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SPECIFICITY IN IMMUNOCYTOCHEMISTRY

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I. INTRODUCTION

Specificity in ICC starts with obtaining some insight into the factors that influence the final outcome of an ICC staining procedure (fig. 1). One of the main difficulties in each ICC study is the complicated set of interrelationships between these factors (indicated by the arrows in fig. 1) leading to the notion that the specific contribution of each individual factor can in most cases never be determined. For instance, a test on the applicability of different tissue fixations for the maintenance of tissue morphology cannot be determined independently from their involvement in the conservation of detectable immunoreactivity of the tissue antigens. At the same time the detection level of immunoreactivity
will be determined by the specificity and potency of the first antiserum both again dependent on the sensitivity of the ICC procedure used. Therefore, in order to gain at least some insight into for instance the possible loss of antigenic determinants during the tissue fixation or in the immunological specificity of the first antiserum, the choice of the control experiments as well as the sequence in which these experiments are carried out is very important. The flowchart given in fig. 2 summarizes the steps usually taken to gain an insight into the specificity of an ICC staining procedure. This 'conventional' approach can be divided into three parts. During the first part, the tissue processing and antiserum incubation conditions are selected in such a way that after incubation of a section (known to contain the component to be localized) with an antiserum raised against that component, a positive reaction is found. If by the start of an ICC study both the quality of the antiserum and the presence of the antigen in
Figure 2. Flowchart of control experiments commonly used to investigate the specificity in an immunocytochemical staining procedure. In this approach, method-specificity is defined as the absence of staining caused by mechanisms other than the interaction between antibodies and the antigen to be localized, and serum-specificity as the situation in which the primary antibodies only react with the antigen to be localized and not with other (tissue) components (Van Leeuwen, 1980).

The tissue are not known, it will be necessary to first use a tissue that may not have one's particular interest, but that is known to contain this component. Only with some insight, both into the immunological characteristics of the antiserum as well as in the procedures necessary to study immunocytochemically the localization of this compound, the 'switch' to unknown tissues can be made.

During the second step the first antiserum is pre-adsorbed with the component to be localized. No reaction of this adsorbed antiserum in the tissue section allows the conclusion that the reaction between the tissue and the antiserum was caused only by antibodies that are able to react with the component used for the ad-
This type of specificity has been called *method specificity* (see legend fig. 2; Van Leeuwen, 1981) and is commonly taken as the ultimate proof for specificity in ICC (Petrusz et al., 1976, 1977). However, in order to proof 'monospecificity in ICC' (see subchapter IX), an additional step is necessary to ascertain that no other tissue components than the antigen used in the adsorption test (cross) react with the antibodies proven to be responsible for the positive ICC reaction (i.e., *serum-specificity*; see legend fig. 4; Van Leeuwen, 1981; Vandesande, 1979). The main reason that this step is often neglected in most ICC specificity test procedures is that usually no information is available about the presence of such cross-reacting compound(s) in the tissue under study (Vandesande, 1979).

The immunocytochemical specificity as ultimately found using this sequence of control experiments is mainly dependent on the specificity of the adsorption in step 2. During this adsorption only the antibodies that react with the component to be localized have to be removed from the antiserum. Therefore, high demands have to be made on the purity of this antigen preparation, demands often impossible to fulfil for antigens isolated from biological material. However, even if an antigen preparation would be available which only contains the component to be localized, the pre-adsorption test still only shows that the immunoreactive compound(s) in the tissue and this component share antigenic determinants. Method specificity obtained this way alone, certainly does not allow definite conclusions with respect to the identity of the antigen(s) present in the tissue. An additional problem may arise during the adsorption step in the flowchart of figure 2 when the animal already produces antibodies capable to react with tissue component(s) prior to immunization. Examples have been described e.g. by Kurki et al. (1977) and Gordon et al. (1978) who were able to characterize immunocytochemically intermediate (10 nm) filaments using 'normal' human or rabbit serum, and Trenchev and Holborow (1976) who described auto-actin antibodies in 80% of their rabbits.

In conclusion, there are at least three drawbacks with respect to the use of the sequence of ICC controls as given in the flowchart in figure 2:

(a) it makes often too high demands on the purity of the antigen to be used for the adsorption in step 3;
(b) it does not supply sufficient information on the identity (or number of) immunoreactive compounds present in the tissue, and (c) it leads to an unsolvable method a-specificity in case of contaminating pre-immune antibodies.

During the last few years in the ICC literature an approach towards specificity has been described differing at least in two important aspects from the procedure described above; firstly, the adsorption test with the antigen to be localized no longer plays such a crucial role, and secondly a positive identification of the immunoreactive compounds actually present in the tissue under investigation forms an intrinsic part of the whole procedure. The se-

Figure 3. Flowchart summarizing the proposed sequence of control experiments to provide specificity in immunocytochemistry (specific only for one defined antigen, the antigen under investigation). This scheme (which is discussed in detail in subchapter II) serves as a guideline to the chapters III-VIII.

For definitions of method specificity and serum specificity see subchapter IX.
sequence of steps necessary for such an ICC specificity study is given in fig. 3. Despite the fact that all problems with respect to the general application of this approach are certainly not yet solved, it was still decided to use this approach as a basis for this chapter on specificity in ICC. Within this frame-work, subchapter II will deal with the theoretical implications of the flow-chart in figure 3, while in subchapters III to VIII the more practical aspects will be described. Subchapter IX, finally, provides a small glossary in which some of the expressions used throughout this chapter are explained.

II. A FLOWCHART OF IMMUNOCYTOCHEMICAL CONTROLS

In this subchapter the flowchart given in figure 3 serving as a guideline to the subchapters III to VIII will be discussed. The various stages are illustrated with control experiments necessary to obtain a specific ICC localization of a component A in a certain tissue.

The initial demands made on the tissue and on the antiserum serving as starting points in the search for specificity in ICC are given in the first frame.

