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Human Serum Dopamine β-Hydroxylase: Correlation of Enzymatic Activity with Immunoreactive Protein in Genetically Defined Samples

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INTRODUCTION

Dopamine β-hydroxylase (DBH; E.C.1.14.17.1) catalyzes the conversion of dopamine (3,4-dihydroxyphenylethylamine) to norepinephrine [1]. It is localized to catecholamine-containing vesicles in the adrenal medulla and sympathetic nerves [2, 3]. Since DBH is released with catecholamines from sympathetic nerves and the adrenal medulla [4–8] and is found circulating in blood [9], it has been suggested that serum DBH activity might be a useful measure of sympathetic nervous system function in man [9, 10].

There is a wide variation of human serum DBH activity in blood samples obtained from a randomly selected population [11, 12]. Genetic factors appear to be of importance in the determination of serum DBH activity. There is a significant sibling-sibling correlation of this circulating enzyme activity [12], and the correlation coefficient is greater in blood samples obtained from monozygotic than dizygotic twins [13]. In addition, the results of population and family studies are compatible with the existence of an allele for very low serum DBH activity, (< 50 U), that is inherited in an autosomal recessive manner [14]. Obligate heterozygotes for this allele have serum enzyme values between those of homozygotes and those from a randomly selected population [14]. The decreased DBH activity in the blood of subjects who carry this allele is not due to different levels of endogenous enzyme inhibitors or activators [14].

Genetically mediated differences in enzymatic DBH activity in human serum might be due either to differences in the quantity of enzyme molecules or to structural alterations of the enzyme molecule that affect its catalytic ability. To test for possible differences in the amount of circulating DBH, antibody to human adrenal DBH was prepared and was used in an immunoprecipitation procedure to measure the quantity of immunoreactive serum DBH. This procedure was carried out on serum samples obtained from randomly selected individuals, obligate

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heterozygotes, and individuals homozygous for the allele responsible for very low serum DBH activity. The results suggest that differences in the quantity of immunoreactive DBH protein explain the different levels of enzymatic activity in blood from subjects of various genotypes.

MATERIALS AND METHODS

Enzymatic Dopamine β-Hydroxylase Assay Procedure

Enzymatic DBH activity was determined by a modification of the method of Molinoff et al. [11, 14, 15]. Serum samples were diluted 1:50 with ice-cold water and assayed in the presence of 3 μM Cu^{2+} at a reaction mixture pH of 5.2. "Blank" samples were serum samples heated to 95°C for 5 min. Samples that contained 100 ng of phenylethanolamine HCl served as internal standards for the phenylethanolamine-*N*-methyltransferase portion of the reaction. One unit (U) of enzyme activity represented the production of 1 nmol of phenylethanolamine per hour per ml of serum incubated at 37°C.

Purification of Human Adrenal Dopamine β-Hydroxylase

DBH was purified from human adrenal glands obtained at autopsy by a modification of the procedure of Geffen et al. [16]. Chromaffin granules were prepared and lysed by freezing and thawing in a hypotonic medium [17]. The lysate was centrifuged at 100,000 *g* for 60 min, and the supernatant was passed over a Sephadex G200 column. A partially purified preparation of DBH obtained from this gel filtration step was used as antigen. Some human adrenal DBH was purified by passage of a vesicle lysate over DEAE Sephadex A-50 followed by elution with a NaCl gradient (0.01 M–0.80 M NaCl) [16].

Preparation of Antibody to DBH

A male New Zealand rabbit was immunized with 0.5 mg of partially purified human DBH in an equal volume of complete Freund adjuvant. Injections were subcutaneous on the rabbit's back. Blood was obtained by venipuncture from the ear before and 7 weeks after immunization. The second bleeding was used as antiserum.

Preparation of Burro Antibody to Rabbit Gamma Globulin

Rabbit gamma globulin was prepared as previously described [18]. A male burro was injected intramuscularly with 1.2 mg of rabbit gamma globulin in complete Freund adjuvant. The injections were repeated 22 days later with incomplete Freund adjuvant. Burro serum from blood obtained 31 days after the original injection was used as the burro antibody to rabbit gamma globulin. The equivalence between the burro antiserum and rabbit serum was reached at 10–20 vol of burro antiserum per volume of rabbit serum.

