By means of the proposed method, depending on the aim of the investigation, the duration of onset of complete muscular fatigue and death of the animals can be shortened or lengthened by changing the water temperature (Fig. 2).

To study the suitability of the model for the screening of substances increasing endurance, substances known to possess adaptogenic properties were used. Amphetamine, piridrol, and liquid extracts of *Rhodiola* and *Eleuterococcus*, freed from alcohol, were injected into rats 10 min before the experiment in doses of 0.3-10 mg/kg. The results of the experiments with the most effective doses of these adaptogens are given in Table 1.

The suggested model of enforced dynamic work is thus suitable for mass screening of compounds in order to study their stimulant properties. The stable experimental conditions and the possibility of setting the precise level of muscular activity mean that this model can be used to study the biochemistry and physiology of endurance.

Because the animals are in a fixed position, their physiological parameters (rectal temperature, blood pressure, pulse rate in the tail of rats) can be recorded and manipulations performed (substances injected) in the course of the experiment.

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IMPROVED HISTOCHEMICAL METHOD OF DEMONSTRATING OPEN CAPILLARIES

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A modification of Pickworth's method for demonstrating open capillaries is described. The distinguishing feature of this method is freezing the organ in situ with liquid propane, cooled with liquid nitrogen, followed by lyophilization of the specimen and fixation with gaseous formaldehyde. The sections are stained strictly in accordance with Pickworth's method.

KEY WORDS: *open capillaries, their demonstration; Pickworth's method, modification.*

Existing methods of detection of capillaries are not free from defects because they require the introduction of foreign materials (suspensions of ink, polymerized plastics, etc.) into the blood stream and use of a high perfusion pressure, outside physiological limits [1,

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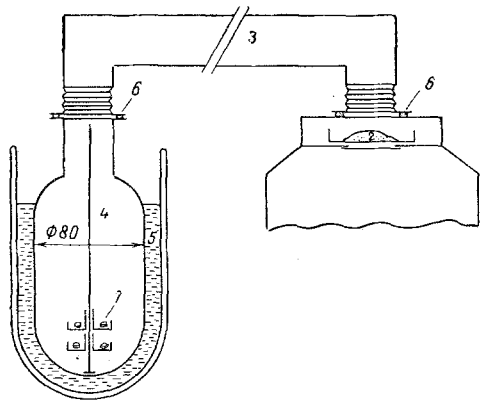

Fig. 1. Scheme of apparatus for freeze-drying of deeply frozen tissues (explanation in text). 1) High-vacuum unit (10^{-6} torr); 2) chamber for P_2O_5; 3) high-vacuum connection (copper, brass); 4) lyophilization chamber (glass); 5) Dewar flasks with cooling mixture; 6) junction between separate parts of apparatus; 7) brass "baskets" with pieces of tissue.

5, 6, 8, 10, 11]. In the writers' opinion, methods not requiring injection of substances of any sort into the blood stream and enabling the tissues to be fixed rapidly without any change in their histological picture are more suitable for the demonstration of open capillaries. One such method is that associated with the name of Pickworth, described in the general textbooks of histological techniques [1], but even this cannot be regarded as ideal, for during removal of the fresh tissue the state of the microcirculation can be changed, and fixation with formalin causes shrinking of the tissue [7, 9].

This disadvantage can be overcome by intravital freezing of the tissue in situ. However, the simplest method of subsequent processing of the preparations (cutting the sections in a cryostat, transferring them to "warm" slides, and drying at room temperature, followed by staining by Pickworth's method) proved to be unsuitable, for during histological treatment red blood cells were destroyed and diffusely scattered small, darkly stained granules, unconnected with the vessels, were found in the sections. Transferring the sections in the cryostat itself, without thawing, into cold formalin solution likewise did not give successful results.

