A simple silver nitrate impregnation of nerve fibres with preservation of acetylcholinesterase activity at the motor end-plate

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Summary. A rapid silver nitrate impregnation of nerve fibres has been devised to maintain a histochemically detectable acetylcholinesterase activity at the motor end-plates.

There are a large number of combined techniques for acetylcholinesterase histochemistry and silver impregnation on motor end-plates[2-4]. In these techniques, the enzymatic activity has always been revealed before the silver impregnation but never after the silver staining. Thus it would be interesting if the combined processes could be reversed, so that the silver impregnation was performed before the enzyme histochemistry. A silver impregnation modified for such a purpose could serve both as a neurocytological and as a histoenzymological tool.

Survival of the enzymatic activity is not expected in conventional silver impregnation methods (Cajal's reduced silver nitrate, Bielschowsky's ammoniacal silver and Bodian's silver protein techniques[5,6]) since the impregnation conditions used are inhibitory for enzymatic activity.

In the present work, a modified silver nitrate impregnation method was devised with respect to the preservation of the enzymatic activity. Special attention was given to the use of a buffered formaldehyde fixative, a not too strong enzyme-inhibitory lipid-releasing solvent or detergent, a mild pH at room temperature with a short impregnation time, and diluted reducing agents.

In order to preserve the enzymatic activity, it was necessary to use only the essential steps of a silver impregnation which finally gives highly reproducible staining of nerve fibres.

Materials and methods. The silver nitrate impregnation technique followed by acetylcholinesterase histochemistry is presented in 10 steps. At the 3rd step, there are 3 different procedures which concern the treatment of the myelin sheath.

1. Fix the muscle fibres (mouse intercostal muscle) with 2–4% para-formaldehyde in 0.1 M phosphate buffer (pH 7.2), at room temperature, at least 1–2 h. The muscle fibres can be kept in the fixative at 4 °C for several days. The paraformaldehyde is dissolved in the buffer by heating.
2. Wash thoroughly in 0.1 M aqueous solution of sodium nitrate, 15 min.
3. Treat the myelin sheath to permit the silver impregnation of the myelinated nerve fibres, a) either by actone diluted in distilled water: 70% (5 min) - 95% (5 min) - 100% (30 min) - 95% (5 min) - 70% (5 min), or b) by 1% Triton X-100 in 0.1 M aqueous solution of sodium nitrate, 1 h, with constant stirring. c) in order to limit the silver impregnation to the unmyelinated part of the nerve terminal only, omit the myelin sheath treatment by acetone or Triton X-100 and go directly to the 5th step.
4. Wash thoroughly in sodium nitrate, 15 min.
5. Impregnate with 0.1 M aqueous solution of silver nitrate, in the dark at room temperature, 2 h.
6. Wash in sodium nitrate, 15 min.
7. Reduce the incorporated silver salts using an aqueous solution containing 0.025 M hydroquinone and 0.025 M sodium sulfite: Rinse in sodium nitrate.
8. Remove the non-reduced silver salts from the tissue with an aqueous solution of 0.1 M sodium thiosulfate, 15 min.
9. Wash thoroughly in sodium nitrate, 15 min.
10. Mount in water mounting medium or use for histochemical reaction to demonstrate acetylcholinesterase.

Results. a) Silver impregnation after acetone. The motor nerve terminal, impregnated after acetone treatment of the myelin sheath, is shown in figure 1. Note that the unmyelinated portion of the nerve terminal is also finely stained.

After this silver impregnation, residual cholinesterase activity was investigated by histochemical techniques. α-naphthol[7] gives a positive red reaction at the motor end-plate after 20 min (figure 2), compared to the 5-min maximum in the muscle which has not been treated with silver impregnation. The reaction is positive with 10^{-5} iso-OMPA. Likewise, Karnovsky's[8] copper-ferrocyanide method is positive with acetylthiocholine (figure 3), but negative with butyrylthiocholine. The results of both methods, after silver impregnation, still confirmed the presence of a specific acetylcholinesterase activity.

However, Karnovsky's reaction, like Koelle's modified reaction[9] interferes with silver staining and is not compatible with fine silver impregnation. Actually, the silver staining tends to disappear, while Karnovsky's precipitate appears.

b) Silver impregnation after Triton X-100. The motor nerve terminal impregnated after Triton X-100 treatment is shown in figure 4. The neurofibrillar staining is comparable to that of acetone treated nerve fibres. Note that the unmyelinated as well as the myelinated portion of the nerve terminal is selectively stained. After silver impregnation, an intense acetylcholinesterase activity was revealed by α-naphthol method (figure 5). (Triton X-100 does not inactivate the end-plate acetylcholinesterase[10].)

