Function of Peroxidase and NADPH Cytochrome C Reductase During the Wolff-Chaikoff Effect

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ABSTRACT. The effects of varying doses of stable iodide on thyroid peroxidase function and NADPH-cytochrome c reductase have been studied in rats. Wolff-Chaikoff effect was induced *in vivo* by injection of 250 or 500 μg of stable iodide, and several enzymatic activities of particulate fractions collected from the same thyroids were measured *in vitro*. There was no difference between the enzymatic activity (peroxidase and NADPH-cytochrome c reductase) of blocked and unblocked thyroid. Blocked thyroids showed increased ^{131}I-PBI formation with NADPH in the same manner as unblocked thyroids. Iodide concentrations 10 times higher than those calculated to be present in the blocked thyroid (3×10^{-5}M–1.2×10^{-3}M) were used in the *in vitro* reconstruction of the Wolff-Chaikoff effect with particulate fraction. Inhibition of iodination was not observed *in vitro*, even when low levels of tyrosine (10^{-5}M) were used in the model system. Kinetic studies of the iodination reaction suggest that it follows a random mechanism, and shows no evidence of competition reaction between iodide and tyrosine for the enzyme. The data are compatible with I^+ serving as the iodinating species formed by membrane bound peroxidase. Our data show no evidence of Wolff-Chaikoff effect *in vitro* at pH 7.0, in particulate fraction from rats receiving pretreatment with various concentrations of stable iodide able to induce iodide block *in vivo*. The easier induction of Wolff-Chaikoff effect in intact thyroid tissue than in an acellular preparation suggests that inhibition of other factors in the iodinating system, such as H_2O_2 generation, may be more important than inhibition of peroxidase. (*Endocrinology* **93**: 822, 1973)

SINCE the first report of the inhibitory effect of stable iodide on thyroid iodide binding, many studies concerning its mechanism have been reported (1–7). It is known that the ratios of MIT:DIT and iodotyrosine:iodothyronine increase during the block, but the enzymatic mechanism of this alteration is not clarified. Recently, Taurog presented evidence that molecular iodine is not the active iodinating agent in the peroxidase system (7); thus the combination of I^- with I_2 to form I_3^- became unlikely as a cause of the Wolff-Chaikoff effect. Taurog proposed an alternative mechanism—competition between iodide and tyrosyl residues of protein for active iodine or for an active site on peroxidase, based on studies with a highly purified thyroid peroxidase (7) and with lactoperoxidase (8). With a less purified enzyme, he could not induce an *in vitro* Wolff-Chaikoff effect at normal pH (9).

High doses of iodide inhibit thyroidal glucose oxidation *in vitro*, suggesting that inhibition of hormonogenesis might be secondary to an alteration of intermediary metabolism related to H_2O_2 generation (10–12). There are, however, no reports on changes of enzymes that may be involved in H_2O_2 generation by excess iodide. Our studies were designed to find whether the activity of enzymes possibly involved in the thyroidal iodinating system (peroxidase and NADPH-cytochrome c reductase) were altered during the process of acute binding block induced by stable iodide. Further, an *in vitro* reconstruction of the conditions present *in vivo* was attempted. Particulate enzyme from blocked rat thyroid was studied in the belief that this may be more physiologic than a purified enzyme.

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Materials and Methods

1. *Induction of Wolff-Chaikoff effect* in Vivo

Studies were performed in male Charles River rats weighing 100 to 120 g, fed Purina Chow for

at least 1 week before experiments were undertaken. Groups of 5 rats were given single ip injections of $Na^{125}I$ together with varying quantities of stable potassium iodide. The dose of ^{125}I varied from 5 to 50 μCi; the dose of stable iodide was 10 to 500 μg. Animals were killed 4 hr after the injection. After rats had been killed with ether, thyroids were quickly removed, freed of connective tissue, pooled, and weighed. Each group of pooled thyroids was homogenized and the total percentage thyroidal uptake of administered ^{125}I was determined in an aliquot of the homogenate (5). The percentage ^{125}I-PBI was assessed by paper chromatography in the B-A-W[1] system.

