AMMONIACAL SILVER METHOD AT ALKALINE pH FOR DEMONSTRATING MORPHOLOGICAL AND FUNCTIONAL DIFFERENCES BETWEEN NERVE CELLS

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In 1958-1966 Black and co-workers [9-11] suggested and substantiated an ammoniacal silver histochemical method (ASHM) for alkaline proteins containing lysine (Lys) and arginine (Arg). It is based on reduction, under the influence of acetate-neutral formalin, of an ammoniacal silver complex which interacts with amino groups of Lys and Arg. As a result of this reaction a product colored different shades of yellow is formed in the case of proteins containing Lys, and brown with proteins containing Arg [10, 11]. However, as other investigations have shown [5, 8, 13], ASHM reveals not only amino groups of Lys and Arg, but also amino groups of nucleic acids DNA and RNA, closely bound with them [3]. It would therefore be more accurate to say that ASHM reveals not only, or not so much, proteins containing Lys and Arg but rather several nucleoprotein complexes (NPC), including these highly basic amino acids.

When testing ASHM on brain tissues we found that if the reaction is conducted at neutral pH (7.0-7.1), as the authors of the method suggest, it considerably limits its potential scope and does not completely reveal amino groups of proteins containing Lys and Arg. The reason is that in a neutral medium, as biochemical studies have shown [14], the amino groups of Lys and Arg are mainly masked and exhibit reactive inertia The degree of their dissociation and, correspondingly, their accessibility to reagents, increases with increasing alkalinity of the solution [12, 15] and reaches a maximum for Lys at pH 10.6 and for Arg at pH values above 12.0 [3].

The aim of this investigation was to increase the sensitivity of ASHM when detecting NPC containing Lys and Arg by enhancing determination of the amino groups of these highly basic amino acids in the alkaline pH range (9.1-12.3), working on the basis of values of dissociation constants of the amino groups of Lys and Arg, and as a result, widening the applications of the ASHM in neuromorphology.

EXPERIMENTAL METHOD

Samples of tissue from the motor cortex (from three chinchilla rabbits) and the anterior parietal cortex (from six Wistar rats with a mirror image epileptogenic focus [7], and from control intact rats were fixed in Carnoy' s fluid and 10% neutral formalin, and embedded in paraffin wax; sections were cut to a thickness of 7, 14, and 21 μ. The animals were decapitated under ether anesthesia. To prepare a 10% solution of ammoniacal silver (AS) [9], a 10% solution of silver nitrate ($AgNO_3$) was added gradually, with continuous mixing, until a slight but stable pale yellow opalescence appeared in the AS solution. A 20% solution of AS was prepared in the same way. Solutions of 20% $AgNO_3$ and 20% AS according to Golgi [6] also were prepared, for which purpose the 20% $AgNO_3$ solution was added to a concentrated solution of ammonia: at first, until the formation of a precipitate, but later until the precipitate dissolved completely. Acetic 10% formalin (ACF) [1] was prepared to strengthen the buffer properties of the

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solution and to make it alkaline in character (pH 8.6) in tap water, free from active chlorine, and in formalin neutralized by $CaCO_3$. Active chlorine was removed by allowing the tap water to stand for 3 days, after which it was boiled for 1-3 min [2]. The formic acid present in the acid formalin, like the active chlorine in the water, has an adverse action on the ASHM reaction. Solutions of 3% ACF were prepared from 10% ACF, by diluting it with tap water free from active chlorine. The preparations were toned with 0.5% gold chloride solution and treated with solutions of 2% oxalic acid and 2% hyposulfite. A freshly prepared solution of 10% AS was applied by a pipet to brain sections after dewaxing and taking through to water; after 5-10 sec the solution was poured off and the sections quickly rinsed in five changes of distilled water, after which, in order to develop the stain, by reduction of the ammoniacal silver complex, the sections were placed in a solution of 3% ACF, and the pH adjusted with 1 N NaOH to the required value within the range 9.1-12.3. The sections were then washed, dehydrated, cleared in eucalyptus oil, and mounted in Canada balsam. To reveal the morphology of the bodies and dendrites of the neurons more clearly the sections were treated, not with 10%, but with 20% AS, prepared after Golgi, for 1 min, and the stain was developed in 3% ACF at pH 10.6. To localize the nerve fibers the sections were kept for 1.5 h in 20% $AgNO_3$ solution at 56°C, transferred without rinsing into 10% or 20% AS for 10-20 sec, and after a short washing they were placed in 3% ACF at pH 10.6, also to develop the stain. Before carrying out the ASHM, control for specificity of the reaction was set up [4]: alkaline proteins were extracted from the tissue with 0.25 N HCl, amino groups were blocked by the deamination reaction, RNA was removed from the tissue by treating it with ribonuclease and 1 N TCA, and DNA was removed with deoxyribonuclease and additional extraction with 5% TCA.

