A Study of the Hemoproteins of Thyroid Microsomes with Emphasis on the Thyroid Peroxidase*

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ABSTRACT: A study of pig thyroid microsomes has revealed the presence of a hemoprotein which complexes with cyanide. In the cyanide difference spectra of the microsomes, there is a peak at 428 mμ and a trough at 402 mμ. It is estimated that the concentration of this component in the microsomes is approximately 0.066 mμmole/mg of protein. Cytochrome b_5 is also present in a concentration of 0.035 mμmole/mg of protein. No cytochrome P-450, P-420, or Fe_x could be detected. Thus, thyroid microsomes contain nc P-450, but in its place have a cyanide-complexing hemoprotein. The thyroid microsomes have high peroxidase activity. The specific activity of this peroxidase is comparable to known peroxidases in ability to catalyze the oxidation of guaiacol and iodide. This activity is several orders of magnitude greater than that of the pseudoperoxidases. The results are discussed as evidence for a thyroid hemoprotein peroxidase.

Recent work has clearly established the presence of an electron transport system in microsomal preparations. The peroxidase activity present in the thyroid tissue has been found to be associated with subcellular particulates. Although there have been conflicting reports (Alexander, 1959, 1965; De Groot, 1965; De Groot and Davis, 1962a,b; Hosoya, 1963a,b; Hosoya *et al.*, 1962; Hosoya and Ui, 1961; Igo *et al.*, 1964; Klebanoff *et al.*, 1962; Mahoney and Igo, 1966; Yip, 1965), the peroxidase has been localized in microsomal preparations. This investigation examines the nature of the hemoproteins present in the thyroid microsomal fraction, with special emphasis on the peroxidase.

Two hemoproteins have been shown to be present in microsomes: cytochrome b_5 and P-450. The P-450, or its altered form, P-420, appears to be the terminal oxidase in the microsomes and is distinguished by the fact that it forms carbon monoxide complexes with absorption maxima at 450 and 420 mμ, respectively. Although early reports indicated that neither P-450 nor P-420 combines with cyanide, more recent evidence (R. E. Estabrook, 1966, personal communication) suggests that this is not the case. The cyanide difference spectrum of this component has a peak at 444 mμ and a trough at 390 mμ. On the other hand, cytochrome b_5 does not combine with either carbon monoxide or cyanide.

All known hemoprotein peroxidases will combine with cyanide to give a distinct spectral complex. In the present work, thyroid microsomes were shown to contain a unique hemoprotein which does complex with cyanide.

Material and Methods

Purified preparations of thyroid peroxidase (Hosoya and Morrison, 1965) and lactoperoxidase (Morrison and Hultquist, 1963) were obtained as previously described. Myeloperoxidase, purified from leucocytes, was kindly provided by Dr. Julius Schultz, and purified horseradish peroxidase *c* by Dr. Robert Shannon and Dr. Ernest Kay. In order to obtain catalase-free hemoglobin, human oxyhemoglobin was purified by Sephadex G-75 gel filtration, and horse oxyhemoglobin was recrystallized four times. Horse heart cytochrome *c*, type 3, was obtained from Sigma Chemical Co. and was further purified, as previously described (Morrison *et al.*, 1960). Commercial horseradish peroxidase was obtained from Worthington.

The subcellular particulate fractionation was carried out by a modification of the method for liver employed by De Duve *et al.* (1955). Connective tissue and fat were removed from glands received fresh from the slaughterhouse. Such glands (15 or 20) were sliced into thin sections with a razor blade. The slices were washed several times with isotonic saline and then blotted on filter paper. About 60–80 g of this material was added to three volumes of 0.25 M sucrose solution containing 1 mM EDTA and 5 mM phosphate buffer, pH 7.4. The suspension was homogenized for 15 sec at 50 v in a Waring Blendor and was then passed through a Potter-Elvehjem homogenizer and centrifuged at 600*g* for 10 min. The sediments and any incompletely homogenized material were combined and suspended in the sucrose solution before repeating the treatment. The supernatants obtained in this manner were combined and the volume was adjusted by adding enough sucrose solution to give a cytoplasmic

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TABLE I: Comparison of Cyanide Difference Spectra of Various Thyroid Microsomes and Hemoproteins.