An attempt to lyophilize deeply frozen tissues also proved unsuccessful. In paraffin sections cut from tissue lyophilized in blocks or in sections cut in a cryostat and then

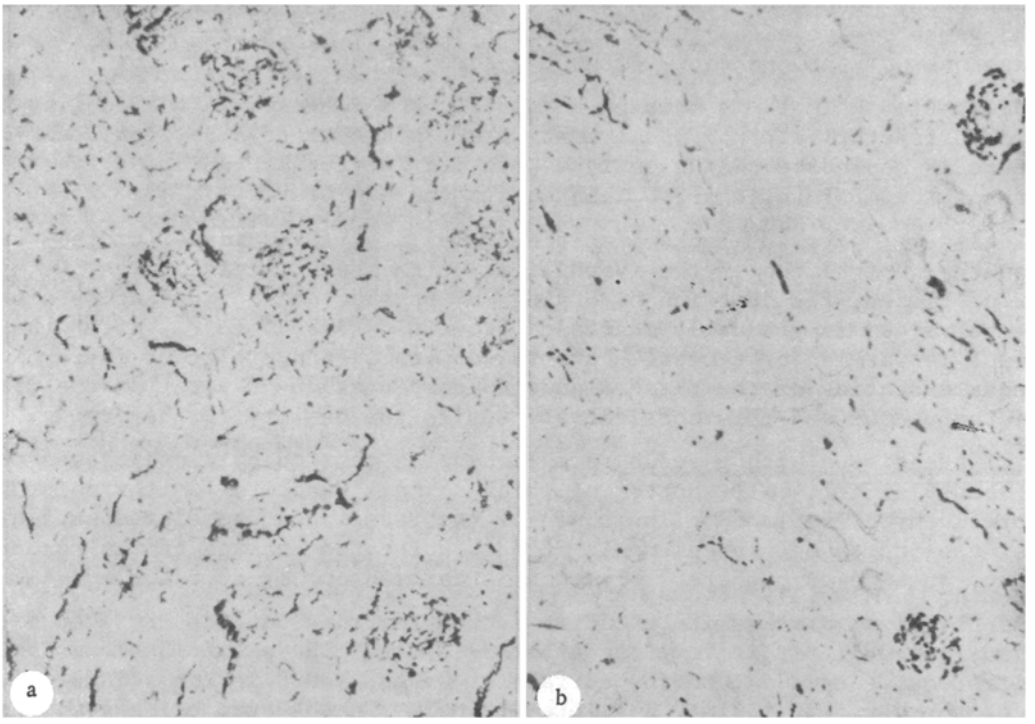

Fig. 2. Photomicrographs of myocardium of intact rat (a) and of rat given injection of noradrenalin (b). Stained by Pickworth's method, 240 ×.

lyophilized and transferred back again to an aqueous medium for the benzidine reaction, the same diffuse small dark granules were observed outside the vessels. It became obvious that to maintain the stability of the red cells the lyophilized tissue would have to be prefixed in a dry medium.

Experiments have shown that during the determination of catecholamines by the method of Carlsson and Hillarp [2, 3], which is based on the reaction of catecholamines with gaseous formaldehyde, fixation of the tissues takes place. It was therefore decided to use this method for the benzidine reaction in order to detect red blood cells in the capillaries. The action of heat on the tissue during the reaction with formaldehyde, as this method requires, had no effect on the pseudoperoxidase activity of the hemoglobin, but it inhibited peroxidase oxidation in other tissue structures, with the result that this reaction was specific for red blood cells.

The basic stages in removal of the material and its processing are listed below.

The animal is anesthetized and the organ to be investigated isolated, and a bath or gutter is formed from the surrounding tissues. A piece of foil is introduced beneath the organ. After a "rest" period of 30 min the organ is flooded with cooled liquid propane–butane (later called propane).

Liquid propane is supplied commercially in small cylinders. One such cylinder (without reducing valve) is turned over with the valve downward and liquid propane is allowed to flow (100-200 ml) into a Dewar flask containing a small quantity of dry ice. The propane is then cooled by immersing it in a copper vessel in liquid nitrogen. Care must be taken to ensure that the boiling nitrogen does not enter the vessel containing propane, for this could cause spillage of the propane.

