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# INHIBITORY EFFECT OF THYROXINE ON CARBONIC ANHYDRASE B ISOZYME BIOSYNTHESIS IN RABBIT RETICULOCYTE LYSATES

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SUMMARY

Biosynthesis of rabbit red cell carbonic anhydrase isozyme B and C was demonstrated in reticulocyte cell-free lysates by the specific immunoprecipitin reaction. Using this homologous protein synthesis system, it was found that $10^{-5}$ to $10^{-7}$ M thyroxine preferentially inhibited the synthesis of carbonic anhydrase B isozyme without affecting that of C isozyme. These results suggested that this inhibitory action of the protein synthesis by thyroxine may be responsible for the decreased level of the B type isozymes in human hyperthyroidism or experimental hyperthyroidism of rabbits.

Red cell carbonic anhydrase isozymes were designated as the B and C types according to the immunochemical (1,2) and genetic (2,3) criteria. Although the level of carbonic anhydrase B varies under some pathological and physiological conditions (4-12), no significant changes occur in the level of carbonic anhydrase C. Many authors reported that the B isozyme level is decreased in hyperthyroid states and increased in hypothyroid states (6,9,10,11). However in experimental animals no inverse relationship between the quantities of carbonic anhydrase B and thyroxine had been confirmed (12). Previous study in our laboratory demonstrated that in experimentally induced thyrotoxicosis of rabbits, the level of carbonic anhydrase B decreased with the increase in the level of thyro-

1) To whom correspondence should be addressed. Abbreviation, HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Definition, $A_{280}$ unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 280 nm.

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xine while the C type did not vary significantly (7). The mechanism of the regulation of the isozyme levels has not been studied as yet. The biosynthesis of hemoglobin by the reticulocytes has been extensively studied and it is well known that hemoglobin is over 90 % of the protein synthesized by reticulocytes. These cells do not contain a nucleus and make no RNA and hence any regulation of protein synthesis must occur at the level of translation. The most abundant of the other red cell proteins is the non-heme protein, carbonic anhydrase, but only a limited number of experiments have been published on the biosynthesis of carbonic anhydrase. Edwards (13), Meyers et al (14) and Desimone et al (15) reported that the reticulocytes of man and the pig-tailed macaque synthesize the carbonic anhydrase B and C isozymes. Stephan et al (16) reported the effect of thyroxine on the biosynthesis differed significantly between the B and C type. However the inhibitory effect of thyroxine on the synthesis of the B type was not clearly demonstrated. The rabbit red cell also contains two major types of isozyme designated as the B type and C types, only one of which is seemed to be sensitive to thyroxine as reported previously (7). Therefore the aim of the present study was to elucidate whether or not the synthesis of rabbit B isozyme is regulated by the level of thyroxine in vitro system. A possible explanation for the regulation of the B isozyme at the level of synthesis is discussed in relation to the level of thyroxine.

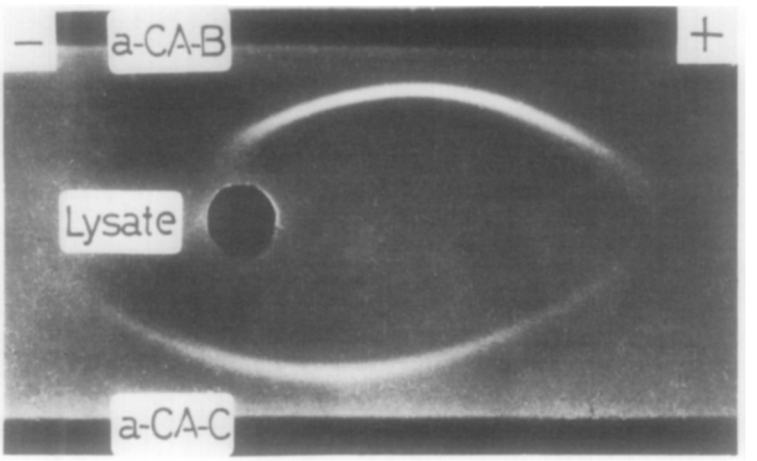
## MATERIALS AND METHODS

Reagents; U-[$^{14}$C]-L-serine was purchased from the Radiochemical Center(Amersham). Na-L-thyroxine was obtained from Sigma Chemical Co. ATP, GTP, dithiothreitol and creatine phosphokinase were obtained from Boehringer Mannheim Yamanouchi Co. Ltd. Japan. Purification of isozymes; Carbonic anhydrase isozymes were purified from rabbit erythrocytes by the method employed for horse enzymes by Deutsch et al (17) with slight modifications described previously (7). Each isozyme obtained gave a single band for

