The Mechanism of Inhibition of Horse Liver Alcohol Dehydrogenase by Thyroxine and Related Compounds*

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SUMMARY
Thyroxine and related compounds have been found to be excellent inhibitors of horse liver alcohol dehydrogenase. The thyroid hormones appear to act by interfering with the normal coenzyme-binding mechanism. Triiodothyronine and thyroxine are uncompetitive inhibitors with regard to the coenzyme. In addition to inhibiting the enzyme activity, the thyroid hormone also quenches the enhanced fluorescence of the bound NADH and greatly reduces the magnitude of a Cotton effect in the optical rotatory dispersion spectrum which is attributed to bound coenzyme. Little effect on the over-all binding of the coenzymes was observed. However, the current findings are consistent with an inhibition scheme in which the thyroid hormones interfere with the binding of the nicotinamide portion of the coenzyme.

EXPERIMENTAL PROCEDURE
Materials—L-Thyroxine, L-3,3',5-triiodothyronine, NAD, and NADH were obtained from Calbiochem. The purity of the pyridine nucleotides used in this study was estimated to be 85 to 90% based on the A260/A190 absorbance ratio of the completely reduced form. Acetaldehyde and 1,10-phenanthroline were purchased from Eastman, and L-3,3',5-triiodothyroacetic acid from Aldrich. Horse liver alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) was purchased as a crystalline suspension in 0.02 M phosphate buffer, pH 7, from Calbiochem. Examination of this material by Sephadex G-100 chromatography showed a single protein band with a specific activity of 6.5 as assayed by the method of Dalziel (6). The concentration of enzyme in solution was determined by measurement of the optical density at 280 nm based on an absorbance index of 0.455 mg−1 cm−1 (7). The value used for the molecular weight in these studies was 84,000 (8).

Assay—The enzyme activity was estimated by the typical spectrophotometric method of measuring changes in the reduced pyridine nucleotide at 340 nm. The reaction was started by the addition of enzyme to a cuvette, and the change in optical density at 340 nm occurring between 15 and 45 sec was recorded.

Electrophoresis—Gel electrophoresis was performed in 7.5% polyacrylamide gel at pH 9.5 and 6 ma per tube for 4 hours (9).
Equilibrium Dialysis—Dialysis of the enzyme was carried out in Visking dialysis tubing at 2°C with an enzyme volume of 1 ml and a dialysate volume of at least 100 ml. A standard dialysis time of 50 hours was used after it was first determined that triiodothyronine, triiodothyroacetic acid, and NADH all pass freely across the Visking casing. Enzyme concentrations were determined in the enzyme solution and in the dialysate by reading the absorbance at 328 mμ with the use of a millimolar extinction coefficient of 5.65 (10). Protein concentrations were determined in the enzyme solutions by the modified Folin method of Rabinowitz and Pricer (11). Ten micrograms of alcohol dehydrogenase in the above assay give an extinction of 0.041 in a cuvette of 1-cm optical depth.

Fluorescence Studies—Fluorescence studies were done in an Aminco-Bowman spectrophotofluorometer equipped with a Mosely X-Y recorder.

Optical Rotatory Dispersion—Studies were carried out with a Cary model 60 spectropolarimeter.

RESULTS

Inhibition Studies—Thyroxine and triiodothyronine are powerful inhibitors of liver alcohol dehydrogenase at pH 7.0. A 1 μM concentration of either thyroxine or triiodothyronine results in nearly 50% inhibition of the enzyme activity, as measured by reduction of acetaldehyde. Triiodothyroacetic acid is also an inhibitor of liver alcohol dehydrogenase; inhibition of acetaldehyde reduction by 50% occurs at a concentration of 50 μM. Fig. 1 shows the effect of inhibitor concentration on acetaldehyde reduction. The inhibition appeared to be both instantaneous and reversible, for the following reasons: (a) preincubation of liver alcohol dehydrogenase with triiodothyronine or triiodothyroacetic acid for 3 hours resulted in the same degree of inhibition as when the inhibitors were added immediately before the activity was measured; (b) when an enzyme solution containing 24 μM liver alcohol dehydrogenase and 140 μM triiodothyronine or 420 μM triiodothyroacetic acid was diluted 1:1500 for assay, there was no apparent inhibition due to the presence of the iodinated compound.