	Peak (mμ)	Trough (mμ)	Ref
Thyroid microsomes	428	402	
Catalase	427	407	Keilin and Hartree (1951)
Metmyoglobin	427	408	Keilin and Hartree (1955)
Methemoglobin	422	404	Keilin and Hartree (1951)
Cytochrome oxidase	432	411	
Lactoperoxidase	433	410	
Myeloperoxidase	460	428	Newton *et al.* (1965)
Horseradish peroxidase	427	402	Keilin and Hartree (1951)

extract, which was 10 ml/g of starting tissue.

The cytoplasmic extract was then divided by differential centrifugation into three particulate fractions which corresponded to the M + L_a fraction obtained by centrifugation at 100,000*g* min, the L_b fraction obtained by centrifugation at 250,000*g* min, and finally, the P fraction obtained by centrifugation at 6,200,000*g* min. The fractions were labeled according to the nomenclature of De Duve *et al.* (1955), where M represents heavy mitochondria; L, light mitochondria; L_a and L_b, subfractions of light mitochondria (Hosoya, 1963a,b); and P, microsome. Both the M + L_a and the L_b fractions were washed twice with sucrose solutions. The P fraction was washed twice with 300 ml of 0.1 M phosphate buffer, pH 7.4, containing 0.25 M NaCl, and was finally suspended in a small volume of 0.1 M phosphate buffer, pH 7.4. Centrifugation was performed on a Servall with rotor 9RA for the M + L_a and the L_b fraction, and on a Spinco Model L-2 with rotor R30. All calculations were based on the average radius for the rotors. Electron microscopy of the P fraction showed that it consisted primarily of rough and smooth endoplasmic reticulum.

Peroxidase activity was measured (Hosoya *et al.*, 1962), using guaiacol or iodide as the hydrogen donor. The reaction was initiated by the addition of peroxide at room temperature (22–24°).

Difference spectra were measured on a Cary Model 14 spectrophotometer with the scatter transmission attachment. Particulate suspensions, containing 7–12 mg of protein/ml in 0.1 M phosphate buffer, pH 7.4, were placed in both sample and reference cuvets. The various difference spectra were obtained and corrected for the base line.

Samples were reduced with dithionite, NADPH,[1] or NADH by adding a small amount of the solid material. Anaerobic conditions were obtained by evacuating a Thunberg-type cuvet. Carbon monoxide spectra were obtained for at least 30 sec. All spectrophotometric measurements were made at room temperature.

Protein was determined by the biuret procedure, while cytochrome b_5 was measured by the method of Omura and Sato (1964).

Results

Difference Spectra of the Microsomal Suspension. All known peroxidases form a cyanide complex, as evidenced by marked spectral changes. As can be seen from curve 1 of Figure 1, the addition of cyanide to the thyroid microsomal fraction produced distinct spectral differences, indicating the presence of a cyanide-complexing pigment. The spectrum, with a peak at 428–430 mμ and a trough at 402–403 mμ, suggests that the pigment is a hemoprotein, although the curve cannot be ascribed to any known cyanide-complexing hemoprotein of mammalian tissue.

Positions of the peaks and troughs of a number of hemoproteins are given in Table I. Catalase and methemoglobin, the most likely contaminants of the microsomal fraction, show peaks and troughs at similar positions to the microsomal suspension. Since no catalase activity could be demonstrated in the fraction, this possibility was eliminated.

The difference spectra obtained when NADH and NADPH were employed as reducing agents are shown in Figure 1 (curve 4.) The addition of NADPH to microsomes results in peaks at 424 and 557 mμ and a trough at 409 mμ. This spectrum is similar to that of the cytochrome b_5 of liver microsomes.

The presence of P-450 in liver microsomes is characterized by a sharp peak at 450 mμ in the reduced *vs.* the reduced carbon monoxide spectrum. Curve 2, which is such a difference spectrum, did not show the 450-mμ peak. A very concentrated microsomal preparation, containing 20 mg of protein/ml, was kindly analyzed for Fe_x by H. S. Mason. No signal corresponding to Fe_x of liver microsomes was detected. These results indicated that P-450 was not present in these particulates. However, P-450 is unstable and can convert to P-420, which has an absorption peak at 420 mμ in this type of difference spectrum. No P-420 was present in the thyroid microsomal fraction, since the microsomes saturated with carbon monoxide and reduced with NADPH under anaerobic conditions

[1] Abbreviations used: NADH, reduced nicotinamide–adenine dinucleotide; NADPH, reduced nicotinamide–adenine dinucleotide phosphate.