EXPERIMENTAL RESULTS

The work described above showed that microstructures of the brain, namely neurons, neuroglia, and neuropil, stained yellow and brown by the ASHM reaction, carried out within the pH range from 9.1 to 12.3 NPC containing Lys characteristically stained different shades of yellow, both containing Arg – brown. If alkaline proteins were removed from the sections before ASHM, or their amino groups were blocked, the characteristic yellow and brown staining of the microstructures could not be obtained. They had a pale grayish-green color, which was abolished by removal of RNA and DNA from the tissue. The reaction also was negative if only nucleic acids were extracted from the sections before testing: their removal destroyed the NPC, including proteins containing Lys and Arg [3].

If ASHM was carried out with successively rising alkalinity of the ACF solution in steps of pH 0.1, the degree of detection of NPC containing Lys and Arg was found to depend clearly on pH. Within the range 9.1-9.5 the brain microstructures showed mainly moderately saturated shades of yellow, the intensity of staining of which increased with increasing alkalinity of the solution, up to a maximum at pH 10.6, after which the intensity decreased. The brown colors characterizing NPC containing Arg, on the other hand, increased rapidly in intensity after pH 10.6, and between pH 11.7 and 12.3 they became the principal colors. However, a further increase in alkalinity of the ACF led to nonspecific grayish-green staining.

ASHM in the alkaline range pH 9.1-12.3 gave stable results in tissues fixed both with Carnoy's fluid and in formalin, and revealed well defined morphology of the nerve cells, especially at pH 10.6 (Fig. 1a-f), under conditions of maximal dissociation of amino groups of Lys and, consequently, optimal proportions of NPC containing Lys and Arg. Meanwhile the suggested modification of ASHM removed the restriction to one pH value and enabled a wide range of alkaline pH values to be used depending on the aims and purposes of the study. It also enabled interaction of the sections with silver salts to be varied. For example, the morphology of the nerve cells, their bodies and processes, was clearly revealed with 20% ASF prepared by Golgi's method (Fig. 1d, e), whereas the localization of the nerve fibers, namely of terminal branches and bundles of myelinated fibers, was clearly demonstrated by treating the sections with silver salts after Bielschowsky [6], i.e., by treatment with 20% $AgNO_3$ and 20% AS, followed by development of the stain in 3% ACF at pH 10.6 (Fig. 1f). The ASHM also showed itself to be a sensitive test for recording histochemical and morphological changes, especially in the neuroglia in the brain of animals subjected to experimental procedures (Fig. 1c).

Because of the different color staining of NPC containing Lys and Arg, their localization in the brain had a mosaic pattern, indicating high heterogeneity of the qualitative composition of proteins both in individual cell components and in individual types of cells. The greatest differences were found between cells differing in morphology and function, namely neurons and neuroglia. On the whole, these can be characterized: the former as lysine-, the