An antiserum raised against A
A tissue known to contain A

After incubation of the antiserum with a section of this tissue there are two possible outcomes:

positive reaction  no reaction

Since the tissue is known to contain the antigen, 'no reaction' can be designated as false-negative. Possible causes of this false negativity may be found at the level of:
(a) the tissue section,
(b) the procedure used to identify the first antibody,
(c) the first antibody,

points which are discussed in subchapter III

Positive reaction on the tissue section

Having arrived at a stage where we obtained a positive reaction with a potent antiserum raised against component A, on a well-preserved section known to contain this antigen, the next step is to examine the reactivity on a section from the same tissue after incubation with a 'non-immune serum' (i.e. serum with no immunoreactivity towards any of the tissue components) while all other steps are kept the same. This 'blank-value' is meant to ascertain whether the immunocytochemical reagents alone cause staining of the tissue, a situation which is called method-specificity (see subchapter IX; Sternberger, 1979).

(Notice that this definition is different from that given in the legend of figure 2.)

After incubation with such a non-immune serum there are again two possible outcomes:

no reaction positive reaction

Since no reactive antibodies are supposed to be present in the non-immune serum, a positive reaction found with this serum can be designated as false-positive.

Causes for false-positive results at this stage may again be found at the level of:

(a) the tissue section,
(b) the procedure used to identify the first antibody, or
(c) the first antiserum itself,

and will be dealt with in subchapter IV.
Having eliminated the factors that may cause false positivity, the following conditions have to be met:

Positive reaction on the action with the antiserum
Negative blank (= method specificity)

At this moment all attempts in an ICC specificity study have to be focussed on the question:

What component(s) in the tissue are exactly responsible for the positive (immunological) reaction of the (first) antiserum?

which is a first step towards a 'serum specific' ICC localization procedure (see subchapter IX).

The criteria for a test which may reveal the serum specificity of an immunocytochemical staining procedure will be discussed in subchapter V. In addition, some of the techniques currently used or being developed for this purpose will be described there.

However, irrespective of what method is used, this step in an ICC serum specificity study should always result in:

a list of components present in the tissue under study, which react with the antiserum in the immunocytochemical procedure

When these compounds have been identified, the unwanted antibodies have to be removed from the antiserum. Some of the techniques currently used for this purpose and the interpretation of the final outcome of such adsorption experiments will be dealt with in subchapter VI.

The moment all necessary antiserum purifications have been carried out we have arrived at a stage where:
According to the test system we have a purified antiserum which reacts only with one tissue component.
This purified antiserum reacts positively on the tissue section.

The final test for serum-specificity in ICC must be performed both on the tissue section and in the model test system and must give an insight into the validity of the serum specificity as was found in the model test system for the ICC reaction on the tissue section. These final tests and the implications of their outcome for the specificity of the ICC reaction on the tissue section will be described in subchapter VII. Ideally this next step in the flowchart should be:

- No reaction on the section and in the test system with the purified antiserum after adsorption with its homologous antigen (A) and
- No reaction with the purified antiserum on a section of the same tissue lacking this antigen.

If at this stage these requirements cannot be fulfilled simultaneously, the staining results on the (normal) section with the purified antiserum (step 3) can be considered 'immunocytological' rather than 'immunocytochemical'. In order to obtain a more immunocytochemical result a jump backwards in the flowchart (see figure 3) will be necessary (see also subchapter VII).

The question whether

A specific immunocytochemical localization of the component (A) in the tissue
can ultimately be obtained using this sequence of control experiments will be discussed in subchapter VIII.

Q.E.D.?

III. FALSE NEGATIVITY

The starting point for the control of specificity in ICC is a positive reaction in a tissue (see step 1 of the flowchart in fig. 3). However, the situation may occur that in a section from tissue which is known to contain a certain component, no staining is found after incubation with an antiserum that is supposed to contain antibodies against that component, a phenomenon which is called false-negativity. As mentioned already in subchapter II, causes for such false negative results may be found at the level of (a) the tissue section, (b) the immunocytochemical procedure used to detect the first antibody, and (c) at the level of the first antiserum itself.

Sub a. There are three possible mechanisms which might result in a false-negative ICC reaction caused at the level of the tissue section, the first being a loss of soluble antigens during the tissue processing or ICC incubation procedures (1), which may be prevented by fixation of the tissue prior to the staining procedure (see contribution R.M.Buijs). However, in addition to an immobilization of the tissue components, fixation may also lead to an impairment of antigenic determinants (2). Many examples can be given in this respect (Swaab et al., 1975; Steinbusch et al., 1978; Sternberger, 1969; Schipper, this manual). In addition, diminution of immunoreactivity has also been reported to occur during embedding procedures (Sternberger, 1979). A third possible cause for false negativity at the tissue section level is the inaccessibility of the antigen during the antiserum incubation steps (3). This is especially important when thick sections are used as for instance
in pre-embedding immunocytochemical staining procedures (Buijs, this manual).

Sub b. The procedure to visualize the first antibody may also be a cause for false negativity. A negative ICC reaction, in spite of an immunoreactive compound in the section and a first antibody which reacts with this antigen, might occur when the PAP procedure is used to visualize the immune complexes. A high concentration of first antibodies in the tissue section caused by a high concentration of the antigen might result in binding both antigenic sites of the (second) bridge antibody and consequently in a failure to bind the PAP complex (4). Dilution of the first antiserum usually solves this problem (Bigbee et al., 1977). However, also in the case of a two-step procedure the antiserum used to visualize the bound first antibodies may be a cause for false negativity. In sections incubated with serum samples from an oxytocin immunized rabbit and subsequently stained with a commercially available horse anti-rabbit IgG fluorescein conjugate (PKF 17-2F3) hardly any reaction was observed with antiserum samples collected at the end of the 1.5 year immunization period. However, when these samples were applied in a PAP procedure using a different anti-rabbit IgG preparation, they turned out to have excellent staining abilities (fig. 4).

![Diagram](image)

**Figure 4.** Diagram representing the 'Bigbee-effect'. Due to a high local concentration of first antibodies in the tissue section both antigenic sites of the second (bridge) antibody are 'used', resulting in a failure to bind the PAP-complex.
This shows that in indirect staining procedures the final ICC-reactivity may differ depending upon the specificity (IgG subclass?) of the second antibody (cf. subchapters VI and VIII).