2. Studies on the alteration of enzymes in the blocked thyroid

Since rats in the high-dose group received about 50 times as much iodide as the low-dose group, a predialyzing procedure was employed in order to make the initial iodide content in the *in vitro* assay system as equal as possible. After the thyroids were dissected, they were cut into 3 pieces, and incubated in 5 ml of cold K-R-phosphate buffer for 15 min 2 times. After leaching, the intrathyroidal iodide in the high-dose group became 2 to 4 times that found in the low-dose group. Then the thyroids were homogenized, diluted to 100 times in volume with K-R-phosphate buffer, and sedimented at 105,000 × *g* for 1 hr. Aliquots of the particulate fraction were incubated for 1 hr with and without 2×10^{-4}M NADPH under standard conditions (13), and ^{131}I-PBI formation was assessed. Activities of peroxidase (measured by activity in iodination of tyrosine) and NADPH-cytochrome c reductase in the particulate fraction were assayed in a standard assay system (14). The percentage of iodotyrosine was assessed with paper chromatography in B-A-W system, and correction was made for non-enzymic iodination. NADPH-cytochrome c reductase was assessed by changes in absorbence of cytochrome c with spectrophotometry at 550 mμ.

3. Reconstruction of Wolff-Chaikoff effect in vitro

The concentration of stable iodide present in the blocked thyroid was calculated from the specific activity of the iodide injected, total percentage thyroidal uptake of administered ^{125}I, and the proportion present in inorganic form. While the extent of intracellular iodide compartmentalization cannot be readily assessed, we assumed that tenfold concentration could occur at the iodinating site. Thus we included assays with an *in vitro* iodide concentration in our model system ten times higher (3×10^{-5}M–1.2×10^{-3}M) than those observed to be present in whole tissue homogenates from the blocked glands. These concentrations of iodide were also used throughout the homogenization and preparation of the particulate fractions. Two different concentrations of tyrosine (10^{-3}M and 10^{-5}M) were used in the model system, and the maximal ratio of iodide to tyrosine could thus reach 100:1. Iodotyrosine formed was assessed by paper chromatography in the B-A-W system after incubation for 20 min at 37 C with glucose and glucose oxidase as H_2O_2 source.

4. Studies on the initial rate of iodination

Various levels of tyrosine concentration and several levels of iodide were used in the studies on the initial rate of iodination. The particulate fraction showed linearity in the iodination reaction at various concentrations of enzyme protein (12–234 μg). Since the iodination reaction system consisted of 3 substrates (tyrosine, iodide, and H_2O_2), 2 substrates were held constant while the third was varied (16). Iodination reaction was started with the addition of H_2O_2, and stopped by adding 2 drops of 2M HCl after 1 min, 2 min and 3 min, respectively. Iodotyrosine formed was assessed by counting radioactivity adsorbed by ion exchange resin. The initial rate of iodination reaction was calculated according to the formula in Table 1. This formula gives the initial rate as a tangent of the

TABLE 1.

$$Vm = 3\,Y_1 + \frac{Y_3}{3} - 1.5\,Y_2$$

Vm represents the initial rate of the reaction, and Y_1, Y_2, and Y_3 show the iodination rate after 1, 2 and 3 min, respectively.

[1] Abbreviations: B-A-W, butanol-acetic acid-water (4:1:5); K-R-phosphate buffer, modified Krebs-Ringer-phosphate buffer (Ca^{++} concentration is decreased by two-thirds).

curve according to a modification of Algranati's method (15). Using this data, Lineweaver-Burke plots were constructed, relating the inverse of the reaction velocity to the inverse of the substrate concentration. Regression lines were calculated to fit the data.

Results

All procedures were carried out in 3 separate experiments, the results of which agreed closely. Typical results obtained in a single experiment are shown in the figures, in which each plot is the mean of duplicated results.

1. Induction of Wolff-Chaikoff effect in vivo *(Fig. 1)*

The percentage thyroidal uptake of ^{125}I decreased progressively when the dosage of stable iodide was increased. In contrast, the percentage of thyroidal unbound ^{125}I increased. This increment became apparent when the dosage of iodide was over 100 μg/animal. The proportionate total organification of thyroidal iodide displayed a biphasic response to progressively increasing doses of carrier. It increased progressively as the iodide dose was moderately increased, but with larger doses it declined. These changes show that a Wolff-Chaikoff effect had been induced *in vivo*.

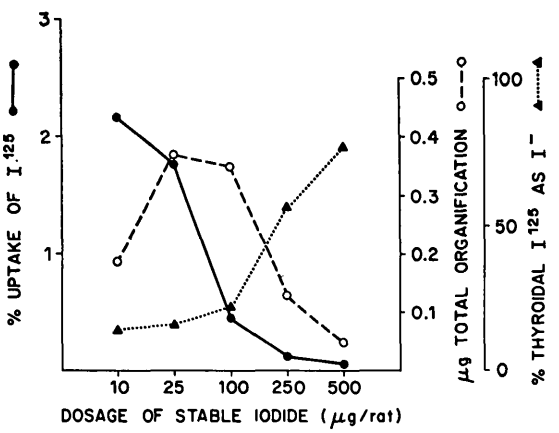

FIG. 1. Effect of graded doses of iodide on the thyroidal metabolism of iodine in rats.