The mechanisms of the triiodothyronine and triiodothyroacetic acid inhibition, however, are completely different. As shown in Fig. 2, triiodothyroacetic acid appears to be a typical competitive inhibitor with respect to NADH. Conversely, triiodothyronine appears to be an uncompetitive inhibitor, altering both the cofactor $K_c$ and $V_{max}$ of the reaction (Fig. 3).

The iodothyronines are most effective when the phenolic hydroxyl is un-ionized and the amine and carboxyl groups are ionized, giving essentially a neutral molecule. The $pK_a$ of the triiodothyronine phenolic hydroxyl is 8.45, and for thyroxine it is 6.73 (12). As shown in Fig. 4, their effectiveness as inhibitors is markedly decreased when the pH is higher than these values.

Since thyroxine is known to exhibit to complex formation with Zn$^{++}$ (1) and liver alcohol dehydrogenase is a zinc-containing enzyme, experiments were done to determine whether the addition of Zn$^{++}$ would reverse the inhibition by triiodothyronine and triiodothyroacetic acid. It had been shown previously (13) that zinc will reverse the inhibition by 1,10-phenanthroline, a zinc chelator, which binds to the zinc site in liver alcohol dehy-
The inhibition of alcohol dehydrogenase by 15 μM triiodothyroacetic acid (●—●), 8 μM thyroxine (○—○), and 10 μM triiodothyronine (▲—▲). The assay system contained 4.8 mM acetaldelyde, 2.2 mM NADH, and 0.082 μM liver alcohol dehydrogenase in 3 ml of 0.2 M sodium phosphate buffer.

**TABLE I**

**Effect of ZnCl₂ on inhibition of liver alcohol dehydrogenase by triiodothyronine and triiodothyroacetic acid**

The assay mixture contained 0.1 M sodium phosphate buffer (pH 7), 130 μM NADH, 18 mM acetaldelyde, and 0.024 μM liver alcohol dehydrogenase in a total volume of 3 ml.

<table>
<thead>
<tr>
<th>Additions to assay mixture</th>
<th>A.D. 1 cm in 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.800</td>
</tr>
<tr>
<td>4.7 μM triiodothyronine</td>
<td>0.410</td>
</tr>
<tr>
<td>4.7 μM triiodothyronine + 210 μM ZnCl₂</td>
<td>0.410</td>
</tr>
<tr>
<td>60.5 μM triiodothyroacetic acid</td>
<td>0.330</td>
</tr>
<tr>
<td>60.5 μM triiodothyroacetic acid + 210 μM ZnCl₂</td>
<td>0.335</td>
</tr>
<tr>
<td>34 μM 1,10 phenanthroline</td>
<td>0.350</td>
</tr>
<tr>
<td>34 μM 1,10 phenanthroline + 210 μM ZnCl₂</td>
<td>0.700</td>
</tr>
</tbody>
</table>

The addition of Zn⁺⁺ to the reaction mixture (Table I) resulted in no change in the degree of inhibition by triiodothyronine, whereas under similar conditions Zn⁺⁺ reversed the inhibition seen with 1,10-phenanthroline. It would appear, therefore, that if triiodothyronine or triiodothyroacetic acid interact at the zinc site of the enzyme, the inhibitor has a much greater affinity for the bound zinc than for free Zn⁺⁺.

**Electrophoretic Studies**—Recent evidence indicates that liver alcohol dehydrogenase is composed of four polypeptide chains (14). Since thyroxine is known to cause dissociation of glutamic dehydrogenase into subunits (3), this possibility was investigated with liver alcohol dehydrogenase. The enzyme was dialyzed overnight against 1.5 μM thyroxine or triiodothyronine and was then subjected to electrophoresis on polyacrylamide gel. The protein was stained with 1% Amido black. There was no change in the electrophoretic mobility of the dialyzed liver alcohol dehydrogenase, nor was there any suggestion of dissociation into subunits with either thyroxine or triiodothyronine.

