme distribution in subcellular fractions of pig thyroid. It was found that the distribution pattern of peroxidase in the fractions was completely different from those of cytochrome oxidase, acid phosphatase, Na^+,K^+–ATPase, and 5′-nucleotidase, and the fractions which showed strong peroxidase activity were always accompanied by vesicles or fragments derived from rough-surfaced endoplasmic reticulum rather than from other cell organelles. These findings, together with evidence obtained histochemically, strongly support the view that the main site of peroxidase is membranes of rough-surfaced endoplasmic reticulum. It is possible that smooth-surfaced endoplasmic reticulum also carry the peroxidase, but it seemed likely that the plasma membranes or microvilli may not be a principal site of the enzyme. No evidence was obtained indicating that the peroxidase exists in follicular lumen. Among other microsomal enzymes, glucose 6-phosphatase, NADH–cytochrome *c* reductase, and NADPH–cytochrome *c* reductase were concentrated in rough microsomes and a hemoprotein, cytochrome b_5, also resembled peroxidase rather closely with respect to its distribution. The physiological significance of these is discussed in connection with the role of the peroxidase.

Recent biochemical studies have clearly established the presence of peroxidase (EC 1.11.1.7) in thyroid tissues of rats (Alexander, 1959; DeGroot and Dunn, 1964; Macho *et al.*, 1965), sheep (DeGroot and Davis, 1962b), pigs (Hosoya and Ui, 1961; Hosoya and Morrison, 1967b; Coval and Taurog, 1967), and cattle (Klebanoff *et al.*, 1962; DeGroot and Dunn, 1964; Yip, 1966; Mahoney and Igo, 1966; Ljunggren and Åkeson, 1968). There is now considerable evidence indicating that the peroxidase might play an essential role in thyroxine formation in the tissues by catalyzing such steps as oxidation of iodide and coupling of diiodotyrosine (for review and reference, see Rall *et al.*, 1964, Ljunggren, 1965, Hosoya, 1968, Blasi *et al.*, 1969, and Taurog, 1970). The site of the enzyme in thyroid follicles, however, has not yet been determined conclusively. Almost all investigations working on the tissue fractions agree that the enzyme is not in a soluble state but firmly bound to unidentified cellular organelles of cytoplasm. In our preliminary work, the enzyme was found predominantly in heavy microsomal fractions which consisted mainly of vesicles or fragments probably derived from rough-surfaced endoplasmic reticulum (Hosoya *et al.*, 1962; Hosoya, 1963).

As long as the enzyme is indispensable to iodination of thyroglobulin in the tissues, studies on the site of binding of iodine to the protein in thyroid follicles should have something to do with the problem of localization of peroxidase. Conflicting reports as to the site of iodination do, however, exist. A number of investigators working on electron microscopic autoradiography favored the view that the iodination does not occur in cells since newly iodinated proteins were located within the lumen and no significant amount of iodine was incorporated in the cells (Stein and Gross, 1964; Nadler and Leblond, 1955; Wollman and Wodinsky, 1955; Lupulescu and Petrovici, 1965; Loewenstein and Wollman, 1967; Fujita, 1969). However, other investigators reported that the grains clearly appeared in epithelial cells (Pitt-Rivers *et al.*, 1964; Schmidt *et al.*, 1965; Takano and Honjin, 1968; Tixier-Vidal *et al.*, 1969; Croft and Pitt-Rivers, 1970), especially Takano and Honjin, and Tixier-Vidal *et al.* suggesting that an important role is played by rough-surfaced endoplasmic reticulum. Meanwhile, the possibility of iodination on cell membranes (microvilli) was proposed on the basis of data obtained biochemically (Benabdeljlil *et al.*, 1967) and by autoradiography (Wollman, 1965). Biochemically, no iodinating activity for tyrosine or thyroglobulin was found in a soluble fraction, but was highest in microsomal fractions (Suzuki *et al.*, 1961; Kondo, 1961; Hosoya *et al.*, 1962; DeGroot and Davis, 1962a; DeGroot and Dunn, 1964; Nunez *et al.*, 1967).

The present investigation was initiated to look for the site of thyroid peroxidase which will shed light upon the abovementioned controversy concerning the site of iodination of thyroglobulin. The present paper deals with the results obtained by the biochemical method and those obtained with the cytochemical technique will be published elsewhere.

Another aim of the present investigation is to gain information about other microsomal enzymes and hemoproteins to understand the physiological role of the membranes of endoplasmic reticulum, because our knowledge in this field is still quite limited.

Experimental Section

Reagent grade chemicals were used whenever possible. The following substrates were used: cytochrome *c* from Sigma Chemical Co.; *p*-nitrophenyl phosphate and AMP from Seikagaku Kogyo Co.; NADH, NADPH, and ATP from Calbiochem. Sodium glucose 6-phosphate was prepared from barium glucose 6-phosphate obtained from NBC. Guaiacol obtained from Wako Junyaku Co. was redistilled under reduced pressure.

Fresh pig thyroids were homogenized and a cytoplasmic extract (E) derived from them in the manner described pre-

* From the Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Ishikawa Prefecture, Japan. *Received February 1, 1971.* A preliminary account of this work has been presented at the 42nd Meeting of the Japanese Biochemical Society, Hiroshima, Oct 1969 (*J. Jap. Biochem. Soc. 41*, 553 (1969)).

† To whom to address correspondence.

viously (Hosoya and Morrison, 1967a) except for the use of a medium. In the present studies, either of two mediums was employed throughout the preparation: medium A, 0.25 M sucrose containing 0.1 mM $MgCl_2$ and 20 mM Tris-HCl buffer, pH 7.4; or medium B, 0.25 M sucrose containing 100 mM KCl, 40 mM NaCl, 10 mM $MgCl_2$, and 20 mM Tris-HCl buffer, pH 7.6. The cytoplasmic extract was centrifuged at 250,000g·min to precipitate particulates which correspond to the sum of the "heavy mitochondrial fraction," M, and the "light mitochondrial fraction," L, of liver tissues (De Duve *et al.*, 1955). The precipitates were washed twice with the medium employed. All the supernatants were combined and centrifuged at 7,000,000g·min to give precipitates (microsomes) and soluble fraction (S). The mitochondria and microsomes were suspended in a small volume of the same medium to make fraction M + L and fraction P, respectively. Centrifugation was performed with a Hitachi Model 55P ultracentrifuge with rotor RP 30 (or rotor 55TA only in washing). All calculations of gravity force were based on the average radius of the rotors. All the steps of the fractionation as well as the subfractionation of the microsomes were carried out mostly at 0–2°.

