oblongata and cerebellum may reflect an increased synthesis of these enzymes, possibly due to changes in retrograde axonal transport, which normally delivers information to the nerve body concerning the state of the axon and its terminals under diabetic conditions[9]. A close correlation between these two enzymes within subsectors of the hippocampal formation with certain pathological implications has been reported[22]. However, the close correspondence between these two enzymes does not hold everywhere in the brain. For example, our data shows a significant elevation of ChAT activity in the hypothalamus, thalamus and pons, while AChE activity was decreased in these brain regions.

The alterations in the cholinergic enzymes associated with diabetes in the brain may, in part, explain the abnormality in hypothalamic function[5], locomotor activity[4], intestinal motility[15], male penile erection, sweating and bladder function[16].

It has been known that diabetes has a profound effect on the brain chemistry[23, 24], particularly its effects on brain neurotransmitters and associated enzymes. For example, a reduction in brain serotonin synthesis rate in diabetic rats has been reported[24], and diabetes resulted in changes in the monoamine oxidase activity of the rat brain[18]. Recently, we have shown that diabetes was associated with concomitant changes in brain beta endorphin and brain insulin[17]. In another study, a decreased rate of dopamine synthesis in the brain of streptozotocin-diabetic rats was reported[19]. More recently, it was found that streptozotocin-induced diabetes was associated with an alteration in the metabolism of brain monoamines[25]. The above studies provide evidence that experimentally induced diabetes may affect the synthesis and/or metabolism of brain neurotransmitter substances. Of particular interest in the present findings is the ChAT activity. ChAT is generally accepted as a specific marker for cholinergic structure in the brain[26]. Thus valuable information can be obtained in studying the activity of this enzyme. In the present investigation ChAT activity is enhanced in most brain regions in diabetic animals. This effect may represent a compensatory mechanism in cholinergic transmission in the brain of diabetic rats.

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## Molecular forms of dopamine beta-hydroxylase in rat superior cervical ganglion and adrenal gland

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*Summary.* Dopamine beta-hydroxylase (DBH) enzyme activity was associated in rat superior cervical ganglion with tetrameric DBH-A (294,000 D) and dimeric DBH-B (147,000 D) and in rat adrenal gland with DBH-A and a novel molecular form of DBH, defined as DBH-C, with a molecular weight of 125,000 D. Pretreatment of the rats with cycloheximide markedly reduced DBH activity without altering the molecular heterogeneity.
*Key words.* Dopamine beta-hydroxylase; molecular forms; enzyme activity; gradient centrifugation; rat.

It has been reported that the enzymatic activity of DBH, determined in non-denaturating conditions, is attributable to multiple molecular forms with different molecular weights. Thus, in sera of different species, tetrameric and dimeric form of the same subunit of DBH, have been described[1–3]. Studies in tissues, however, are complicated by

the occurrence of a membrane-linked form of the enzyme. The biochemical characterization of this form often requires the use of ionic detergents which may provoke desactivation of the enzyme as well as the appearance of low molecular weight structures due to the denaturating conditions[3-6]. Hence, information about the physiological situation can be obscured.

We therefore investigated the heterogeneity of DBH activity in rat superior cervical ganglion and adrenal gland, using rate zonal centrifugation and avoiding denaturating conditions.

*Materials and methods.* Albino rats, weighing 250–300 g and pretreated or not with cycloheximide (4.5 mg/kg orally, 19 h before) were used. The superior cervical ganglion and adrenal gland were dissected free under pentobarbital anesthesia, minced and homogenized (Dual 21 glas/teflon homogenizer) in ice-cold 0.25 M sucrose buffered with 10 mM potassium phosphate buffer pH 6.8. The homogenate was submitted to an initial centrifugation (Sorvall SS-34) at 3000 × g for 10 min in order to remove unbroken material. The suspension obtained was centrifuged (Beckman T65) at 140,000 × g for 60 min to yield a first supernatant, defined as sucrose supernatant (SS).

The 140,000 × g pellet was rinsed twice and lysed by freezing and thawing twice in 10 mM potassium phosphate buffer pH 6.8. After centrifugation at 140,000 × g for 60 min, a second supernatant was obtained and defined as the hypotonic lysate (LYS).

The pellet was again rinsed twice and resuspended in 10 mM potassium phosphate buffer pH 6.8 containing 0.15 M NaCl and 0.2% triton X-100 (PBS-T), stored for 20 h at 4 °C and centrifuged at 140,000 × g for 60 min, to yield a third supernatant defined as the triton soluble membranous fraction (TMS).

In separate experiments, the whole tissue was homogenized in PBS-T and centrifuged at 140,000 × g for 60 min. The DBH activity in this supernatant, defined as TS, was considered to represent the maximal amount of enzyme activity which could be extracted from the tissue.

