Membrane Proteins of Chromaffin Granules

DOPAMINE β -HYDROXYLASE, A MAJOR CONSTITUENT

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1. Soluble lysates and membranes were prepared from chromaffin granules isolated from bovine adrenal medulla. The detergent *N*-cetylpyridinium chloride was used for solubilizing the membrane proteins, including the membrane-bound dopamine (2,4-dihydroxyphenethylamine) β -hydroxylase. The solubilized proteins were fractionated by Sephadex chromatography in the presence of *N*-cetylpyridinium chloride. The major component of the membrane proteins, i.e. chromomembrin A, was identified as the enzyme dopamine β -hydroxylase. 2. The addition of *N*-cetylpyridinium chloride to the soluble lysate caused precipitation of up to 96% of the proteins, but only a small proportion of the dopamine β -hydroxylase activity was precipitated. The only protein demonstrable in the supernatant by polyacrylamide-gel electrophoresis was the protein that has a lower mobility than chromogranin A in disc gel electrophoresis. This component has been identified previously as dopamine β -hydroxylase. Thus, this method provides an extremely simple isolation procedure for dopamine β -hydroxylase. 3. A comparison of the membrane-bound and soluble dopamine β -hydroxylases revealed the identity of these two preparations. Both were activated by *N*-cetylpyridinium chloride, they migrated identically in polyacrylamide-gel electrophoresis, their amino acid composition was very similar and an immunological cross-reaction could be demonstrated.

In adrenal medulla the enzyme dopamine β -hydroxylase is localized in chromaffin granules (Kirshner, 1959; Oka *et al.*, 1967). The proteins of these organelles can be divided into two groups: (i) the chromogranins (Blaschko *et al.*, 1967), which are released into the supernatant when the particles are lysed by hypo-osmotic shock; (ii) the chromomembrins (Winkler, 1971), which are recovered in the insoluble residue, which consists, as demonstrated by electron microscopy, of the granule membranes (Helle, 1971a; Winkler *et al.*, 1972).

The activity of dopamine β -hydroxylase is distributed equally between the soluble chromogranins and the insoluble membrane proteins (Duch *et al.*, 1968; Belpaire & Laduron, 1968; Winkler *et al.*, 1970). When the catecholamines are secreted from the adrenal medulla by exocytosis, only that part of dopamine β -hydroxylase activity which is soluble is released, whereas the membrane-bound enzyme activity remains behind in the cell (Viveros *et al.*, 1969). Dopamine β -hydroxylase has already been purified from a particulate fraction containing intact chromaffin granules (Kaufman & Friedman, 1965; Goldstein *et al.*, 1965) and from the soluble granule proteins (Sage *et al.*, 1967; Geffen *et al.*, 1969). However, a comparison of the soluble and membrane-bound dopamine β -hydroxylase has not been reported.

The membrane proteins of chromaffin granules

have been characterized by polyacrylamide-gel electrophoresis (Winkler *et al.*, 1970). The two major components, which have been named chromomembrin A and B (Winkler, 1971), have been purified (Hörtnagl *et al.*, 1971). Since these proteins were isolated after solubilization with sodium dodecyl sulphate, which inactivates dopamine β -hydroxylase, no enzymic activity could be attributed to them. The present paper reports the isolation of an active preparation of dopamine β -hydroxylase from membranes and its identification as chromomembrin A. This study was initiated by the finding that a purified sample of dopamine β -hydroxylase (kindly given to us by Professor Kirshner) behaved on electrophoresis like chromomembrin A. A comparison of the membrane-bound dopamine β -hydroxylase with the soluble enzyme indicates the identity of the enzymes from the two sources. A preliminary account of these results has been given at the Meeting of the International Society of Neurochemistry in Budapest, 1971 (Winkler *et al.*, 1972).

Experimental

Methods

Isolation of soluble lysates and of membranes from chromaffin granules. Soluble lysates were obtained by lysing isolated chromaffin granules (Smith & Winkler,

1967b) in tris-sodium succinate buffer (pH 5.9, *I* 0.015; Smith & Winkler, 1967a), followed by the removal of the insoluble residue by high-speed centrifugation (160000g for 45 min). When desired, soluble lysates were dialysed against tris-sodium succinate buffer (pH 5.9, *I* 0.015). Washed membranes of isolated chromaffin granules were obtained as previously described (Hörtnagl *et al.*, 1971).

Enzyme assay. Dopamine β -hydroxylase was assayed with [3 H]tyramine as substrate (see Friedman & Kaufman, 1965). The composition of the incubation medium and the method of radioactivity counting have been described (Hörtnagl *et al.*, 1969). The substrate concentration was 0.023 μ M. The detergent *N*-cetylpyridinium chloride activated dopamine β -hydroxylase (see the Results section). To ensure optimum activation, the samples (0.1 ml) to be assayed were first incubated in ice for 30 min with several appropriate concentrations (in 0.1 ml of the tris-sodium succinate buffer) of *N*-cetylpyridinium chloride. After this incubation the assay mixture was completed and then incubated at 37°C for the usual 20 min.