Microsomes were subfractionated mainly by the two different methods (methods I and II). Method I is essentially the same as that of Imai (1965) with certain modifications. In this method, illustrated in Figure 1, 2 ml of the microsomal fraction (equivalent to 2–3 g wet tissues) was layered on top of 6 ml of 1.23 M sucrose solution which was previously placed on 2 ml of 2.1 M sucrose as a cushion in a 10-ml tube. After 15-hr centrifugation at 105,000g (Hitachi 55TA rotor, tube angle 26°), which resulted in the appearance of two milky bands at the interface of the discontinuous sucrose density and faint precipitates at the bottom, the content was divided into five fractions as shown in the figure. Usually three to six tubes were run at the same time. Corresponding fractions from each tube were combined and subjected to chemical analysis and enzyme assay.

Method II is a modification of that of Dallner (1963) which made use of CsCl. From pellets of microsomes, a suspension was prepared to contain 15 mM CsCl and 0.5% protein by adding 1 M CsCl (dissolved in 0.25 M sucrose) and 0.25 M sucrose solution. The suspension (6 ml) was layered on top of 4 ml of 1.3 M sucrose–15 mM CsCl. After centrifugation at 105,000g for 230 min (Hitachi 55TA rotor), a fluffy white layer at the interface, *i.e.*, smooth microsomes (S_m) and brown pellets at the bottom, *i.e.*, rough microsomes (R_o), were collected. The pellets of rough microsomes were homogenized in 0.25 M sucrose. In few cases, smooth microsomes were further fractionated into Mg^{2+}-binding and Mg^{2+}-free microsomes according to Dallner (1963). The smooth microsomal suspension obtained above was adjusted by the addition of distilled water and $MgCl_2$ solution to become 0.25 M in sucrose and 10 mM in $MgCl_2$. The suspension (6 ml) was layered on 4 ml of 1.15 M sucrose and centrifuged at 105,000g for 180 min. The resulting pellets, after being suspended in 0.25 M sucrose, were referred to as S_m-a and the fluffy layer at the interface was referred to as S_m-b.

The activity of peroxidase was measured using guaiacol as hydrogen donor as described previously (Hosoya and Morrison, 1967b) in an Hitachi Model 124 spectrophotometer with recorder attachment. Cytochrome oxidase (EC 1.9.3.1) was assayed by the method of Smith (1955) except that reduced cytochrome c was prepared by the addition of sodium dithionite followed by passage through Sephadex G-25 with distilled water. Acid phosphatase (EC 3.1.3.2) was assayed employing p-nitrophenyl phosphate as substrate (Axelrod, 1947). Tissue fractions were always preincubated at 0° for 15 min in the presence of 0.1% Triton X-100 (De Duve *et al.*, 1955). NADH–cytochrome c reductase (EC 1.6.2.1) or NADPH–cytochrome c reductase (EC 1.6.2.3) was measured as described by Ernster (1958). For the assays of Mg^{2+}–ATPase[1] (EC 3.6.1.3) and Na^+,K^+–ATPase (EC 3.6.1.3) the following two systems were run at the same time. The one contained 2 mM Tris-ATP, 5 mM $MgCl_2$, and 50 mM Tris-HCl buffer (pH 7.4), and the tissue fractions, in a final volume of 2 ml. The other contained, in addition to all the components described above, 0.1 M NaCl and 5 mM KCl, also in a final volume of 2 ml. Both were incubated at 37° for 20 min and 1 ml of 15% trichloroacetic acid added to stop the reaction. P_i liberated was measured by a modified Martin–Doty method (Lindberg and Ernster, 1956). The result of the first system represents the activity of Mg^{2+}–ATPase and the difference between the two systems reveals tat of Na^+,K^+–ATPase. Glucose 6-phosphatase (EC 3.1.3.9) and 5′-nucleotidase (EC 3.1.3.5) were assayed by the methods of Swanson (1955) and of Heppel and Hilmore (1955) using sodium glucose 6-phosphate and AMP as substrates, respectively. P_i liberated was determined as in the case of ATPases.

Appropriate blanks were determined in all these assays and subtracted from the observed values. The validity of the methods were verified in control experiments which showed that measured activity was proportional to both enzyme concentrations and time. The unit of enzymes except peroxidase and cytochrome oxidase was expressed in terms of micromoles of substrate converted per minute under the conditions specified. One unit of cytochrome oxidase was defined as the amount of enzyme causing the logarithms of the concentration of reduced cytochrome c to decrease by 1 unit/min per the reaction mixture (3 ml). The unit of peroxidase was expressed by GU as described previously (Hosoya *et al.*, 1962; Hosoya and Morrison, 1967b).[2]

Protein was determined by the method of Lowry *et al.* (1951). Extraction of phospholipid and RNA was performed by a modification of Schmidt and Thannhauser (1945). Lipid was extracted and treated as described by Folch *et al.* (1957) and phospholipid–phosphorous was determined according to Marinetti (1962). A factor of 25 was used to convert the weight of phosphorous into phospholipid. RNA was estimated according to Mejbaum's orcinol method (1939). Ribose was used as the standard and a factor of 3.76 was employed to calculate the amount of RNA (Dallner, 1963). Cytochrome b_5 was measured as described previously (Hosoya and Morrison, 1967a) in an Hitachi Model 356 dual-wavelength spectrophotometer.

For electron microscopical examination of microsomal subfractions an aliquot of the suspension (protein concentration 5–10 mg/ml) was fixed at 2° for 90 min by the addition of an equal volume of cold 2% OsO_4 in 0.25 M sucrose containing 0.1 M sodium phosphate buffer (pH 7.4). After centrifugation at 20,000 rpm for 20 min, compact pellets produced were dehydrated in graded ethanol, embedded in Epon 812, and sectioned on a Porter-Blum microtome. The sections were

[1] Abbreviations used are: Mg^{2+}–ATPase, Mg^{2+}-stimulated adenosine triphosphatase; Na^+,K^+–ATPase, Mg^{2+},Na^+,K^+-stimulated adenosine triphosphatase.