Immunoprecipitation

Immunoprecipitation was carried out in two steps: (1) diluted human serum was incubated with varying quantities of rabbit anti-DBH and preimmune rabbit serum; and (2) burro anti-rabbit gamma globulin (BARGG) was added to the reaction mixture to precipitate rabbit gamma globulin. After centrifugation the enzymatic DBH activity that remained in the supernatant was measured.

The incubation tubes for step one contained 50 μl of human serum diluted 1:6 and 50 μl of rabbit serum diluted 1:250. The rabbit serum contained varying proportions of antiserum to DBH and preimmune rabbit serum in order to provide different points in the immunotitration. The dilutions in this step and all subsequent dilutions were made with 0.25% bovine serum albumin in 0.9% NaCl buffered to pH 7.4 with 10 mM potassium phosphate buffer. The components in the first step were mixed, incubated at 37°C for 60 min, and refrigerated at 4°C for 20 hr. Step two was initiated by the addition

of 100 μl of BARGG (diluted 1:25) to the reaction mixture. The tubes were placed in a cold room at 4°C for 16 hr and then centrifuged at 6,000 *g* for 15 min to remove the precipitate. From each tube 100 μl of supernatant was pipetted into a reaction tube that contained 100 μl of a solution of 0.25% bovine serum albumin, 9 μM $CuSO_4$, and 1 mM potassium phosphate buffer, pH 7.4. Enzymatic DBH activity was then measured. The final dilution of these samples was 1:48. pH and $CuSO_4$ concentration were the same as those used to measure native human serum DBH activity.

Human Serum Samples

Randomly selected samples. Blood was obtained by venipuncture from 247 subjects of high school age in the morning after an overnight fast. Only those subjects whose parents gave written consent were included in the study. Blood samples obtained from 38 consecutive subjects were selected for immunoprecipitation studies.

Genetically defined samples. Serum samples from individuals who were homozygous for the allele responsible for very low serum DBH activity and obligate heterozygotes for this allele (i.e., parents of individuals with activity of < 50 U who themselves had activity > 50 U) were selected from samples obtained in the course of previous family studies of the inheritance of serum DBH activity [14]. There were 12 families represented by 12 homozygotes. Twenty-one heterozygotes were from 16 families.

RESULTS

Characterization of Antibody to DBH

The rabbit anti-human DBH antibody was characterized by immunodiffusion, immunoelectrophoresis, and immunoprecipitation studies. Immunodiffusion studies of preimmune rabbit serum vs. a crude lysate of human adrenal chromaffin granules showed no precipitin arcs. Similar experiments carried out with immune serum showed a single precipitin arc (fig. 1*A*). An apparently identical precipitin band formed during immunodiffusion of rabbit anti-DBH against partially purified human adrenal DBH at two different stages of purity (fig. 1*A*). The partially purified preparations had either been passed over a Sephadex G200 column or had been purified by ion exchange chromatography on DEAE Sephadex A-50.

Immunoelectrophoresis of human adrenal chromaffin granule lysates and of partially purified human adrenal DBH vs. rabbit anti-human DBH yielded only a single precipitin arc (fig. 1*B*). These results were compatible with the presence of a monospecific antibody in the immunized rabbit serum that was probably directed against DBH.

Immunoprecipitation studies were carried out to determine whether an antibody against DBH was present in the immune serum. In these experiments using human adrenal DBH purified through the G200 gel chromatography step, the enzyme activity in the purified preparation could be removed by exposure to immune rabbit serum. When immune rabbit serum was used to immunoprecipitate DBH activity in human serum, serial increments of DBH activity were removed from the serum after incubation with increasing quantities of immune serum, and 100% of the human serum DBH activity could be titrated by mixing of human serum with appropriate concentrations of immune rabbit serum. Therefore, the immune rabbit serum appeared to contain a monospecific antibody directed against both human adrenal and human serum DBH.

