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The influence of salts on the reaction between hGH and several of its monoclonal antibodies in fixed, paraffin-embedded human pituitary as a model system

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SUMMARY

A study of the influence of three salts of monovalent cations (Nacl, KCl, and CsCl) and three salts of divalent cations ($MgCl_2.6H_2O$, anhydrous $CaCl_2$, and $SrCl_2.6H_2O$), on the specific reaction between human hGH and three of its monoclonal antibodies has been carried out, using human pituitary glands obtained at autopsy as a model system. This study is an attempt to find out the physico-chemical laws ruling the immunohistochemical reaction in the special circumstance of fixed specimens, where the antigen (Ag) is immobilized in the tissue substrate, while the antibody (Ab) is mobile. Our interpretation of the results points to a reaction of the salts with side groups of aminoacids in the paratope (specific saline effect) and a mass salt effect on the reaction kinetic proper that is dependent on the salt concentration. The high affinity coefficient of the in situ Ag-Ab reaction is shown, at the immunohistochemical level, by the high molarity necessary to make the reaction negative. Also, it seems there is a direct relationship between the critical molarity values obtained and the affinities of the monoclonal antibodies used in this study.

INTRODUCTION

There are data in the literature to assert that the nature of the physical forces responsible for maintaining the epitope and the paratope bonded are short ranged (van der Waals, hydrogen bonds, hydrophobics) and that the electrostatic forces acting at a greater distance capable of approaching both surfaces locally have to act against the high dielectric constant of water. Consequently, the two surfaces (epitopic and paratopic) have to be complementary, so as to be able to permit the closest approach between them over a large area (Novotny *et al.*, 1987). The work of many authors has helped to understand the basis of such a great attraction (see Saira Mian *et al.*, 1991, for a review) and the general consensus on the structural features determining the binding of Ag and Ab have been summarized by Braden and Poljak (1995).

It may be anticipated that: (1) the Ag-Ab reaction specificity cannot have the same character in fixed tissues as in vivo or in vitro, (2) a study of the alterations introduced in protein side groups by fixation may lead to a better understanding of the Ag-Ab reaction in this material, (3) one way to do this may be by the blockade of the histochemical procedures for the demonstration of side groups of proteins and their effect on the two Ag-Ab reaction. We have made two initial studies with the blockade of amino groups (Montero and Segura, 1989), and the benzyl blockade of the guanidium group of arginine (Montero *et al.*, 1991). Although there is still much to be done in this area, we have now used another approach.

Inspired by the work of Scott and Dorling (1965) and the theoretical exposition of Scott (1973), we thought it might be interesting to study the influence of adding increasing concentrations of various cations to the dilutions of the primary antiserum before doing the Ag-Ab reaction. We assumed that much higher concentrations of the salts than those used by Scott and Dorling (1965) were going to be needed, considering the very high affinity between Ag-Ab, due to the short ranged physical forces that act there. We used hGH and various of its monoclonal antibodies on sections of human pituitaries, as a model system. Our aim was twofold: to make a more detailed study of the action of electrolytes on the Ag-Ab reaction, and to follow the assertion of Avrameas (1992), that "...more knowledge is needed to clarify the differences between specific and non-specific binding...", which we eagerly support.

On the other hand, high background staining is frequently found, even with the use of monoclonal antibodies. Jones *et al.* (1987) suggested increasing the ionic strength of the buffer used to dilute the primary antiserum, and in washing to eliminate the background staining, with great success in their procedures, but without a theoretical explanation of the underlying physicochemical mechanism. We have attempted an explanation as a consequence of our results.

MATERIALS AND METHODS

The material used in this work was 30 human pituitaries, obtained from autopsies done in the Department of Pathology of the University Hospital. Portions of each gland were fixed in methacarn (Puchtler *et al.*, 1970), for 20-24 hours at room temperature (25-27°C). The specimens were then treated with three changes of methanol, two hours each; next, they were immersed in methylbenzoate overnight, cleared in xylene and embedded in paraffin. Five-micron sections were obtained in a rotary microtome and adhered to slides that had been covered with a chromalum-gelatine mixture (Pappas, 1971). After drying the slides overnight at 37°C, they were ready for processing.