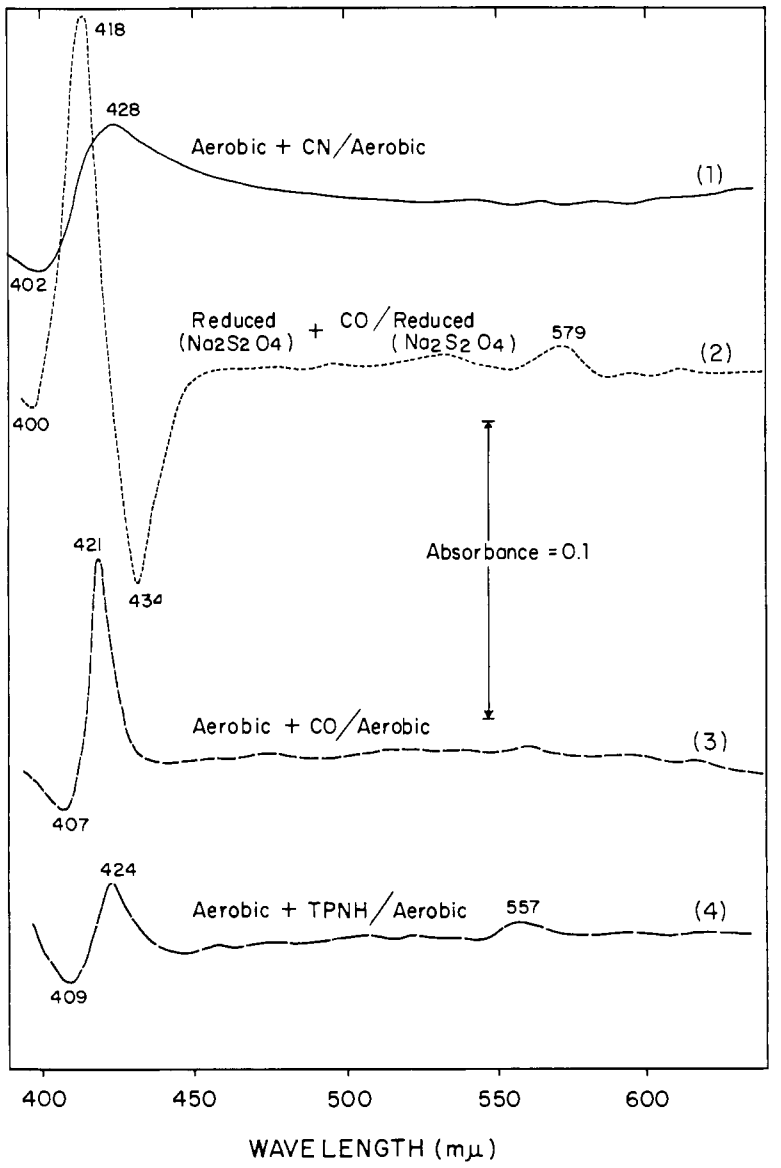

FIGURE 1: Difference spectra of thyroid microsomes. The spectra were recorded as indicated in the text. (curve 1) Cyanide difference spectrum. Both the sample and reference cuvet contained a microsomal suspension of approximately 7 mg of protein/mg of 0.1 M phosphate buffer, pH 7.4. The sample cuvet was 4×10^{-2} M with respect to cyanide. (curve 2) Reduced carbon monoxide difference spectrum. Both cuvets contained a suspension of microsomes as described above, reduced with dithionite. The sample cuvet was saturated with carbon monoxide just prior to recording the spectrum. (curve 3) Aerobic carbon monoxide difference spectrum. Both cuvets contained a suspension of microsomes, as described for curve 1. The sample cuvet was saturated with carbon monoxide just prior to recording the spectrum. (curve 4) NADPH difference spectrum. Both cuvets contained a suspension of microsomes, as described for curve 1. Approximately 1 mg of solid NADPH was added to the sample cell just prior to recording the spectrum.

gave no spectral shifts. Curve 2, thus, can be interpreted as representing the difference spectrum of the carbon monoxide complex of a hemoprotein such as hemoglobin, which is not affected by NADPH or NADH.

In order to detect the presence of hemoglobin, a number of difference spectra were employed. Curve 3 was obtained when one of the cuvets was saturated with carbon monoxide under aerobic conditions and is the difference spectrum of carboxyhemoglobin *vs.* oxyhemoglobin. The presence of oxyhemoglobin was confirmed by deoxygenation of the preparation in a Thunberg cuvet. The difference spectrum obtained of the deaerated *vs.* aerated microsomal preparation could be attributed to oxyhemoglobin *vs.* hemoglobin. Using preparations of human hemoglobin as a stand-

TABLE II: Estimation of the Content of Contaminating Hemoglobin in Thyroid Microsomes.