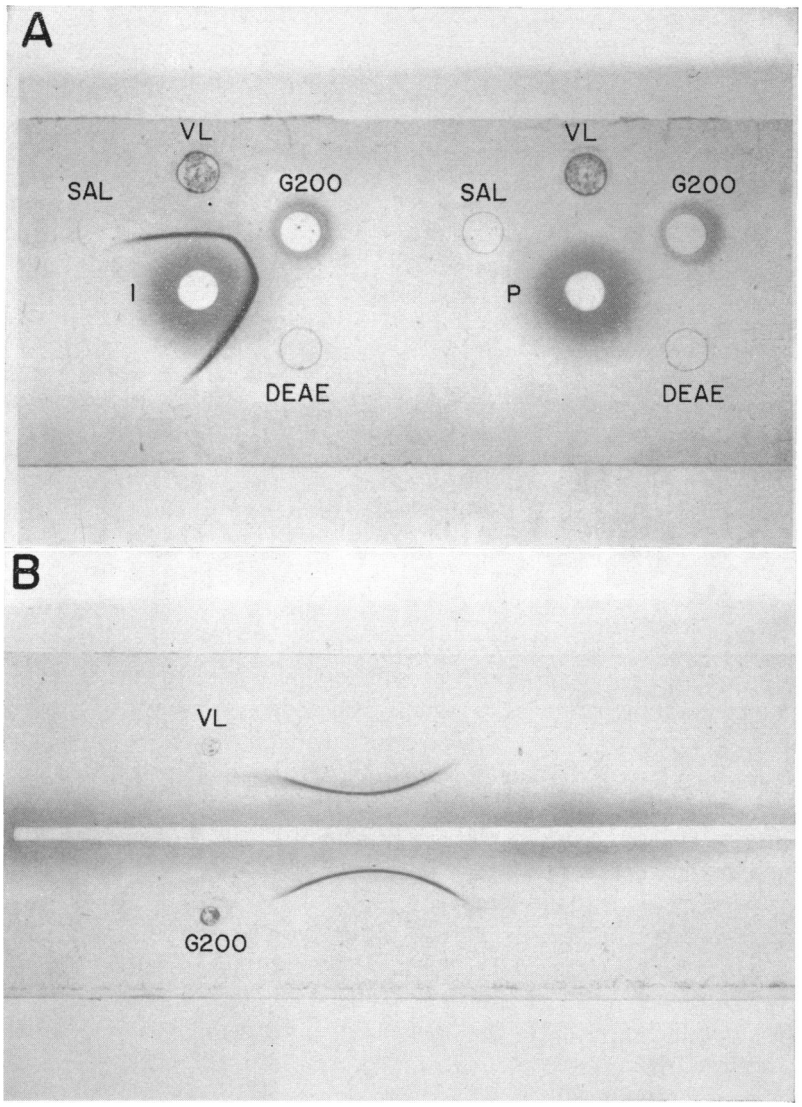

FIG. 1.—*A*, Immunodiffusion of rabbit anti-DBH (*I*) and rabbit preimmune serum (*P*) against saline (*SAL*), lysate of human adrenal chromaffin vesicles (*VL*), human adrenal DBH purified over Sephadex G200, (G200) and human adrenal DBH purified over DEAE Sephadex (DEAE). *B*, Immunoelectrophoresis of lysate of human adrenal chromaffin vesicles (*VL*) and human adrenal DBH purified over G200. (G200) Rabbit anti-DBH was placed in the center trough.

Frequency Distribution of DBH Activity in Randomly Selected Population

The percentage frequency distribution of values of enzymatic serum DBH activity in a randomly selected population of 247 subjects between the ages of 16