The multiple molecular forms of DBH in the different supernatants were separated by centrifugation through a 12-ml linear (5–20%) PBS-buffered sucrose gradient at 36,000 rpm (SW 40 rotor Beckman) for 20 h[7]. Triton (0.1%) was included in the gradient solutions for the analysis of the TMS fractions. From the supernatants (protein concentration less than 5 mg/ml), 700 µl were applied to the gradients. The gradients were collected in 24 fractions of 0.5 ml at a flow of 1 ml/min.

$S_{20w}$ values were estimated using bovine serum albumin (4.2 S), aldolase (7.3 S) and catalase (11.3 S) as standards. Migration distances were found to be unaffected by the presence of triton. The variability of the separation method, expressed as variability of the migration distance of the standard molecules through the gradient, was found to be at most one fraction in the distribution pattern. Proteins were determined by the method of Bradford[8].

Dopamine beta-hydroxylase was assayed as described before[3]. The samples were diluted either in PBS or in PBS-T. Cycloheximide at a concentration of 1 mM did not affect DBH activity.

All values are expressed as mean ± SEM. Statistical differences are calculated using the Student's two-tailed t-test.

*Results.* In rat superior cervical ganglion, 21.0 ± 1.8 U/ganglion (N = 4) and in adrenal gland 46.7 ± 4.0 U/gland (N = 4) was present in the TS supernatant. The DBH activity recovered in the three subfractions (SS + LYS + TMS) represented 36.2% for the ganglionic tissue and 104.5% for the adrenal gland of the activity in the TS fraction.

In rat superior cervical ganglion and adrenal gland, particle bound (LYS + TMS) DBH activity accounted for 35.5 and 72.5% respectively of total DBH activities (table 1). In both tissues, about 1/3 of this activity could be recovered, after lysis, in the LYS fraction.

Table 1. DBH activities, expressed in percentage of total, in subcellular fractions of rat superior cervical ganglion and adrenal gland

| Fraction | | Superior cervical ganglion | Adrenal gland |
|---|---|---|---|
| SS | (N = 6) | 64.5 | 27.5 |
| LYS | (N = 3) | 10.5 | 26.2 |
| TMS | (N = 3) | 25.0 | 46.3 |

Upon rate zonal centrifugation, the major part of DBH activity was found to be confined in all subcellular fractions, both in the ganglion and the adrenal gland, to a symmetrical peak in tubes 10 to 14 defined as DBH-A (fig. 1).

Some DBH activity was found in tubes 15 to 17, defined as DBH-B in the superior cervical ganglion, and in tubes 17 to 19, defined as DBH-C in the SS fraction of the adrenal gland. The relative amounts of the various DBH forms in the three subcellular fractions, together with their respective $S_{20w}$ values, are given in table 2.

Pretreatment of the animals with cycloheximide resulted in a significant ($p < 0.05$) decrease of DBH activity in the TS fractions of the superior cervical ganglion to 9.9 ± 0.3 U/ganglion and in the TS fraction of the adrenal gland to 10.5 ± 2.4 U/adrenal. This represents 47 and 23% of normal values, respectively (see table 1). Despite these dramatic changes in total activity, the gradient distribution pattern remained essentially unchanged. Figure 2 shows the distribution pattern for the TS fraction with and without cycloheximide treatment. A similar result is obtained in all subcellular fractions (results not shown).

*Discussion.* Our observations show that DBH enzyme activity in both rat superior cervical ganglion and adrenal gland is mainly associated with DBH-A (294,000 D) and to a smaller extent with DBH-B (147,000 D) in rat superior cervical ganglion and with a hitherto undescribed DBH-C (125,000 D) in rat adrenal gland.

It has been suggested that cytoplasmatic DBH activity is either derived from newly formed, more vulnerable vesicles[9,10] or corresponds to the presence of a supplementary DBH pool[11-13]. Whereas our results do not allow to conclude in this respect, they do exclude the possibility that the low molecular weight forms of DBH are precursors in the biosynthetic pathway of DBH-A. Indeed, pretreatment with the protein synthesis inhibitor cycloheximide did not decrease the relative amount of low molecular weight DBH forms, in contrast to what is found for acetylcholinesterase[14]. These forms are therefore most likely due to a degradation process of DBH-A which is, moreover, tissue-specific. The possibility that artifacts are introduced during the manipulations is considered unlikely as no effect of physical forces on the molecules were expected to occur[9] and acidic conditions (pH < 5.7) which favor the dissociation of the tetrameric form into the dimeric form[15] were avoided. Neither is proteolytic activity considered to be of any importance since all experiments were performed at low tempera-

Table 2. Relative amounts of the DBH forms, expressed as the percentage of the total activity in the gradient, in subcellular fractions of rat superior cervical ganglion and adrenal gland, together with their respective $S_{20w}$ value.

