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First demonstration of highly specific and sensitive antibodies against dopamine

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Dopamine was coupled to bovine serum albumin (BSA) with glutaraldehyde, precautions were taken in order to preserve the catechol ring. After injection of this immunogen into rabbits, antidopamine antibodies were obtained and tested using radioimmunochemical binding studies and adsorption to catecholamine covered sepharose beads. A good correlation was found between the results of the different test systems, allowing us to visualize dopamine specifically in glutaraldehyde-fixed rat brains.

Since dopamine was biochemically found in the brain^{2,4,21}, the fluorescent Falck-Hillarp and the glyoxilic acid techniques^{5,6,9,17,18,24} have been used for its intraneuronal localization. More recently the visualization of the catecholamine synthesizing enzymes was used for this purpose^{12–16,22,23}. Using immunocytochemical techniques, catecholamines were never demonstrated directly nor specifically, because of the difficulty in raising antibodies against small-sized haptens as dopamine (DA). Initially, an attempt was made to raise antibodies to DA by injecting rabbits with a DA conjugate coupled to albumin with formalin¹⁰. A specific staining of dopaminergic neurons has been obtained in the goldfish (*Carassius auratus*) brain. However, the formaldehyde fixation of DA turned out to be highly dependent upon the nature of biological specimen, and to be poorly efficient in mammalian brain. This prompted us to synthesize a new immunogen coupled by glutaraldehyde, known to be a good histological fixative.

In the present report, we describe a method by which dopamine (DA) has been coupled to a protein carrier by glutaraldehyde in such a way that antigenic determinants of DA were fully protected. Subsequently the obtained antibodies recognized DA with high affinity and good specificity. These antibodies

were used in glutaraldehyde-fixed brain tissue for specific immunocytochemical demonstration of DA neurons and their fine processes.

To obtain the immunogen, 10 mg dopamine hydrochloride (Sigma) in 1 ml of distilled water were mixed with 30 mg bovine serum albumin (Sigma) in 1 ml of 3 M acetate buffer (pH 7.5). Then, 1 ml of a 5% glutaraldehyde solution (Fluka) was added. This dialdehyde is known to couple irreversibly and predominantly the ϵ -amino group of lysine in proteins¹ and the amino group of DA⁷. The reaction time lasted 3 min at room temperature. The end of the coupling reaction was signalled when the solution became orange-yellow in color. Since, after coupling, the remaining double bonds would lead more to the production of antibodies against imines than to the intended DA hapten, we solved this problem by reducing the double bonds. Consequently, 1 ml of sodium borohydride (Merck) 10 mM was added to the mixture to saturate the double-bonds. The mixture became translucent at the end of the reaction. The solution was dialyzed at 4 °C and separated by centrifugation and 1 ml of lyophilized aliquot was used to determine the conjugate concentration. Then, the molar coupling ratio was calculated after spectral analysis to be 40. The immunogen was administered to rabbits according to the method of Vaitukaitis²⁵ with

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Fig. 1. Displacement curves of $[^3\text{H}]\text{DA-G-ALM}$ by unconjugated catecholamines: (1) L-DOPA, octopamine, tyramine; (2) noradrenaline; (3) dopamine; (4) dopamine-glutaraldehyde-ALM (DA-G-ALM). From the non-displacement, it appears that the antibody site does not recognize unconjugated compounds.

some slight modifications. For each immunogen, 2 or 3 rabbits were injected subcutaneously into their back with 500 μg of an immunogen emulsified in 500 μl of 0.15 M NaCl and 750 μl of complete Freund's adjuvant (Difco). One booster injection per month was made during 5 months. In our radioimmunochemical system, antibodies began to appear after the second booster injection.

The antibody specificity and affinity were demonstrated quantitatively by a radioimmunochemical method using a radiolabelled ligand mimicking the antigenic determinant recognized by anti-DA antibodies. The catecholamine derivatives were synthesized by glutaraldehyde condensation reaction which coupled the ϵ -amino group of N- α -acetyl-L-lysine N-methylamide (ALM) (Sigma) to the amino group of either tritiated DA $[^3\text{H}]\text{DA}$ (NEN) or of non-radiolabelled DA, NA, tyramine (TA), L-DOPA and octopamine (OA). These conjugated catecholamines mimicked the configuration of these substances after fixation in the tissue. The reduction of double-bonds was always carried out by using sodium borohydride. Each semi-synthetic compound was purified on an SP C25 Sephadex column ($1 \times 48 \text{ cm}$) which was eluted by a $2 \times 200 \text{ ml}$ of a 10 mM–0.8 M NaCl gradient in 10 mM phosphate buffer, pH 6.2. After chromatography, the conjugate concentration was calculated by spectral analysis. Thereafter these derivatives

were used to evaluate the antibody specificity by equilibrium dialysis.