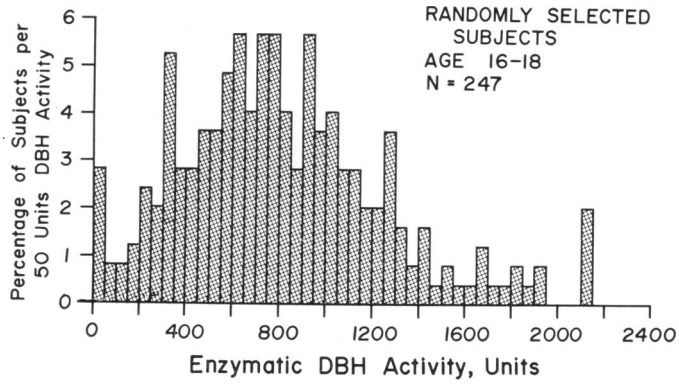

FIG. 2.—Percentage frequency distribution of human serum enzymatic DBH activity from 247 randomly selected subjects aged 16–18.

and 18 is shown in figure 2. The randomly selected samples described below were drawn from this population. This distribution is very similar to that found in large randomly selected populations of adults and children between the ages of 6 and 12 [12, 14]. Previous studies showed that DBH values of less than 50 U are found in 3%–4% of a randomly selected population, and family studies demonstrated that such "very low" enzyme activity is inherited in an autosomal recessive fashion [14]. Obligate heterozygotes for the allele for very low DBH had mean serum DBH activity of 518 ± 70 U (mean ± SEM; $N = 32$), a value between that of homozygotes for the allele for very low activity and that of a randomly selected population [14].

Analysis of Data from Immunoprecipitation Studies

The results of a series of immunoprecipitation studies of human serum DBH are shown in figure 3. Human serum samples that contained different endogenous DBH activity were mixed with varying quantities of immune serum. The immunoprecipitation procedure was carried out in the presence of three different quantities of rabbit anti-DBH. The enzymatic activity that remained in the sample was compared with the enzymatic activity in the same serum sample that had been exposed only to preimmune rabbit serum. Exposure of human serum to preimmune rabbit serum resulted in no inhibition of endogenous DBH activity. When plotted in a linear fashion, the results of immunoprecipitation studies were exponential in character (fig. 3*A*). The use of a semilog plot yielded a linear relationship between the quantity of antiserum added and the percentage inhibition of DBH activity (fig. 3*B*). This phenomenon with immunotitration procedures for DBH and other enzymes has been previously described [19, 20].

The correlation coefficients for each of the sets of data in fig. 3*B* were between —.988 and —1.0 when they were plotted in semilog fashion. To avoid any systematic bias in the results described below, linear least square lines were calcu-