| Fraction | Superior cervical ganglion | | Adrenal gland | |
|---|---|---|---|---|
| | DBH-A | DBH-B | DBH-A | DBH-C |
| SS (N = 4) | 57.9 ± 2.9 | 40.1 ± 3.8 | 77.1 ± 5.6 | 22.7 ± 5.5 |
| LYS (N = 4) | 84.2 ± 1.5 | 13.9 ± 1.4 | 92.8 ± 2.4 | – |
| TMS (N = 4) | 88.8 ± 1.2 | – | 91.5 ± 1.1 | – |
| $S_{20w}$ | 11.0 | 7.0 | 11.2 | 5.8 |

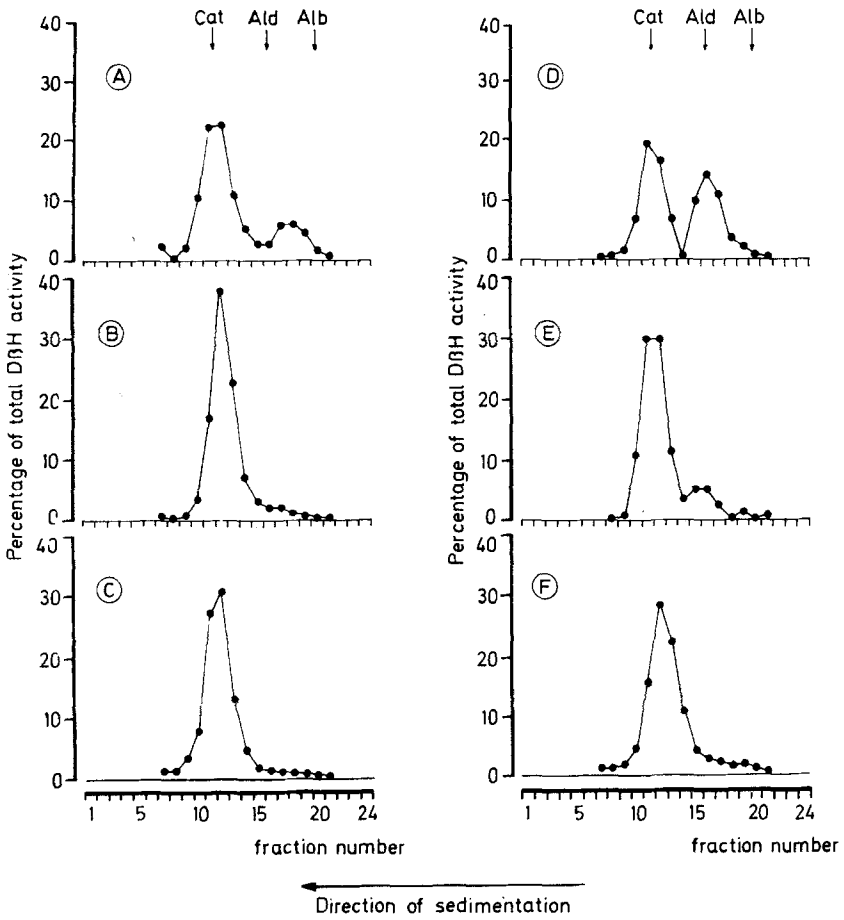


Figure 1. Gradient distribution patterns of DBH activities in subcellular fractions of rat adrenal gland (*A, B, C*) and superior cervical ganglion (*D, E, F*). *A, D*: SS fractions; *B, E*: LYS fractions; *C, F*: TMS fractions. Cat, Ald, Alb: bovine catalase, aldolase and albumin, respectively.

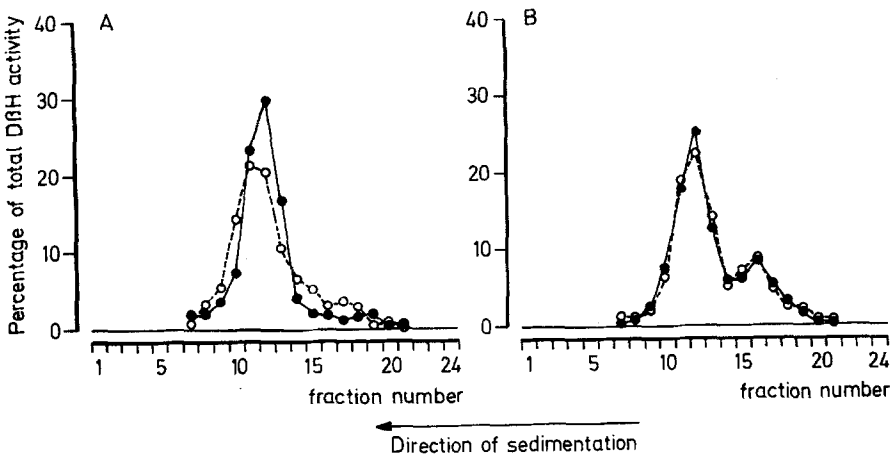


Figure 2. Gradient distribution patterns of DBH activities in the TS fraction of *A:* adrenal gland and *B:* superior cervical ganglion. ●: tissues from control rats; ○: tissues from rats pretreated with cycloheximide.