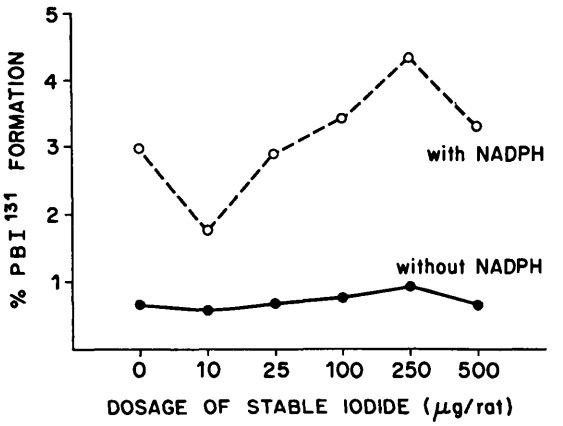

FIG. 2. ^{131}I-PBI formation by thyroid particulate fraction was assayed with and without the addition of 2×10^{-4}M NADPH. Each iodinating system contained 80 μg enzyme protein, 0.5 ml K-R-phosphate buffer at pH 7.0, 1 nmole KI and 1 μCi ^{131}I, in 1 ml. Incubation was performed at 37 C for 1 hr.

2. Studies on alteration of enzymes in the blocked thyroids

The function of ^{131}I-PBI formation by aliquots of the particulate fraction with and without NADPH is shown in Fig. 2. There was no difference in ^{131}I-PBI formation on comparison of blocked and unblocked thyroids. Blocked thyroids also showed increased ^{131}I-PBI formation on addition of NADPH, in the same manner as unblocked thyroids. Fig. 3 shows peroxidase and NADPH-cytochrome c reductase activity after the predialyzing procedure. There was no decrease in peroxidase activity of the particulate fraction from rat thyroids which received pretreatment with excess iodide to induce Wolff-Chaikoff effect *in vivo*. In the high dose group, enzymatic activity showed a small but statistically insignificant increment. No difference was found in NADPH-cytochrome c reductase activity between blocked and unblocked animals.

3. Attempted reconstruction of Wolff-Chaikoff effect in vitro

^{131}I-PBI formation was assessed in particulate fraction of glands homogenized in buffer with iodide added at a level 10 times that calculated to be present *in vivo*, as de-

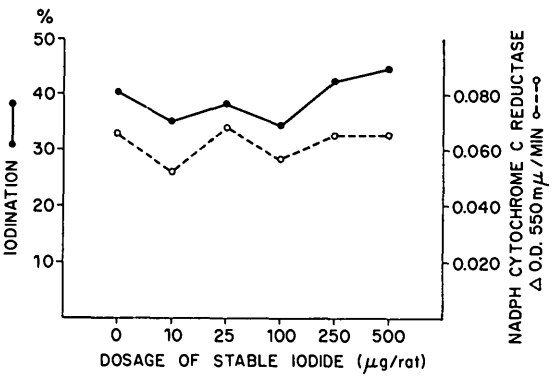

FIG. 3. The activity of peroxidase and NADPH-cytochrome c reductase was measured with rat thyroid particulate fraction. The iodinating system contained 80 μg enzyme protein, 0.5 ml K-R-phosphate buffer at 7.0, 11 nmoles KI, 1 μCi ^{131}I, 1 μmole glucose, 0.1 mg glucose oxidase, and 1 μmole tyrosine, in 1 ml. Incubation was started by adding glucose oxidase and stopped after 20 min at 37 C. Reductase assay system consisted of 80 μg enzyme protein, 0.4 ml, 0.2M phosphate buffer at pH 7.0, 1 μmole KCN, 50 nmoles cytochrome c and 100 nmoles NADPH, in 1 ml. The reaction was started by adding NADPH and read by spectrophotometer at 550 mμ.

scribed above. The final iodide concentrations in the incubation flasks were thus 7×10^{-6}–1.22×10^{-4} for the various groups of rats. Assays of PBI formation, PBI formation with added NADPH, NADPH-cytochrome c reductase, and peroxidase gave no indication of iodide inhibition.