Solubilization of granule membranes. A suspension of granule membranes (440 μ g of protein/0.5 ml) was mixed with an equal volume of *N*-cetylpyridinium chloride solution of various concentrations. This suspension was well mixed by pipetting. After 1 h in ice the suspension was centrifuged at 160000g for 45 min. The supernatant and the sediment (resuspended in 1 ml of the tris-sodium succinate buffer) were assayed for protein (micro biuret method as described by Winkler *et al.*, 1970) and for dopamine β -hydroxylase. The enzyme assay was performed with different concentrations of added *N*-cetylpyridinium chloride to ensure optimum activity (see above).

Treatment of soluble lysate with *N*-cetylpyridinium chloride. Various concentrations of *N*-cetylpyridinium chloride were added in a small volume (up to 30 μ l) to 0.5 ml (2 mg of protein) of dialysed soluble lysate. After shaking and 30 min in ice, the mixture was centrifuged for 45 min at 160000g. Protein was determined in the supernatant and sediment. Dopamine β -hydroxylase was usually measured (again with various concentrations of *N*-cetylpyridinium chloride) in the sediment resuspended in 0.5 ml of 1 M-KCl solution. In some experiments the enzyme was also determined in the supernatant.

Polyacrylamide-gel electrophoresis. Disc electrophoresis was usually done in a gel containing sodium dodecyl sulphate or in an acid medium containing phenol-acetic acid-urea as described previously (Winkler *et al.*, 1970). In some experiments the tris-glycine buffer system described by Clarke (1964) was used.

Sephadex chromatography. A column (100 cm long $\times$ 1.5 cm diam.) was used. Sephadex G-200 was swollen in tris-sodium succinate buffer (pH 5.9, *I* 0.015)

containing 0.01% (w/v) *N*-cetylpyridinium chloride. The same buffer was used for elution of 1-ml fractions. For chromatography, membranes of chromaffin granules (up to 18 mg of protein) were solubilized in 1.5 ml of the tris-sodium succinate buffer containing *N*-cetylpyridinium chloride in a concentration to give 2 mg of detergent/mg of membrane protein. The suspension was left for 1 h in ice and then was centrifuged for 45 min at 160000g. The supernatant was applied to the column. Chromatography was performed at 2–4°C. The eluted fractions were assayed for cholesterol (Zlatkis *et al.*, 1953), for total phosphorus (Bartlett, 1959), for dopamine β -hydroxylase activity and for protein by the biuret method after precipitation with 5% (w/v) (final concn.) trichloroacetic acid. For electrophoresis the fractions were concentrated by ultrafiltration and then dialysed against water.

Amino acid analysis. Dopamine β -hydroxylase was purified from the soluble lysate. To portions (3 ml) of soluble lysate 0.2 ml of *N*-cetylpyridinium chloride solution was added to a final concn. of 0.55 mg of detergent/mg of protein. After 30 min in ice, the precipitate was removed by high-speed centrifugation (45 min, 160000g). The supernatant was concentrated by ultrafiltration and then dialysed against water for 72 h at 2°C. After this prolonged dialysis some of the protein came out of solution. The successful isolation of dopamine β -hydroxylase was tested by polyacrylamide-gel electrophoresis in phenol-acetic acid-urea, which solubilized the precipitate. The sample was then hydrolysed (see Hörtnagl *et al.*, 1971) and analysed in an EEL 194 amino acid analyser.

Immunological experiments. Dopamine β -hydroxylase was isolated as described above for the amino acid analysis. However, the sample, after ultrafiltration, was dialysed against the tris-sodium succinate buffer (pH 5.9) for 24 h. Rabbits were immunized with 700 μ g of the purified protein for each injection according to the schedule given by Schneider *et al.* (1967). Immunoelectrophoresis and immunodiffusion on Ouchterlony plates (for 48 h at 2°C) were carried out by standard procedures (see Huber *et al.*, 1972; pp. 281, 285). Micro-complement fixation was done as described by Schneider *et al.* (1967).

To locate the antigen in polyacrylamide gels, 100 μ l of soluble lysate (4.8 mg of protein/ml) were run on gels by the method of Clarke (1964). After electrophoresis, the gels were cut into small pieces (0.3 cm), to which 0.1 ml of 0.1 M-KCl in tris-sodium succinate buffer (pH 5.9, *I* 0.15) was added. The pieces were then mashed with a glass rod. After 18 h at 2°C the supernatants were tested against the antisera on Ouchterlony plates.