disc gel electrophoresis and starch gel electrophoresis. Antisera; γ-Globulin was prepared by DEAE cellulose chromatography by the method of Fahey (18). γ-Globulin thus obtained contained 20-40 unit per ml with a $A_{280}$ : $A_{260}$ ratio of 0.53. One $A_{280}$ unit of γ-globulin would precipitate 10 and 20 ug of rabbit carbonic anhydrase B and C respectively at the equivalent point. Immunoelectrophoresis: This was carried out on 1.2 % agarose gel in 0.05 M barbital buffer, pH 8.6. Reticulocyte lysates: Reticulocytes were obtained from male rabbits given 5 daily injections of neutralized phenylhydrazine after one day rest. Reticulocyte rich blood was collected in heparin by cardiac puncture. The cells were washed three times with a solution containing 145 mM NaCl, 5 mM KCl and 15 mM magnesium chloride and centrifuged at 12,000 g for 15 min and lysed for 10 min by dilution with 1.5 fold volumes of 3 mM glutathione in water. Carbonic anhydrase synthesis in reticulocyte extracts: The standard reaction mixture contained 1.8 ml of reticulocyte lysate, 2.5 umoles of ATP, 0.5 umole of GTP, 150 umoles of KCl, 20 umoles of dithiothreitol, 30 umoles of HEPES, pH 7.5, 50 umoles of magnesium acetate, 5 uCi of U-[$^{14}$C]-L-serine, 0.3 ml of 19 amino acids mixture (19) except serine (0.4 umole of each) and with or without $10^{-5}$ to $10^{-8}$ M Na-L-thyroxine, which had been previously solubilized in 0.1 ml of 0.01 N NaOH. Reaction mixtures were incubated in polypropylene centrifuge tubes on a mechanical shaker at 30 °C. Aliquots of 0.1 ml were taken at intervals into one ml plastic tube containing 0.1 ml of 10 mM L-serine and immediately mixed with 0.1 ml of specific anti-carbonic anhydrase B or C γ-globulin. The contents were allowed to stand at 37 °C for one hour and then at 4°C overnight. The precipitates were removed by centrifugation at 10,000 g for 10 min. The supernatant was withdrawn completely. The precipitates were broken up with a small glass rod, and the ice cold 145 mM NaCl was added and centrifuged again. The precipitated protein was washed with cold 145 mM NaCl for more three times and then with ice cold water three times. The washed precipitate was suspended in 500 ul of Soluen TM 100 (Packard) and heated at 60°C for 10 min in a small glass tube. The tube was placed directly in a scintillation counter vial and added 10 ml of dioxan based scintillator by Bray (20). After mixing well, the vials were counted in a ALOKA Scintillation Counter (ALOKA Co Ltd., Japan) with an efficiency of 80 %. The amount of radioactivity precipitated by the control γ-globulin was subtracted from the amount precipitated by anti-rabbit carbonic anhydrase B or C.

## RESULTS

The specificity of antisera was checked by immunoelectrophoresis. The immunoelectrophoresis gave a single precipitin arc between the purified carbonic anhydrase B and anti-carbonic anhydrase B as well as between the purified carbonic anhydrase C and anti-carbonic anhydrase C goat serum. A single precipitin arc was also observed between reticulocyte lysate and the specific antisera of B type as well as the C type as shown in Figure 1. The

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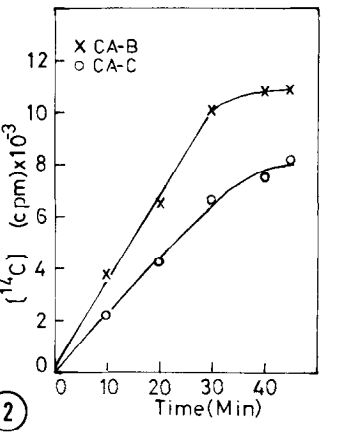


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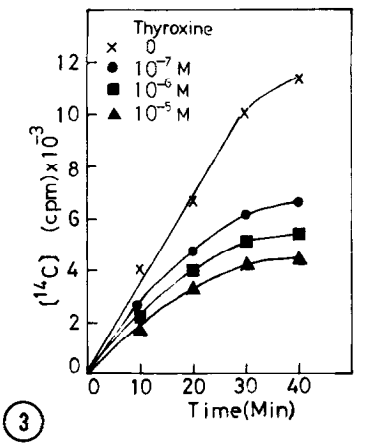
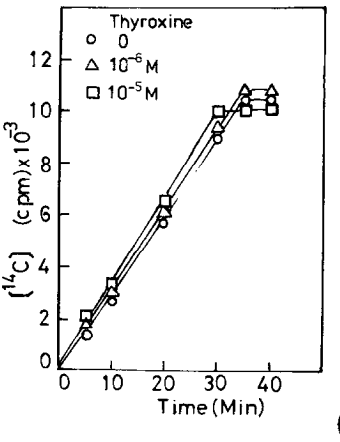
Figure 1. Immunoelectrophoresis of rabbit carbonic anhydrase B and C. a-CA-B; anti-carbonic anhydrase B goat serum, a-CA-C; anti-carbonic anhydrase C goat serum, Lysate; rabbit reticulocyte lysate.

