Effects of phenylbutazone on thyroid iodine metabolism in vitro

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Abstract. Several alterations of thyroid function parameters have been reported in patients treated with phenylbutazone and we have studied the effect of this drug on the intrathyroidal iodine metabolism. An inhibition of the iodide transport expressed in terms of T/M ratios was observed in bovine thyroid slices incubated with high phenylbutazone concentrations. 10⁻³M produced 72% inhibition whereas lower concentrations showed no significant difference as compared with controls. Iodotyrosine synthesis was affected by 10⁻⁴M and 10⁻⁵M phenylbutazone. Formation of iodothyronine synthesis was markedly affected between 10⁻⁴M and 10⁻³M phenylbutazone concentrations. Thyroid peroxidase activity was measured by tyrosine-iodinase, triiodide and guaiacol assays. Soluble, pseudosolubilized and crude peroxidase preparations from bovine glands, as well as the soluble enzyme from human thyroids, have shown inhibition of tyrosine-iodinase activity when incubated with phenylbutazone in concentrations ranging from 10⁻³M to 10⁻⁸M, with a Ki of 4 x 10⁻⁶M for bovine thyroid peroxidase and of 6 x 10⁻⁶M for human soluble peroxidase. Formation of triiodide was affected between 10⁻³M and 10⁻⁸M phenylbutazone concentrations. Guaiacol peroxidation was scarcely affected by the action of the drug.

We have concluded that phenylbutazone affects the intrathyroidal iodine metabolism through the inhibition of thyroid peroxidase in concentrations which are usually present in the sera of patients treated with this drug.

Some patients treated with phenylbutazone have presented alterations of thyroid function (Green et al. 1953; Morgans & Trotter 1955; Linsk et al. 1957; Hansen 1962; Abiodun et al. 1973). Although a variety of hypotheses have been postulated to explain the antithyroid action of the drug (Scott et al. 1953; Green et al. 1953; Morgans & Trotter 1955; Linsk et al. 1957; Hansen 1962; Abiodun et al. 1973), its real nature remains unclear.

Several investigators have observed that phenylbutazone produced an inhibitory effect on thyrodial ¹³¹I uptake both in man and rat, and this effect was assumed to be located at the level of the pituitary gland (Linsk et al. 1957; Hansen 1962) or at the level of the serum transport of iodide (Scott et al. 1953; Green et al. 1953).

Morgans & Trotter (1955) have suggested a direct inhibitory effect of phenylbutazone on the thyroid gland since a patient with a diffuse goitre had an organification defect, demonstrated by a positive perchlorate tests.

A significant fall in total and free thyroxine levels suggesting a defect of thyroid function was also observed in other patients treated with phenylbutazone (Abiodun et al. 1973).

Since the mechanism of action of phenylbutazone on the thyroid gland has not as yet been

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clarified we have developed in vitro studies to elucidate the effects of phenylbutazone on the intrathyroidal iodine metabolism.

Materials and Methods

Tissue sources
Thyroid tissue was obtained from patients who underwent thyroidectomy for Graves' disease. Bovine thyroid tissue was obtained at a nearby abattoir.

Solubilization of phenylbutazone
Phenylbutazone was solubilized in ethanol before dilution in the different incubation mediums. The final ethanol concentrations, even in the more concentrated solutions of phenylbutazone, were sufficiently low so as not to affect the different parameters which have been studied.

Thyroid iodide transport
Studies of the iodide transport were made in fresh bovine thyroid slices of 0.5 mm thickness, 40 to 240 mg of wet weight, obtained by means of a Steady-Riggs microtome. These slices were incubated in an appropriate medium (Wolff 1960) containing methimazole 10⁻⁵M and ¹³¹I plus Ki 10⁻⁵M (Niepomnische et al. 1975), with the addition of several phenylbutazone concentrations ranging from 10⁻⁸M to 10⁻⁵M. Six slices for each concentration of phenylbutazone, including 6 slices without the drug, and 3 slices with 10⁻²M KCIO₄ instead of phenylbutazone, were incubated in separate vials containing 3 ml of medium each. Incubation was performed in a Dubnoff metabolic shaking incubator for 90 min. with 60 cycles/min and air as the gaseous phase. After incubation the slices were washed, blotted and weighed. Finally, ¹³¹I radioactivity was measured in each thyroid slice as well as in 1 ml of each incubation medium. T/M (tissue/medium) ratios were obtained as described previously (Niepomnische et al. 1975).

