AMPEROMETRIC TITRATION OF MILLIMICROGRAM QUANTITIES OF SH-COMPOUNDS IN A TRIS BUFFER

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The argentometric amperometric titration of SH-groups of proteins and of mercaptans of low molecular weight in a tris buffer, as suggested by Benesch and co-workers [2], permits the quantitative determination of SH-groups to be made in more favorable conditions than by previously described methods [3, 4] and in the presence of denaturing agents. This method has been used by the authors to determine the number of SH-groups in crystalline proteins and enzymes, and more recently Ungar and Romano [6] have used it successfully in physiological experiments to evaluate the structural changes in the proteins of nervous tissue during stimulation. However, this method can only be used with comparatively large amounts of nervous tissue, which, having regard to the complexity of the structure of the nervous system, makes it difficult to assess biochemically the functional state of its individual formations.

In this paper we describe an ultramodification of the method of Benesch, Lardy, and Benesch, suitable for analysis of millimicrogram quantities of SH-compounds and enabling structural changes, such as those of denaturation, in proteins in individual nervous formations during stimulation to be detected.

Apparatus and Reagents

A platinum electrode (Fig. 1, B, d), 0.5 mm in diameter and 30 mm long, is soldered into a glass tube (a) and wound around it for a few turns. The upper end of the tube is fixed by Mendeleev's paste inside a coupling (f), inserted into a metal socket (h) and secured by a screw (g). The spindle of the socket is joined by a rubber tube to the shaft (l) of the motor. This method of fixing the electrode makes it easily changed if it is damaged or if it is necessary to replace it by an electrode for macrotitration. The platinum electrode is connected to a microammeter through a mercury contact and iron wire, soldered to the body of the coupling, through the body of the socket, through a thin steel spring (k) pressed by the rubber tube against the spindle of the socket and the shaft of the motor, and thence through the motor shaft. The rate of revolution of the platinum electrode is 750-800 rpm, and it is regulated by a rheostat.

As comparison electrode we used a Hg-saturated $HgO-Ba(OH)_2$ electrode, having a potential of 0.1 V against a saturated calomel electrode. To prepare it, a known excess of HgO and $Ba(OH)_2$ were shaken up in water for a short time, and the resulting saturated solution, together with the undissolved mercuric oxide and barium hydroxide, was poured over a layer of mercury. The electrode is stable for 5-6 months if protected from the light. The comparison electrode is connected electrolytically with the liquid to be titrated by means of an agar bridge, the end of which dips into the solution for titration in the form of a glass capillary tube, 0.2 mm wide.

For the titration a capillary ultramicroburet (UMB) is used, and the liquid is aspirated into it and expelled from it by means of a pneumatic regulator (Fig. 1, A), consisting of 2 metal plugs, separated by a round rubber membrane. The upper part of the capillary tube of the UMB (a) fits into the upper plug and is secured with Mendeleev's paste. The lower plug (c), together with the micrometer screw inside it, are screwed into the upper plug, firmly compressing the membrane between them (d). To provide a smoother and more gradual change in the position of the membrane, and to avoid crumpling it, a cylindrical insert (e) is placed in the canal of the lower plug between the membrane and the micrometer screw; the upper part of this insert is hemispherical and is attached to the membrane, and the lower part is conical and rests on the rod of the micrometer screw. As the micrometer screw is turned, the membrane is given only a progressive movement in a vertical direction. The capillary tube of the UMB, bent to a right angle, has an external

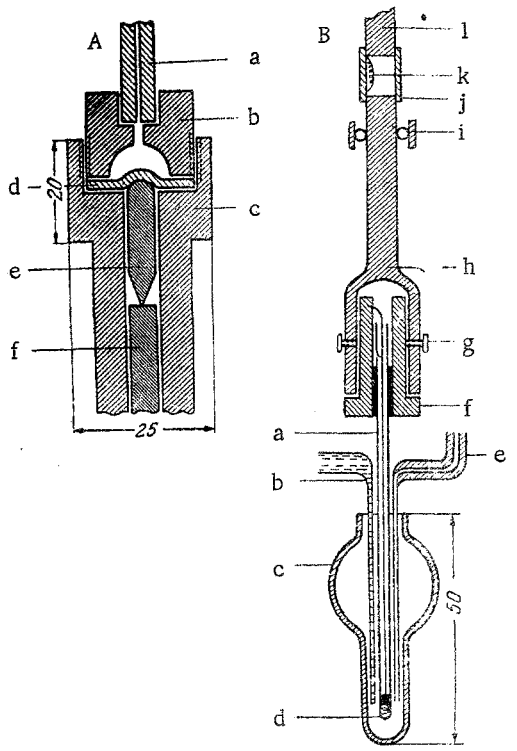

Fig. 1. Scheme of the individual parts of the titration apparatus. A) Pneumatic regulator of the ultramicroburet: a) capillary tube of the UMB; b) upper plug; c) lower plug; d) rubber membrane; e) insert; f) rod of micrometer screw; B) a) tube of platinum electrode; b) capillary tube of agar bridge; c) electrolyzer; d) platinum electrode; e) capillary tube of UMB; f) coupling; g) screw; h) socket; i) bearing; j) rubber tube; k) contact spring; l) shaft of motor. Dimensions are given in millimeters.