The unlabelled antibody enzyme method of Sternberger (1970) was used as follows: briefly, the hydrated sections were covered with a 1:1000 dilution of the anti-GH monoclonal antibody being tested, using 0.05 M Tris buffer, pH 7.5, plus 0.8% NaCl (TBS) or the selected molarity of the salt in question dissolved in 0.05 M Tris, pH 7.5. Incubation was overnight (18-20 hours) at 4°C. The sections were then washed with TBS, incubated with a 1:100 dilution of rabbit anti-mouse immunoglobulin (DAKO A/S Produktionsvej 42, 2600 Glostrup, Denmark), at room temperature, for 30 minutes, washed again with TBS and incubated for 30 minutes, at room temperature, with monoclonal PAP (DAKO), diluted 1:100 in TBS. Finally, the sections were washed with 0.05 M Tris buffer, pH 7.5 and the peroxidase activity developed in a solution of 3% diaminobenzidine (DAB) in Tris buffer, with a drop of 3% hydrogen peroxid added. The brown colored reaction was monitored under the microscope for 3 to 4 minutes.

Controls for the immunohistochemical reaction

A check of the method was always carried out. Substitution of TBS for the primary antibody, as well as for the secondary antibody and PAP was done periodically during the course of the experiments. The immunological control consisted of the absorption of the primary antiserum with its specific antigen overnight and incubation of one section with this serum. An aliquot of a solution containing 20 µg hGH/ml was absorbed with an aliquot of a 1:1000 dilution of the anti-GH monoclonal antiserum in question. A control experiment, to assure that the antigen-antibody reac-

tion worked between the pH range of 5 to 9, was done as follows: the antibody against GH was diluted 1:1000 in a series of 0.1 M phosphate buffers of pH 5.13, 5.79, 6.43, 7.03, 7.69, and 8.72. Sections were incubated overnight at 4°C and were then revealed as usual.

Procedures for the use of salts

The following salts were tried: NaCl, KCl, and CsCl, among the monovalents cations, and $MgCl_2.H_2O$, anhydrous $CaCl_2$, and $SrCl_2.6H_2O$, among the divalent cations.

A series of solutions of graded molarities of the different salts were prepared in 0.05 M Tris, pH 7.5. A decreasing or increasing value of the pH, depending on the salt used, was obtained upon addition of increasing amounts of the salt. However, all these pH's fell within the range of pH where the Ag-Ab reaction was found to work.

Reagents

All chemical reagents used in this work were analytical grade (Merck, Darmstadt, FRG). The monoclonal antibodies were obtained from Chemicon (El Segundo, CA, USA) and the reference number in their catalog (MAB 650, 651, and 652) will be used in this paper. Tris reagents were obtained from Sigma Co. (St. Louis, MO, USA).

RESULTS

The final reaction schemes for the three monoclonal antibodies (650, 651, 652) were similar, except that MAB 652 gave an overall less intense immunocytohistochemical reaction in the control sections. When similar sections were submitted to the PAP method using 1:1000 dilutions of the anti-GH antibody in 0.05 M Tris, pH 7.5, and the addition of one of the divalent cation salts in ascending molarities from 0.5 M up to and including 3.0 M, 4.0 M, and 4.5 M, depending on the solubility of each salt, the intensity of the final reaction decreased towards a critical molarity where the Ag-Ab reaction was completely negative.

The final pH of the antibody-salt solution in 0.05 M Tris, pH 7.5 was always measured (Table I) to make sure that it fell within the range of pH where the antigen-antibody reaction regularly worked, as tested in the control experiment explained in MATERIALS AND METHODS.

The immunocytohistochemical reaction for any of the three monoclonal antibodies studied (MABs 650, 651, 652) was not affected by the addition of the monovalent cation salts (Na, K, Cs), at least in the range of the molarities studied. However, concentrations over 3.0 M for CsCl, gave a pH higher than 9, which might have

Table I
pH of the salt solutions of ascending molarities in 0.05 M Tris, pH 7.53

	MOLARITIES								
Salt	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
$MgCl_2$	7.50	7.33	7.11	6.97	6.76	6.52	5.95	5.81	5.47
$CaCl_2$	7.38	7.20	7.04	6.91	6.77	6.59	6.46	6.31	6.16
$SrCl_2$	7.50	7.33	7.12	6.92	6.76	6.57	–	–	–
NaCl	7.58	7.58	7.58	7.58	7.58	7.58	7.58	7.58	7.58
KCl	7.72	7.80	7.83	7.91	7.94	7.98	8.01	–	–
CsCl	8.30	8.55	8.65	8.72	8.79	8.85	8.88	–	–

NOTE: The solutions were prepared in 0.05 M Tris, pH 7.53 as a solvent. It may be seen that every pH value obtained falls into the range where the antigen-antibody reaction is regularly obtained as explained in the pertinent control experiment (see text). KCl and CsCl are not soluble beyond 3.5 M and $SrCl_2.H_2O$ beyond 3.0 M.

affected the result of the Ag-Ab reaction; consequently, this salt was not used above this concentration.