Exptl Condn	Microsomal Suspension Observed Spectrum: Peak (mμ)	Trough (mμ)	δ OD	Hemoglobin Observed Spectrum: Peak (mμ)	Trough (mμ)	δε (mM)	Hemoglobin Content of Microsomes (mμmoles/mg of protein)[a]
I. Aerobic *vs.* aerobic saturated with CO HbO_2 *vs.* HbCO	421	408	0.0578	421	407	107	0.069
II. Aerobic CO *vs.* anaerobic HbCO *vs.* Hb	418	434	0.097	418	433	167	0.075

[a] Calculated on the basis of the extinction coefficient determined for hemoglobin.

ard, extinction coefficients for the various difference spectra were obtained for comparison. Using these extinction coefficients, calculations of the hemoglobin content of the microsomal fractions were made. The values given by the various procedures are in reasonable agreement and are shown in Table II.

Estimation of the Hemoproteins in the Microsomes. The content of oxyhemoglobin was calculated from the difference spectrum of HbCO *vs.* HbO_2. Cytochrome b_5 was estimated from curve 4, employing the molar extinction coefficient as described previously. A molar extinction coefficient for the cyanide-complexing pigment of 102×10^3 cm^{-1} was employed. This extinction is based on studies of the purified thyroid peroxidase (T. Hosoya and M. Morrison, unpublished data). The values are presented in Table III. The

TABLE III: Quantitative Estimation of Hemoproteins in Thyroid Microsomal Fractions.[a]

Hemoprotein	Quantity (mμmoles/mg of protein): Prepn 1	Prepn 2	Prepn 3
Cyanide-complexing hemoprotein	0.061	0.072	0.065
Cytochrome b_5		0.026	0.040
Oxyhemoglobin	0.033	0.066	0.069

[a] Values were determined as described in text.

cyanide-binding pigment in three different preparations is constant, and slightly higher than the cytochrome b_5 present. The hemoglobin, which is a contaminant in the preparation, varies greatly and is a function of how thoroughly the preparations were washed.

The specific activity of the microsomal peroxidase, other peroxidases, and pseudoperoxidases are compared in Table IV. The concentration of microsomal peroxidase was determined from the cyanide-binding complex, as described above. On this basis, the microsomal peroxidase can be clearly distinguished from the pseudoperoxidases and is similar to other known peroxidases in its ability to catalyze the oxidation of guaiacol and iodide.

The L_b fraction contained a considerable amount of peroxidase; hence, the cyanide difference spectrum of this fraction was also examined. Since this fraction contained mitochondria, the spectra reflect the presence of cytochrome *c* oxidase. This, together with the other cytochromes present in the preparation, made an estimate of the peroxidase difficult. However, the magnitude of the peak at 428 mμ and the trough at 402 mμ did indicate that the peroxidase activity of this particulate was comparable to that of the cyanide pigment in microsomes.

Discussion

Most previous workers (Alexander, 1959, 1965; De Groot, 1965; De Groot and Davis, 1962a,b; Hosoya *et al.*, 1962; Hosoya and Ui, 1961; Igo *et al.*, 1964; Mahoney and Igo, 1966; Yip, 1965), in attempting to localize the peroxidase in subcellular particulates, have done so on the basis of peroxidase activity. Since numerous oxidative systems have been found in the microsomal fractions of other tissues, the presence of the thyroid peroxidase was not unexpected. The fact that it is not readily dissociated from the particulate clearly indicates that the enzyme is not derived from either the soluble fraction or the other organelles.

The role of pseudoperoxidase activity has rarely been adequately assessed in previous work on the thyroid peroxidase. Not only do the naturally occurring hemoproteins show pseudoperoxidase activity (Hosoya and Morrison, 1965; Morrison *et al.*, 1965), but the addition of heme to protein will inevitably give rise to a protein hemochromogen of variable pseudoperoxidase activity.

Based on pyridine hemochromogen, all of the heme in the microsomes is accounted for by the three hemoproteins: cytochrome b_5, the cyanide-complexing hemoprotein, thyroid peroxidase, and the contaminating hemoglobin. Although the microsomal fraction

TABLE IV: Comparison of Specific Activity of a Variety of Peroxidases and Other Hemoproteins.