Materials

Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; *N*-cetylpyridinium

chloride and Triton X-100 were from E. Merck A.-G., Darmstadt, Germany; [^3H]tyramine (5–10 Ci/mol) was from NEN Chemicals, Dreieichenhain, Germany; freeze-dried complement (guinea pig) was from Sclavo, Siena, Italy; and haemolysin was from Serotherapeutisches Institut, Vienna, Austria. Complete Freund's adjuvant was from Difco Laboratories, Detroit, Mich., U.S.A. Sheep blood in alsever solution (see Huber *et al.*, 1972; p. 318) was from a local source.

Results

The specific activity (per mg of protein) of dopamine β -hydroxylase in the membrane preparations was 3.1 ± 0.18 (S.D.; $n=6$) times that in the soluble lysate of chromaffin granules. This was expected, since the enzyme is divided equally between the soluble lysate and the membranes (Duch *et al.*, 1968; Belpaire & Laduron, 1968; Winkler *et al.*, 1970) and the membrane proteins represent about 25% of the total granule proteins.

Activation of dopamine β -hydroxylase by *N*-cetylpyridinium chloride

Soluble lysates and membranes of chromaffin granules were treated with increasing concentrations of *N*-cetylpyridinium chloride as described in the Experimental section. The activity of dopamine β -hydroxylase was increased considerably by this treatment (see Fig. 1). The soluble and the membrane-bound dopamine β -hydroxylase were activated to a similar degree. However, higher concentrations of the detergent were needed to give full activation of the membrane-bound dopamine β -hydroxylase, which may be explained by a binding of *N*-cetylpyridinium chloride to membrane lipids. The mechanism of this activation is unknown. To exclude the possibility of an artifact, *N*-cetylpyridinium chloride was added in various concentrations to assay mixtures after incubation at 37°C. This addition had no effect on the results. The activation of dopamine β -hydroxylase by *N*-cetylpyridinium chloride was not shared by other detergents. Treatment with Triton X-100 (tested range: 500 μg –25 mg/mg of protein) or sodium deoxycholate (tested range 1–9 mg/mg of protein) caused no activation of the enzyme. The higher concentration of these detergents gave, in fact, a nearly complete inhibition.

Membrane-bound dopamine β -hydroxylase

N-Cetylpyridinium chloride, which activates dopamine β -hydroxylase, was used for solubilizing the membrane-bound enzyme. Fig. 2 shows the results of a representative experiment. The membrane proteins and the dopamine β -hydroxylase activity were

Fig. 1. Activation of dopamine β -hydroxylase by *N*-cetylpyridinium chloride

Increasing concentrations of *N*-cetylpyridinium chloride were added to the soluble lysate (16 μg of protein/tube) and to membranes (6 μg of protein/tube) of chromaffin granules 30 min before the incubation for the assay of dopamine β -hydroxylase was started. The activation of dopamine β -hydroxylase resulting from this procedure is given on the ordinate as the percentage increase above control values. The absolute enzymic activity of the untreated soluble lysate was 0.038 nmol of substrate conversion/h per mg of protein, whereas that of the untreated membranes was 0.12 nmol of substrate conversion/h per mg of protein. Each point (○, soluble lysate; ●, membranes) is a mean value of at least two experiments. Concentrations of *N*-cetylpyridinium chloride higher than 11 mg/mg of protein were not used to avoid complications caused by the relatively low solubility of this detergent at low temperature.

solubilized together. With 2.5 mg of *N*-cetylpyridinium chloride/mg of protein a maximal solubilization was obtained, i.e. 80% of the protein and 90% of the dopamine β -hydroxylase activity were found in the supernatant. Polyacrylamide-gel electrophoresis of the supernatant and the sediment revealed that all components of the total membranes were found both in the supernatant and in the sediment. The component that migrated like chromogranin A on gel electrophoresis (see Winkler *et al.*, 1970; Hörtnagl *et al.*, 1971) was relatively concentrated in the sediment. This can be taken as further evidence that this component is chromogranin A; it will be shown below that the chromogranins are precipitated by *N*-cetylpyridinium chloride.

For Sephadex chromatography, membranes were treated with 2 mg of *N*-cetylpyridinium chloride/mg of protein, which solubilized 80% of the proteins and 90% of the dopamine β -hydroxylase activity (see Fig. 2). The solubilized membranes were fractionated

Fig. 2. Solubilization of membrane proteins of chromaffin granules by *N*-cetylpyridinium chloride

Membranes of chromaffin granules (440 μ g of protein) were mixed with increasing concentrations of *N*-cetylpyridinium chloride. After 60 min the suspension was centrifuged. The protein and the activity of dopamine β -hydroxylase (after addition of optimum amounts of *N*-cetylpyridinium chloride) were determined in the supernatant and sediment. The ordinate gives the percentage of protein (●) and of dopamine β -hydroxylase activity (○) solubilized by this procedure.