**Equilibrium Dialysis Studies**—Solutions of liver alcohol dehydrogenase (1 ml) were dialyzed against various concentrations of NADH in the presence and absence of the inhibitors, triiodothyronine and triiodothyroacetic acid. As can be seen in Fig. 5, in the absence of inhibitor liver alcohol dehydrogenase binds 2 moles of NADH per mole of enzyme. As would be expected from the kinetic studies, triiodothyroacetic displaces NADH from the enzyme while triiodothyronine does not. Since triiodothyronine does not appear to displace NADH from liver alcohol dehydrogenase, it is likely that triiodothyronine can form a ternary NADH-enzyme-triiodothyronine complex. A possible source of error in the equilibrium dialysis would arise if liver alcohol dehydrogenase interacts with the ionized form of triiodothyronine. Triiodothyronine has a molar extinction coefficient of 4089 at 295 nm. If the phenolic hydroxyl is ionized, the extinction maximum is shifted to 320 nm with a molar extinction...
Fig. 6. The quenching of the fluorescence of the binary liver NADH-alcohol dehydrogenase complex by triiodothyronine. The assay solution contained 2.0 μM enzyme, 3.1 μM NADH, and various amounts of triiodothyronine in 0.1 M sodium phosphate buffer, pH 7, in a 1-cm cuvette. Curve 1, enzyme and NADH; Curve 2, enzyme, NADH, and 2.5 μM triiodothyronine; Curve 3, enzyme, NADH, and 5.0 μM triiodothyronine; Curve 4, enzyme, NADH, and 7.5 μM triiodothyronine. Unlabeled curves show the effect of increasing amounts of triiodothyronine up to 50 μM. Curve 6 shows the fluorescence of an equivalent amount of NADH in the absence of enzyme. Triiodothyronine has no effect on the fluorescence of free NADH. The wave length of the excitation light was 328 μm.

Coefficient of 4685 (12). Table II shows that at pH 7 liver alcohol dehydrogenase interacts with only the un-ionized form of triiodothyronine, which has little or no absorbance at 328 μm.

Fluorescence Studies—NADH exhibits fluorescence with an emission maximum at 462 μm when irradiated by light in the 340 μm region. When NADH is bound to liver alcohol dehydrogenase, it can be activated at either 328 μm or 280 μm (15). The emission maximum of bound NADH is shifted from 462 to 440 μm and is increased in intensity (15). Triiodothyronine quenches the enhanced fluorescence of bound NADH when activated at 328 μm, whereas it exerts no effect on the fluorescence of free coenzymes (Fig. 6). Furthermore, the quenching of the NADH-liver alcohol dehydrogenase complex by triiodothyronine appears to be independent of NADH concentration. A large molar excess of NADH does not reverse the quenching of the bound NADH (Fig. 7). Table III shows that a definite correlation exists between the ability of an iodinated compound to quench the enhanced fluorescence of the binary NADH-liver alcohol dehydrogenase complex and its ability to inhibit the holoenzyme.

Optical Rotatory Dispersion Studies—Ulmer, Li, and Vallee (16) made extensive use of optical rotatory dispersion in studying the binding of NADH and 1,10-phenanthroline to liver alcohol de-
The optical rotatory dispersion spectrum of liver alcohol dehydrogenase in the spectral range of 220 to 440 nm shows a single negative trough at 233 nm (Fig. 8), with a specific levorotation of 2720°. This is in good agreement with the specific levorotation of 2650° reported by Ulmer and Vallee (17) and is typical of helical proteins. Addition of triiodothyronine (6 moles per mole of liver alcohol dehydrogenase) results in little change in the over-all dispersion curve, indicating little or no change in the conformation of the protein. Examination of the spectral curve in the range of 290 to 390 nm on an expanded scale, however, shows a slight shift in the rotation, the maximum effect being at 315 nm (Fig. 9A). The degree of helicity in the presence and absence of triiodothyronine was therefore determined with the Moffitt-Yang equation. Fig. 10 shows the linear Moffitt-Yang plots obtained for the native enzyme and the enzyme in the presence of triiodothyronine. A λs value of 212 nm was assumed, and b0 was obtained from the slope by use of the equation

\[ b_0 = \frac{\text{mean residue weight} \times (\pi^2 - 3) \times 100(2)}{\text{slope}} \]