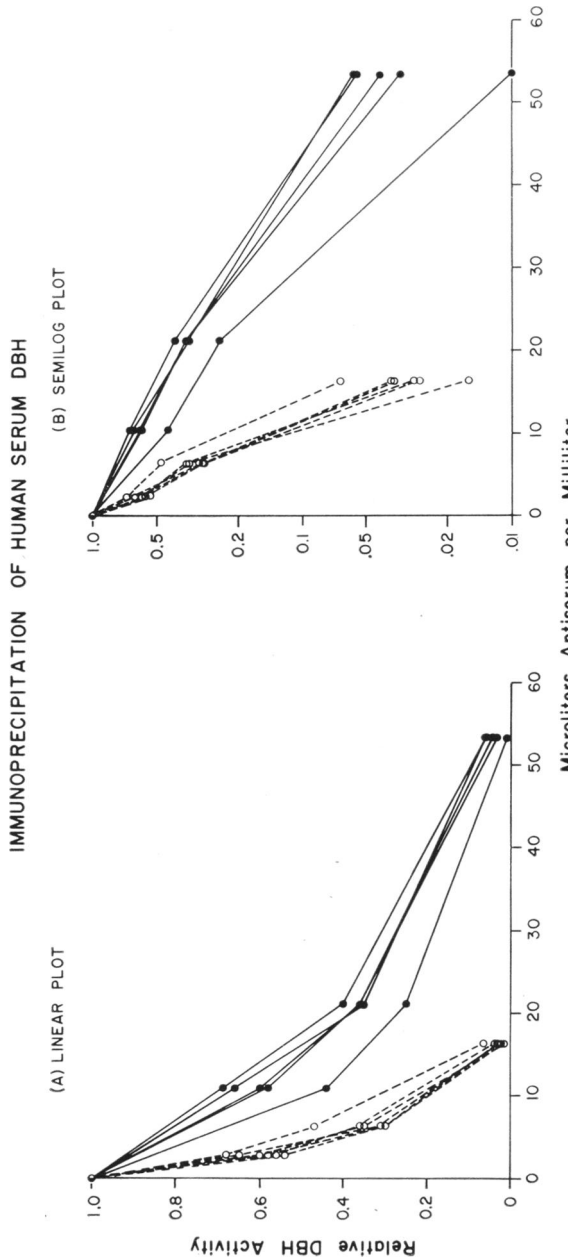

FIG. 3.—Immunoprecipitation of 11 separate samples of human serum graphed in linear (*A*) and in a semilog (*B*) fashion. Six samples with relatively low activity (453–677 U, O- - -O) were diluted 1:5 for the immunoprecipitation, and five samples with high activity (1,377–2,203 U, ●——●) were diluted 1:8.

TABLE 1

REPLICATE IMMUNOPRECIPITATION STUDIES OF HUMAN SERUM DBH

	Enzymatic DBH (U)	AD50 (μl/ml)	N
Low	451 ± 16	3.98 ± 0.28	4
High	1985 ± 110	18.17 ± 1.03	3

NOTE.—The values are mean ± SEM. N = no. of replicate determinations.

lated for immunoprecipitation data plotted in semilog fashion, and the amount of antiserum necessary to inhibit endogenous serum DBH activity by 50% (antibody dose 50% or AD50) was used as a measure of the quantity of immunoreactive DBH present in the sample. Although determinations at three concentrations of antiserum and one concentration of preimmune serum are shown in figure 3, for most of the studies described below three immunoprecipitation points were determined per sample, one uninhibited and two inhibited with appropriate quantities of antibody. Table 1 shows the results of repeated determinations of DBH enzymatic activity and AD50 values for two human serum samples that contained

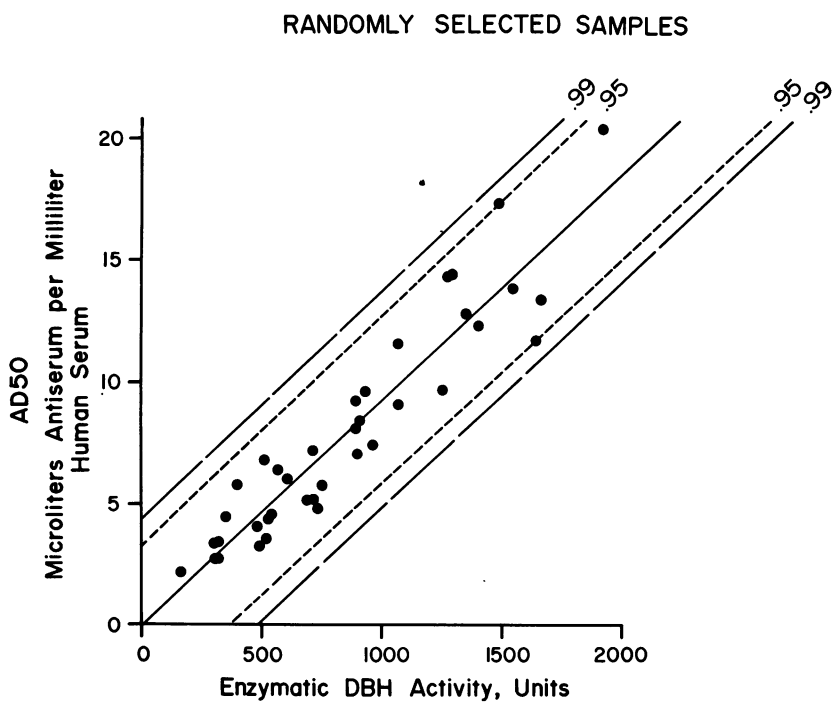

FIG. 4.—Results of immunoprecipitation of DBH in 38 samples of human serum from randomly selected subjects. The regression line of the AD50 values against enzymatic DBH activity with its 95% and 99% confidence limits is shown.

low and high baseline enzyme activity. The standard errors of the means of replicate determinations of AD50 values were between 5% and 10% of the mean values.