Sub c. False negativity may also be due to a first antiserum that is less potent than supposed, for which several possible causes can be mentioned:

The immunisation procedure may be the cause for such a low potency antiserum. The most commonly used animal for immunization purposes is the rabbit. Important considerations in choosing an animal for this purpose are the source and availability of the immunogen. Rabbits, for instance, may produce low (or no) titer antisera when immunized with a 'rabbit-related' antigen. The use of other animals (sheep, goat, rat, chicken) then has to be considered as well as the use of denatured antigens (Lazarides, 1976; Jokusch et al., 1978). Peptides (or other compounds with a molecular weight below 4000 D) generally have poor immunogenic properties. In order to obtain 'high titer' antisera these compounds should be coupled covalently to large proteins like serum albumin or thyroglobulin (Skowsky and Fisher, 1974). Thyroglobulin seems to be superior in terms of percentage of rabbits that respond and in the radioimmunoassay (RIA) titer which is obtained (Skowsky and Fisher, 1972). In order to avoid — later in the ICC staining procedure — interference due to the antibodies raised against the carrier protein (Vandesande, 1980; Steinbusch et al., 1979), it seems appropriate to use proteins that are as little as possible related to the proteins of the tissue studied. Also in this respect thyroglobulin or even better, sunflower globulin (Grouselle et al., 1978) seems to be a better choice than for instance serum albumin.

False negativity caused by the first antiserum may also be due to the selection of an improper antiserum sample. In immunocytochemical papers precipitation techniques (Ouchterlony, Immunoelctrophoresis) or radio-immunoassays (RIA) are frequently mentioned as methods to select antisera for ICC studies. However, the general validity of these techniques for the determination of potency and/or specificity (see subchapter V) of an antiserum in an ICC procedure is non-existent and may therefore lead to the selection of an antibody that is potent in one of these techniques but
Figure 5. The course of an antibody development in a rabbit (0-2) immunized with oxytocin (Sigma Grade V; lot 103C-2910) coupled to bovine thyroglobulin according to Skowsky and Fisher (1972). Each arrow indicates one immunization whereby the length represents the amount of oxytocin that was injected. Plasma samples were collected one week after each antigen injection. In each sample radio-immuno assay (a,b) and immunocytochemical (c,d) properties were measured:
(a) maximal binding (% of oxytocin tracer to an excess of antibody;
(b) titer (the antibody dilution to which 50% of the oxytocin tracer was bound) (see also Docterom et al., 1976);
(c) the potency in an indirect immunofluorescence procedure, and
(d) the potency in a PAP-procedure.

were measured on neurohypophysial sections of male Wistar rats using the different antiserum samples in the same dilution (1:80 for the IMF- and 1:200 in the PAP-procedure).

causes a false negative result in an ICC staining procedure. The considerable discrepancy between the reactivity of an antiserum in an indirect immunofluorescence procedure, in a PAP procedure, and in a RIA, is shown in figure 5. Seven weeks after the start of the (oxytocin) immunization an excellent antiserum is obtained for the use in both ICC staining procedures, while the same antiserum samples are worthless for radio-immunoassay. However, after 10-12 weeks the (high) potency in the RIA remains, the reactivity of the antiserum in the PAP procedure diminishes only slightly, while on the other hand the immunofluorescence values rapidly drop to useless values. Two conclusions have to be drawn from these findings:
(1) the characteristics of an antiserum vary during the immunization period. This means that the reactivity in an ICC staining procedure can be enhanced by using serum amounts collected at a proper time. Pooling of antisera collected at different time intervals during an immunization decreases thus mostly their capacity in ICC. In order to obtain large samples of antiserum, it is our experience that 3 times 50-60 ml blood can easily be collected from the same rabbit within 5 days, if intraperitoneal plasmaphoresis is performed (see Appendix A-1).

(2) whether an antiserum will be qualified as 'good' depends largely on the technique used. The 'best' antisera for immunocytochemical studies therefore have to be selected with the same ICC procedure as will be used on the tissue sections.

IV. METHOD SPECIFICITY

The moment a positive reaction in a tissue section is obtained after incubation of an antiserum followed by an ICC staining procedure, the question arises whether this reaction is only due to an immunological binding of antibodies from this serum (= method specificity; see subchapter IX), or (also) based on a false positive reaction of the immunocytochemical reagents.

The causes for false positivity may again be found at the level of (a) the tissue section, (b) the procedure used to visualize the first antibody and (c) the first antiserum itself. In order to evaluate each of these possibilities, different control incubations should be carried out.

Sub a. The role of false positivity caused by the tissue section will become obvious after inspecting an unstained section. Possible causes described for this type of false positivity are: (1) the presence of a pigment in the cell which resembles the DAB precipitate (when an immunoperoxidase method is used) like neuromelanin in the substantia nigra; (2) autofluorescence (in case of an immuno-
fluorescence procedure), as e.g. caused by lipofuscin which is often dominant in tissue of older human beings. One might either try to solve this problem by using a different fluorochrome and/or filter combination (although other conjugates (rhodamin) usually exhibit a lower emission than the FITC conjugates and the emission spectrum of lipofuscin is extremely large) or switch to an immuno-enzymatical procedure.

Sub b. False positivity due to the procedure used to demonstrate the first antibody will become obvious when the first antiserum incubation is omitted. Some known examples are:

(1) *Pseudoperoxidase activity* (in case of an immunoperoxidase procedure) caused by the haem groups in erythrocytes. This can be prevented effectively by perfusing the animal or, if this is not possible (e.g. for human tissue), by pre-incubation with a methanol hydrogen peroxide mixture (Streefkerk, 1972), or 100% methanol alone which has the same effect in our hands (Buijs, this manual; Van Leeuwen, this manual). Although Geyer (1973) mentioned lipofuscin as a source of pseudoperoxidase activity we did not find it to cause this type of false positivity in formalin fixed human brains.

(2) *Endogenous peroxidase activity* as present e.g. in neurons of the subfornical organ (Buijs, 1978). This activity may be inhibited by methanol nitroferricyanide containing 1% sodium and 1% acetic acid (Strauss, 1971).

(3) In immunoelectron microscopy, in case of an immunoperoxidase staining, the osmiophilia of cell organelles (e.g. lipid droplets) (Van Leeuwen, 1982).