Iodine organification
Except for the absence of methimazole, incubation mixtures were identical to those utilized in the iodide transport studies.

After incubation the slices were pooled in groups of two and homogenized in 0.1 M phosphate buffer, pH 7 (1:4 w/v); five homogenates were then obtained for each concentration of phenylbutazone besides those corresponding to the control which were incubated without the drug.

Fig. 1.
Effect of several concentrations of phenylbutazone on the iodide transport by bovine thyroid slices as measured by T/M (tissue/medium) ratios. Drug concentrations are expressed in moles per litre. Each bar is the mean ± sd. The perchlorate group represented passive diffusion of iodide. P values (Student's t-test for difference between means of each phenylbutazone concentration against control) are also stated above each bar. N.S. = P > 0.05.
The homogenates underwent Pronase digestion (Protease Sigma type VI) during 16 h (Taurog & Howells 1966). The digest were chromatographed in butanol, acetic acid, water (4:1:2) by ascending paper chromatography. Chromatograms were counted for $^{131}I$ to obtain the percentage of the formed iodoamino acids.

**Enzyme preparations**

Several preparations were employed to obtain samples with peroxidase activity.

1. **Particulate fraction.** Bovine thyroid tissue was homogenized with 0.12 M KCl in 0.02 M sodium phosphate buffer, pH 7 (1:4 w/v) in a Potter-Elvehjem type homogenizer. The supernatant obtained after centrifugation at 700 g for 10 min was then centrifuged at 30 000 g for 60 min. The precipitate was re-suspended in 0.1 M sodium phosphate buffer, pH 7.

2. **Digitonin pseudosolubilized preparations.** Homogenates of the bovine glands were centrifuged at 30 000 g for 60 min. The precipitate obtained was re-suspended in a solution containing 1% digitonin in a 0.1 M sodium phosphate buffer, pH 7, and kept at 4°C for 30 min with continuous shaking. The digitonin treated suspension was centrifuged at 30 000 g for 60 min. The pseudosolubilized enzyme activity was found to be present in the supernatant (Nieponniszcze et al. 1969).

3. **Soluble peroxidase.** Pellets obtained from human and bovine glands were after centrifugation at 105 000 g during 1 h treated with sodium deoxycylate, trypsin and acetone. The soluble enzyme was further purified by precipitation at 45% of saturation with ammonium sulphate, followed by dialysis and chromatography through Sephadex G-200 equilibrated in 0.1 M sodium phosphate buffer (Nagasaka & DeGroot 1971).

**Assay of peroxidase activity**

Enzyme activity was assayed by 3 different methods.

1. **Iodide assay.** Formation of triiodide absorbing at 287.5 nm was measured with iodide and H$_2$O$_2$ as substrates. The final concentrations of iodide and H$_2$O$_2$ in the standard conditions were $1.33 \times 10^{-2}$M and $5.33 \times 10^{-4}$M, respectively, in 3 ml of the final volume. The reaction was started with the addition of H$_2$O$_2$ (Alexander 1962).

2. **Guaiacol assay.** Guaiacol peroxidation was measured by spectrophotometry at 470 nm, the absorption maximum of the oxidation product of guaiacol. The final concentrations of guaiacol and H$_2$O$_2$ in the standard conditions were $1.33 \times 10^{-2}$M and $5.33 \times 10^{-4}$M, respectively, in 3 ml of the final volume. The reaction was started with the addition of H$_2$O$_2$.

3. **Tyrosine iodinase assay.** This is based on formation of iodothyrosine when the enzyme is incubated with iodide, tyrosine, and H$_2$O$_2$ source, such as the glucose-glucose oxidase system. The activity was measured by $^{131}I$ incorporation into tyrosine. In some studies H$_2$O$_2$ was provided by direct addition. The final concentrations of H$_2$O$_2$ were $2.5 \times 10^{-4}$M (DeGroot & Davis 1962).