on Sephadex G-200 in the presence of 0.01 % *N*-cetylpyridinium chloride. Owing to the low solubility of *N*-cetylpyridinium chloride in the cold, higher concentrations of *N*-cetylpyridinium chloride could not be used. Fig. 3 shows the results of a representative experiment. A large peak absorbing at 280 nm was eluted first. The fractions of this peak were slightly turbid. Further investigations revealed that this peak contained cholesterol (see Fig. 3) and phospholipid. Apparently, the lipids were eluted as large micelles, which was quite different from our previous study with sodium dodecyl sulphate where the lipids were eluted after the proteins (see Hörtnagl *et al.*, 1971). The lipid micelles present in these fractions could be sedimented by high-speed centrifugation (160000g for 2 h). Dopamine β -hydroxylase activity was eluted as a definite peak, which coincided with the main protein peak. Gel electrophoresis of the fractions from this peak showed that the enzyme was eluted together with the protein component that we have named chromomembrin A. The fractions from this protein peak were contaminated with lipids, which in electrophoresis give the fastest-moving band (cf.

Hörtnagl *et al.*, 1971). The activity of the eluted enzyme was not very stable and decreased considerably within hours. The other protein components were eluted after chromomembrin A. A similar elution pattern of the proteins has been obtained with membranes solubilized in sodium dodecyl sulphate (Hörtnagl *et al.*, 1971).

Some experiments were performed with membranes after diethyl ether extraction, which removes most of the cholesterol and the phospholipids (see Hörtnagl *et al.*, 1971). The fractions eluted from the Sephadex column did not exhibit the large peak absorbing at 280 nm, which was found in experiments with unextracted membranes (see above). This confirms the conclusion that this peak is due to lipid micelles. Dopamine β -hydroxylase activity was eluted with the first peak absorbing at 280 nm. Gel electrophoresis demonstrated again the presence of chromomembrin A in the fractions of this peak.

Soluble dopamine β -hydroxylase

When *N*-cetylpyridinium chloride was added to dialysed soluble lysates of chromaffin granules a surprising observation was made. The addition of this detergent led to an immediate precipitation of protein. Fig. 4 shows that *N*-cetylpyridinium chloride in increasing concentrations precipitates up to 96% of the protein in the soluble lysate. Only a small proportion of the dopamine β -hydroxylase activity, on the other hand, is precipitated. The values for dopamine β -hydroxylase activity in Fig. 4 were obtained by measuring the enzymic activity in the sediment, since the dopamine β -hydroxylase activity in the supernatant was very unstable making reliable measurements difficult. In a few experiments, which were done as quickly as possible, the enzymic activity in the supernatant (obtained after treatment of soluble lysate with 0.45 mg of *N*-cetylpyridinium chloride/mg of protein) was compared with that of the soluble lysate. The dopamine β -hydroxylase activity in the supernatant (after the addition of optimum amounts of *N*-cetylpyridinium chloride) was 1.15 nmol of substrate conversion/h per mg of protein, whereas that of the soluble lysate was 0.15 nmol of substrate conversion/h per mg of protein (mean values of four experiments). Polyacrylamide-gel electrophoresis (Clarke, 1964) revealed (see Plate 1a) that the supernatant remaining after the precipitation of protein with 0.5 mg of *N*-cetylpyridinium chloride/mg of protein contained only one protein component, which migrated more slowly than chromogranin A in disc electrophoresis. All the components of the soluble lysate were found in the sediment, although there was little of the protein which characteristically remained in the supernatant after *N*-cetylpyridinium chloride treatment. The protein migrating in gel electrophoresis more

Fig. 3. *Sephadex chromatography of membranes solubilized with N-cetylpyridinium chloride*

Membranes (18mg of protein) solubilized in buffer containing *N*-cetylpyridinium chloride were applied to a column of Sephadex G-200. The elution buffer contained 0.01 % (w/v) *N*-cetylpyridinium chloride. The abscissa indicates the elution volume of the fractions. The ordinates give the protein content (●), the cholesterol content (Δ) and the dopamine β -hydroxylase activity (○) of the fractions. The proteins eluted from the column were characterized by polyacrylamide-gel electrophoresis. The arrows (---→) indicate the fractions which were subjected to gel electrophoresis. Three fractions were combined, concentrated by ultrafiltration and dialysed against water to remove the detergent *N*-cetylpyridinium chloride. For electrophoresis in the alkaline-gel system (see Winkler *et al.*, 1970), sodium dodecyl sulphate was added. The electrophoretic patterns obtained are shown on the figure. Chromomembrins A and B are indicated by A and B.