Randomly Selected Samples

Immunoprecipitation was carried out on serum samples from 38 randomly selected individuals age 16–18. AD50 values were calculated, and the results were plotted against enzymatic serum DBH activity (fig. 4). A highly significant correlation was present ($r = .94$; $N = 38$; $p < .001$). The least squares line for the regression of AD50 values against enzymatic DBH activity was calculated. This line with its 95% and 99% confidence intervals is shown in figure 4 [21]. The ratio of enzymatic to immunologic DBH appears to be constant across the distribution of values in blood samples from randomly selected subjects.

Heterozygotes

Serum samples from obligate heterozygotes for the allele for very low serum enzymatic DBH activity were subjected to immunotitration, and AD50 values were calculated. With one exception these samples fell within the 95% confidence interval calculated for randomly selected samples (fig. 5). Although most heterozygotes had relatively low enzyme activity, a few had activity greater than 1,000

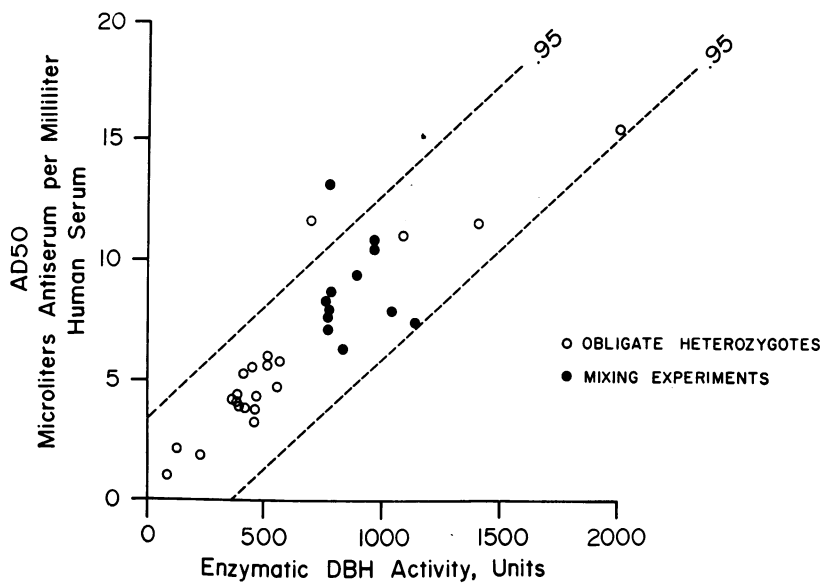

FIG. 5.—Result of immunoprecipitation of DBH in serum of obligate heterozygotes and subjects homozygous for the allele for very low serum enzymatic DBH. *Open circles,* results of studies on serum from 21 obligate heterozygotes; *closed circles,* results of 12 experiments in which serum from subjects with very low activity (< 50 U) was mixed 50:50 (vol/vol) with serum of high DBH activity (> 1,500 U). The 95% confidence limits about the regression line shown in figure 4 are also shown.

U, and three of these subjects were included among the 21 individuals studied. Although heterozygotes carry the allele for very low serum enzymatic DBH, the relationship between their AD50 values and serum enzymatic DBH levels is the same as that found in a randomly selected population.

Homozygote Mixing Experiments

Serum samples from homozygotes for the allele for very low serum DBH have so little enzyme activity that the results of immunoprecipitation studies are not reliable. To overcome this problem immunoprecipitation may be carried out on samples that consist of mixtures of serum from homozygous individuals and serum that contains high DBH activity. If very low activity serum contains immunoreactive DBH protein that is not enzymatically active, presumably this protein will compete with enzymatically active DBH for antibody binding sites. The result of such competition would be a displacement of the point from the regression line shown in figure 4 to a higher apparent ratio of AD50:DBH enzyme activity.