**Statistics**

All data were analyzed by Student's $t$-test.

**Results**

**Iodine transport**

An inhibition of iodide transport, expressed in terms of T/M ratios, was observed in slices incubated with high phenylbutazone concentrations, since $10^{-3}$M produced inhibition of 72%, whereas lower concentrations showed no significant differences from the controls (Fig. 1). Simple diffusion can be seen in the perchlorate group.

**Iodine organization**

Thyroidal proteinbound iodine was affected by $10^{-4}$M and $10^{-5}$M phenylbutazone with inhibitions of 72% and 51%, respectively (Fig. 2).

![Fig. 2.](image)

Effect of several concentrations of phenylbutazone on the iodine organization by bovine thyroid slices as measured by the percentage of $^{131}I$ bound to proteins. Drug concentrations are expressed in moles per litre. Each bar is the mean ± SD. $P$ values (Student's $t$-test for difference between means of each phenylbutazone concentration against control) are also stated above each bar.

N.S. = $P > 0.05$. 

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Fig. 3.
Effect of phenylbutazone in the iodothyrosine synthesis by bovine thyroid slices. The two lines (— and ——) show MIT and DIT formation at different concentrations of phenylbutazone. Upper and lower areas represent the normal formation of MIT and DIT, respectively. (⋯⋯) shows normal MIT/DIT ratio, while (— —) are the ratios obtained at different concentrations of phenylbutazone.

Fig. 4.
Effect of phenylbutazone in the iodothyronine synthesis by bovine thyroid slices. The solid line (——) shows iodothyronine formation at different concentrations of phenylbutazone. The area represent the normal formation of iodothyronines. (⋯⋯) shows normal iodityrosines /iodothyronines ratio, while (— —) are the ratios obtained at different concentrations of phenylbutazone.
The iodotyrosine synthesis was affected by $10^{-4} \text{M}$ and $10^{-5} \text{M}$ phenylbutazone (Fig. 3). Formation of iodothyronines was markedly affected between $10^{-4} \text{M}$ and $10^{-7} \text{M}$ phenylbutazone concentrations (Fig. 4).

**Thyroid peroxidase**

**Tyrosine iodinase assay.** Soluble, pseudosolubilized, and crude peroxidase preparations from bovine glands, as well as the soluble enzyme from human thyroids, have shown inhibition of tyrosine-iodinase activity when incubated with phenylbutazone in concentrations ranging from $10^{-3} \text{M}$ to $10^{-8} \text{M}$, with a $K_i$ (concentration causing a 50% inhibition) of $4 \times 10^{-6} \text{M}$ for bovine thyroid peroxidase (3 preparations average) and of $6 \times 10^{-6} \text{M}$ for human soluble peroxidase (Figs. 5 and 6). No difference in enzymatic inhibition was observed when the glucose-glucose oxidase system was replaced by direct addition of $\text{H}_2\text{O}_2$, with a $K_i$ of $4.5 \times 10^{-6} \text{M}$ and $6.5 \times 10^{-6} \text{M}$ for human and bovine soluble peroxidases, respectively.

**Triiodide assay.** Formation of triiodide was affected between $10^{-3} \text{M}$ and $10^{-8} \text{M}$ phenylbutazone concentrations. $K_i$ for human soluble peroxidase is $2 \times 10^{-6} \text{M}$ (Fig. 6).

**Guaiacol assay.** Guaiacol peroxidation was scarcely affected by the action of the drug (Fig. 7). $K_i$ for human soluble peroxidase is $7 \times 10^{-4} \text{M}$ (Fig. 6).
Oxidase during the Trotter Effect.

Scott (Green et al., 1955; 1973) explained that the Abiodun (Green et al., 1955) mechanism of iodide uptake.