slowly than chromogranin A has been identified by Smith *et al.* (1970) as dopamine β -hydroxylase and this was confirmed by the present study. The peculiar shape of the precipitation curve for dopamine β -hydroxylase in Fig. 4 deserves comment. It seems likely that the precipitation of the enzyme is due to a trapping of the enzyme molecules in the considerable precipitate formed immediately after the addition of the detergent. Between the effect of the addition

of 0.3 and 0.4mg of *N*-cetylpyridinium chloride/mg of protein there was a clear-cut and reproducible difference in the formation of the precipitate. With the lower detergent concentrations the solution turned immediately cloudy and a fine precipitate formed, whereas with the higher detergent concentrations the precipitate became immediately flocculent. This flocculent precipitate is likely to trap dopamine β -hydroxylase very effectively, which may

Fig. 4. Effect of the addition of *N*-cetylpyridinium chloride to a soluble lysate of chromaffin granules

N-cetylpyridinium chloride in increasing concentrations was added to a soluble lysate. After 30 min the mixture was centrifuged. The protein in the supernatant and sediment was determined. Dopamine β -hydroxylase activity was only determined in the sediment, since the activity in the supernatant quickly decreased. The ordinate gives the percentage of protein (●) and of dopamine β -hydroxylase activity (○) recovered in the sediment.

explain the higher percentage of dopamine β -hydroxylase precipitated with 0.4 mg of *N*-cetylpyridinium chloride/mg of protein. In any case the method described above seems suitable for the isolation of dopamine β -hydroxylase from the soluble lysate in a pure form. A disadvantage of the method is that the enzymic activity in the supernatant is quickly lost. An advantage, on the other hand, is the extreme simplicity of the isolation, and the fact that the antigenic properties of the purified enzyme are well retained (see below).

Comparison of soluble and membrane-bound dopamine β -hydroxylase

The procedures described above led to the isolation of pure dopamine β -hydroxylase both from the soluble lysate and from the membranes of chromaffin granules. These two preparations were compared by polyacrylamide-gel electrophoresis. Plate 1(b) shows that the two enzyme preparations migrate identically in the acid-gel system. Analogous results were obtained in the alkaline-gel system. Thus, these electrophoresis results indicate the identity of the two enzyme preparations.

Further evidence for this suggestion can be derived from the amino acid analysis. Table 1 shows the composition of chromomembrin A (Hörtnagl *et al.*, 1971), of chromogranin A (Smith & Winkler, 1967b) and of soluble dopamine β -hydroxylase. The composition of the latter is very similar to that of chromo-

Table 1. Amino acid composition of soluble dopamine β -hydroxylase, chromomembrin A and chromogranin A

The amino acid compositions are expressed in g of amino acid/100 g of protein. The data for soluble dopamine β -hydroxylase are mean values of two experiments. The amino acid compositions of chromomembrin A and of chromogranin A are taken from previous publications (Hörtnagl *et al.*, 1971; Smith & Winkler, 1967b).

	Soluble dopamine β -hydroxylase	Chromomembrin A	Chromogranin A
Lys	5.8	5.6	9.4
His	3.2	3.1	2.4
Arg	6.8	7.9	8.5
Asx	9.3	9.4	8.4
Thr	6.0	4.9	2.5
Ser	6.6	5.4	6.2
Glx	13.5	13.5	26.0
Pro	5.4	6.0	8.6
Gly	4.5	4.4	3.9
Ala	4.7	5.3	5.0
Cys	1.9	1.1	0.3
Val	6.0	5.3	3.3
Met	2.1	2.3	2.2
Ile	3.9	4.2	1.0
Leu	8.6	10.6	7.3
Tyr	5.7	4.7	1.7
Phe	5.8	6.2	2.1

EXPLANATION OF PLATE I

(a) Polyacrylamide-gel electrophoresis of soluble lysate and of purified dopamine β -hydroxylase

The gel system of Clarke (1964) was used. Electrophoretic patterns were of: (1) the soluble lysate of chromaffin granules; (2) the supernatant obtained by treatment of a soluble lysate with 0.5mg of *N*-cetylpyridinium chloride/mg of protein. The supernatant was concentrated by ultrafiltration before electrophoresis.

(b) Polyacrylamide-gel electrophoresis of purified dopamine β -hydroxylase

Dopamine β -hydroxylase was purified from soluble lysate and from membranes of chromaffin granules. The dialysed enzyme preparations were mixed with phenol-acetic acid-urea and were applied to gels equilibrated with this solvent (see Winkler *et al.*, 1970). Electrophoretic patterns were of: (1) enzyme purified from membranes; (2) enzyme purified from soluble lysate; (3) total membranes of chromaffin granules. Chromomembrin A and B (see Winkler, 1971) are indicated by A and B.

