PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 143–146 (1976)

Thyroid Status and Carbonic Anhydrase Levels in Mouse Erythrocytes[1] (39497)

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Mammalian carbonic anhydrase occurs in two forms, carbonic anhydrase I (CA I) and carbonic anhydrase II (CA II), which appear to be the products of two closely linked genetic loci (1). The two isoenzymes differ in a number of ways; in particular, the hydration of CO_2 is catalyzed more rapidly by CA II than it is by an equivalent amount of CA I (2). In human erythrocytes, carbonic anhydrase levels are influenced by the levels of thyroxine in the plasma. In hyperthyroid states the concentration of CA I is decreased and in hypothyroid individuals it is increased (3–9). To date, only a limited number of reports has appeared concerning the effects of thyroid status on erythrocyte carbonic anhydrase in experimental animals (10–12). In this report, we describe an experimental model wherein alterations of thyroid status produce marked changes in erythrocyte CA I concentrations of the house mouse, *Mus musculus*.

Materials and Methods. Experimental rationale and animals. Our experiments involved the manipulation of thyroid status in mutant and normal mice and the assessment of changes in erythrocyte CA I and CA II levels. DW/J is an inbred strain segregating for the recessive gene *dw*; homozygous *dw/dw* mice are panhypopituitary dwarfs. Hypothyroidism has been documented in this mouse (13). To show that carbonic anhydrase alterations in *dw/dw* mice were due to the hypothyroidism, some DW/J *dw/dw* mice were fed thyroid powder. Since 0.066% (w/w, i.e., weight of supplement/weight of food) thyroid powder in a diet is sufficient to prevent goiter formation in mice on a low iodine diet (14), this quantity should be the replacement dose for a severely hypothyroid mouse. It has previously been shown that DW/J *dw/dw* mice respond to thyroid hormone by an increase in weight (13), and this was also observed here.

C57BL/6J was chosen as a standard laboratory strain. A 0.1% (w/w) thyroid powder dietary supplement was arbitrarily chosen as a dose likely to produce hyperthyroidism. Propylthiouracil (0.1%) has been used previously in mice to produce hypothyroidism (14).

Two sets of experiments were performed, each with several groups of mice. In the first set, one group of six C57BL/6J females was kept on its regular diet (Jax 234 pellets, Old Guilford) while another group of six C57BL/6J females was fed the same diet supplemented with 0.1% thyroid powder (USP). For each group, the pellets were ground to a powder and mixed with the appropriate supplement in both sets of experiments. A final group of six C57BL/6J females was fed its regular diet supplemented with 0.1% (w/w) propylthiouracil.

In the second set of experiments, DW/J mice were used. Six DW/J ?/+ mice (three females and three males) and six DW/J *dw/dw* (three males and three females) were fed Jax 234 pellets, while seven DW/J *dw/dw* (three females and four males) were fed this diet supplemented with 0.066% thyroid powder (USP).

Feeding experiments were performed at the Jackson Laboratory, which is fully accredited by the American Association of Laboratory Animal Care. C57BL/6J mice were obtained from production colonies and DW/J mice were from the Mouse Mutant Stock Center. All mice were 2 to 3 months old at the start of the experiments. At the end of 46 days, the mice were exsanguinated.

Qualitative assessment of carbonic anhydrase. A sample of the blood from each

[1] This work was supported by grants to R. E. T. (NIH GM-15419), to Dr. Elizabeth Russell (NIH CA-01074), and to Dr. Samuel Boyer (NIH 5R01-HL15026-09).

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animal was withdrawn and hemolysates were prepared from washed cells and examined by electrophoresis on Titan III cellulose acetate strips (Helena Laboratories) in a pH 9.1 buffer (25.2 g of Tris, 2.5 g of EDTA (acid) and 1.9 g of boric acid per liter) for 30 min at 300 V at room temperature. Protein was stained with Ponceau S. Since electrophoretic patterns from mice within a given treatment group appeared the same, blood from individuals of a group was pooled. Although no sex differences were visible, blood from males and females was pooled separately. Hemolysates were prepared and frozen at −80° for later quantitative measurement of each carbonic anhydrase.