In a third series of experiments, the pooled thyroids were homogenized with added KI as described above, the particulate fraction was suspended in buffer with added KI, and all assays were conducted with final iodide concentrations tenfold the level calculated to be present in the particular group of animals *in vivo* (3×10^{-5}–1.2×10^{-3}M). With progressive increments of iodide, the percent of PB^{131}I declined with and without NADPH, but total organic iodine formation (% incorporation × concentration) increased (Fig. 4).

Peroxidase assays were similarly conducted with increments of added iodide, but tenfold those actually found *in vivo*. Fig. 5 shows iodination in the *in vitro* model system during 20 min with glucose and glucose oxidase as H_2O_2 source. Inhibition of iodination was not observed even with the low level of tyrosine. On the contrary, at the 10^{-5}M level, tyrosine was completely iodinated to at least the level of MIT. In other studies (data not shown), 5×10^{-5}M H_2O_2 was directly added in the assay system, and the reaction was stopped after three minutes. Again, we could not obtain a Wolff-Chaikoff effect in this acellular system.

4. Studies of the initial rate for iodination

The calculated initial rate of the iodination reaction showed no evidence of iodide inhibition, or tyrosine-iodide competition. The Lineweaver-Burke plots converge at a common point on the base line when either tyrosine or iodide is the varied substrate, and the other two substrates are at different concentrations but in constant ratio (Fig. 6A, 6B). Also, when iodide is the varied substrate with different levels of tyrosine and fixed H_2O_2 concentration, the lines converge at a common point (Fig. 6C). The

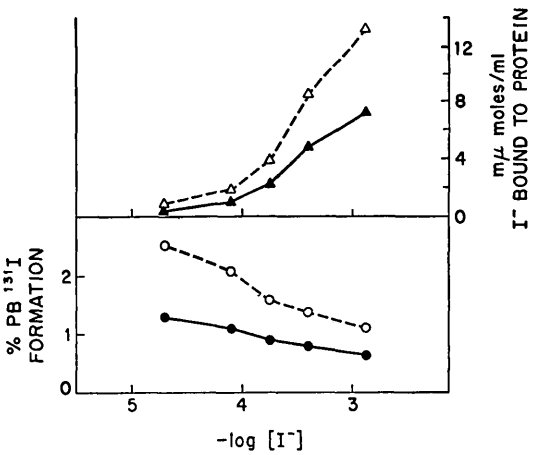

FIG. 4. ^{131}I-PBI formation with and without NADPH (2×10^{-4}M) was assayed with different concentrations of iodide. Iodinating system was the same as Fig. 2 except that concentrations of iodide were different, as shown in the figure. ●——● % ^{131}I-PBI without NADPH. ▲——▲ I^- bound to protein without NADPH. ○----○ % ^{131}I-PBI with NADPH. △----△ I^- bound to protein with NADPH.

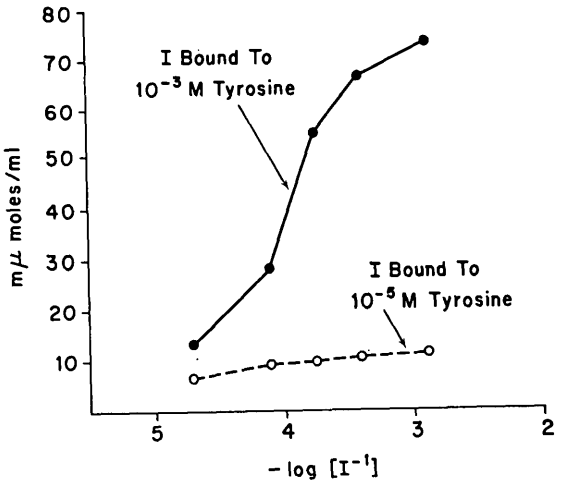

FIG. 5. The activity of peroxidase was assayed in the model system with 2 different concentrations of tyrosine and varying doses of iodide. Ten times higher concentration of iodide than those calculated to be present in whole homogenates from the blocked glands were used. Conditions were the same as for Fig. 3, except that different concentrations of tyrosine and iodide were used as shown in the figure.

data from Figs. 6A and 6B show that the reaction involves a quaternary complex and that it follows the "random" mechanism (17). Since the Lineweaver-Burke plots with different ratios of tyrosine and H_2O_2 (Fig. 6C) show the same pattern as the reaction using the fixed ratio of tyrosine and H_2O_2 (Fig. 6B), it is suggested that the mechanism is partial "random" (17).