EXPLANATION OF PLATE 2

Immunodiffusion experiment with an antiserum against soluble dopamine β -hydroxylase

The antiserum was placed in the centre well. The other wells contained: (A) dopamine β -hydroxylase isolated by polyacrylamide-gel electrophoresis (see the Experimental section); (B) soluble lysate of chromaffin granules; (C) membranes of chromaffin granules solubilized in an excess of Triton X-100 (50 mg/mg of membrane protein); (D) chromomembrin A isolated by Sephadex chromatography. The relevant fractions eluted from the column were concentrated by ultrafiltration, dialysed against water and then treated with an excess of Triton X-100. Immunodiffusion was carried out for 48 h at 2°C. Before staining with Amido Black the plate was washed in NaCl (0.9%, w/v) for 15h.

membrin A. The minor differences can be attributed to methodological variation ($\pm 10\%$). The composition of chromogranin A is clearly different. Thus, these results are consistent with the suggestion that chromomembrin A (membrane-bound dopamine β -hydroxylase) and the soluble dopamine β -hydroxylase are identical.

Finally, immunological results were obtained. An antiserum was produced against the purified preparation of soluble dopamine β -hydroxylase. When this antiserum was tested against soluble lysates only one precipitin line was obtained (see Plate 2). In immunoelectrophoresis the precipitin arc stayed near the origin, as has been described for the soluble dopamine β -hydroxylase by Sage *et al.* (1967). This antiserum was also tested against eluates from gel pieces obtained after electrophoresis of the soluble lysate (see Plate 2). It gave only a precipitin line with the eluate of that region of the gel where the dopamine β -hydroxylase activity was localized. Thus, the purification procedure was sufficient to give an antiserum only directed against dopamine β -hydroxylase. Quite extensive purification procedures were used by other authors (Geffen *et al.*, 1969; Hartman & Udenfriend, 1970) to achieve the same result.

Plate 2 demonstrates that the antiserum gave identical precipitin lines with soluble lysate, with dopamine β -hydroxylase purified by electrophoresis, with Triton X-100-solubilized membranes and with chromomembrin A (membrane-bound dopamine β -hydroxylase) purified by Sephadex chromatography.

Micro-complement fixation was carried out with the soluble lysate and total membranes. When related to mg of protein, the membranes gave about the same complement fixation (1.01 times as high; $n = 13$) as the soluble lysate. However, as judged from the specific activity of the enzyme, they should contain three times as many enzyme molecules. The most likely explanation seemed to be that the membrane-bound dopamine β -hydroxylase was not completely accessible for the interaction with the antibody. Therefore experiments with Triton X-100 were performed. Pretreatment of membrane suspensions (0.1 ml containing 0.2 mg of protein) with Triton X-100 (from 0.250 mg of Triton X-100/mg of protein upwards) for 60 min at 20°C caused an increased micro-complement fixation. The maximal effect was obtained with 0.7 mg of Triton X-100/mg of membrane protein. Treatment of soluble lysate with an analogous concentration of Triton X-100 was without effect. When related to mg of protein the micro-complement fixation of Triton X-100-treated (2 mg of Triton X-100/mg of protein) membranes was 4.8 ± 1.2 (s.d.; $n = 8$) times that of the soluble lysate. Since the enzymic activity of the membranes is only three times that of the soluble lysate these immunological results indicate that the specific enzymic activity of dopamine

β -hydroxylase when bound to the membrane is lower than that of the soluble, unbound enzyme.

Discussion

Dopamine β -hydroxylase was isolated from both the soluble lysate and the membranes of chromaffin granules. The identity of the enzymes isolated from the two sources could be demonstrated. Both enzymes were activated by the detergent *N*-cetylpyridinium chloride, they migrated identically in two electrophoresis systems, there was immunological identity and their amino acid composition was identical, if we assume that minor differences were due to methodological variation. During the preparation of the present paper, two reports appeared in which it was also concluded that the soluble and membrane-bound dopamine β -hydroxylases are identical (Foldes *et al.*, 1972; Kuzuya & Nagatsu, 1972). These results raise the important question of whether the membrane-bound dopamine β -hydroxylase is an integral part of the membrane or just an adsorbed soluble enzyme. We consider that the available evidence is in favour of the first alternative. The results of Viveros *et al.* (1969) already exclude the possibility that the appearance of membrane-bound dopamine β -hydroxylase is an artifact *in vitro*. Secretion of catecholamines is accompanied only by the release of part of the dopamine β -hydroxylase, whereas the other portion of this enzyme remains inside the cell bound to the membranes. *In vitro*, the membrane-bound enzyme is always a fixed proportion of the total granule enzyme and it cannot be decreased by further washes of the membranes (Winkler *et al.*, 1970). The solubilization of the enzyme by detergents parallels the solubilization of the other membrane proteins. It should also be emphasized that the isolated granule membranes are relatively poor in proteins, containing only 0.45 mg of protein/mg of lipid (Winkler *et al.*, 1970). This protein content is much lower than that of other membranes (0.7–4.0; see Korn, 1969); it approaches the very low content (0.25) of myelin. In the present study it has been shown that the membrane-bound dopamine β -hydroxylase is the major protein of our membrane preparation. If this protein were not an integral part of the membrane we would be left with a membrane nearly devoid of proteins, which seems unlikely. If we accept these arguments, then the major protein of the granule membrane can be considered to be an enzyme. Structural proteins without catalytic activity were thought to be the major building blocks of membranes (see Green *et al.*, 1968). This does not seem to be the case for membranes of chromaffin granules. It has also been shown for sarcoplasmic reticulum membranes that one of the major components is an enzyme, namely a Ca^{2+} -activated ATPase (see