[2] When a calculation is made on the basis of the molar extinction coefficient of oxidized guaiacol at 470 mμ, 5.57 × 10^3 cm^{-1} (Hosoya, 1960a,b), it is found that the peroxidase with 1 GU oxidizes 32.5 μmoles of guaiacol per min under the conditions specified.

TABLE I: Distribution of Enzymes in Fractions Obtained by Differential Centrifugation.[a]

Protein and Enzymes	Absolute Values of E^b	Percentage Values[c]				Absolute Values of N[d]
		M + L	P	S	Recov	
Protein	98.4	8.7	4.9	83.6	96.9	ND[e]
Peroxidase	0.0704	42.0	44.0	1.6	87.6	ND
Cytochrome oxidase	15.5	132.6	3.2	0.7	136.5	ND
Acid phosphatase	2.99	47.2	8.7	33.2	89.1	ND
5′-Nucleotidase	0.372	49.3	27.0	13.7	96.0	0.065
Mg^{2+}–ATPase	0.139	155.5	100.5	0	256.0	0.030
Na^+,K^+–ATPase	0.213	263.1	23.4	0	286.5	0.027

[a] The homogenization and differential centrifugation were performed as described in Experimental Section employing medium A. The number of experiments was 9 for protein, and, for enzymes, 9, 7, 9, 3, 2, and 2 from top (peroxidase) to bottom (Na^+,K^+–ATPase), and the mean values are presented. [b] Absolute values of E and N are expressed in milligrams per gram wet tissues for protein, and in units per gram wet tissues for enzymes. [c] The activity of E was taken as 100. [d] Fraction N was obtained as described by De Duve *et al.* (1955). [e] Not determined.

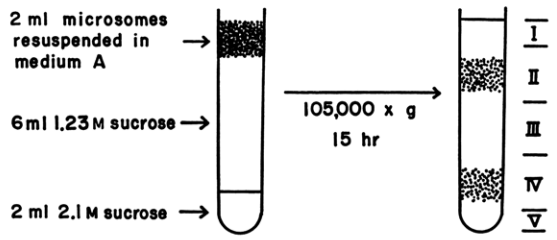

FIGURE 1: Schematic representation of a procedure for subfractionation of thyroid microsomes, method I.

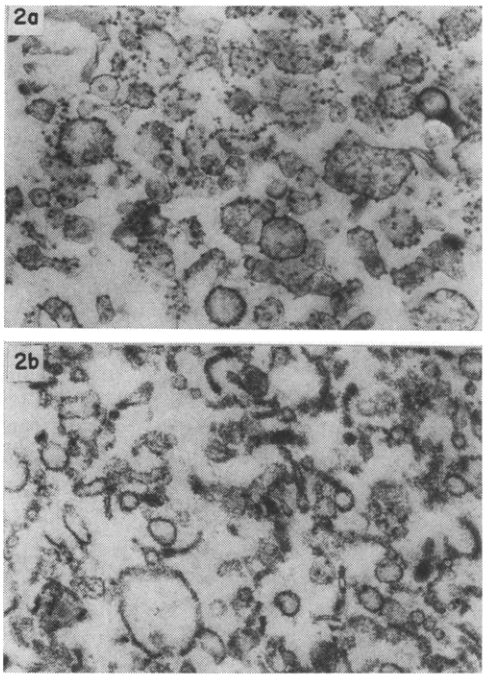

FIGURE 2: Electron micrograph of submicrosomal fractions prepared with method I, fraction IV (Figure 2a) and fraction II (Figure 2b). ×25,000.

stained with uranyl acetate and lead citrate and examined in an Hitachi HU 11P electron microscope.

Results

Table I shows the distribution of enzymes and protein in subcellular fractions obtained employing medium A. The assay of peroxidase, cytochrome oxidase, and acid phosphatase in the nuclear fraction was omitted because of their weak activity. The activity of cytochrome oxidase and acid phosphatase contained in fraction P represented only about 2.5 and 10% of that in cytoplasmic extract (E), indicating that contamination of mitochondria and lysosomes in fraction P was not serious. Total and specific activity of peroxidase was greatest in fraction P. This confirms the previous view (Hosoya *et al.*, 1962; Hosoya, 1963) that peroxidase is not a mitochondrial or lysosomal enzyme, but a microsomal enzyme.[3] Fraction M + L possessed almost an amount of peroxidase equal to that of fraction P, but this may be explained by the fact that a considerable amount of microsomes contaminated the fraction, as will be described later. Since microsomes contain small vesicles derived from various part of cellular organelles, although most of them are fragments of endoplasmic reticulum (Palade and Siekevitz, 1956a), an attempt has been made to fractionate further them to find peroxidase-bound constituents. First, microsomes were separated into five subfractions (fractions I–V) by method I (Figure 1). Figure 2 shows typical electron micrographs of fractions IV and II. Inspection of these figures shows that fraction IV (Figure 2a) consists mainly of vesicles or membrane fragments with electron-dense particles (10–15 mμ in diameter), whereas fraction II (Figure 2b) is composed mostly of smooth-surfaced vesicles. From comparison to the microsomes of liver and pancreas (Palade and Siekevitz, 1956a,b), it is evident that main components of fraction IV are derived from endoplasmic reticulum, the small electron-dense particles being ribosomes. Fraction IV contained very few dense body (probably lysosomes) and smooth-surfaced vesicles but practi-

[3] Peroxidase also does not seem to be an enzyme of peroxisomes because the activity of catalase and aliphatic α-hydroxy acid oxidase was not found in fraction P. The assay of the latter enzyme was performed by Dr. Minoru Nakano using the procedure he employed (Nakano *et al.*, 1968), to whom the authors' thanks are due.