Hemoprotein Preparation	GU[e]/mμmole[a]	IU[e]/mμmole[a]	GU/IU
Cyanide-complexing hemoprotein in thyroid microsomes	0.50	0.29	1.72
Purified thyroid peroxidase	1.15	0.60	1.90
Lactoperoxidase	4.4	7.0	0.63
Myeloperoxidase[b]	2.1	0.34	6.28
Horseradish peroxidase *c*[c]	1.5	0.015	100
Horseradish peroxidase[d]	2.2	0.02	110
Horse hemoglobin	0.000006		
Human hemoglobin	0.000045	0.000000	
Horse heart cytochrome *c*	0.000017	0.000084	

[a] Based on heme content. [b] Purified sample kindly provided by Dr. J. Schultz. [c] Purified sample kindly provided by Drs. L. M. Shannon and E. Kay. [d] Obtained from Worthington. [e] GU is guaiacol units obtained as described in text. IU is iodide units obtained as described in text.

was washed thoroughly, it is difficult to remove hemoglobin completely. In contrast to the other components whose concentration was relatively uniform, the amount of hemoglobin per milligram of protein varied from one preparation to another.

On a protein basis, the cytochrome b_5 in the thyroid microsomal fraction is very low, amounting to only about 3 or 4% of that found in liver. Garfinkel (1963), who has investigated the cytochrome b_5 content in various organs of different species, has reported a wide variation in values. M. Suzuki (personal communication, 1966) has recently isolated the cytochrome b_5 of thyroid microsomes and has shown it to have a molecular weight of 14,500.

In liver and adrenal microsomes, cytochrome b_5 is accompanied by another hemoprotein, P-450, or its degradation product, P-420 (Estabrook *et al.*, 1963; Garfinkel, 1958; Hashimoto *et al.*, 1962; Klingenberg, 1958; Mason *et al.*, 1965; Omura and Sato, 1964; Omura *et al.*, 1965; Sato *et al.*, 1965). In the present study, no evidence was found for either P-450, P-420, or Fe_x, the component detected by its electron spin resonance signal. These components were detectable in the microsomes of other tissues. Although a P-450 cyanide spectrum was not reported in earlier work, more recent studies have indicated that this component does form a cyanide complex (R. W. Estabrook, 1966, personal communication). This complex would, however, be readily distinguished from that of the cyanide-binding pigment in the thyroid microsomes.

In place of the P-450, the thyroid microsomal fraction contains the cyanide-complexing compound which appears to be unique to the thyroid. Recently, Maloof and Soodak (1966) have confirmed our findings (Hosoya and Morrison, 1965) and reported a cyanide-complexing component in calf thyroid microsomes. Although similar to those of catalase and methemoglobin or metmyoglobin, the difference spectra of the cyanide-complexing compound are distinguishable. Catalase could be eliminated from consideration as a cyanide-binding pigment, since little or no catalase activity was present in the preparation. The cyanide difference spectrum of cytochrome a_3 eliminates it from consideration, since it has peaks and troughs at 430 and 412 mμ, respectively. In addition, no cytochrome oxidase activity was present in the microsomal preparation.

There are several indications that the amount of cyanide-complexing hemoprotein is directly related to the peroxidase activity. First, the number of guaiacol units per cyanide-complexing unit is reasonably constant throughout the procedures employed in the present study and is similar to that obtained in the purified thyroid peroxidase (Hosoya and Morrison, 1965). Equally important is the fact that the specific activity, based on the cyanide difference spectrum of the peroxidase in the thyroid microsomes, is comparable to that of the known peroxidases and is several orders of magnitude greater than the pseudoperoxidase activity of other hemoproteins. The pseudoperoxidase activity of metmyoglobin, methemoglobin, and hemoglobin is similar and very low. The activity of the microsomes is far too high to ascribe to these compounds. Further, the cyanide difference spectrum of the purified thyroid peroxidase is very similar to that found in the thyroid microsomal fraction. On a spectral basis, the activity in the purified enzyme and in the thyroid microsomal fraction is the same.

As Table IV shows, the specific activity of all the true peroxidases is comparable and very high. This means that in assaying for the heme prosthetic group of a peroxidase, a relatively large amount of peroxidase activity is required to obtain a detectable amount of heme for the hemochromogen assay.

Since it has been suggested that iodination in the thyroid may not be directly related to the peroxidase activity, an effort was made to evaluate both the guaiacol- and iodide-oxidizing activity. The ratio of these activities at all stages of the cell fractionation and in

the purified enzyme is very similar. This strongly suggests that either both activities reside in a single enzyme, or that if more than one enzyme is involved, they are not separated.