ture[16] with freshly prepared tissue. It is known, moreover, that the tetrameric form of DBH is relatively resistant to proteolytic activity[17]. Previous experiments with mouse neuroblastoma DBH have also shown that precautions such as adding proteolytic activity inhibitors are superfluous[3]. The calculated molecular weight values are compatible with the view that DBH-A is the tetrameric and DBH-B the dimeric form of the enzyme. The structure of DBH-C remains essentially unknown. Whereas it is clear that in view of the number of DBH-derived polypeptides which may be formed under experimentally induced denaturing and desactivating conditions, a large number of combinations remain possible[18–21], it should be stressed that these conditions were avoided in the present experiments. Furthermore, dif-

ferences in glycosylation pattern could explain differences in molecular weight. In addition, although we accept that only one single molecule DBH-C exists this cannot be proven. Indeed, the resolution of the separation method is too low to distinguish between closely related forms, hence if different degradation products exist, their molecular weight should be within the experimental error of the method.

We therefore suggest that the presence of low molecular weight forms of DBH, DBH-B and DBH-C, are most likely the consequence of a tissue-specific, naturally occurring degradation process of DBH-A. The structure of DBH-C and its relation to DBH-A and DBH-B remain to be elucidated with further experiments which will require denaturating and deactivating conditions.

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## Aldolase C is localized in neuroendocrine cells

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*Summary.* To elucidate the localization of the subunit C of aldolase (aldolase C) in peripheral neuroendocrine cells, we made an immunohistochemical study with monospecific antibodies against human aldolase C. Aldolase C was found to be localized in various types of neuroendocrine cells; in the pituitary gland, thyroid, pancreas, adrenal gland, bronchus, and gastrointestinal tract.
*Key words.* Aldolase; subunit; isozyme; neuroendocrine cell; immunohistochemistry.

Fructose-1,6-diphosphate aldolase (EC 4.1.2.13), a glycolytic enzyme, has a tetrameric form [1] with three immunologically distinct subunits; A, B, and C [2]. Subunit A (aldolase A) is a fetal form of aldolase, and is mainly present in muscle [1], while subunit B (aldolase B) is predominantly distributed in liver and kidney [1]. Subunit C (aldolase C) was considered for a long time to be localized mainly in the brain, in a five-membered A–C hybrid set which could be separated by electrophoresis [1]. In a study employing immunohistochemical methods, it was reported that aldolase C is localized in Purkinje cells of the cerebellum and some neurons in the central nervous system [3, 4].

Recently, we developed a highly sensitive immunoassay of human aldolase C, and reported that peripheral tissues such as adrenal gland, jejunum, and other tissues contained significant amounts of aldolase C [5].

We describe here the localization of aldolase C in peripheral neuroendocrine cells, and discuss its usefulness as a biomarker for neuroendocrine cells, as compared with γ-enolase (neuron-specific enolase), another glycolytic enzyme which is known to be a specific marker for those cells [6 – 10].

*Materials and methods. Antibodies.* Antibodies were raised in New Zealand white rabbits by i.c. injections of purified aldolase C, and further purified by immunoaffinity chromatography as described previously [5]. The specificity of the antibodies has been reported [5]. For secondary antibodies, horseradish peroxidase(HRP)-labeled goat IgG Fab' fragments against rabbit IgG were prepared as previously described [11].

*Tissue specimens and preparations.* Two or three pieces of each adult tissue (thyroid, pancreas, bronchus, stomach, small intestine, and large intestine) were obtained from surgical operations. Adrenal and pituitary glands were obtained at autopsies within 2 h after the patients' deaths. They were fixed in periodate-lysine-4% paraformaldehyde (PLP) for 6 h, washed in phosphate-buffered saline (PBS, pH 7.2) containing increasing concentrations of sucrose, embedded in OCT compound (Lab-Tek Products, Miles Laboratories Inc., Naperville, Ill), and frozen quickly in dry ice and ethanol.

Normal pancreatic tissues were fixed in formalin for 12 h and embedded in paraffin in order to compare the preservation of the antigenicity under the conditions used.

*Immunohistochemistry.* The indirect immunoperoxidase method according to Nakane [12] was used. In brief, cryostat sections were placed on albumin-coated slides and dried at room temperature. The sections were treated with 5 mM periodic acid solution, to inactivate endogeneous peroxi-