TABLE II: Amounts of Protein, Phospholipid, and RNA in a Microsomal Fraction (Fraction P) and Their Distribution in Submicrosomal Fractions Prepared by Method I.[a]

	Absolute Values in Fraction P[b]	Percentage Values[c]					
		I	II	III	IV	V	Recov
Protein	4.99	2.7	38.4	20.4	30.7	6.7	98.6
Phospholipid	0.92	4.9	52.4	14.1	24.5	1.5	97.3
RNA	0.52	5.6	10.0	9.2	50.5	20.8	95.2

[a] The procedure for preparation of fraction P and its subfractions is described in Experimental Section. The volume of the subfractions varied somewhat from preparation to preparation, but usually 1.4, 2.5, 1.8, 2.6, and 0.9 ml per one centrifuge tube, for fractions I, II, III, IV, and V, respectively. The number of experiments was 10, 9, and 5 for protein, phospholipid, and RNA, respectively. Mean values are presented. [b] Absolute values are given in mg in an aliquot of fraction P which is equivalent to 1 g wet tissues. [c] Amounts of protein, phospholipid, and RNA in an aliquot of fraction P employed for the subfractionation (about 2–3 g wet tissue equivalent for each tube) were taken as 100.

TABLE III: Total and Specific Activity of Enzymes in a Microsomal Fraction (Fraction P).[a]

Enzymes	Total Act.[b]	Protein-Based Sp Act.[c]	Phospholipid-Based Sp Act.[d]
Peroxidase	0.0328	9.16	26.2
Cytochrome b_5[e]	0.334	70.1	406
NADH–cytochrome *c* reductase	0.686	176	852
NADPH–cytochrome *c* reductase	0.130	36.0	164
Glucose 6-phosphatase	0.008	2.52	11.0
5′-Nucleotidase	0.094	17.8	178
Mg^{2+}–ATPase	0.179	40.3	218
Na^+,K^+–ATPase	0.119	24.6	122

[a] Fraction P was obtained as described in the legend of Table I. The number of experiments was 7, 5, 9, 5, 4, 7, 8, and 7 from top to bottom. Mean values are presented. [b] Expressed in units contained in an aliquot of fraction P equivalent to 1 g wet tissues. [c] Expressed in milliunits per milligram of protein. [d] Expressed in milliunits per milligram of phospholipid. [e] Expressed in terms of micromoles in the column of total activity and in terms of millimicromoles in the columns of specific activity.

cally no mitochondria. Smooth-surfaced vesicles in fraction II vary in size and shape. Tubular formations resemble those derived from the Golgi apparatus of liver (Fleischer *et al.*, 1969). Fraction II contained also ribosome-studded membranes as minor components.

The results of the electron microscopic observations coincide closely with the data obtained by chemical analysis of these subfractions (Table II). Fraction II possessed more than 50% of phospholipid of the original sample, whereas RNA was concentrated in fraction IV. RNA in fraction V is due to free ribosomes.

The activity of the enzymes and also the amount of cytochrome b_5 in fraction P is shown in Table III. Distribution of these enzymes and cytochrome b_5 in the five submicrosomal fractions was determined, but only the ratio of total activity and specific activity of fraction IV to those of fraction II are set out here (Table IV) because a great part of the activity (about 70–80%) was confined to these two fractions and information about the ratio is most important in this work. In these tables, the values of specific activity are presented not only on a protein basis but also on a phospholipid basis. Strictly speaking, the phospholipid-based specific activity represents rather the distribution of enzymes in the membrane portion of microsomes by eliminating the effect due to proteins of ribosomes as discussed by Chauveau *et al.* (1962) and Imai *et al.* (1966), and enzymes are presented in decreasing order of the ratio of phospholipid-based specific activity.

Table V shows the results obtained by another method of subfractionation, method II, which is based on Dallner's finding that the density of ribosome-studded membranes increases in the presence of CsCl. The microsomal fraction used in the experiments was prepared employing medium B as described below. The values of ratio of fraction R_o (rough microsomes) to fraction S_m (smooth microsomes) with respect of both total and specific activity are presented and arranged in the same manner as in Table IV.

These tables show that the ratio with respect to specific activity of peroxidase is always two to three times of that of Na^+,K^+–ATPase and similar to that of cytochrome b_5 although the values shown in the two tables are somewhat different from each other. The difference may be ascribed mostly to the difference of preparation of microsomes. The preparation of microsomes used in Table V was obtained from experi-

TABLE IV: Comparison of Total and Specific Activity of Fraction IV with That of Fraction II.[a]

Enzymes	Ratio of Fraction IV to Fraction II with Respect to: Total Act.	Protein-Based Sp Act.	Phospholipid-Based Sp Act.
Glucose 6-phosphatase	1.25	2.40	8.72
NADPH–cytochrome *c* reductase	1.16	2.06	5.30
NADH–cytochrome *c* reductase	1.23	1.18	4.18
Peroxidase	0.84	1.31	2.78
Cytochrome b_5[b]	2.04	1.65	2.69
Mg^{2+}–ATPase	0.49	0.42	0.97
Na^+,K^+–ATPase	0.37	0.54	0.92
5′-Nucleotidase	0.34	0.42	0.70

[a] Fractions IV and II were obtained as described in the legend of Table II. Mean values are presented. (The number of experiments is the same as in Table III.) [b] Total and specific content (in terms of micromoles) was taken instead of total and specific activity.

TABLE V: Comparison of Total and Specific Activity of Fraction R_o with That of Fraction S_m.[a]

Enzymes	Ratio of Fraction R_o to Fraction S_m with Respect to: Total Act.	Protein-Based Sp Act.	Phospholipid-Based Sp Act.
Cytochrome b_5[b]	3.55	4.72	1.95
Peroxidase	2.71	3.92	1.71
NADH–cytochrome *c* reductase	2.40	3.41	1.42
Na^+,K^+–ATPase	1.04	1.50	0.66
5′-Nucleotidase	0.91	1.23	0.53
Mg^{2+}–ATPase	0.82	0.88	0.50

[a] Fraction R_o and fraction S_m were obtained from fraction P which was prepared employing medium B as described in the Experimental Section. Mean values of three experiments are presented. [b] See the legend of Table IV.