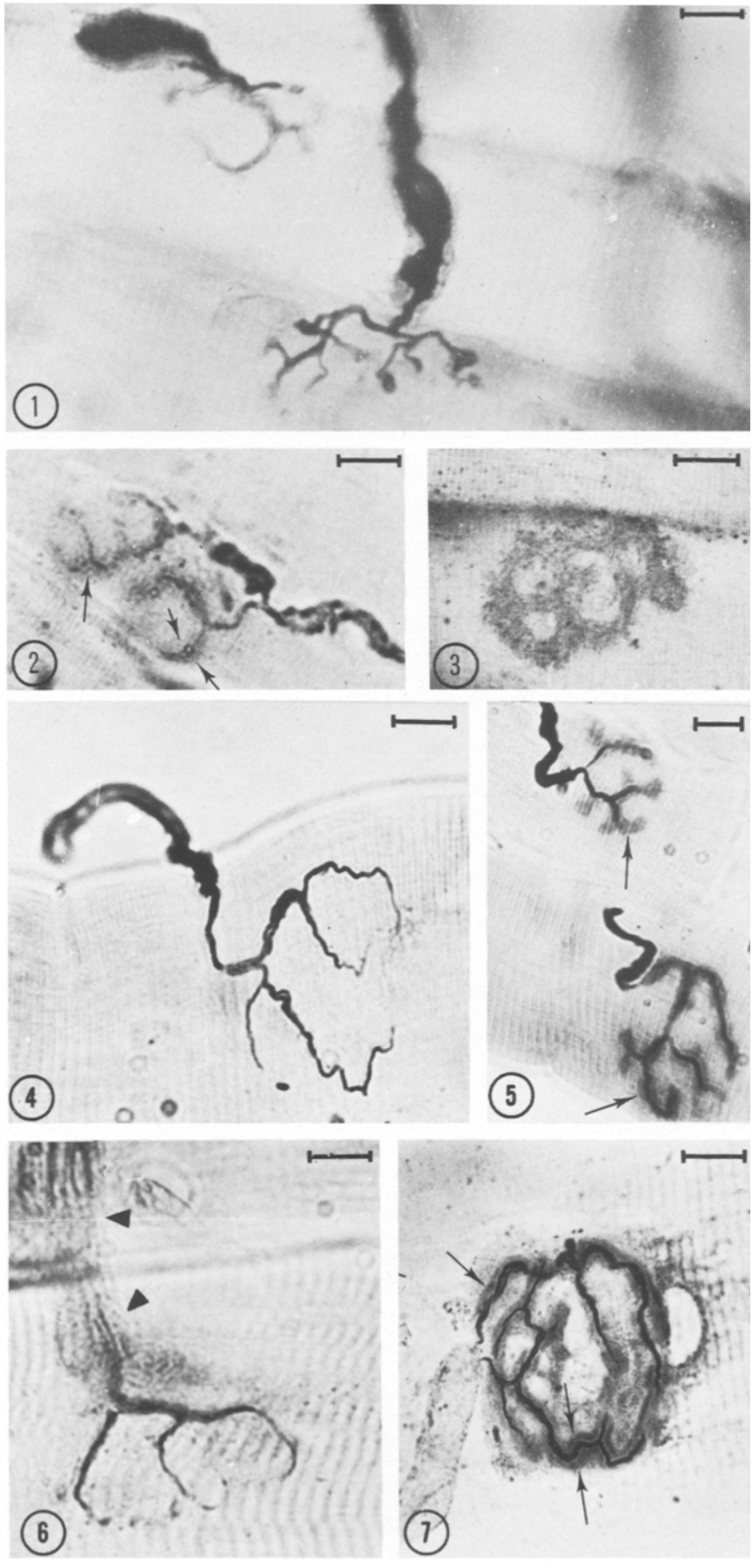
In comparison to Triton X-100, a 'capricious' inactivation of the enzyme due to an inhibitory action of acetone[11] (may be also due to residual impurities of acetone) limits the acetone treatment for a histoenzymological purpose.

c) Silver impregnation with neither acetone nor Triton X-100. It was observed that neither acetone nor Triton X-100 is needed, if one impregnates only the unmyelinated portions of motor nerve terminals (figure 6). In this case, the enzymatic activity is as intense as that of Triton X-100 treated muscle (figure 7).

The incubation time necessary for a positive reaction with α-naphthol histochemistry was determined on finely isolated muscle fibres after silver impregnation. For a control, well isolated muscle fibres without silver impregnation were used. These 2 kinds of muscle fibres were put together in the histochemical incubation medium and were monitored under a light microscope. Within 1 min a red positive reaction appeared on the motor end-plates of both kinds of isolated muscle fibres. In this short lapse of time (1 min), it is practically impossible to observe a possible enzymatic inactivation due to silver impregnation.

Discussion. The present technique consists of rapid formaldehyde fixation followed by acetone or Triton X-100 treatment and silver nitrate impregnation. These impregnations are highly reproducible and can be considered as practical techniques in neurocytology. If both acetone and Triton X-100 are omitted, fine silver impregnation can be obtained only on unmyelinated portions of nerve terminals.

Our combined technique, silver nitrate impregnation followed by α-naphthol cholinesterase histochemistry, shows clearly that, if acetylcholinesterase is inactivated by silver nitrate[12], this inhibition must be reversible. Our technique



Simple silver nitrate impregnation. Material: Mouse intercostal muscle. Scale bar: 10 µm for all figures.