It appeared that the anti-DA antibodies only recognized DA coupled to a protein. Antibodies had a very low affinity for uncoupled DA (Fig. 1), as low as 10^{-6} M . DA-glutaraldehyde-ALM (DA-G-ALM) yields a conjugated DA with an immunogen configuration comparable to that found in the tissue. Thus, adsorption tests using free DA or any other uncoupled compound are not suitable in a procedure using glutaraldehyde or formaldehyde coupled compounds. So, tests for specificity could only be done with amines coupled by glutaraldehyde to a protein carrier or amino acid containing a free amino group (e.g. ALM). When employing these conjugates in our experiments, the antibody affinity appeared to be the best for DA-G-ALM, the self-displacement occurring between 10^{-9} and 10^{-7} M , indicating a rather high affinity (Fig. 2). The other glutaraldehyde-conjugated catecholamines exhibited a slight immunoreactivity, far from that found with DA-G-ALM. The next most immunoreactive compound was NA-G-ALM which was 50 times less reactive than DA-G-ALM. L-DOPA-G-ALM, OA-G-ALM and TA-G-ALM show even less immunoreactivity. The radiolabelled $[^3\text{H}]\text{DA-G-ALM}$, fully recognized by anti-DA antibodies, allowed us to firmly establish the antibody specificity by competition experiments in equi-

Fig. 2. Displacement curves of $[^3\text{H}]\text{DA-G-ALM}$ by conjugated catecholamines: (1) tyramine-G-ALM; (2) octopamine-G-ALM; (3) L-DOPA-G-ALM; (4) NA-G-ALM; (5) DA-G-ALM. These curves demonstrate the results of radioimmunological incubations performed by equilibrium dialysis, as described elsewhere¹⁸. The dialysis cells were fitted with large pore cellulose membranes (Sartorius SM 115 33 or Schleicher and Schull RC 50). Each catecholamine or conjugated catecholamine was mixed with an equal volume of radiolabelled ligand before being placed on one side of the membrane. On the other side the antibody dilution was 1/500. The final volume was 300 μl ($2 \times 150 \mu\text{l}$), and the dialysis lasted 20 h at 4 °C. After dialysis, 100 μl of the solution were prelevied from each membrane side and put in vials containing 1 ml of Instagel. Beta-radioactivity was evaluated and the binding ratio calculated as bound/total (B/T).

librium dialysis. But to complete specificity studies for immunocytochemical applications, a test system was used in which the catecholamines were coupled with glutaraldehyde to the ϵ -amino group of lysine bound to sepharose beads. An evaluation of the catecholaminergic coupling ratio on sepharose beads has been done by using radiolabelled catecholamines. The anti-DA antibody was incubated with 'catecholamine sepharose' beads while for the detection of the bound anti-DA antibodies the same peroxidase-labelled secondary antibody was used as in immunocytochemistry. Peroxidase activity was quantitatively measured using phenyldiaminobenzidine, which yields a soluble staining in the incubation buffer. The optical density of the reaction was measured. With this procedure, it was also found that the antibody had a very high affinity for DA coupled to the lysine agarose beads, while the antibody appeared to bind with a much lower affinity to NA-lysine beads. In addition, it appeared that after adsorption of the antibody to NA-lysine beads, the anti-DA antibody affinity for NA-lysine beads disappeared while it was hardly diminished for the DA-lysine beads. These results are in close agreement with those obtained from radioimmunochemical tests.

Using the present antibodies in an immunocytochemical procedure on glutaraldehyde-fixed rat brain, at a 1/3000 dilution, a very intense staining was observed in the dopamine-containing cell bodies of the substantia nigra (Fig. 3, parts 1 and 2). In addition, a light staining was observed in the cell bodies of the locus coeruleus (Fig. 3, part 3) which persisted after adsorption of the antibody with NA-lysine beads. It can be concluded that this staining is due to the presence of DA which acts as a precursor in noradrenaline-containing neurons²⁰. The DA antiserum enabled the visualization of dopaminergic fibers in the lateral septum, the prefrontal cortex and the dendrites of the substantia nigra (Fig. 3, parts 2 and 4) which is in good agreement with the hitherto described projections^{13,15,17,18,24}. On no occasion was a staining observed in the noradrenergic fiber system such as in the cerebral or cerebellar cortex. After adsorption of the anti-DA antibodies with glutaraldehyde-conjugated DA-protein, the specific staining disappeared. The immunochemical detection of dopamine eliminated some of the problems of the previous techniques, such as the histofluorescence technique⁸ and its derivatives^{3,19}. These techniques allowed the specific demonstration of dopamine neu-

Fig. 3. Parts 1-4: vibratome transversal sections of a rat brain, perfusion fixed with 5% glutaraldehyde in 0.05 M cacodylate buffer containing 1% $\text{Na}_2\text{S}_2\text{O}_5$, pH 7.5, incubated with a dopamine antiserum diluted 1:3000. Parts 1 and 2 demonstrate the dopaminergic cell bodies in the substantia nigra pars reticulata, the arrows point to the dendrites extending into the pars compacta. Part 3 demonstrates the weak staining of the noradrenaline (and dopamine) containing cells of the locus coeruleus (LC). Arrows point to thin dopaminergic fibers in this area. Part 4 shows a part of the dopaminergic fiber system in the lateral septum (L.S.). The arrows point to perineuronal fibers; E = ependyma of the third ventricle. (Bars = 100 μm .)

rons only when subsequent microspectrofluorimetric analysis could be performed distinguishing dopamine from other catecholamines³. Up until now, however, these techniques have not allowed an easy localization of dopamine especially not in areas densely innervated with NA-containing fibers. In addition the visualization of dopamine in glutaraldehyde-fixed brains will also permit the direct localization of this amine at the electron microscopical level.

It can be concluded that it is possible to raise sensitive and specific antibodies to amines, provided that: (1) the necessary precautions to preserve the molecular form of these substances are taken; (2) when a fix-

ative is used to couple the antigen to a protein carrier, the same fixative should be used to retain the antigen in the tissue; and (3) for the specificity and affinity tests the antigen should be fixed to a protein carrier using the same fixation procedure. If the above-mentioned procedures are taken into account, it is herewith demonstrated that also for small amines it is possible to raise sensitive and specific antibodies.

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