Figure 2. The effect of time on the incorporation of [$^{14}$C]-L-serine into carbonic anhydrase isozymes.

formation of carbonic anhydrase isozymes in reticulocyte lysates as a function of time is shown Figure 2; up to 30 min a linear increase in the [$^{14}$C]-L-serine incorporation was observed for each isozyme. At temperatures above 30°C the incorporation was not linear with time. The incorporation of [$^{14}$C]-L-serine was inhibited almost completely in the presence of puromycin at a concentration of 1 mM or at low temperature at 4°C. The facts support the de novo synthesis of these isozymes. The effect of thyroxine on the rate of [$^{14}$C]-L-serine incorporation into the B and C isozymes was studied at the initial phase of the curves. As shown in Figure 3, the rate of [$^{14}$C]-L-serine incorporation into the B isozyme was inhibited with increasing thyroxine concentration. However the incorporation of [$^{14}$C]-L-serine into C isozyme was not affected even at concentrations above $10^{-5}$ M. The initial rate of biosynthesis of the B isozyme as a function



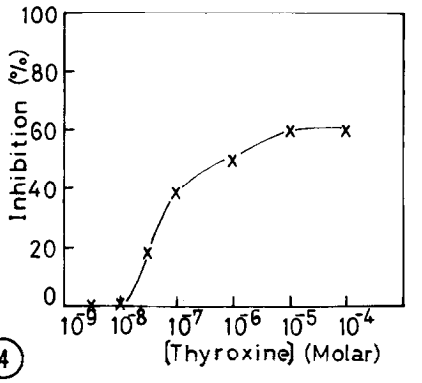




Figure 3. $[^{14}C]$-L-serine incorporation into carbonic anhydrase isozyme after addition of thyroxine to rabbit reticulocytes. Incorporation of $[^{14}C]$-L-serine into B type isozyme (left hand panel) and C type (right hand panel).

Figure 4. Concentration dependence of the inhibition of the biosynthesis of B isozyme by thyroxine. The inhibition of the rate of incorporation into B type isozyme was tested at varying concentrations of thyroxine in the assay system as described in Methods using a 30 min incubation.

of the concentration of added thyroxine is shown in Figure 4. At a concentration as low as $10^{-7}$ M there was a considerable decrease of $[^{14}C]$-L-serine incorporation into the B isozyme. In these experiments the reticulocytes were incubated with thyroxine for 3 min prior to the addition of $[^{14}C]$-L-serine. In other experiments thyroxine and $[^{14}C]$-L-serine were added simultaneously and in both experiments the inhibition of carbonic anhydrase B biosynthesis was observed following the initial rate as well as the rate at 10 to 15 min after the addition of thyroxine.

## DISCUSSION

The present study describes immunochemical methods capable of analyzing the biosynthesis of carbonic anhydrase B and C type, and demonstrates that rabbit reticulocyte cell-free extracts

synthesize carbonic anhydrase B and C. Each isozyme was purified from rabbit red cells and monospecific antibody against each isozyme was used for the specific incorporation of [$^{14}$C]-L-serine into each isozyme. The function and genetic interpretation of the presence of isozymes are not clearly understood, and this technique may provide a useful approach to the physiological significance of the isozymes. Furthermore, a limited number of experiments have been reported on the regulation of isozymes at the level of biosynthesis. Thyroxine in most cases has an inducing effect on protein synthesis both in vitro and in vivo, as reported by Krause and Sokoloff (21). The observed inhibition of the rate of synthesis by thyroxine clearly involves post-transcriptional events in either synthesis or degradation of carbonic anhydrase isozymes. Desimone et al (15) reported that there was no evidence of degradation of carbonic anhydrase isozymes within reticulocytes during a chase of more than 10 hours. Assuming that the degradation of nascent isozymes was not observed at the initial phase of incubation, it is postulated that one stage of biosynthesis is rate limiting in the presence of thyroxine. The present study is considered to be the first to indicate clearly in an in vitro system that thyroxine inhibits the rate of synthesis of carbonic anhydrase B. The results are consistent with the observations reported from our laboratory on the experimental thyrotoxicosis of rabbits, in which an inverse correlation between the quantities of serum thyroxine and rabbit carbonic anhydrase B was observed. The primary site of inhibition in the process of carbonic anhydrase biosynthesis is under study in this laboratory.

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