(4) Conjugates or other secondary antibodies may bind electrostatically to basic proteins in the tissue section (protein/protein interaction) (see e.g. Vandesande, 1980). Such *non-specific binding* appeared difficult to eliminate and may even be enhanced during the preparation of labelled antibodies. Hebert et al., (1967), for instance, showed that non-specific staining of FITC-conjugates increased linearly with the FITC/protein ratio. At least some of this non-specific reactivity may be prevented by pre-incubation of the tissue with pre-immune serum from the animal which served as a source for the second antibody (Sternberger, 1979).
Sub c. The same protein/protein interactions may of course occur during the incubation with the first antiserum and can only be recognized by incubating the sections with a non-immune serum (see Introduction), while all further incubation steps are kept unchanged. Pre-immune serum, antiserum raised against an antigen not present in the tissue under study, or the (first) antiserum pre-adsorbed with the injected immunogen may be used for this purpose. These control serum incubations approximate probably best the non-immune serum conditions when applied in a dilution, such that the concentration of immunoglobulin is the same as in the first antiserum used for the tissue incubation.

False positive staining observed after incubation with a 'non-immune' serum may be caused by:

1. Free aldehydes still present in the tissue due to the fixation procedure. For this reason, Sternberger (1979) proposed a pre-incubation step with a (non-related) non-immune serum (see before) while incubations with Na-borohydrate (Lillie and Pizzolato, 1972; De Brabander et al., 1979) or with small molecules or proteins containing free amino groups have also been applied for this purpose (Buijs, this manual).

2. Ionic interactions of IgG's with basic proteins (e.g. Fc binding sites; Aarli et al., 1975; Kraehenbuhl and Jamieson, 1974) might be reduced by enhancing the ionic strength of the buffer which is used to dilute the antiserum and for the rinsing steps (Capel, 1974; Grube, 1980).

3. Hydrophobic interactions, e.g. of immunoglobulins with embedding media may be prevented by the addition of a detergent like TWEEN or Triton X-100 to the incubation and washing media (Buijs, Van der Sluis, Pool and Diegenbach, all this manual).

Staining which persists after a non-immune serum incubation (e.g. with an adsorbed (first) antiserum) may point either to (1) the presence of contaminating antibodies induced by impurities in the immunogen in quantities sufficient to induce antibody formation but insufficient to allow a complete removal of these antibodies, or (2) to contaminating antibodies already present in the serum before the immunization procedure started. However, since in both
cases this staining is based on an immunological reaction between antibodies of the first antiserum and the tissue, it can, according to the definition of method specificity given above (and in subchapter IX), not be called false-positive but falls within the framework of serum specificity (subchapter V) and should be studied accordingly.

V. SERUM SPECIFICITY

In this subchapter attention will be paid to the question what tissue components are immunocytochemically stained in the tissue under study, the first step towards a type of specificity which is called serum specificity (step 3 of figure's 3 flowchart; subchapter IX; Sternberger, 1979).

As has already been mentioned in the introduction, antibodies are directed against antigenic sites of the immunogen and not against the molecule as an entity. Therefore, apart from the difficulties caused by the possible presence of small (highly antigenic) contaminations (see subchapter III), ICC serum specificity can never be derived solely from the characteristics of the injected immunogen. Related (but not different) molecules in the tissue may share antigenic sites with this immunogen and therefore be a cause for a serum aspecific reaction. For the same reason, a positive immunocytochemical reaction with a monoclonal- or 'affinity purified' antibody (see subchapters VI, VIII) or no staining after adsorption of the antiserum with the immunogen, perhaps provide some information on the characteristics of the antibodies responsible for the ICC staining, but certainly not about the nature of the immunoreactive compound(s). It is therefore that in order to proof serum specificity in ICC the use of other ICC serum specificity tests will be necessary enabling a positive identification of the ICC reactive compounds present in the tissue itself.
Requirements for an ICC serum specificity test

Since apart from the characteristics of the first antiserum a great number of additional factors influence the intensity and specificity of an ICC reaction (fig. 1), serum specificity in ICC should be studied under conditions that are as much as possible comparable to those used during the immunocytochemical staining of the tissue. An ICC serum specificity test should, in addition, allow the control for improved specificity after an antiserum purification. On the basis of these conditions the following criteria for an ICC serum specificity test can be formulated:

1. The (antiserum) incubation conditions have to be identical to those used in the tissue section.

This condition is very important, especially since it has been shown that e.g. precipitation and radioimmunoassay (RIA) techniques (frequently used to define serum specificity) may not in all cases reveal the potency and specificity of a (first) antiserum in an ICC procedure (subchapter IV, fig. 5); Swaab et al., 1975; Van Raamsdonk et al., 1979; Vandesande, 1979; Cumming, 1980; Schipper, this manual).

2. The condition of the antigens in the test should be identical to that in the tissue.

Changes in immunoreactivity of the tissue compounds can be induced by differences in the pre-treatment of the tissue (see e.g. Schipper, this manual), which implies that in an ICC specificity test the tissue should be processed in a way similar to that used for the preparation of the tissue sections. As will be shown later, this condition is often very difficult to fulfil in model systems and the final outcome given by each type of ICC serum specificity test should, therefore, be checked afterwards in the tissue section itself (see subchapter VI).

3. All tissue antigens should be included.

In order to gain an insight into the possible immunoreactivity of all compounds present in the tissue under study, no prior selection (e.g. by an extraction procedure) should be made in the test.

4. Non-immunological characterization of the antigens should be possible.

When an immunoreactive tissue compound has been found, the next step will be to establish at least some of its characteristics
such as: is it a protein, what is its molecular weight or isoelectric point? In some cases it may also be possible to characterize the antigen on the basis of biological activity (hormones, enzymes). The establishment of these characteristics is necessary to prove the identity of the immunoreactive tissue compound for comparison with the antigen of primary interest (Geuze et al., 1979). Moreover, some insight into the characteristics of the immunoreactive compounds may be convenient when they have to be isolated e.g. in order to be able to use them in an antiserum purification procedure (see subchapter VI).

5. Quantification should be possible.

Even if the primary question (the localization of a certain component in a tissue section) is a qualitative one, quantification of the antigen/first antibody reaction in the test is important, for instance, to allow an accurate determination of the changes in ICC reactivity after purification of an antiserum (subchapter V; p. this manual).