Tyrosine and iodide might follow a "random" mechanism while H_2O_2 follows an "ordered" mechanism forming a complex before or after tyrosine and iodide take seat on the enzyme.

Discussion

Biosynthesis of iodotyrosines requires generation of H_2O_2, oxidation by peroxidase of iodide, and its attachment to tyrosine to form MIT and DIT. The nature of the iodinating intermediate has not been defined. Administration of excess iodide to animals or man inhibits the iodination reaction (Wolff-Chaikoff effect) (1). It has been presumed that this effect is transient and

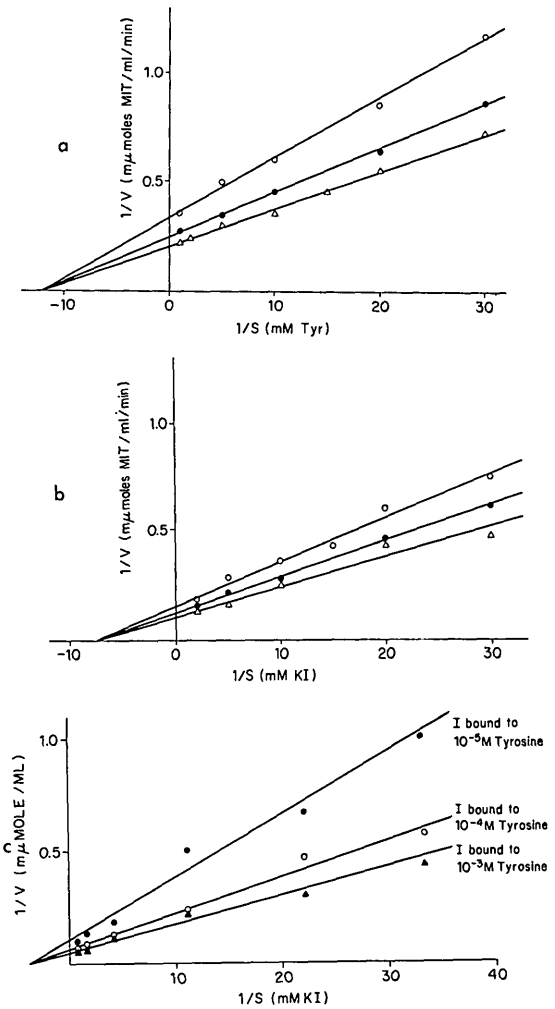

FIG. 6. Lineweaver-Burke plots were constructed, relating the inverse of the reaction velocity to the inverse of the substrate concentration. Initial rate of iodination reaction was calculated with the formula shown in Table 1. Iodinating system contained 80 μg enzyme protein, 0.5 ml K-R-phosphate buffer at pH 7.0, 1 μCi ^{131}I and different concentrations of tyrosine, iodide, and H_2O_2 in 1 ml. (A) L-tyrosine concentration was varied with several concentrations of KI and H_2O_2 in a fixed ratio (○———○) 1.0×10^{-5}M KI and H_2O_2, (●———●) 2.0×10^{-5}M KI and H_2O_2, (△———△) 5.0×10^{-5}M KI and H_2O_2. (B) KI concentration was varied with several concentrations of tyrosine and H_2O_2 in a fixed ratio (○———○) 1.0×10^{-5}M tyrosine and H_2O_2, (●———●) 2.0×10^{-5}M tyrosine and H_2O_2, (△———△) 5.0×10^{-5}M tyrosine and H_2O_2. (C) KI concentration was varied with several concentrations of tyrosine. As the concentration of H_2O_2 was fixed at 5×10^{-5}M, the ratio of tyrosine and H_2O_2 varied in each case.

involves some direct interference in the iodinating process by intrathyroidal excess iodide (2,3,6). Fawcett and Kirkwood (4) proposed that the inhibitory effect of excess iodide could be explained by I_3^- formation, on the assumption that the active iodinating agent in the thyroid is molecular iodine. Taurog argued against this mechanism, giving as evidence that molecular iodine is not the active agent in the iodination reaction, and he proposed a competition mechanism alternatively (7). Since, with less purified enzyme, he did not find inhibition with excess iodide (9), it must be considered that results using the purified enzyme relate to changes produced during the processing. In this study, rat thyroid particulate fraction was used in the belief that this may be more physiologic than a purified enzyme. Peroxidase in the thyroid is thought to be a membrane bound form.