MacLennan *et al.*, 1971). For mitochondrial membranes it has been demonstrated that the preparation considered to be a structural protein contained in fact denatured ATPase (Schatz & Saltzgaber, 1969; Senior & MacLennan, 1970). Thus, it would appear that at least in some membranes the same molecule may combine enzymic and structure-providing properties.

Hartman & Udenfriend (1970) demonstrated an immunological cross-reactivity between dopamine β -hydroxylase from several species. This can now be linked with our previous studies on proteins of granule membranes (Hörtnagl *et al.*, 1971), in which we showed by electrophoresis that chromomembrin A (membrane-bound dopamine β -hydroxylase) can be found in the granule membranes of several species, including man. Dopamine β -hydroxylase is also present in the catecholamine-storing vesicles (large dense core vesicles) of sympathetic-nerve trunks (Hörtnagl *et al.*, 1969; De Potter *et al.*, 1970). It has been shown by immunological methods (Asamer *et al.*, 1971; Winkler *et al.*, 1972) that these nerve vesicles also contain chromomembrin B. Since these two proteins are the major proteins of chromaffin-granule membranes it seems that the membranes of nerve vesicles are very similar to those of chromaffin granules.

Helle (1971a,b) has published results on dopamine β -hydroxylase and chromogranin A, which are in obvious contrast with the results presented here. We therefore have to discuss her results in some detail. Two conclusions drawn by Helle (1971a,b) are of particular relevance to the present results. (i) The band preceding chromogranin A in disc electrophoresis is an aggregation product of chromogranin A. (ii) After electrophoresis of soluble lysates of granules, dopamine β -hydroxylase activity can be found throughout a disc gel, indicating enzymic activity of several chromogranins. This latter finding is in clear contrast with the results of Sage *et al.* (1967) and of Geffen *et al.* (1969). Both these groups demonstrated that the soluble dopamine β -hydroxylase is eluted from ion-exchange columns as a defined peak well separated from the other chromogranins. Further, Smith *et al.* (1970) have shown that the soluble dopamine β -hydroxylase activity is confined to the band preceding chromogranin A, when soluble lysates are resolved by polyacrylamide-gel electrophoresis. This result is confirmed by the present study where, after precipitation with *N*-cetylpyridinium chloride, the only protein of the soluble lysate remaining in the supernatant together with most of dopamine β -hydroxylase activity was this protein. These *N*-cetylpyridinium chloride experiments indicate that all chromogranins behave similarly, except for dopamine β -hydroxylase. It has been shown (Strieder *et al.*, 1968; Helle & Serck-Hanssen, 1969) that the amino acid composition of the total

chromogranins is characterized by a preponderance of acidic amino acids giving an excess of negative charges (Smith & Winkler, 1967b). It seems likely that this acidic nature of the proteins is responsible for the precipitation by the detergent *N*-cetylpyridinium chloride, since it is well known (see Scott, 1960) that *N*-cetylpyridinium chloride can precipitate polyanionic molecules. Dopamine β -hydroxylase is not precipitated to any great extent by *N*-cetylpyridinium chloride and, in fact, the amino acid composition of this protein differs from that of the chromogranins precisely in its lower content of acidic amino acids. The amino acid composition of dopamine β -hydroxylase, which is clearly different from that of the chromogranins, excludes the possibility that this protein is an aggregation product of chromogranin A, as concluded by Helle (1971a,b). Further, we have shown that an antiserum against dopamine β -hydroxylase does not cross-react with chromogranin A.

Kirshner (1962) has presented evidence that dopamine β -hydroxylase is localized on the inner side of the granule membranes. If this is correct, it is likely that the outer face of the granule membranes is not completely covered by proteins. Secretion from the chromaffin granules in the adrenal medulla is achieved by exocytosis involving a fusion of the granule membrane with that of the cell. To ensure the specific attachment of the granules to the cell membrane and the subsequent fusion, a protein of the granule membrane may be expected to act as a receptor (see Winkler, 1971). It seems now to be excluded that this 'receptor' protein can be chromomembrin A. It will be relevant to determine whether chromomembrin B or one of the minor proteins is localized on the outer face of these granules.