At the present moment no antiserum test procedure is available which meets all these requirements but some of the successful attempts to develop ICC antiserum test systems which fulfil at least most of the criteria mentioned above will be discussed.

In general, these serum specificity tests are aimed at either (1) characterizing immunocytochemically the coupling abilities of the first antiserum to a number of antigens known, supposed or expected to be present in the tissue, or (2) determining directly what compounds from the tissue of investigation are actually coupling with the first antiserum. Two of the most commonly used model tests which follow the first approach are the defined antigen substrate sphere (DASS) system, as originally described by Capel (1974) and the enzyme-linked immuno-sorbent assay (ELISA) as developed by Engvall and Perlman (1971). Recently two promising new methods have been described by Larsson and Schipper, which will be discussed in their own contributions to this manual (pp. 339 and 159). In the DASS and ELISA techniques, the proteinaceous compound to be tested is immobilized on a matrix (CNBr activated agarose and polyethylene tubes, respectively) and immunostaining is performed identical to that on a tissue section (fig. 6). Both techniques allow a quantification of the first antiserum binding (condition 5). For
Figure 6. Serum specificity tests using isolated antigens. (a) In the defined antigen substrate sphere (DASS) system, proteinaceous compounds are covalently coupled to agarose beads which subsequently can be used in routine immunocytochemical staining procedures. In case of an immunofluorescence procedure the immunoreactivity can be determined by microfluorometric readings on individual beads while after an immunoperoxidase staining procedure either microspectrophotometer readings on the beads or spectrophotometer determination of the absorbance of the supernatant may be used. (b) In the enzyme-linked immunosorbent assay (ELISA), the proteinaceous compound is adsorbed to a polyethylene surface, whereafter an immunoperoxidase staining is performed. The presence of an immune reactivity can be detected by spectrophotometer readings with a variety of soluble chromogens (see for reviews O'Beirne and Cooper, 1979, and Smith and Gehle, 1980).

DASS this has been done by microfluorometric readings after an immunofluorescence procedure (Capel, 1974; Deelder and Ploem, 1975) and by (micro)spectrophotometric determination of the peroxidase enzyme activity after HRP-conjugate (Streefkerk et al., 1975; Pool, 1980; Pool and Diegenbach, this manual), and PAP-incubation (Pool and Diegenbach, this manual). ELISA has also been performed both using enzyme conjugated antibodies or the PAP-procedure.

In connection with the determination of serum specificity in
Figure 7. Schematic representation of possible changes in immunoreactivity upon tissue treatment. Cleavage of the original antigen can lead to the 'production' of two or more immunoreactive compounds (e.g. dependent upon the number of antigenic sites originally present on this antigen), or to total loss of immunoreactivity when the antigenic sites themselves are influenced by this cleavage. Conformational changes of the antigen can lead to sterical hindrance for the antibodies resulting in a false negative immunoreaction.

ICC, the DASS system has, for instance, successfully been applied by Swaab and Pool (1975) to determine quantitatively the reactivity of an oxytocin antiserum with the structurally related vasopressin (see also Pool and Diegenbach, this manual). The DASS and ELISA techniques fulfil the requirements 1 and 5 described above, and consequently allow the determination of the reactivity of the antiserum in an ICC procedure towards compounds known or supposed to be present in the tissue under study. However, the immunoreactivity towards unknown tissue antigens will never be recognized this way. A test for the positive identification of antigens actually present in the tissue under investigation requires the solubilization and subsequent separation of all tissue components followed by a test on their reactivity in an immunocytochemical staining procedure.

Tissue material can probably be solubilized by the use of SDS (sodium dodecyl sulfate) and/or urea. Large proteins and hydro-
phobic components are then included as well. Moreover, such a procedure appears not to disturb the antigen/antibody reaction (Stumph et al., 1974). However, a single solubilization procedure does not allow the control on possible fragmentation of immunoreactive compounds, either resulting in a loss of immunoreactivity or (in case of two or more antigenic determinants on the same molecule) in the production of immunoreactive compounds originally not present in the tissue (fig. 7). Preferably different tissue processing procedures should therefore be used: for instance, a combination of an ethanol/acetic acid or HCl extraction followed by an SDS/urea treatment of the remaining insolutes.

So far, the techniques used as ICC specificity test applying the steps of solubilization of the tissue, separation of the tissue compounds followed by an ICC reaction (fig. 8) all make use of an SDS electrophoresis in the second step. They differ, however, in the way the separated compounds are immobilized in order to perform the ICC staining procedures. An immobilization of a discontinuous spectrum of tissue components by slicing the gel after an SDS-electrophoresis and coupling the tissue components from these slices onto polystyrene test tubes, followed by ELISA (SDS gel electrophoresis derived ELISA: GEDELISA) has been described by Lutz et al. (1979) (fig. 8a). The immobilization of the continuous spectrum of SDS electrophoretically separated tissue compounds in the gel itself followed by a routine ICC staining procedure (SDS gel electrophoresis immunoperoxidase method: SGIP) (fig. 8b) has been described by Van Raamndonk et al. (1977) (see also Van Raamndonk, this manual). And finally the transfer of the separated tissue compound, from the SDS-gel onto filter paper (blot-technique) followed by ICC staining or an autoradiographic procedure using I-labelled protein A (fig. 8c) has been described by Renart et al. (1979) and Towbin et al. (1979), respectively (see De Jong and Eng, this manual).