Quantitative assessment of carbonic anhydrase. Concentrations of CA I and CA II were measured by radial immunodiffusion analysis, with the use of a modification of the technique described by Mancini (15). Antisera specific for mouse CA I and CA II were prepared by immunization of rabbits with purified enzymes. Mouse CA I and CA II were isolated from pooled hemolysates by chromatography on a CM-32 cellulose column equilibrated with 0.02 *M* phosphate buffer, pH 6.6. After addition of the hemolysate to the column, CA I and CA II were eluted with a linear 0 to 0.2 *M* NaCl gradient. Specific CA I and CA II antisera were prepared by injecting rabbits with the purified preparations of CA I and CA II as previously described (16). Specificity was established by showing the absence of crossreactivity between each carbonic anhydrase and antisera prepared against the other isozyme. An agarose solution was prepared by dissolving 0.225 g of agarose (MCI Biomedical, Rockland, Maine) in 14.625 ml of borate saline [prepared by mixing 1 vol of 0.125 *M* fused boric acid (B_2O_3), 0.075 *M* sodium chloride with approximately 0.5 vol of 0.125 *M* sodium borate ($Na_2B_4O_7 \cdot 10H_2O$), 0.075 *M* sodium chloride until the pH was 8.37] containing 0.1 mg/ml of thimerosal in a boiling water bath. After cooling the agarose solution to 50°, 0.375 ml of antisera was heated to 50° and quickly mixed with it. The resulting solution was quickly poured into the bottom (i.e., the wrong side) of an immunodiffusion plate (Miles Laboratories, No. 42-150-1), uniformly distributed by tilting the plate, and allowed to cool to room temperature. The plate was allowed to sit overnight in a water-saturated atmosphere at room temperature. Excess fluid was then removed and wells of about 10-μl vol were cut with a trochar (No. 10, Becton-Dickinson). The agarose plug was rimmed and removed with a 25-gauge syringe needle. Eight microliters of sample (standard or unknown) was placed in the wells. Radii of precipitin rings were measured after a 3-day incubation at room temperature in a water-saturated atmosphere. Measurement was facilitated by the use of a dissecting microscope fitted with an eyepiece reticle. A standard series of purified mouse CA dilutions was included in each plate. A regression line of precipitin ring area on carbonic anhydrase concentration of the standards was fitted to these data and used to calculate carbonic anhydrase concentration in the samples. This value was divided by the hemoglobin concentration of the hemolysates (determined by the absorption of cyanmethemoglobin at 545 nm) and the results were reported as μg of CA/mg of hemoglobin. Multiple determinations were made for each experimental group.

Results. The results are shown in Table I. The experiments with C57BL/6J and DW/J mice were analyzed by one-way and two-way analysis of variance, respectively. The only significant results were treatment effects for CA I. Erythrocyte CA I concentrations in control C57BL/6J mice were significantly less than those in C57BL/6J mice fed propylthiouracil ($P < 10^{-4}$) and significantly greater than those in C57BL/6J mice fed thyroid powder ($P < 10^{-2}$). Erythrocyte CA I concentrations in untreated DW/J *dw/dw* mice were significantly greater than those in DW/J +/? mice ($P < 10^{-7}$) and those in DW/J *dw/dw* mice fed thyroid powder ($P < 10^{-8}$). These multiple comparisons are not statistically independent (i.e., orthogonal).

Discussion. The data show that hypothyroidism, whether produced genetically or pharmacologically, elevates CA I levels. Moreover, feeding genetically hypothyroid mice thyroid powder lowered their elevated CA I levels. Similarly, thyroxine injections

TABLE I. EFFECT OF THYROID POWDER AND PROPLYTHIOURACIL (PTU) ON ERYTHROCYTE CARBONIC ANHYDRASE ISOZYMES, CA I AND CA II, IN DIFFERENT MOUSE STRAINS.