A mean residue weight of 108 was used (18). It would appear from Fig. 10 that triiodothyronine does not alter the helical content of liver alcohol dehydrogenase.

The addition of 2 moles of NADH per mole of enzyme is known to result in the appearance of a single negative Cotton effect at 327 nm (16). This phenomenon is also shown in Fig. 9A. The

\[ \Delta [\beta]_\lambda^\infty \]

A, effect of triiodothyronine upon the optical rotatory dispersion of the binary liver NADH-alcohol dehydrogenase complex. The spectra were taken in a 30-mm cuvette with a protein concentration of 1 mg per ml in 0.1 M sodium phosphate buffer, pH 7. Curve 1, enzyme and 25 μM NADH; Curve 2, enzyme, 25 μM NADH, and 75 μM triiodothyronine; Curve 3, enzyme and 75 μM triiodothyronine; Curve 4, enzyme. B, constructed difference optical rotatory dispersion of the binary liver NADH-alcohol dehydrogenase complex in the presence and absence of 75 μM triiodothyronine. Curve 1, difference between Curves 1 and 4 of A; Curve 2, difference between Curves 2 and 3 of A; Curve 3, an intermediate value of triiodothyronine (36 μM) not shown in A.
addition of triiodothyronine to this binary complex results in a diminution of the Cotton effect. This can be seen more clearly by the difference spectrum constructed in Fig. 9B.

The zinc chelator 1,10-phenanthroline is known to be bound at the zinc moiety of liver alcohol dehydrogenase to give an optically active liver alcohol dehydrogenase-zinc-1,10-phenanthroline chromophore (16).

The optical rotatory spectrum of the chromophore shows a peak at 297 μm (Fig. 11A). Upon titration of the chromophore with triiodothyronine, the peak at 297 μm disappears, and a new peak appears at 313 μm (Fig. 11A). This also is more clearly seen in the difference spectrum (Fig. 11B). Although the nature of this new peak is not understood, it indicates that triiodothyronine alters the ternary liver alcohol dehydrogenase-zinc-1,10-phenanthroline chromophore by a mechanism other than direct displacement of 1,10-phenanthroline from the zinc-liver alcohol dehydrogenase.

**DISCUSSION**

Thyroxine, triiodothyronine, and triiodothyroacetic acid are all instantaneous and reversible inhibitors ofhorse liver alcohol dehydrogenase. Triiodothyronine is a more potent inhibitor of activity than triiodothyroacetic acid. The concentration required for 50% inhibition is about 1 μM for triiodothyronine as compared to about 50 μM for triiodothyroacetic acid. Inhibition by the triiodothyronines is also pH-dependent and is most effective when the phenolic hydroxyl groups are un-ionized. Although the hydroxyl groups of triiodothyronine and triiodothyroacetic acid are both un-ionized at pH 7, the mechanism of inhibition is entirely different for the two compounds. The log dose-response curves of Fig. 1 suggest that triiodothyronine binds at multiple sites which have different affinities for the inhibitor, while triiodothyroacetic acid would appear to bind at only one site. Furthermore, triiodothyroacetic acid is a competitive inhibitor of liver alcohol dehydrogenase with respect to NADH, while triiodothyronine appears to be uncompetitive with respect to NADH. Triiodothyronine and triiodothyroacetic acid both quench the enhanced fluorescence of the binary NADH-liver alcohol dehydrogenase complex. The equilibrium dialysis and kinetic studies indicate that the quenching by triiodothyroacetic acid can be explained as a displacement of the coenzymes from the protein by triiodothyroacetic acid.