From these techniques GEDELISA seems to meet most of the criteria for an ICC specificity test as given above. Its use is mainly limited by the shortcomings of the ELISA technique. The physical adsorption of the antigen to the solid phase will not be the same for all proteinaceous compounds, and up to 30% of the antigen or antigen/antibody complexes may be lost during the washing and incubation steps (Engvall, 1980), while also the limited capacity of
Figure 8. Serum specificity tests based on the detection of ICC reactive compounds in an SDS gelelectrophoretically separated tissue homogenate.
(a) In GEDELISA, the separating gel is sectioned, fractions are subsequently eluted and an ELISA is performed to detect immune reactivity (see also fig.10). A second gel routinely stained with commassie blue is necessary to identify the antigens (Lutz et al., 1979).
(b) In the SGIP procedure, longitudinal cryostat sections are made of the separating gel and the proteins in the section are immobilized by a cold ethanol/acetic acid treatment. Gel sections flattened on glass slides can then be proceeded in an immunoperoxidase staining procedure in which the immune complexes can be visualized with oxidized diaminobenzidine. The remaining part of the unsectioned gel, stained with commassie blue, serves as the identification matrix. Several samples (lanes) can be treated simultaneously from the same gel, allowing the direct comparison with 'standard' proteins or other tissue samples.
(c) The blotting with diaminobenzylxymethyl (DBM) -paper allows the covalent binding of proteins diffusing out of the gel. The paperbound components are then tested for immunoreactivity with a 125-I-protein A method using autoradiography. A second - routinely stained - gel is used for comparison (Renart et al., 1979).

the adsorption of the (plastic) tubes may be a disturbing factor. An additional disadvantage of GEDELISA may be the loss of the high resolution of the SDS-electrophoresis due to the slicing of the gel. This problem does not occur in the SGIP technique in which the
separated proteins are immobilized directly in the gel matrix by fixation with an ethanol acetic acid mixture. However, also in this case it is not known whether this fixation procedure immobilizes all types of antigens. Unlike GEDELISA, the SGIP technique itself is not suitable for a quantification of the reactivity of the first antiserum (Van Raamsdonk et al., 1977). This quantification can, however, be introduced by combining SGIP with the DASS system. Antigens can be eluted from sliced gels and coupled covalently to CNBr-activated agarose beads. With these beads the reactivity of the antiserum can be determined quantitatively towards the same antigens initially identified in a SGIP procedure, thereby combining the advantages of a continuous spectrum of tissue components and avoiding the difficulties due to a physical adsorption of the antigens to the solid phase (Pool, 1980). A circumstantial advantage of SGIP is that it allows the use of the same protein spectrum (successive gel sections) for incubation with different antisera (Van Raamsdonk et al., 1977; Van Raamsdonk, this manual). The assessment of an antiserum affinity towards a continuous separation spectrum of tissue components is also inherent in the blot-technique. Although, in principle, this approach can be made quantitative, so far it has not been worked out.

The SDS gel electrophoresis which is used in the three techniques described above, however, does not allow enough separation of proteinaceous material with molecular weights below 5kD. Consequently, neither of these techniques can be used to gain an insight into e.g. the distribution of immunoreactive peptides present in the tissue homogenates. Although this lack of resolving power may be overcome by the use of other separation techniques such as gel isoelectric focussing (Boer, 1979; Van der Sluis et al., 1983) or even two-dimensional electrophoresis, also the immobilization of these small compounds is a serious problem to be encountered (Boer, 1979; Van der Sluis et al., 1983). A new fixation technique using glutaraldehyde-impregnated filter paper is promising in this respect, and will be demonstrated during this course by P.J. van der Sluis.
VI. ANTISERUM PURIFICATION

The moment it has been assessed that an antiserum reacts with more than one component present in the tissue under study, the decision should be made whether this 'not-wanted' reactivity has to be removed from the antiserum or not, for instance on the basis of the necessity for each particular study to use a 'one-tissue component specific' ICC localization procedure. Basically such an antiserum purification (step 4 in fig. 3) can be performed in two different ways: (1) by elution of antibodies from immune complexes formed with the antigen to be localized ('affinity purified antibodies') or (2) by the removal of the unwanted antibodies by (pre-)incubation with the appropriate contaminating antigens. The main difference between these two approaches is that the immunoabsorbent in the first method has to be a preparation in which the antigen to be localized should be the only immunoreactive compound while the second method requires antigen preparations that do not contain the antigen to be localized. This implies that in all cases prior to each adsorption the immunocytochemical purity of the immunoabsorbent has to be known, which can be obtained by one of the antigen identification techniques described in subchapter V (see also the contributions to this manual of Van Raamsdonk and van der Sluis in this respect). In case 100% pure antigen preparations are available in sufficient amounts, both antiserum purification techniques might equally well be used. However, since this situation is an exception rather than a rule, the strategy followed for the antiserum purification will largely be dependent upon the (immunological) purity of the antigen preparations available. The conditions for the use of the method (2) (the removal of not-wanted antibody populations) are the ones to be met most easily, while in addition cross-reacting antibodies (i.e. antibodies that react next to the antigen to be localized also with another antigen in the tissue) cannot be removed using the first method. For these reasons we will confine ourselves in the second subchapter only to the second type of antiserum purification.

In order to remove a distinct population of antibodies from an antiserum, a solid phase immunoabsorbent should be used since
otherwise the tissue antigen may compete successfully with the free antigen and the antigen present in soluble immunocomplexes in the adsorbed serum. This is especially important for small antigens (such as peptides), since they usually do not form precipitating immunocomplexes at all. The solid-phase medium most commonly used for proteinaceous antigens are CNBr-activated agarose beads (see e.g. VandeSande, 1979). These antigen-coupled beads exhibit also an additional advantage: the amount of antibody bound to the beads can be determined quantitatively using the procedures described for the DASS system. This means that the amount of beads necessary minimally for an adsorption as well as the final outcome of each adsorption can be assessed quantitatively. The former may be of importance in the case of scarcely available antigens, while the latter enables a direct comparison of the reactivities of the non-adsorbed, adsorbed and control serum towards this antigen (Pool and Diegenbach, this manual). However, in order to evaluate the effect of this changed immunoreactivity for the tissue situation, the reactivity of the adsorbed antiserum should be checked on a tissue section and in the model ICC serum specificity test (e.g. with the GEDELISA technique or the SGIP/DASS combination) where the reactivity towards each of the tissue antigens should be expressed in quantitative terms. Table 1 illustrates the possible outcomes of such a purified antiserum test procedure. When the antiserum after the adsorption with B still reacts with this component (table 1; possibility 1), the adsorption apparently was not carried out properly. When, on the other hand, the adsorbed antiserum no longer reacts with component B (table 1; possibilities 2, 3 and 4) the outcome of the antiserum test procedure may show a discrepancy between the results of the model test system and the tissue section (table 1; possibility 2). According to the model test the adsorbed antiserum should contain antibodies against A, but in the tissue section (known to contain A) no reaction is observed, the apparent conclusion being that all antigenic determinants on component A in the tissue section are lost or masked (e.g. due to the tissue fixation procedure) and that thus probably the tissue processing has to be re-evaluated. When after a successful adsorption with B the reactivity with A is unchanged and also the tissue staining remains (table 1; possibility 3) apparently two different populations of antibodies are present in the anti-
**TABLE 1**