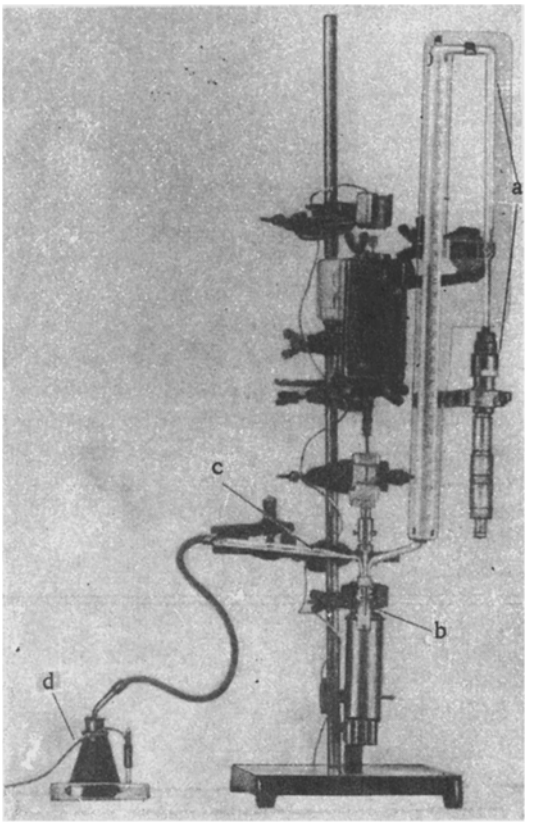

Fig. 2. Titration apparatus. a) Ultramicroburet; b) electrolyzer; c) agar bridge; d) comparison electrode.

diameter of 7 mm and a bore of 0.3 mm; the calibrated part is 40 cm long and its volume is 5 μl. The UMB is calibrated gravimetrically. Readings are taken from a vertical scale, graduated in millimeters. One scale division is equivalent to 0.0125 μl. The parts of the UMB are assembled and fixed on organic glass panels.

Titration is carried out in a glass electrolyzer (Fig. 1, B, c). Into its lower, narrow part are poured the buffer mixture and solution of SH-compound; the upper part is spherical and is fixed to the stand. This type of electrolyzer can be firmly fixed, but at the same time allows regulation of the position of the platinum electrode, the tip of the capillary tube of the UMB, and the capillary tube of the agar bridge in the liquid during titration.

The holder of the electrolyzer is connected with a toothed lifting mechanism enabling the position of the electrolyzer to be altered vertically.

The titration apparatus is mounted on an ordinary laboratory stand (Fig. 2). The strength of the current during titration is measured by a microammeter M-91 with a scale reading of 1 μA and the value of the scale division 0.01 μA to 1°.

In principle, the electrical design of the apparatus does not differ from that described by Benesch and coworkers.

For the work we used ordinary 0.1 and 0.2 ml micropipets, and for the precise measurement of very small volumes of solution semiautomatic micropipets of the Levy [5] type of a capacity of 2-10 μl, calibrated gravimetrically. Titration was carried out with a 0.001 M solution of $AgNO_3$, prepared immediately before the experiment. The titer of the stock 0.1 M solution of $AgNO_3$ was determined by Mohr's method.

Tris buffer, pH 7.4, was prepared immediately before titration by mixing 1 ml of a 1 M solution of trioxymethylaminomethane and 0.85 ml of 1 M HNO_3. All solutions were made up in double distilled water. The $AgNO_3$ and tris were twice recrystallized.

Technique of Titration and Testing the Accuracy of the Method

Into the lower, narrow part of the electrolyzer is placed 5-10 μl of the test thiol solution, and to it are added 74 μl of the buffer mixture and water to bring the total volume up to 0.3 ml. The final concentration of tris is 0.133 M. During titration of nonprotein SH-groups, in order to obtain more reproducible results a 0.01% solution of gelatin may be added instead of water.