Strain	Genotype[a]	Supplement	Sex	CA I[b] (μg/mg Hb)	CA II[b] (μg/mg Hb)
C57BL/6J	+/+	None	Female	3.7 (3.4-4.0)	7.1 (6.2-7.8)
C57BL/6J	+/+	PTU[c]	Female	7.2 (6.0-8.4)	7.1 (6.8-7.3)
C57BL/6J	+/+	Thyroid	Female	1.9 (1.6-2.1)	7.4 (6.7-8.3)
DW/J	+/?	None	Male	3.5 (3.2-3.9)	6.1 (5.8-6.4)
DW/J	+/?	None	Female	2.7 (2.4-3.1)	6.2 (5.9-6.4)
DW/J	*dw/dw*	None	Male	7.8 (6.2-8.8)	5.9 (5.8-6.0)
DW/J	*dw/dw*	None	Female	7.9 (6.3-9.1)	5.7 (5.6-5.9)
DW/J	*dw/dw*	Thyroid	Male	2.1 (2.0-2.1)	5.7 (5.3-5.9)
DW/J	*dw/dw*	Thyroid	Female	1.7 (1.6-1.8)	6.0 (6.0-6.1)

[a] Refers to genotype at *dw* locus.
[b] Values given are means of three or four determinations. Values in parentheses refer to the range of determinations on the pooled samples.
[c] Propylthiouracil.

have been reported to decrease erythrocyte CA I levels in the rabbit (10). On the other hand, the injection of thyroxine for up to 34 weeks into pig-tailed macaques (*Macaca nemestrina*) failed to produce any reduction in red cell carbonic anhydrase levels (12). In experiments where the synthesis of CA I and CA II was followed in reticulocytes of the rhesus macaque (*Macaca mulatta*) and *M. nemestrina*, no alteration in the translation of CA II or two allelic forms of CA I was observed on addition of thyroxine to the reticulocyte systems (12, 16). It was of interest, however, that the synthesis of one genetic variant of CA I in *M. nemestrina* was reduced with thyroxine treatment (2). In the present study, mouse red cell CA II levels did not appear to be altered by any of the test procedures, a finding which seems to be in contrast to an apparent positive correlation between the altered levels of human red cell CA I and CA II in hyper- and hypothyroid states (3, 6, 7, 10). This difference between the effect of thyroxine on CA II in mice and humans may be due to differences in their regulatory control mechanisms.

The effects described here are probably submaximal since studies in man indicate that alterations in erythrocyte carbonic anhydrase concentrations occur during formation and/or maturation of the erythrocyte (4). By analogy, maximal results should appear after 60 days, the lifetime of a mouse erythrocyte, slightly later than the 46 days of treatment used here. Furthermore, different dietary protocols might produce larger alterations in erythrocyte CA I.

Summary. Carbonic anhydrase I (CA I) levels in mouse erythrocytes are influenced by thyroid status, while carbonic anhydrase II (CA II) levels appear to be unaffected. Adding propylthiouracil to the diets of normal mice (C57BL/6J) resulted in elevated erythrocyte CA I levels, while thyroid powder produced decreased levels. Panhypopituitary mutants (DW/J *dw/dw*) have increased levels of erythrocyte CA I in comparison to their normal litter mates (DW/J +/?). These increased levels were decreased by adding thyroid powder to their diets.

We are indebted to Drs. Elizabeth Russell and Samuel Boyer for providing research facilities and comments on the manuscript, to Drs. Wes Beamer and Gary Chase for advice, and to Ms. Sharon K. Stroup for research assistance.

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Received April 28, 1976. P.S.E.B.M., 1976, Vol. 153.