Possible (ICC) reactivities of an adsorbed antiserum determined in a (model) specificity test and in the tissue section

<table>
<thead>
<tr>
<th></th>
<th>reaction with A</th>
<th>reaction with B</th>
<th>reaction on the tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-adsorbed</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>possibility 1</td>
<td>≥ 100%</td>
<td>≥ 100%</td>
<td>≥ 100%</td>
</tr>
<tr>
<td>possibility 2</td>
<td>≥ 100%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>possibility 3</td>
<td>100%</td>
<td>0</td>
<td>≥ 100%</td>
</tr>
<tr>
<td>possibility 4</td>
<td>≥ 100%</td>
<td>0</td>
<td>≥ 100%</td>
</tr>
</tbody>
</table>

An antiserum (raised against A) reacts with two components (A and B) both present in the tissue under study. Since the aim was a specific ICC localization of component A in that tissue, the antiserum was adsorbed with component B. The final outcome of this adsorption experiment was tested in isolated A and isolated B (for instance with the DASS system) and on tissue sections (the reactivities of the non-adsorbed antiserum were set at 100% in each case).

serum: one directed against A and one directed against B. In this case, the antibodies directed against B are called contaminating antibodies (see subchapter IX). Such contaminating antibodies may already be present in the serum prior to immunization (subchapter IV) or can be induced by impurities in the injected antigen or especially in the case of small haptens by the carrier protein used to prepare the antigen (Steinbusch et al., 1978).

The last possible reaction of the purified antiserum described in table 1 is that after a successful adsorption with B also the reactivity with A has changed (table 1; possibility 4). Apparently in this case at least part of the antibodies against A react also with B. Such antibodies are called cross-reacting antibodies and point to structural similarities between (in our case) A and B (see subchapter IX). Such cross-reacting antibodies have frequently been observed in ICC studies in the brain as for example the cross-reactivity of anti-ACTH with α-MSH or anti-AVP with oxytocin (see also contributions of Schipper and Pool and Diegenbach to this...
manual). In some cases two antigens may be structurally so similar that with an antiserum raised against one of these compounds 100% crossreactivity towards the other may be found. For instance, in an attempt to remove the vasopressin (cross-)reacting antibodies from an anti-vasotocin serum, all reactivity of this antiserum towards vasotocin disappeared (Swaab, unpublished observation). This means that using this antiserum no distinction can be made, immunocytochemically, between vasotocin and vasopressin.

VII. SPECIFICITY IN IMMUNOCYTOCHEMISTRY

When an antiserum has sufficiently been purified - which means it only reacts with one tissue compound in the antiserum specificity test (step 4 of the flowchart in fig. 3) - one might conclude that this antiserum consequently allows a monospecific localization of this compound in the tissue under study (see subchapter IX). However, this presupposes a full predictive value of the specificity test system for the reaction of that antiserum with the tissue section, which has to be proven in each case again for all currently used ICC serum specificity model systems (step 5 of the flowchart in fig. 3). The tissue section itself is the only final control in this respect, since the consequences of the in most cases inevitable different processing of the tissue compounds in the specificity test and in the tissue fixation procedure can never be determined in model specificity tests alone (see contribution on antigen quantification of Schipper, this manual). Theoretically, such a final test on the 'monospecificity' of an immunocytochemical localization procedure must give negative staining (1) in the serum specificity test and on the tissue section after removal of the specific antibodies (adsorption test) and (2) in the same tissue lacking this particular antigen (mutant test).

This pre-adsorption test is different from the commonly used adsorption test which has been discussed in the introduction and subchapter IV to prove the method specificity. At that stage in the flowchart all immunological activity has to be removed, in
an attempt to obtain a 'non-immune' serum, however, without any prior knowledge with respect to what components are actually stained immunocytochemically in the tissue. Since the adsorption-test mentioned here is not carried out to determine the 'monospecificity of the antiserum reaction', but to test the validity of the specificity of the ICC reaction as determined with the model serum specificity test (step 4 in the flowchart), it also differs fundamentally from that currently mentioned in a great number of ICC papers to prove a 'monospecific' ICC localization (e.g. Petrusz et al., 1977).

The most direct and probably also the only correct way to obtain an antigen that can be used in the final adsorption test is to isolate it from the tissue itself by using the same separation technique as used in the antiserum specificity test (see subchapter V). For instance, after SDS gel electrophoresis, as used in GEDELISA and SGIP, the antigen can be eluted from the gel and coupled to CNBr-activated sepharose beads and subsequently processed in the adsorption procedure (subchapter VI; Pool, 1980). Of course, if an antigen preparation is available that in the test system behaves identically as the remaining single immunoreactive compound from the tissue, this preparation may also be used.

As stated above, the second possibility to prove the validity of the antiserum specificity test used involved an incubation of the purified antiserum with tissue lacking the antigen to be localized. It assumes either the presence of a mutant deficient for that antigen or the possibility to remove this component specifically from the tissue under study. Unfortunately, both are only available for a very limited number of antigens. The example 'par excellence' of such a mutant is the diabetus insipidus Brattleboro rat which is unable to synthetize the peptide arginine vasopressin. In fact, the neural lobe of the pituitary of this animal has been used to demonstrate the specific ICC vasopressin localization both at LM (Swaab and Pool, 1975) and EM level (Van Leeuwen and Swaab, 1977). When either in the adsorption or in the 'mutant-test' positive reactions are found, the monospecificity of the immunocytochemical staining procedure — as was suggested on the basis of single immunoreactive tissue compound in the serum specificity test — must be questioned. In practically all
cases this erroneously supposed monospecificity will be caused by limitations of the test system. For instance, the test may not allow the detection of tissue amounts of antigen. An antigen present in a few high local concentrations within the tissue, i.e. a few densely stained cells (μ-range) may cover a larger area in the test situation (mm-range), (for instance in the SGIP procedure) and therefore not be 'seen'. Also the antigen immobilization may be less efficient in the test or the preservation of immunoreactivity may be reduced. Such situations may lead to the necessity of processing larger amounts of tissue or more sensitive techniques to detect the immunostaining in the test.