TABLE VI: Distribution of Enzymes in Subfractions of Smooth Microsomes (Fraction S_m).[a]

Protein, Phospholipid, and Enzymes	Percentage Values[b]: Fraction S_m-a	Fraction S_m-b	Ratio of Fraction S_m-a to Fraction S_m-b
Protein	21.4	23.6	0.91
Phospholipid	38.7	16.3	2.38
Cytochrome b_5	69.9	4.7	14.7
Peroxidase	60.8	16.5	3.68
Na^+,K^+–ATPase	50.3	40.3	1.25
5′-Nucleotidase	33.6	41.9	0.80

[a] The procedure for preparation of smooth microsomes, fraction S_m, and its subfractions, fraction S_m-a and fraction S_m-b, is described in the Experimental Section. Mean values of two experiments are presented. [b] The values of fraction S_m added to each centrifuge tube was taken as 100.

ments of enzyme distribution employing medium B instead of medium A. The distribution pattern did not differ greatly from the previous one, but the fraction P recovered more peroxidase with concomitant increase of acid phosphatase activity. In fact, electron microscopic examination of the fraction P and its subfractions, fraction R_o and fraction S_m, showed that fraction P, although it also consisted mainly of rough and smooth microsomes, contained more membranous materials like dense body and mitochondria, which were found after all in larger quantities in fraction R_o than in fraction S_m. The distribution of protein and phospholipid in fractions R_o and S_m showed an inverse relationship to the results of Table II, the R_o/S_m ratio being 0.72 and 1.76, respectively. These circumstances may account for the ratio of phospholipid-based specific activity becoming lower than that of protein-based specific activity in Table V. As for the total activity, the R_o/S_m ratio of peroxidase was as high as 2.7, indicating superiority of rough microsomes to smooth microsomes in quantity also. The IV/II ratio of total activity of peroxidase, on the contrary, was below 1 (Table IV), but this may be related to the fact that fraction M + L possessed an almost equal amount of peroxidase (Table I) and that fraction M + L is contaminated with rough microsomes.

Both tables (Tables IV and V) agree inasmuch as they indicate that other microsomal enzymes, *i.e.*, NADPH–cytochrome *c* reductase, NADH–cytochrome *c* reductase, and glucose 6-phosphatase are definitely rich in rough microsomes.

The smooth microsomes (fraction S_m) was further fractionated in the presence of Mg^{2+} into two components: Mg^{2+}-binding vesicles (S_m-a) and Mg^{2+}-free vesicles (S_m-b). According to Dallner (1963), the former originates in endoplasmic reticulum whereas the latter is not related to it in the case of liver. The results of the present experiments (Table VI) indicated that cytochrome b_5 and, to a lesser degree, peroxidase were definitely concentrated in S_m-a while Na^+,K^+–ATPase and 5′-nucleotidase were distributed in both subfractions.

The activity of Na^+,K^+–ATPase in the samples used in the experiments described so far represented only a small portion of the total activity in the tissues. Since the majority of the activity appeared in fraction M + L (Table I), it was examined, employing a mitochondrial fraction, to see whether Na^+,K^+–ATPase-associated vesicles reveal peroxidase activity or not (Table VII). In this series of experiments, most of peroxidase-binding vesicles settled to the bottom through the layer of 1.20 M sucrose to form a sediment, whereas most of Na^+,K^+–ATPase activity appeared at the interface between the two layers. Accordingly, this indicates again that there is a difference in density between Na^+,K^+–ATPase-binding vesicles and peroxidase-binding vesicles in the fraction, the latter's density being greater than $d = 1.16$.[4]

Discussion

The present investigation clearly indicates that thyroid peroxidase is not involved in such cell organelles as mitochondria, lysosomes and microbody but bound to smaller vesicles contained in microsomal fractions. Subfractionation of microsomes disclosed that both total and specific activity were high in rough microsomes rather than in smooth microsomes. The former consisted mainly of vesicles or fragments derived from rough-surfaced endoplasmic reticulum when examined with the electron microscope. The study on sucrose density gradient centrifugation also revealed that peroxidase is associated with vesicles whose density is similar to rough-surfaced endoplasmic reticulum of liver. These findings, together with the previous one that ribosomes do not possess peroxidase activity (Hosoya *et al.*, 1962), strongly support the view that the main site of peroxidase is the membranous part of rough-surfaced endoplasmic reticulum. This is in accord with our electron

[4] This suggests also that the contaminating microsomes in fraction M + L are not smooth but mostly rough microsomes. It is clear from the results of Table IV that the density of rough microsomes is greater than $d = 1.16$, but it may be more pertinent to note the results of a sucrose 1.0–1.8 M linear density gradient centrifugation (75,000*g*, 36 hr) which brought about two bands of peroxidase activity, the peak of the first band being high and at $d = 1.20$ and the other being low and at $d = 1.14$. In the case of liver, the values of 1.22–1.27 and 1.06–1.18 were reported for rough- and smooth-surfaced endoplasmic reticulum, respectively (Rothschild, 1963).

microscopic observation on histochemically stained pig thyroid slices which found dense reaction products mainly in the area of endoplasmic reticulum[5] (N. Sasaki and T. Hosoya, to be published).

It is unlikely, however, that the site of peroxidase is confined only to rough-surfaced endoplasmic reticulum. Smooth microsomes had a considerable amount of peroxidase activity, although the specific activity was low, which was too much to be ascribed to such small amount of contaminating rough microsomes. When the smooth microsomes were further separated into two fractions in the presence of Mg^{2+}, most of the peroxidase activity was bound to Mg^{2+}-binding smooth vesicles which were thought to be related to endoplasmic reticulum. Because of the continuity of membranes of rough- and smooth-surfaced endoplasmic reticulum (Palade and Siekevitz, 1956a), it may be permissible to consider that some of the activity in smooth microsomes is due to peroxidase associated to membranes of smooth-surfaced endoplasmic reticulum. Whether Golgi-like fragments in the fraction carry peroxidase or not was not determined in the present study, but clear reaction products have not been obtained histochemically so far in the Golgi apparatus although sometimes outermembranes of Golgi lamellae were stained.