The salts of the divalent cations (Mg, Ca, Sr) always produced a negative reaction in a range of molarities that varied from one monoclonal antibody to the other for the three cations (Table II).

There was an oscillation of the critical molar concentration needed to make the Ag-Ab reaction negative from one case to the other, and, in every case, the values given are the average of six experiments. This is not surprising considering the many variables involved in this kind of experiment. However, despite all this, the results obtained were homogeneous and all point to a similar value for the molar concentration needed to make the Ag-Ab reaction negative, after a gradual decrease of its intensity.

An interesting result of the addition of the divalent cation salts used in these experiments was the elimination of background "staining" (Figs. 1 and 2), observed in the control sections, even at a 0.5 M concentration of the salt. A similar result, though in a biotin-avidin system, was found by Jones *et al.* (1987).

DISCUSSION

The binding of two biomacromolecules, as it occurs in an immunocytohistochemical reaction,

Table II
Critical molarity for the three monoclonal antibodies and the three salts of the divalent cations used

Type of salt	MAB 650	MAB 651	MAB 652
$MgCl_2.6H_2O$	4.5 M	2.0 M	0.6 M
$CaCl_2$ anhydrous	3.5 M	1.8 M	1.0 M
$SrCl_2.6H_2O$	3.0 M*	2.5 M	0.5 M

* Immunohistochemical results were almost negative at this molarity; they are assumed to be negative at higher molarity; however, 3.0 M is just around the saturation point for the strontium salt and could not be tried.

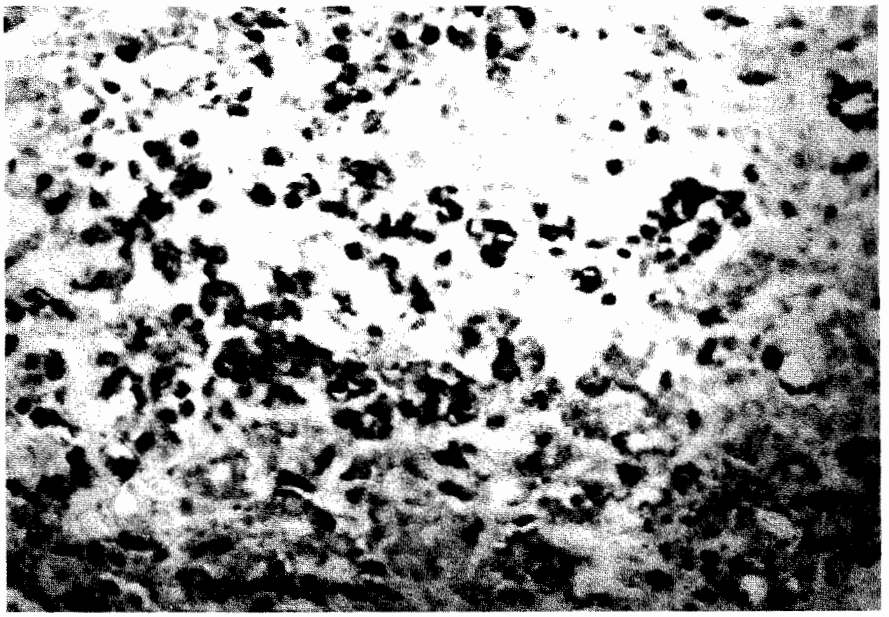

Fig. 1 - Immunohistochemical demonstration of GH in human somatotrophs using MAB 650 1:1000 in 0.5 M Tris, pH 7.53. showing some background staining. PAP method. Original magnification 20X.

is subject to physicochemical laws, as is true of any other reaction between any two kinds of macromolecules (Gutierrez, 1990, 1991; Avrameas, 1992), but it must be under some limitations due to the special location of one of the participating biomacromolecules; in fact, one of these is fixed in the tissue, while the other is mobile, and any interpretation of the reaction between them must take this fact into account. We assume this must affect the results of any kinetic study that might be undertaken.

A methodology similar to that used by Scott and Dorling (1965) has been applied here because the Ag-Ab reaction consists of a series of preliminary steps of the same character as those that take place in dye/substrate binding. The reaction differs in that the tight binding between Ag and Ab is established by the matching conformation between epitope and paratope which permits the formation of a series of hydrogen bonds, as well as van der Waals forces and hydrophobic binding. We wondered to what extent the interference of salts with the preliminary electrostatic binding would prevent the subsequent conformational match, if this is the way it actually works.