The presence in the titration mixture of a 0.01 M solution of KCl, as recommended by Benesch and co-workers for the macromethod, is not necessary in the case of titration of ultramicro-quantities of SH-groups, as our experiments have shown, and it may even lower the accuracy of the method. After the electrolyzer has been filled it is fixed in the holder and raised smoothly until the capillary tube of the agar bridge, the tip of the UMB, and the platinum electrode are immersed in the titration fluid, the motor is started, and titration begins, the micrometer screw of the regulator of the UMB being turned. At the end of titration the electrolyzer is lowered and taken from its holder. The volume of $AgNO_3$ solution used in titration of the SH-compounds is obtained by extrapolation in the ordinary way.

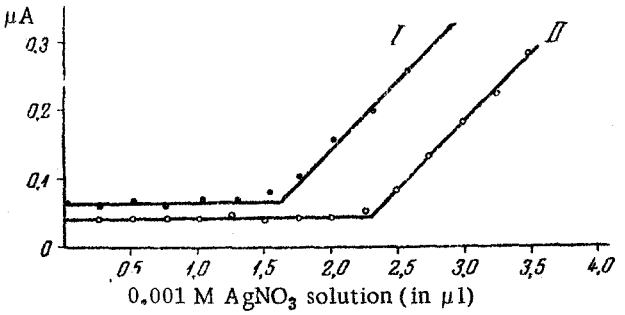

Fig. 3. Titration curves of 4.6 μl of rabbit's serum (I) and 2.3 mμmole of SH-glutathione (II).

In order to study the accuracy and the possibilities of the method, we titrated a $0.5 \cdot 10^{-3}$ M solution of SH-glutathione and unithiol. The titration curve of 4.6 μl of SH-glutathione solution, containing 2.3 mμmole thiol, is shown in Fig. 3, II. The results obtained in 8 parallel experiments show that by means of the suggested method it is possible to determine 2.231 mμmole or 97.0% of the SH-glutathione. Of the 2.3 mμmole of unithiol taken for analysis, 2.254 mμmole or 98.0% can be determined. The titration curve of the SH-groups in 4.6 μl of rabbit's serum is shown in Fig. 3, I.

An example of the application of this method is given by our determination of SH-groups in tissue extracts of the superior cervical sumpathetic ganglion of the cat in states of rest and excitation.

TABLE 1. Content of SH-groups in Fractions of a Tissue Extract of the Superior Cervical Sympathetic Ganglion of a Cat in States of Rest and Excitation (in mμmoles/mg fresh tissue)

Type of SH-groups	Functional state						Difference between content of SH-groups in excitation and rest			
	Rest			Excitation						
	content of SH-groups	σ	N	content of SH-groups	σ	N	content of SH-groups	σ	P	N
Total	3.867	±0.556	18	4.969	±0.448	18	1.102	±0.269	<0.001	18
Nondialyzable	2.883	±0.448	18	3.468	±0.516	18	0.635	±0.374	<0.001	18
Dialyzable*	1.034	±0.192	18	1.501	±0.295	18	0.467	±0.319	<0.001	18

*Obtained mathematically by the difference between the total and nondialyzable SH-groups.

An operative approach to the superior cervical sympathetic ganglia was made in decerebrate cats. The preganglionic trunk was then divided and the central cut end stimulated by an electric current (3-4 V, frequency 10 impulses/sec, duration of impulse 10 msec) for 20 minutes. The state of excitation of the ganglion was demonstrated by a kymographic recording of the contraction of the nictitating membrane of the eye. As controls we used the opposite ganglia. At the end of stimulation the ganglion was quickly excised, its connective tissue coverings were removed, and it was weighed on a torsion balance with a scale reading to 10 mg. The tissue was homogenized with a small volume of Ringer's solution in a Potter's glass homogenizer at 2-4°, and the concentration of the homogenate was brought to 10 mg/ml. In order to extract the tissue proteins, the homogenate was kept on a water bath at 38° for

30 minutes, and centrifuged, and 20.3 μl of the supernatant fluid was taken for titration of the SH-groups. Before titration, part of the extract was first dialyzed against 25 volumes of Ringer's solution containing $3 \cdot 10^{-5}$ M ethylenediamine tetra-acetic acid.

The results of these experiments, given in Table 1, show that during excitation of the nervous tissue there is an increase in the content of SH-groups, both total and nondialyzable, presumably bound with proteins. This increase in determinable SH-groups, found during excitation of the superior cervical sympathetic ganglion of the cat, agrees with the findings of Ungar and Romano [6] obtained in the cerebral cortex of the rat in response to electrical stimulation.

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ERRATUM

In the article by V. A. Parnes entitled "LEUKEMIA IN MICE OF LINE Afb," published in No. 1 (1962), the following error occurs.

Page	Actually printed	Should read
127 (Contents)	Leukemia in mice caused by a factor contained in the blood of patients with homocytoblastosis	Leukemia in mice of line Afb