The microsomal fraction may contain some vesicles derived from plasma membranes in view of Na^+,K^+-ATPase activity found in the fraction (Emmelot *et al.*, 1964; Emmelot and Bos, 1966). However, the activity in the microsomal fraction represented only a small portion of total activity in thyroid (Table I) and its distribution patterns in subfractions and density of the vesicles were very different from those of peroxidase (Tables IV–VII). Moreover, plasma membranes purified from a mitochondrial fraction revealed practically no peroxidase activity, having high activity of Na^+,K^+-ATPase (unpublished data). These findings do not favor the view that peroxidase exists in plasma membranes, although we do not deny the possibility altogether. It may be said at least that plasma membranes are not the main site of the enzyme.

Peroxidase is firmly bound to membranes of endoplasmic reticulum, splitting of which requires such treatment as digestion by trypsin (Hosoya and Morrison, 1967b; Taurog, 1970), and does not seem to be an enzyme being secreted into colloid but to be a constituent of the membranes. Therefore it is possible that the peroxidase standing ready for on the membranes takes a role *in situ* in iodination of thyroglobulin which is moving from polysomes to the Golgi apparatus through the lumen of endoplasmic reticulum. This is in accord with the observation of grains of radioactive iodine in the endoplasmic reticulum region (Takano and Honjin, 1968; Tixier-Vidal *et al.*, 1969). The so-called "ring structure" found by means of autoradiography would be, at a glance, against the view, but the discrepancy would be reconciled if it is true that transportation and secretion of iodinated thyroglobulin to colloid lumen is as rapid a process as suggested by the results of involving pulse labeling of [^{3}H]leucine (Cheftel *et al.*, 1968) and in a recent autoradiographic observation on incorporation of radioiodine (Croft and Pitt-Rivers, 1970). Alternatively, owing to a great quantity of thyroglobulin in the colloid lumen in contrast to a very small amount of the protein in the lumen of endoplasmic reticulum, incorporation of radioiodine into thyroglobulin may become marked on the cell colloid interface during the early phase even if only a small amount of peroxidase is located on the cell surface or near the cell surface inside the cell.

[5] Recently it has been also reported by Nakai and Fujita (1970) and Strum and Karnovsky (1970) that the region of endoplasmic reticulum of rat thyroid was stained histochemically.

TABLE VII: Distribution of Enzymes in Subfractions Obtained by Sucrose Density Gradient Centrifugation from a Mitochondrial Fraction.[a]

Protein and Enzymes	Percentage Values[b]		Ratio of Fraction A to Fraction B
	Fraction A	Fraction B	
Protein	52.1	14.0	3.72
Cytochrome oxidase	118.3	1.8	65.5
Peroxidase	116.7	13.3	8.75
Na^+,K^+-ATPase	14.6	79.0	0.19

[a] The mitochondrial fraction used in this experiment was a suspension of precipitates obtained by centrifugation (6000*g*, 10 min) from the cytoplasmic extract, E. The suspension (5 ml, 6 mg/ml) was layered over 10 ml of 0.32 M sucrose placed on 20 ml of 1.2 M sucrose in a 38.5-ml Spinco tube. After centrifugation at 25,000 rpm (81,500*g*) for 1 hr in a Spinco SW 27 rotor, a pellet at the bottom (fraction A) and a milky band at the interface between the 0.32 and 1.2 M sucrose (fraction B) were collected. Mean values of two experiments are presented. [b] The values in the mitochondrial suspension added in each centrifuge tube were taken as 100.

NADH–cytochrome *c* reductase, NADPH–cytochrome *c* reductase, and glucose 6-phosphatase are generally considered to be microsomal enzymes (Siekevitz, 1963; De Duve *et al.*, 1962), and therefore, were surveyed as to their content in the microsomal fraction of thyroid and their distribution in submicrosomal fractions. The finding that NADPH–cytochrome *c* reductase was distributed much more in rough microsomes than in smooth microsomes is of interest in view of the possibility of its participation in hydrogen peroxide generation in the tissues on the basis of the following facts. (1) Iodination reaction by thyroid preparation was enhanced when suppled with NADPH (Schussler and Ingbar, 1961; DeGroot and Davis, 1961). (2) NADPH–cytochrome *c* reductase of liver microsomes was found to be identical with its NADPH oxidase with FAD as the prosthetic group and to be able to produce hydrogen peroxide through aerobic oxidation of NADPH (Nishibayashi-Yamashita and Sato, 1969). NADH–cytochrome *c* reductase may work in a similar fashion, but the physiological meaning of glucose 6-phosphatase is obscure. The role of cytochrome b_5 is also unknown, but it is worthwhile to note that the distribution rather resembled that of peroxidase. It is possible to suppose its role in collaboration with peroxidase, for example, cytochrome b_5 may participate in regulating generation of hydrogen peroxide in view of the fact that above-mentioned NADPH oxidase catalyzes also NADPH-linked reduction of cytochrome b_5 even though the rate is low (about 10% of that of cytochrome *c* in the case of liver).

Acknowledgments

The authors are exceedingly grateful to Professor R. Honjin (School of Medicine, Kanazawa University) and Professor Y. Kurata (Cancer Institute, Kanazawa University) for help-

ing in the electron microscopy part of this work. Thanks are also due to Misses S. Mizukami, M. Shintaku, M. Nagaoka, and N. Sasaki for their technical assistance.

Added in Proof

The assay procedure employed for acid phosphatase was actually a modification of Axelrod's (Axelrod, 1947). The assay system contained 0.1% *p*-nitrophenyl phosphate–0.05 M acetate buffer (pH 5.0), and a suitable amount of the tissue fraction, in a final volume of 1.0 ml. After incubation at 37° for 20 min, the reaction was stopped by the addition of 4 ml of 0.2 N NaOH, and the absorbance of the mixture at 400 mμ was measured. The amount of *p*-nitrophenol liberated was calculated using a molar extinction coefficient of 1.80×10^4 cm^{-1}.

References

Alexander, N. M. (1959), *J. Biol. Chem. 234*, 1530.

Axelrod, B. (1947), *J. Biol. Chem. 167*, 57.

Benabdeljlil, C., Michel-Bechet, M., and Lissitzky, S. (1967), *Biochem. Biophys. Res. Commun. 27*, 74.