Monoclonal antibodies were used because, differently from polyclonal antibodies, they are homogeneous in their affinities; consequently, the kinetics of the Ag-Ab reaction is simpler to study and the lessons learned with the monoclonal antibodies could be used, in the future, in an analysis of the Ag-Ab reaction with polyclonal antibodies.

It has been proposed by one of us (Gutierrez, 1990, 1991) that we are dealing here with a "saline effect", for interactions chiefly of an electrostatic nature of various kinds, that may take place between macromolecules. However, Paverit (1990), in a study of the salting effect on the kinetics of enzymatic reactions, noticed that the addition of salts to the reaction medium may produce a specific salt effect, i.e. the binding of saline ions to the reactant. A similar phenomenon might take place in the Ag-Ab reaction by the binding of saline ions to the antibody macromolecules.

In the present experiments, we measured the reactions of both anions and cations with amino acid residues on the paratopes, which may establish a kind of equilibrium with the ions present in the medium (the specific salt effect); and following this, the reactions to equilibrium between the paratopes, encumbered by the cations and anions present in the surrounding atmosphere (the unspecific salt effect), and their corresponding epitopes. Considering the high affinity coefficient of the reaction between the antigen and the antibody, the molar concentration of the salt to be

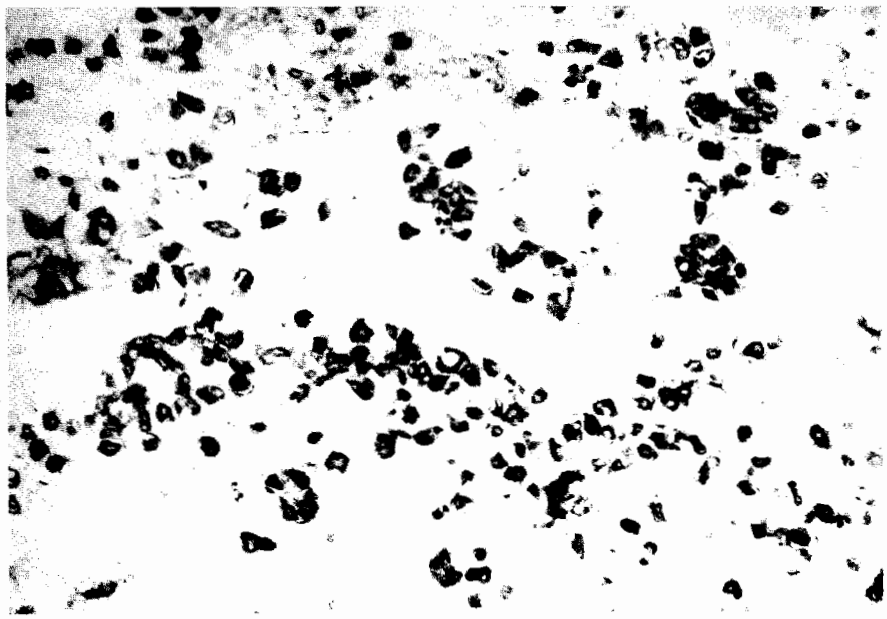

Fig. 2 - Immunohistochemical demonstration of GH in human somatotrophs using MAB 650 1:1000 in 0.5 M $MgCl_2.6H_2O$ in 0.05 M Tris. There is complete absence of background staining. PAP method. Original magnification 20X.

added was thought to have to be sufficiently high to alter the equilibrium between the breaking of bonds and the possibility of establishing them. This conclusion seems to have been confirmed.

The supersaturation of the amino acid side group in the paratope, which might take place during the preparation of the dilution of the primary antibody, at the molar solution of the respective salt, may be effective, at a specific molar value, to block the reaction with the epitopic electrophylic side groups and, subsequently, prevent the conformational match.

There is a general trend of decreasing values of the critical molarity needed to make the immunohistochemical reaction negative from MAB 650 down to MAB 652 and this is good for the three salts studied. The apparently obvious interpretation is that of a decreasing affinity of each one of these antibodies in the same order; in other words, the critical molarity seems to be directly proportional to the affinity values.

Our interpretation of the elimination of the background staining, even with the lowest molarity of the salt used (0.5 M), is that this staining is due to the binding of the macromolecules to the proteins in the tissue by electrostatic bonds only. The elimination of the unspecific reaction, that is found as a background staining, by low molar values of the salt used, is an exponent of its electrostatic nature with a low affinity value, and may be used to enhance the specific staining reaction. This observation is coincident with that of Jones *et al.* (1987).

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