By flooding with cold liquid propane it is possible to freeze organs such as the rat heart or kidney. To hasten the subsequent freeze-drying, it is useful to break off a thin flake from the frozen organ with a chisel cooled in liquid nitrogen or in a vessel with dry ice.

Freeze-drying was carried out in an apparatus of the writers' own design (Fig. 1), consisting of a high-vacuum unit, including rotary and diffusion pumps (VS-35, Laboratorni Pristroje, Prague) connected to a chamber for P_2O_5 (2), a high-vacuum connection pipe (3) 35-40 mm in diameter and 750 mm long, and a glass lyophilization chamber (4) with a capacity of about 1 liter. The junctions between the separate parts of the apparatus have flat metal ground ends and gaskets made of silicone and elastic rubber. The external pressure ensures adequate airtightness of the junctions without a screw connection.

The lyophilization chamber is mounted vertically and immersed in the cooling mixture consisting of 3 parts ethyl alcohol and 1 part water, poured into a 5-liter Dewar flask (5). The mixture is cooled by adding solid CO_2 to it. At the beginning of lyophilization the temperature of the mixture is –65°C, and at its end –25°C.

Blocks of tissue (up to 40 blocks simultaneously) are placed in perforated brass baskets (7), mounted on the central rod of the lyophilization chamber. Drying takes 10 days (with a smaller number of blocks, the duration of drying may be shortened). During freeze-drying the degree of the vacuum is gradually increased from 10^{-4} to 10^{-5} torr. After the end of drying the tissue is warmed for several hours to room temperature without disturbing the vacuum. Inadequate heating of the tissue leads to condensation of water on the blocks after disturbance of the vacuum and this considerably spoils the quality of the drying.

Fixation of the tissue with gaseous formaldehyde is carried out in a closed vessel with a capacity of about 1 liter, on the bottom of which a small quantity (on the point of a knife) of powdered formaldehyde is placed. The mouth of the vessel must be closed sufficiently tightly so that the increase of pressure in it does not cause leakage of the gaseous formaldehyde into the outside atmosphere. Fixation is carried out at 80°C for 1 h.

Soaking of the fixed blocks with paraffin is best carried out at a reduced pressure (a water pump is sufficient). It is complete after 8-10 min. The paraffin blocks are cut on an ordinary microtome, spread out on the surface of water, and fixed on slides with egg white. After removal of the paraffin (xylol, absolute ethanol, and 50% ethanol) and moistening of the sections with water, the benzidine test is carried out as described by Pickworth.

To carry out this reaction two solutions (A and B) are used. To prepare solution A, 0.5 g benzidine is diluted in 50 ml 96% ethanol (solution I) and the resulting solution is filtered. Next, 0.1 g sodium nitroprusside is dissolved in 10 ml distilled water (solution II). Solutions I and II are mixed and made up to 100 ml with distilled water.

To prepare solution B, 0.1 g sodium nitroprusside is dissolved in 10 ml distilled water (solution III), and 2 ml of glacial acetic acid and 5-10 ml 3% hydrogen peroxide are dissolved in 50 ml 96% ethanol (solution IV). Solutions III and IV are mixed and made up to 100 ml with distilled water.

The sections are treated for 5 min with solution A, gently rinsed in distilled water, and then treated with solution B for 5 min. The sections are then again rinsed with water and taken through alcohol and xylol, then mounted in balsam.

The product of the benzidine reaction has a dense black color. It is insoluble in water, alcohol, and xylol and, for that reason, the tissue can be counterstained both with hematoxylin and with cytoplasmic stains. However, staining with hematoxylin is undesirable, for in some cases (for example, investigation of skeletal muscles) the nuclei of the muscle cells, stained with hematoxylin, may interfere with counting of the open capillaries [10]. Better results are obtained by counterstaining the tissues with 5% fuchsin. Sections stained in this way are suitable for quantitative estimation of the relative volume of active capillaries [3].

Photomicrographs of the myocardium of an intact rat (Fig. 2a) and of a rat after preliminary injection of noradrenalin (Fig. 2b) are shown as an example.

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