Blasi, F., Fragomele, F., and Covelli, I. (1969), *Endocrinology 85*, 542.

Chauveau, J., Moulé, Y. and Rouiller, C., and Schneebell, J. (1962), *J. Cell Biol. 12*, 17.

Cheftel, C., Bouchilloux, S., and Chabaud, O. (1968), *Biochim. Biophys. Acta 170*, 29.

Coval, M. L., and Taurog, A. (1967), *J. Biol. Chem. 242*, 5510.

Croft, C. J., and Pitt-Rivers, R. (1970), *Biochem. J. 118*, 311.

Dallner, G. (1963), *Acta Pathol. Microbiol. Scand., Suppl.* 166.

De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955), *Biochem. J. 60*, 604.

De Duve, C., Wattiaux, R., and Baudhuin, P. (1962), *Advan. Enzymol. 24*, 291.

DeGroot, L. J., and Davis, A. M. (1961), *J. Biol. Chem. 236*, 2009.

DeGroot, L. J., and Davis, A. M. (1962a), *Endocrinology 70*, 492.

DeGroot, L. J., and Davis, A. M. (1962b), *Endocrinology 70*, 505.

DeGroot, L. J., and Dunn, A. D. (1964), *Biochim. Biophys. Acta 92*, 205.

Emmelot, P., and Bos, C. J. (1966), *Biochim. Biophys. Acta 120*, 369.

Emmelot, P., Bos, C. J., Benedetti, E. L., and Rümke, Ph. (1964), *Biochim. Biophys. Acta 90*, 126.

Ernster, L. (1958), *Acta Chem. Scand. 12*, 600.

Fleischer, B., Fleischer, S., and Ozawa, H. (1969), *J. Cell Biol. 43*, 59.

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Fujita, H. (1969), *Virchows Arch. B 2*, 265.

Heppel, L. A., and Hilmore, R. J. (1955), *Methods Enzymol. 2*, 546.

Hosoya, T. (1960a), *J. Biochem. (Tokyo) 47*, 794.

Hosoya, T. (1960b), *J. Biochem. (Tokyo) 48*, 178.

Hosoya, T. (1963), *J. Biochem. (Tokyo) 53*, 86.

Hosoya, T. (1968), *Gunma Symp. Endocrinol. 5*, 219.

Hosoya, T., Kondo, Y., and Ui, N. (1962), *J. Biochem. (Tokyo) 52*, 180.

Hosoya, T., and Morrison, M. (1967a), *Biochemistry 6*, 1021.

Hosoya, T., and Morrison, M. (1967b), *J. Biol. Chem. 242*, 2828.

Hosoya, T., and Ui, N. (1961), *Nature (London) 192*, 659.

Imai, Y. (1965), *Tampakushitsu Kakusan Koso 10*, 170.

Imai, Y., Ito, A., and Sato, R. (1966), *J. Biochem. (Tokyo) 60*, 417.

Klebanoff, S. J., Yip, C., and Kessler, D. (1962), *Biochim. Biophys. Acta 58*, 563.

Kondo, Y. (1961), *J. Biochem. (Tokyo) 50*, 210.

Lindberg, O., and Ernster, L. (1956), *Methods Biochem. Anal. 3*, 1.

Ljunggren, J.-G. (1965), *Biochim. Biophys. Acta 107*, 434.

Ljunggren, J.-G., and Åkeson, Å. (1968), *Arch. Biochem. Biophys. 127*, 346.

Loewenstein, J. E., and Wollman, S. H. (1967), *Endocrinology 81*, 1074.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lupulescu, A., and Petrovici, A. (1965), *Curr. Top. Thyroid Res. Proc. Int. Thyroid Conf., 5th*, 85.

Macho, L., Poór, J., Palkovič, M., and Mitro, A. (1965), *Curr. Top. Thyroid Res. Proc. Int. Thyroid Conf., 5th*, 55.

Mahoney, C. P., and Igo, R. P. (1966), *Biochim. Biophys. Acta 113*, 507,

Marinetti, G. V. (1962), *J. Lipid Res. 3*, 1.

Mejbaum, W. (1939), *Hoppe Seyler's Z. Physiol. Chem. 258*, 117.

Nadler, N. J., and Leblond, C. P. (1955), *Brookhaven Symp. Biol. 7*, 40.

Nakai, N., and Fujita, H. (1970), *Z. Zellforsch. 107*, 104.

Nakano, M., Ushijima, Y., Saga, M., Tsutsumi, Y., and Asami, H. (1968), *Biochim. Biophys. Acta 167*, 9.

Nishibayashi-Yamashita, H., and Sato, R. (1970), *J. Biochem. (Tokyo) 67*, 199.

Nunez, J., Mauchamp, A. J., Jerusalmi, A., and Roche, J. (1967), *Biochim. Biophys. Acta 145*, 127.

Palade, G. E., and Siekevitz, P. (1956a), *J. Biophys. Biochem. Cytol. 2*, 171.

Palade, G. E., and Siekevitz, P. (1956b), *J. Biophys. Biochem. Cytol. 2*, 671.

Pitt-Rivers, R., Niven, J. S. F., and Young, M. R. (1964), *Biochem. J. 90*, 205.

Rall, J. E., Robbins, J., and Lewallen, C. G. (1964), *in* The Hormones, Vol. V, Pincus, G., Thimann, K. V., and Astwood, E. B., Ed., New York, N. Y., Academic Press, p 159.

Rothschild, J. A. (1963), *Biochem. Soc. Symp. 22*, 4.

Schmidt, H. J., Hennig, N., Scheiffarth, F., Witte, S., Wolf, F., Kleyensteiber, G., and Zicha, L. (1965), *Curr. Top. Thyroid Res. Proc. Int. Thyroid Conf., 5th*, 95.

Schmidt, G., and Thannhauser, S. J. (1945), *J. Biol. Chem. 161*, 83.

Schussler, G. C., and Ingbar, S. H. (1961), *J. Clin. Invest. 40*, 1394.

Siekevitz, P. (1963), *Annu. Rev. Physiol. 16*, 15.

Smith, L. (1955), *Methods Biochem. Anal. 2*, 427.

Stein, O., and Gross, J. (1964), *Endocrinology 75*, 787.