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References

- Asamer, H., Hörtnagl, H. & Winkler, H. (1971) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **270**, 87–89
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Belpaire, F. & Laduron, P. (1968) *Biochem. Pharmacol.* **17**, 411–421
- Blaschko, H., Comline, R. S., Schneider, F. H., Silver, M. & Smith, A. D. (1967) *Nature (London)* **215**, 58–59
- Clarke, J. T. (1964) *Ann. N. Y. Acad. Sci.* **121**, 428–436
- De Potter, W. P., Smith, A. D. & De Schaepdryver, A. F. (1970) *Tissue & Cell* **2**, 529–546
- Duch, D. S., Viveros, O. H. & Kirshner, N. (1968) *Biochem. Pharmacol.* **17**, 255–264
- Foldes, A., Jeffrey, P. L., Preston, B. N. & Austin, L. (1972) *Biochem. J.* **126**, 1209–1217

- Friedman, S. & Kaufman, S. (1965) *J. Biol. Chem.* **240**, 4763–4773
- Geffen, L. B., Livett, B. G. & Rush, R. A. (1969) *J. Physiol. (London)* **204**, 593–605
- Goldstein, M., Lauber, E. & McKereghan, M. R. (1965) *J. Biol. Chem.* **240**, 2066–2072
- Green, D. E., Haard, N. F., Lenaz, G. & Silman, H. I. (1968) *Proc. Nat. Acad. Sci. U.S.* **60**, 277–284
- Hartman, B. K. & Udenfriend, S. (1970) *Mol. Pharmacol.* **6**, 85–94
- Helle, K. B. (1971a) *Biochim. Biophys. Acta* **245**, 80–93
- Helle, K. B. (1971b) *Biochim. Biophys. Acta* **245**, 94–104
- Helle, K. B. & Serck-Hanssen, G. (1969) *Pharmacol. Res. Commun.* **1**, 25–29
- Hörtnagl, H., Hörtnagl, H., & Winkler, H. (1969) *J. Physiol. (London)* **205**, 103–114
- Hörtnagl, H., Winkler, H., Schöpf, J. A. L. & Hohenwallner, W. (1971) *Biochem. J.* **122**, 293–304
- Huber, H., Pastner, D. & Gabl, F. (1972) *Laboratoriums Diagnose hämatologischer und immunologischer Erkrankungen*, Springer-Verlag, Berlin, Heidelberg and New York
- Kaufman, S. & Friedman, S. (1965) *Pharmacol. Rev.* **17**, 71–100
- Kirshner, N. (1959) *Pharmacol. Rev.* **11**, 350–357
- Kirshner, N. (1962) *J. Biol. Chem.* **237**, 2311–2317
- Korn, E. D. (1969) *Annu. Rev. Biochem.* **38**, 263–288
- Kuzuya, H. & Nagatsu, T. (1972) *Biochem. Pharmacol.* **21**, 740–742
- MacLennan, D. H., Seeman, P., Iles, G. H. & Yip, C. C. (1971) *J. Biol. Chem.* **246**, 2702–2710
- Oka, M., Kajikawa, K., Ohuchi, T., Yoshida, H. & Imaizumi, R. (1967) *Life Sci.* **6**, 461–465
- Sage, H. J., Smith, W. J. & Kirshner, N. (1967) *Mol. Pharmacol.* **3**, 81–89
- Schatz, G. & Saltzgaber, J. (1969) *Biochim. Biophys. Acta* **180**, 186–189
- Schneider, F. H., Smith, A. D. & Winkler, H. (1967) *Br. J. Pharmacol. Chemother.* **31**, 94–104
- Scott, J. E. (1960) *Methods Biochem. Anal.* **8**, 145–197
- Senior, A. E. & MacLennan, D. H. (1970) *J. Biol. Chem.* **245**, 5086–5095
- Smith, A. D. & Winkler, H. (1967a) *Biochem. J.* **103**, 480–482
- Smith, A. D. & Winkler, H. (1967b) *Biochem. J.* **103**, 483–492
- Smith, A. D., De Potter, W. P., Moerman, E. J. & De Schaepdryver, A. F. (1970) *Tissue & Cell* **2**, 547–568
- Strieder, N., Ziegler, E., Winkler, H. & Smith, A. D. (1968) *Biochem. Pharmacol.* **17**, 1553–1556
- Viveros, O. H., Arqueros, L. & Kirshner, N. (1969) *Mol. Pharmacol.* **5**, 342–349
- Winkler, H. (1971) *Phil. Trans. Roy. Soc. London Ser. B* **261**, 293–303
- Winkler, H., Hörtnagl, H., Hörtnagl, H. & Smith, A. D. (1970) *Biochem. J.* **118**, 303–310
- Winkler, H., Hörtnagl, H., Asamer, H. & Plattner, H. (1972) in *Structural and Functional Proteins of the Nervous System* (Davison, A. N., Morgan, I. G. & Mandel, P., eds.), Plenum Press, New York, in the press
- Zlatkis, A., Zak, B. & Boyle, A. J. (1953) *J. Lab. Clin. Med.* **41**, 486–492