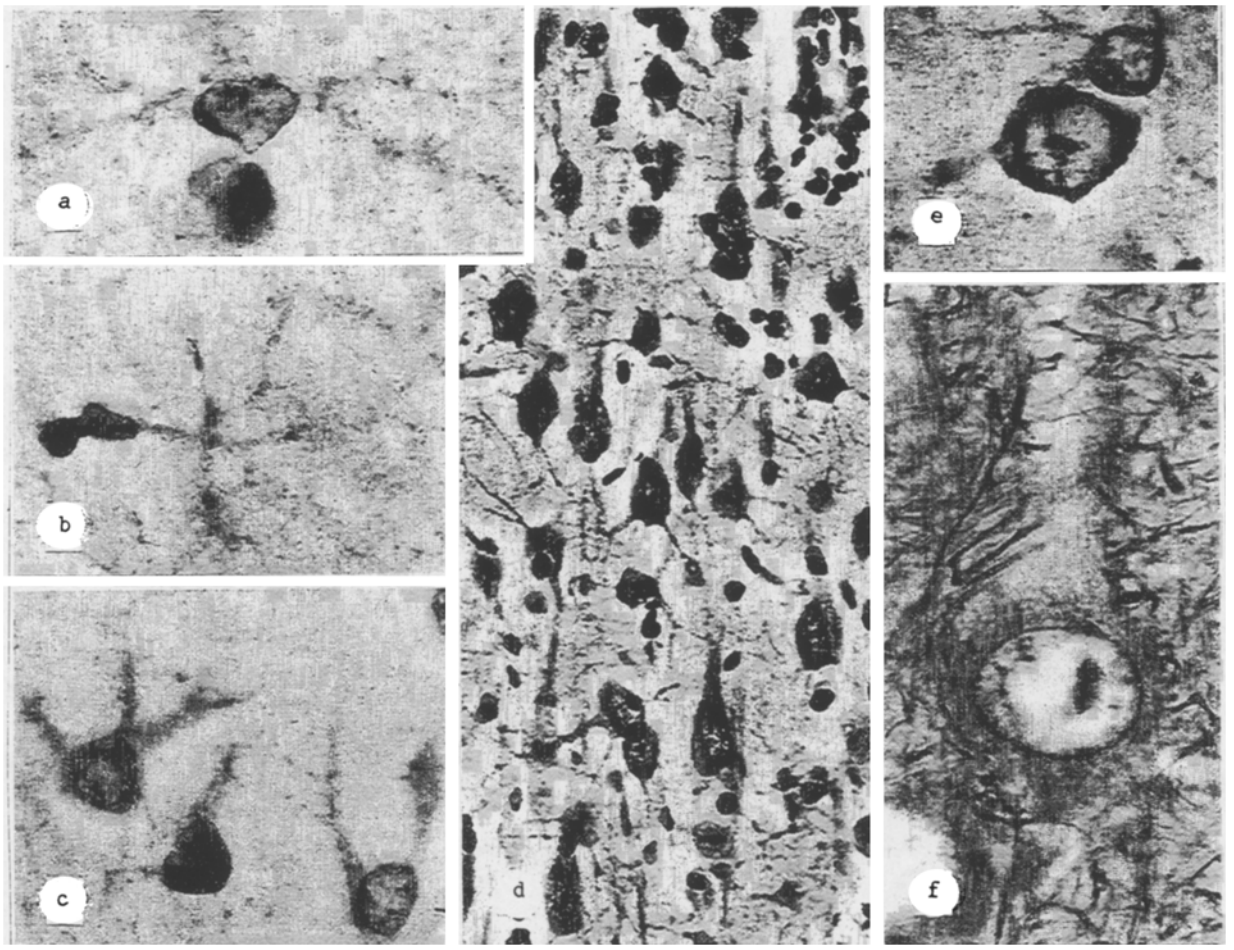

Fig. 1. Detection of neurons, neuroglia, and fibrous brain structures by ammoniacal silver method in alkaline pH range. a, b, c) Reaction by ammoniacal silver method: sections treated with 10% ammoniacal silver followed by development of stain at pH 10.6. Neuroglial cells in layer I of motor cortex of rabbit brain (a, b) – control animal, and in anterior parietal region of rat brain, c) mirror image epileptogenic focus. 700×; d, e) Reaction by ammoniacal silver method: sections treated with 20% ammoniacal silver, prepared after Golgi, followed by development of stain at pH 10.6. Microstructures of rabbit motor cortex (control animal); d) cortical layers IV-V 140×; e) Granular neuron and oligodendrocyte in cortical layer IV. 700×; f) Reaction by ammoniacal silver method: treatment with 20% silver nitrate and 20% ammoniacal silver, followed by development of stain at pH 10.6. En passant fibers and terminal branches of fibers on large pyramidal neuron in layer V of rabbit motor cortex (control animal). 700×.

latter as arginine-containing. In turn, depending on their morphology and function, differences in qualitative composition of the proteins also could be seen among neurons and neuroglia: NPC containing Lys predominated in neurons of association type in the cerebral cortex, NPC containing Arg in neurons of afferent and efferent type (layers IV and VI), whereas neurons of projection-efferent type (layer V) occupied an intermediate position. Different types of glia also were heterogeneous in composition: NPC containing Lys predominated in some (astroglia), NPC containing Arg in other types (microglia).

The use of an alkaline pH range (9.1-12.3) in ASHM to detect NPC containing Lys and Arg can thus make the ASHM stable when testing for neurons, neuroglia, and nerve fibers, and can demonstrate the high degree of heterogeneity of the qualitative composition of proteins in brain microstructures differing in their morphology and function; in this way, not only can existing known morphological and functional features of nerve cells be added to, but their identification can be facilitated.

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ULTRASTRUCTURAL AND CYTOCHEMICAL CHANGES IN THE RAT LIVER DURING ADAPATION TO HYPOXIA

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Normobaric hypoxia is currently being used on an ever-increasing scale in curative and preventive medicine in connection with various pathological conditions [2]. Hypoxia leads to rapid disappearance of intracellular glycogen in the liver and other organs, due to its mobilization as a source of energy during anaerobic glycolysis. Under these circumstances fatty acids (FA) become more important as energy substrates [4, 5], although for their combustion in the Krebs cycle under these conditions considerable obstacles are created in the form of a deficient oxygen supply to the cells and associated disturbances of the structure and function of the main energy-producing structures, namely the mitochondria. The question arises: what structural and functional mechanisms in animal and human tissues are responsible for maintaining energy homeostasis, and thereby ensuring a favorable preventive and therapeutic effect through the use of interrupted normobaric hypoxia?

The aim of this investigation was to answer the above question by parallel cytochemical and electron-microscopic methods. The organ chosen as the test object was the liver – the main organ in which carbohydrates and FA, the principal energy substrates of the body, are formed and stored during metabolism.

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