During chronic administration of the drug, phenylbutazone may directly bind iodine, precluding its thyroidal uptake. During prolonged administration of phenylbutazone, a steady state would be reached, the iodide binding capacity being fully saturated; in this moment the uptake of iodide would be recovered. Linsk et al. (1957) have explained the fall of the $^{131}$I uptake produced by the drug as a direct inhibition of the TSH secretion; considering that the uptake inhibition is neutralized when the patient receives TSH and phenylbutazone at the same time. Hansen (1962) has observed an elevation of the percentage of dialyzable thyroxine and has related this finding to a possible increase of the free serum thyroxine produced by displacement of the thyroxine from its protein carrier, resulting in an inhibition of the TSH secretion and a fall of the $^{131}$I thyroid uptake. The previous hypothesis were weakened when Abiodun et al. (1973) observed that both the values of TSH and the absolute concentrations of free thyroxine were normal during the first week of treatment.

When phenylbutazone is prescribed at a daily dose of 800 mg, the blood concentration of the drug is about $2.66 \times 10^{-3}$M (Linsk et al., 1957). The 98% of phenylbutazone is bound to the serum proteins (Brodie et al., 1954), while the remaining 2%, which represents a blood concentration of $5.32 \times 10^{-3}$M, would be directly available for distribution in peripheral tissue.

Our studies in vitro on the active transport of iodide by thyroid slices have shown a 72% inhibition with $10^{-3}$M. Lower concentrations produced no significant differences from the controls. Consequently, it appears unlikely that there is a direct inhibition of the iodide transport by the drug in vivo. On the other hand, it is well-known that phenylbutazone impairs iodine organification since Morgans & Trotter (1955) have observed a diffuse goitre in a patient treated for 17 months with phenylbutazone, and this patient had a positive perchlorate test during the administration of the drug.

The studies from Abiodun et al. (1973) would support the hypothesis of a direct effect of the drug on the thyroid gland associated with a displacement of the thyroxine from its binding serum proteins, as suggested by the increase of the dialyzable percentage of thyroxine with low values of the total thyroxine, and normal values of the absolute concentration of free thyroxine and TSH in the first week of treatment.

After 14 days of treatment the investigators observed a significant decrease of the absolute concentration of free thyroxine while, the dialyzable percentage becomes normal, and the total thyroxine remains low, pointing out an aggravation of the thyroid failure, while TSH remains within the normal values.

According to our studies on the iodine organification, phenylbutazone inhibits the synthesis of both iodotyrosines and iodothyronines, with a
stronger effect on the latter. This coupling inhibition produces an increase of the iodotyrosines/iodothyronines ratio even at concentrations of \(10^{-7}\)M.

Our studies with thyroid peroxidase, an enzyme involved in the oxidation and incorporation of iodide to the tyrosil residues of thyroglobulin and the coupling of iodotyrosines, show the important inhibitory effect of phenylbutazone on peroxidase. This inhibitory effect would be more important on the coupling mechanism than on the other steps of the thyroid hormone biosynthesis. Phenylbutazone, in the tyrosine iodinase and triiodide assays, inhibits the peroxidase activity. The \(K_i\) value in the tyrosine iodinase assay was \(6 \times 10^{-6}\)M, and \(2 \times 10^{-6}\)M in the triiodide assay for soluble human peroxidase. Recently, Nagasaka & Hidaka (1976) have found \(K_i\) values for methimazol (MMI) inhibiting thyroid peroxidase that resemble those obtained in the present work with the phenylbutazone. However, in the guaiacol assay phenylbutazone has shown a slight inhibition, while MMI is a well-known inhibitor in this assay. For this reason it appears that phenylbutazone and methimazol, although both acting on thyroid peroxidase, have some differences in their mechanism of action. While the latter drug fully inhibits the enzyme activity phenylbutazone would only be able to block the enzymatic site of action, where iodide and probably tyrosine can bind it. Moreover, the coupling reaction seems to be the most vulnerable step in which phenylbutazone exerts its inhibitory effect on thyroid peroxidase.

To conclude, for the first time the inhibitory effect of phenylbutazone on thyroid peroxidase was demonstrated, and this type of inhibition is likely to explain the well-known antithyroid activity of phenylbutazone observed in clinical practice. Future studies on enzyme kinetics will allow the understanding of these effects and at the same time obtain additional data on the mechanism of action of this newly found thyroid peroxidase inhibitor.

References


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