Fig. 1. Silver impregnation after acetone. Fig. 2. Silver impregnation after acetone, followed by the α-naphthol reaction (arrows) for cholinesterase activity. Fig. 3. Silver impregnation followed by Karnovsky's copper-ferrocyanide method for cholinesterase histochemistry with acetylthiocholine as substrate. Fig. 4. Silver impregnation after 1% Triton X-100. Fig. 5. Silver impregnation after Triton X-100 followed by α-naphthol reaction (arrows) for cholinesterase activity. Fig. 6. Silver impregnation without either acetone or Triton X-100. The unstained myelin part of nerve fibre is pointed out by arrowheads. Fig. 7. Silver impregnation without either acetone or Triton X-100, followed by α-naphthol histochemical reaction (arrows) of cholinesterase.

gives a better resolution of nerve terminal and acetylcholinesterase localizations than that obtained by conventional combined techniques on the same material.

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Localization of human A, B and H isoantigens in Cynomolgus monkey tissues

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Summary. The presence and distribution of human A, B and H isoantigens were demonstrated in Cynomolgus monkey *(Macaca fascicularis)* by means of red cell adherence test. Although no human antigens were found on primate erythrocytes, various epithelial tissues revealed the presence of A, B or H antigenic substance. The distribution and localization was similar to that found in human tissues. Majority of specimens from each individual animal possessed only 1 human type isoantigen with the exception of the salivary and sweat glands, where all animals showed the presence of H antigen in addition to other specificity, and of Brunner's gland, where all sections reacted positively also for A antigen.

Blood group isoantigens A, B and H are present in human body fluids and tissues other than erythrocytes and their distribution has been well established[1,2]. In non-human primates, the presence of these antigens on red blood cells was also extensively described[3–5]. However, most of the Old-World monkeys do not possess the A-B-H factors on their erythrocytes, but the factors appear as soluble blood group substances in some of their secretions, as described by Wiener et al.[5], who were able to demonstrate all 4 A-B-0 groups in *Macaca fascicularis* by using the inhibition test on saliva. Franks et al.[6] showed the presence of the B antigen on buccal and kidney cells, using the mixed agglutination technique.

In the present study we have tried to establish the presence and distribution of human A, B and H antigens in various tissues obtained from Cynomolgus monkeys *(Macaca fascicularis)*, using the red cell adherence (RCA) test.

Materials and methods. RCA technique. The principle of the red cell adherence test is a modification of mixed cell agglutination reaction adapted for use in paraffin-embedded histologic sections[7]. Briefly, the tissue sections are de-paraffinized, transferred into tris-buffered saline and reacted with human anti-A or anti-B agglutinin for type A or B antigen, or with an extract of *Ulex europeus* seeds for type 0. After incubation at room temperature, the unfixed antibody or lectin is washed off and the sections are covered with erythrocyte suspension of the appropriate blood group. Following the incubation, the sections are inverted on supports in a layer of tris-saline in a petri dish. The non-attached red blood cells fall to the bottom of the dish and the erythrocytes adhering to the section show the localization of the antigen.

The RCA reaction is evaluated as: a positive reaction (+), where the attached erythrocytes demonstrate the presence of an antigen; a negative reaction (−), which indicates the absence of an antigen; and a weak positive reaction (±), where only some cells within a microscopic field demonstrate small amounts of antigenic material.

After the RCA test, each histologic section is stained with hematoxylin and eosin and the exact localization of the antigen is established.

Results. Several examples of a red cell adherence test on histologic sections of Cynomolgus monkey tissues are presented in the following photographs. Figure 1 demonstrates

Distribution of human type A-B-H antigens in Cynomolgus monkey

Animal No.	1			2			3			4			5			6		
Antigenic type	A	B	H	A	B	H	A	B	H	A	B	H	A	B	H	A	B	H
Esophagus	+	−	−	−	−	+	−	+	−	−	−	+	−	+	−		NA	
Stomach	+	−	±	−	−	+	−	+	±	−	−	+	−	+	±	−	+	−
Duodenum	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−		NA	
Brunner's gland	+	−	−	+	−	+	+	+	−	+	−	+	+	+	−		NA	
Small intestine	+	−	−	−	−	−	−	+	−	−	−	+	−	+	−		NA	
Large intestine		NA		−	−	−	−	+	−	−	−	+	−	+	−	−	+	−
Pancreas	+	−	−	−	−	+	−	+	−	−	−	+	−	+	−		NA	
Salivary gland	+	−	+	−	−	+	−	+	+	−	−	+	−	+	+	−	+	+
Oral epithelium	+	−	±		NA		−	+	±		NA			NA			NA	
Bronchus	+	−	−	−	−	+	−	+	−		NA			NA			NA	
Skin (keratin)	+	−	−	−	−	+	−	+	±	−	−	+	−	+	−		NA	
Sweat gland	+	−	+	−	−	+	−	+	+	−	−	+	−	+	+		NA	
Urinary bladder	+	−	−	−	−	+	−	+	−	−	−	+	−	+	−	−	+	−

NA, not available for evaluation; +, positive RCA, antigen is present; −, negative RCA, antigen is absent; ±, weak positive RCA, antigen is present in very small quantity.