The inhibition of liver alcohol dehydrogenase by triiodothyronine presents a more complex and interesting model for study. Both the optical rotatory dispersion and fluorescence studies indicate that triiodothyronine may displace NADH from liver alcohol dehydrogenase, while the equilibrium dialysis and kinetic studies suggest that triiodothyronine does not compete with NADH for a binding site on the protein. An alternative hypothesis would be that triiodothyronine can form a ternary NADH-enzyme-triiodothyronine complex which is catalytically inactive and in which the nicotinamide moiety of the bound NADH shows no enhanced fluorescence.

Theorell, Ehrenberg, and deZalenski (5) have recently proposed that NADH binds to liver alcohol dehydrogenase by a two-step mechanism. This proposition was based on x-ray analysis in Branden’s laboratory (19) and on stop-flow fluorescent studies (6). Although the latter experiments proved to be inaccurate (20), and Gerachi and Gibson (21) failed to obtain kinetic data to support such a mechanism, it is of interest to speculate on the current results in the light of a two-step mechanism. In Theorell’s model, the adenosine diphosphate ribose moiety of NADH binds to the enzyme in a site distinct from the nicotinamide-binding site. This binary complex is catalytically inactive, and the nicotinamide moiety shows no enhanced fluorescence. It is believed, however, that the binding of the adenosine diphosphate ribose moiety to the enzyme induces conformational changes within the enzyme, which then allow the nicotinamide moiety of the bound NADH to bind at or near the zinc of the enzyme. This process is accompanied by an enhancement of the fluorescence of the nicotinamide moiety of the
bound NADH and the formation of a catalytically active holoenzyme.

Consideration of the aforementioned hypothesis in conjunction with the current study suggests a possible mechanism for the inhibitory properties of the thyroid hormones. This mechanism is proposed in Scheme 1. Part A schematically represents the two-step binding process for NADH as proposed by Theorell et al. (5). As proposed in Part B, however, the presence of triiodothyronine may prevent the formation of the holoenzyme by not permitting the second step of the binding process. The over-all binding of the coenzyme probably depends on the first step, i.e. the binding of the adenosine diphosphate ribose portion of the NADH, since it is believed that this step has a much lower dissociation constant (5). Triiodothyronine does not appreciably affect this step, as determined by equilibrium dialysis and the lack of competitive inhibition with regard to the coenzyme. The enzyme, however, in the presence of bound thyroid hormone, the nicotinamide portion of the NADH may not bind. The inhibitor either may prevent the conformation change necessary for the nicotinamide binding or may block the binding site on the protein. Since the second step is necessary for both the enhanced fluorescence and the catalytic activity of the holoenzyme, such a scheme would be consistent with the inhibition of activity, the quenching of the enhanced fluorescence, and the changes in the optical activity of the bound NADH. It has been proposed (22-24) that the nicotinamide-binding site is at or near the zinc of the enzyme molecule. It is therefore significant that the thyroid hormones altered the optical rotatory dispersion of the enzyme-zinc-phenanthroline complex. This observation is consistent with the proposed mechanism of inhibition and the role of the enzyme-bound zinc in the coenzyme-binding mechanism.

It should be noted that in general the proposed inhibition mechanism need not be limited to a two-step coenzyme-binding process. The results presented would also be consistent with the single step coenzyme-binding mechanism, if the two steps of Scheme 1A are pictured as occurring as simultaneous reaction.

\[
\text{Enzyme} + \text{ADPR-NA} \rightarrow \text{enzyme-ADPR-NA} \rightarrow \text{ADPR-NA} \quad (A)
\]

\[
\text{Enzyme} + \text{triiodothyronine} + \text{ADPR-NA} \rightarrow \text{triiodothyronine ADPR-NA} \quad (B)
\]

**SCHEME 1.** ADPR-NA = NADH

**REFERENCES**

The Mechanism of Inhibition of Horse Liver Alcohol Dehydrogenase by Thyroxine and Related Compounds
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