^{131}I-PBI formation *in vitro* by particulate fraction from blocked thyroid did not differ from iodination by unblocked thyroid, and added NADPH increased ^{131}I-PBI formation in a normal manner. It is believed that NADPH may participate in an endogenous H_2O_2 generating system, as incubation of NADPH, NADPH-cytochrome c reductase, and cytochrome c leads to H_2O_2 generation and increased iodination by thyroid peroxidase *in vitro* (18). The increment in ^{131}I-PBI formation by particulate fraction from blocked thyroid with added NADPH shows that peroxidase and NADPH-cytochrome c reductase are normally reactive, although iodination had been "inhibited" *in vivo*. Direct assay of these enzymes also showed no changes in their function. In an attempt at reconstructing a Wolff-Chaikoff effect *in vitro*, we included assays with iodide concentration ten times higher than those observed to be present in the blocked thyroid. Two different concentrations of tyrosine (10^{-3}M and 10^{-5}M) were used with an expectation of inducing the Wolff-Chaikoff effect easily with a lower tyrosine level, if the "competition" mechanism prevailed in our tissues.

Contrary to our expectations, the Wolff-Chaikoff effect was not observed with an acellular system. These results prompted us to make studies on the kinetics of the iodination reaction of particulate fraction. Our data from the Lineweaver-Burke plots show that this reaction follows the "random" mechanism, and suggest that the active sites for iodide and tyrosine in the enzyme are different. The same apparent Km for iodination with different concentrations of tyrosine and iodide shows that there is no competition between iodide and tyrosine for the enzyme.

The work of Nunez with horseradish peroxidase (19), and of Yip with myeloperoxidase and purified beef peroxidase (20), have suggested a model of tyrosine iodination involving oxidation of both iodide and tyrosine to the free radical state, and indicates competition between iodide and tyrosine. Morrison's data (21) with lactoperoxidase have also suggested that both iodide and tyrosine are oxidized by the peroxidase in a "ping pong" reaction mechanism. Our data suggest a different model for the reaction of the membrane bound enzyme. Iodide and tyrosine probably have different active sites on the enzyme, as shown in Fig. 7. Iodide may be oxidized to iodinium ion, which then binds to tyrosine held appropriately at a second active site on the peroxidase molecule. In the absence of tyrosine, I_2 might be formed non-enzymatically by I^+ and I^-. In the case of purified enzyme, it is supposed that the active site for tyrosine might be changed

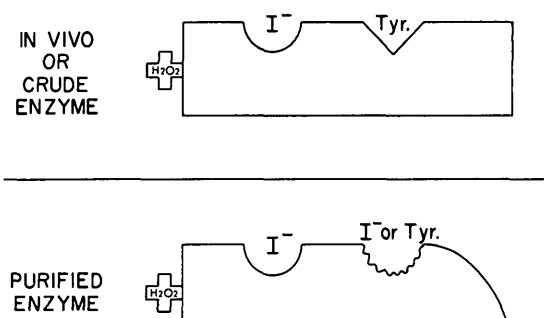

FIG. 7. Scheme of possible active sites in peroxidase.

during processing (trypsin digestion, detergent treatment, detaching protein by column chromatography, etc.), and then iodide could bind to and compete with tyrosine for the altered active site. While the interpretation of reaction kinetics for enzymes with several substrates, and presumably in an impure state, involves great uncertainty, our studies are most compatible with a mixed "random" and "ordered" mechanism with formation of a quaternary complex of enzyme—H_2O_2—I^-—and tyrosine.

It has been reported that the Wolff-Chaikoff effect is induced *in vitro* using rat thyroid lobes (22), bovine thyroid slices, and isolated bovine thyroid follicular cells (23). Since we found neither evidence of altered activity of enzymes by excess iodide nor of a competitive mechanism in the iodination reaction, the easier induction of Wolff-Chaikoff effect in intact thyroid tissue than in an acellular preparation suggests that the effects of excess iodide on the other factors in the iodinating system, such as H_2O_2 generation, might be more important than inhibition of peroxidase. Maayan and Ingbar reported that excess iodide decreases thyroid pyridine nucleotide levels *in vivo* (24). Also, Ogata *et al.* showed that iodide caused oxidation of thyroid pyridine nucleotides *in situ,* using an organ-microfluorometer (25). These changes in reduced pyridine nucleotide levels might result in decreased H_2O_2 generation. Thus the Wolff-Chaikoff effect could be explained without changes of thyroid peroxidase and NADPH-cytochrome c reductase.

Acknowledgments

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