Strum, J. M., and Karnovsky, M. J. (1970), *J. Cell Biol. 44*, 655.

Suzuki, M., Nagashima, M., and Yamamoto, K. (1961), *Gen. Comp. Endocrinol. 1*, 103.

Swanson, M. A. (1955), *Methods Enzymol. 2*, 541.

Takano, I., and Honjin, R. (1968), *Okajimas Folia Anat. Jap. 44*, 173.

Taurog, A. (1970), *Recent Progr. Horm. Res. 26*, 189.

Tixier-Vidal, A., Picart, R., Rapaport, L., and Nunez, J. (1969), *J. Ultrastructure Res. 28*, 78.

Wollman, S. H. (1965), *Curr. Top. Thyroid Res. Proc. Int. Thyroid Conf., 5th*, 1.

Wollman, S. H., and Wodinsky, I. (1955), *Endocrinology 56*, 9.

Yip, C. (1966), *Biochim. Biophys. Acta 128*, 262.

Phototransformation of 4-Thiouridine in *Escherichia coli* Valine Transfer Ribonucleic Acid to Uridine, Cytidine, and N^4-Methylcytidine*

M. G. Pleiss† and P. A. Cerutti‡

ABSTRACT: The minor nucleoside 4-thiouridine in *Escherichia coli* tRNA^{Val} is transformed selectively to uridine upon irradiation of cetyltrimethylammonium salt of tRNA^{Val} in *tert*-butanol with light of wavelengths longer than 300 mμ. The four major nucleosides and the minor nucleosides pseudouridine, 1-methyladenosine, N^6-isopentenyladenosine, 5,6-dihydrouridine, 2-thiomethyl-N^6-isopentenyladenosine, and glucosyl-2-thiouracil (used as a model for 2-thiouridine), were found to be inert under these conditions. In a *tert*-butanol solution containing methylamine or ammonia, 4-thiouridine in tRNA^{Val} is converted into a mixture of N^4-methylcytidine and uridine or cytidine and uridine, respectively. At 93% total conversion of 4-thiouridine in tRNA^{Val} in the presence of 0.57 M methylamine, 54% uridine and 46% N^4-methylcytidine had been formed; at 92% total conversion in the presence of 0.72 M ammonia, 69% uridine and 31% cytidine had been formed.

The ratio of N^4-methylcytidine and cytidine over uridine formed increases with increasing amine concentrations. Uridine formation, however, cannot be suppressed even at high amine concentrations. Transformation of 90% of the original 4-thiouridine in tRNA^{Val} to uridine led to a 19% reduction in the valine acceptance. Retention of a substantial portion of the aminoacylation activity suggests that 4-thiouridine *per se* is not required as a specific recognition site for valyl-tRNA synthetase.

While the primary structures of numerous tRNAs have been elucidated and the major biological functions of tRNA understood, surprisingly little is known about the relation between the two. To be able to carry out its multifaceted functions as the translator molecule in protein synthesis considerable information and specificity must be built into the structure (primary, secondary, and tertiary) of each individual tRNA molecule, *i.e.*, ultimately into the sequence of 75–85 nucleotide residues. A highly characteristic feature of tRNA sequences is the presence of a variety of simple derivatives of the major nucleotides, the rare or minor nucleotides. Almost any derivation of a major nucleotide will result in some change of its base-pairing and -stacking properties. A large number of subtle structural variations become possible by changing the distribution, number, and kind of minor components in the nucleotide sequence of a tRNA. Such a "fine structure" may be needed for the specific interaction of tRNA with enzymes, ribosomes, and mRNA, for the regulation of protein synthesis or for functions of tRNA which are not yet appreciated.

One such minor component, 4-thiouridine, has been identified in purified, unfractionated tRNA from *Escherichia coli* (Lipsett, 1965). Subsequent sequencing studies on several highly purified, fractionated tRNAs from *E. coli* have demonstrated that this nucleoside occupies the eighth nucleoside site removed from the 5′-terminal end (Zachau, 1969). A number of chemical procedures for the modification of 4-thiouridine have been developed. Transformation of 4-thiouridine to uridine or, in the presence of added nucleophiles such as ammonia or methylamine, to uridine plus cytidine derivatives has been accomplished with $KMnO_4$, $NaIO_4$, and OsO_4 (Hayatsu and Ukita, 1967; Hayatsu and Yano, 1969; Ziff and Fresco, 1968; Burton, 1970). The usefulness of these reactions for the modification of 4-thiouridine in tRNA is limited since the 3′-terminal ribose and possibly other components are also altered under these conditions. Iodine oxidation of 4-thiouridine to disulfides (Carbon *et al.*, 1965; Lipsett, 1966; Lipsett and Doctor, 1967) and sodium borohydride reduction of 4-thiouridine have also been investigated (Cerutti *et al.*, 1968).

More selective approaches to the study of the functional role of 4-thiouridine have emerged recently. Modification of 4-thiouridine with cyanogen bromide has been demonstrated to yield uridine as the sole transformation product (Walker and RajBhandary, 1970; Saneyoshi and Nishimura, 1970). *N*-Ethylmaleimide has also been shown to react selectively and quantitatively with 4-thiouridine in tRNA. tRNA species partially deficient in 4-thiouridine have been investigated in an attempt to implicate the nucleoside as essential for tRNA synthetase recognition (Johnson *et al.*, 1970; Kaiser, 1969), while the biological properties of mutant tRNA^{Tyr} have been studied (Abelson *et al.*, 1970; Smith *et al.*, 1970). A method for the selective photochemical transformation of 4-thiouridine in *E. coli* tRNA to uridine or uridine plus cytidine (or N^4-methylcytidine) has been developed in our laboratory and

* From the Department of Biochemical Sciences, Frick Chemical Laboratory, Princeton University, Princeton, New Jersey 08540. *Received March 26, 1971.* This work was supported by Grant GM-14090 from the National Institutes of Health, and an unrestricted grant from Hoffmann-La Roche, Inc., Nutley, N. J.

† A National Institutes of Health postdoctoral fellow.

‡ Present address: Department of Biochemistry, College of Medicine, University of Florida, Gainesville, Fla. 32601.