In these experiments each of the samples subjected to immunoprecipitation consisted of a 50:50 (vol/vol) mixture of serum from an individual with very low enzymatic DBH activity with serum from an individual with high activity ($>$ 1,500 U). In one experiment serum from six individuals with very low activity was mixed with serum from six individuals with high activity. In another experiment serum from six subjects with very low activity was mixed with serum from a single individual with high DBH. The results of these experiments did not differ. AD50 values were determined for all mixing studies and were plotted against enzymatic DBH activity (fig. 5). With one exception the results fell within the 95% confidence limits calculated for randomly selected samples. These results support the conclusion that the sera of individuals with very low enzymatic DBH activity do not contain a disproportionate quantity of immunoreactive DBH protein.

DISCUSSION

Genetic factors are important in the determination of levels of enzymatic serum dopamine β-hydroxylase activity in man. Family studies strongly suggest the existence of an allele for very low serum DBH enzymatic activity that is inherited in an autosomal recessive manner [14]. The results of the experiments described here are compatible with true differences in immunoreactive DBH protein in the blood of individuals with different genotypes for this allele. A direct positive correlation between enzymatic and immunoreactive DBH in blood samples obtained from a randomly selected population is present, and the results of immunoprecipitation studies carried out on blood samples from obligate heterozygotes for this allele and samples in which serum from homozygous recessive individuals and subjects with high enzyme activity are mixed are all compatible with this conclusion. The results of other workers who have attempted to correlate enzymatic with immunoreactive DBH protein in human serum from subjects who were not genetically characterized have been contradictory. Ebstein et al. [22] demonstrated a positive correlation of enzymatic with immunoreactive serum DBH in randomly

selected samples in a study with a radioimmunoassay that used anti-human pheochromocytoma DBH antibody and human pheochromocytoma antigen. These same workers have demonstrated a positive correlation between immunoreactive and enzymatic DBH in family studies of patients with torsion dystonia and Down syndrome [23, 24]. Similar results in a randomly selected population were obtained by Ciaranello and Wooten [19] using an immunoprecipitation procedure with an antibody against bovine adrenal DBH. Rush et al. [25], on the other hand, have reported a "complete lack of correlation" of enzymatic with immunoreactive DBH measured by radioimmunoassay with an antibody to bovine adrenal DBH. The antibody used by these latter workers had a much lower affinity for human DBH than for bovine DBH.

Experiments in which circulating proteins are quantitated immunologically must be interpreted with caution since different rabbits may produce antibodies to the same antigen with different specificities [26]. This fact may help explain discrepancies between the results obtained in different laboratories on the quantitation of immunoreactive DBH. Our results on subjects who have been characterized genetically by family studies, when combined with those of other investigators, are compatible with the existence of an allele for very low human serum DBH activity that results in a decrease in blood levels of DBH protein.

Several possible mechanisms by which the allele for very low serum DBH might act to influence serum enzymatic activity have been suggested [14]. Included among these was the possibility that there might exist a structural variant of DBH with very low enzymatic activity per molecule. The results of the studies described here make this possibility less likely as an explanation for the biochemical basis of the action of this allele. However, it cannot be assumed that the allele for very low DBH is necessarily a regulatory gene rather than a structural gene merely because of the correlation between enzyme activity and immunoreactive protein described here [27]. In addition, these results do not exclude the possibility that other familial factors, either genetic or environmental, might also affect serum DBH activity.

SUMMARY

An antibody against human adrenal dopamine β-hydroxylase (DBH) was used to quantitate immunoreactive DBH protein in human serum by an immunoprecipitation technique. A significant correlation was found between DBH enzyme activity and immunoreactive DBH protein in randomly selected serum samples ($r = 0.94$; $N = 38$; $p < .001$). Studies of sera from obligate heterozygotes and individuals homozygous for the allele responsible for very low serum DBH enzymatic activity were compatible with a genetically mediated decrease in the quantity of circulating DBH protein in these subjects.

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Erratum

In the paper "A $^{G}\gamma$-Type of the Hereditary Persistence of Fetal Hemoglobin with β Chain Production in Cis" by T. H. J. Huisman, A. Miller, and W. A. Schroeder (*Am J Hum Genet* 27:765–777, 1975), the β should *not* be deleted in schemes 4 and 4a on page 775.