Recent evidence has suggested that the iodination reaction in the thyroid takes place on the preformed peptide chain (Alexander, 1964; Curtouzou *et al.*, 1964; Maloof *et al.*, 1964; Nunez *et al.*, 1965; Seed and Goldberg, 1963; Taurog and Howells, 1964). Since the sites of peptide chain formation and iodinating enzymes are both located on the reticula endothelium, it may well be that the iodination could take place during or immediately after the formation of thyroglobulin chains, but prior to the secretion of the thyroglobulin to the colloid.

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References

Alexander, N. M. (1959), *J. Biol. Chem. 234*, 1530.
Alexander, N. M. (1964), *Endocrinology 74*, 273.
Alexander, N. M. (1965), *in* Current Topics in Thyroid Research, Casseno, C., and Andreoli, M., Ed., New York, N. Y., Academic, p 43.
Alexander, N. M., and Corcoran, B. J. (1962), *J. Biol. Chem. 237*, 243.
Cooper, D. Y., Estabrook, R. W., and Rosenthal, O. (1963), *J. Biol. Chem. 238*, 1320.
Curtouzou, G., Aquaron, R., and Lissitzky, S. (1964), *Biochem. Biophys. Res. Commun. 15*, 2.
De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955), *Biochem. J. 60*, 604.
De Groot, L. J. (1965), *New Engl. J. Med. 272*, 243, 297, 355.
De Groot, L. J., and Davis, A. M. (1962a), *Endocrinology 70*, 492.
De Groot, L. J., and Davis, A. M. (1962b), *Endocrinology 70*, 505.
Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. (1963), *Biochem. Z. 338*, 741.
Garfinkel, D. (1958), *Arch. Biochem. Biophys. 77*, 493.
Garfinkel, D. (1963), *Comp. Biochem. Physiol. 8*, 367.
Hashimoto, L., Yamano, T., and Mason, H. S. (1962), *J. Biol. Chem. 237*, PC3843.
Hosoya, T. (1963a), *J. Biochem. (Tokyo) 53*, 86.
Hosoya, T. (1963b), *J. Biochem. (Tokyo) 53*, 381.
Hosoya, T., Kondo, Y., and Ui, N. (1962), *J. Biochem. (Tokyo) 52*, 180.
Hosoya, T., and Morrison, M. (1965), *Biochem. Biophys. Res. Commun. 20*, 27.
Hosoya, T., and Ui, N. (1961), *Nature 192*, 659.
Igo, R. P., Mahoney, C. P., and Mackler, B. (1964), *J. Biol. Chem. 239*, 1893.
Keilin, D., and Hartree, E. F. (1951), *Biochem. J. 49*, 88.
Keilin, D., and Hartree, E. F. (1955), *Biochem. J. 61*, 153.
Klebanoff, S. J., Yip, C., and Kessler, D. (1962), *Biochim. Biophys. Acta 58*, 563.
Klingenberg, M. (1958), *Arch. Biochem. Biophys. 75*, 376.
Mahoney, C. P., and Igo, R. P. (1966), *Biochim. Biophys. Acta 113*, 507.
Maloof, F., Sato, G., and Soodak, M. (1964), *Medicine 43*, 375.
Maloof, F., and Soodak, M. (1966), *Endocrinology 78*, 661.
Mason, H. S., Yamano, T., North, J. C., Hashimoto, Y., and Sakagishi, P. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 879.
Morrison, M., Allen, P. Z., Bright, J., and Jayasinghe, W. (1965), *Arch. Biochem. Biophys. 111*, 126.
Morrison, M., Hollocher, T., Murray, R., Marinetti, G., and Stotz, E. (1960), *Biochim. Biophys. Acta 41*, 334.
Morrison, M., and Hultquist, D. (1963), *J. Biol. Chem. 238*, 2847.
Newton, N., Morell, D. B., and Clarke, L. (1965), *Biochim. Biophys. Acta 96*, 476.
Nunez, J., Jacquemin, C., Brun, D., and Roche, J. (1965), *Biochim. Biophys. Acta 107*, 454.
Omura, T., and Sato, R. (1964), *J. Biol. Chem. 230*, 2370, 2379.
Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W. (1965), *Federation Proc. 24*, 1181.
Sato, R., Omura, T., and Nishibayashi, H. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 861.
Seed, R. W., and Goldberg, I. H. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 275.
Taurog, A., and Howells, E. M. (1964), *Federation Proc. 23*, 149.
Yip, C. (1965), *Biochim. Biophys. Acta 96*, 75.