VIII. SPECIFICITY IN IMMUNOCYTOCHEMISTRY: SOME CONCLUDING REMARKS

Starting from the idea that one of the conditions for specificity in immunocytochemistry is the proof that the component of interest is indeed the only immunoreactive compound present in the tissue under investigation, it will be clear that a positive identification of all immuno(cytochemical)reactive components in the tissue section should be an intrinsic part of an ICC specificity test procedure. In contrast to this 'defined antigen specificity' it has been shown in the introduction that the commonly used ICC specificity tests (e.g. the adsorption test) will ultimately yield a (possibly very good) characterization of the antibodies that are responsible for the ICC reactivity but fail to give this insight into the binding capacities to unknown compounds in the tissue section. The type of specificity obtained in this 'classical' way may therefore best be termed 'defined antibody specificity'.

The 'defined antigen specificity' as ultimately found using the sequence of control experiments as described in subchapters II-VII and schematically visualized in figure 3 will mainly be dependent on the procedures used for the identification of the immunoreactive tissue components. It is therefore not surprising that in all currently used ICC serum specificity tests the tissue components are separated by an SDS electrophoresis (see subchapter V). But
at the same time this means that the limitations of these ICC serum specificity tests will be set by the resolution of this type of electrophoresis. For instance, two tissue antigens with (approximately) the same molecular weight will never be recognized as such. In this respect the approach based on a two-dimensional electrophoresis (isoelectric focusing in one direction and SDS electrophoresis in the second dimension) to separate the tissue antigens will definitely be an improvement.

A second point which has to be taken into account in every ICC specificity study is that the finally found 'specific ICC reactivity' will always be a procedure-dependent 'specific ICC reactivity'. This is e.g. illustrated by the second antibody-dependent ICC reactivity with the oxytocin antiserum as given in figure 5. An example of the role of the first antiserum incubation conditions on the outcome of an ICC staining procedure may be illustrated by the 'first antiserum dilution dependent specificity' as described by e.g. Keefer et al. (1976), Van Leeuwen et al. (1977) and Petrusz et al. (1980), while an illustration of a tissue dependent ICC reactivity has been given by Vandesande during the EMBO course on Immunocytochemistry in 1980: In hypothalamic preparations from normal rat he found a maximal staining of the vasopressin containing cells, with an anti-vasopressin serum applied in a PAP procedure at a dilution of 1/200. However, using the same ICC staining procedure for a study in rats from which the hypothalamic magnocellular neurosecretory system had been activated by dehydration combined with a blocking of the axonal transport by colchicine, no 'vasopressin' reaction in the hypothalamus of these animals was observed. Starting with an ICC procedure which has proven to allow the specific ICC localization of vasopressin, an apparent conclusion may be that the hypothalamic magnocellular neurons from dehydrated, colchicine-treated rats do not contain vasopressin. However, after using a different dilution (1/1000) of the (same) vasopressin antiserum, an intense reaction was observed in the vasopressin cells in these rats. The maximal staining intensity that could be obtained after a renewed optimization of the ICC staining procedure even turned out to be much higher in the experimental (dehydrated, colchicine-treated) animals than in the normal rats.
These three examples all illustrate the procedure-dependent ICC reactivity: a change in one of the components (the tissue, the antisera) or conditions (tissue fixation, antiserum incubation, the ICC staining procedure) used to define the specificity of a given ICC localization means that the specificity of the ICC staining procedure has to be re-evaluated.

As in a number of other papers dealing with immuno(cyto)chemical methodology, also this chapter cannot silently pass over the question: does the application of monoclonal antibodies provide an answer to the specificity problems in immunocytochemistry? It will be clear that indeed this type of antibodies will abolish all specificity problems caused by the presence of contaminating antibodies (see subchapters VI, IX), but beforehand crossreactivity of a monoclonal antibody (see subchapters VI, IX) cannot be excluded and moreover, if present, it will involve the entire antibody population and a purification as described in subchapter VI will be impossible.

Theoretically, a clone can be selected that produces an antibody that does react in an ICC staining procedure with no other component in the tissue under study than the antigen of interest. However, practically this implies that a test on the fulfilment of this condition should be an intrinsic part of the clone selection procedures. Since this type of selection procedures will and probably can only be realized in a limited number of cases, the same series of ICC specificity tests as described in this chapter for polyclonal antibodies will be necessary to prove a 'defined antigen specific' immunocytochemical localization with a monoclonal antibody.

IX GLOSSARY

Method-specificity
The absence of staining in an immunocytochemical localization procedure by mechanisms other than an immunological interaction between antibodies from the first antiserum and the tissue.
Serum-specificity
The absence of immunological interactions in an immunocytochemical staining procedure between antibodies from the first antiserum with other tissue components than the antigen of interest.

Monospecific immunocytochemical staining procedure
A staining procedure proven to result in the immunocytochemical localization of one defined antigen in a given tissue.

Contaminating antibodies
Antibodies present in the first antiserum which react in an immunocytochemical staining procedure with tissue component(s) which share no antigenic sites with the antigen of interest.

Cross-reacting antibodies
Antibodies present in the first antiserum which react in an immunocytochemical staining procedure with the antigen of interest but also with (an)other tissue component(s).

XI. APPENDIX

Plasmaferesis in the rabbit
- 50 ml blood is sampled from the ear artery using a 'winged' infusion system with a needle diameter of 1.2 mm, and collected in a sterile, pyrogene free siliconized bottle containing 8 ml of a solution of 2.7% di-potassium citrate and 2.3% glucose.
- Centrifugation of the bottle for 10-15 min at 3000 rpm.
- Removal of the plasma from the bottle under sterile conditions using a 20 cm long needle with a diameter of 3 mm.
- Collection of the blood cells in a 20 ml syringe and undiluted injected, under sterile conditions, intraperitoneally back into the rabbit using a needle with a diameter of 1.2 mm. The needle is connected by a small tube with the syringe.
XI. REFERENCES


Fink, R.P., Heimer, L. (1967) : Two methods for selective silver impregnation
of degenerating axons and their synaptic endings in the central nervous system. Brain Res. 4, 369.


