β-ALANINE RELEASE FROM FROG SKELETAL MUSCLE DETERMINED BY THIN-LAYER CHROMATOGRAPHY

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The amino acid β-alanine, which is not found as a component of proteins, is, however, a component of muscle dipeptides and, in particular, of carnosine in amphibians [3, 8]. Since β-alanine is concentrated in muscle, it can be postulated that, on being released from muscles which are in an active state, it might perhaps play the role of a simple and sufficiently specific signal from muscle fibers to, for example, motor nerve endings [6], or to other targets.

In this investigation β-alanine in the incubation medium of muscles at rest and during contractile work was determined qualitatively and quantitatively by the method of high-efficiency two-dimensional thin-layer chromatography (HETLC) of dansyl derivatives of amino acids [2]. The results of this investigation were read at the 14th Congress of the All-Union Physiological Society [7].

EXPERIMENTAL METHOD

Experiments were carried out on the sartorius muscles of *Rana temporaria* at room temperature. The muscle was placed in a chamber filled with a certain volume (1.5 or 2 ml) of Ringer's solution. The muscle was activated with a frequency of 2 Hz by series of stimuli lasting 20 min, separated by intervals of 10 min. The total duration of stimulation was determined by the working capacity of the muscle (the duration of salt-free contractile activity); it varied in different experiments from 80 to 160 min. The symmetrical muscle of the same frog which was not stimulated (control) was kept for the same length of time in the same volume of solution.

In the experiments of series I the muscle was activated through the sciatic nerve, which was introduced into a glass suction electrode. In the experiments of series II the muscle, which was dissected without the sciatic nerve, was stimulated by means of silver electrodes immersed in the surrounding solution (total stimulation, addressed mainly to the intramuscular part of the nerve). In both series of experiments stimuli of supramaximal strength, and from 0.1 to 0.3 msec in duration, were used. In the experiments of series III total stimulation of the preparations was applied while synaptic transmission was blocked with D-tubocurarine (DTC, 3×10^{-4} g/ml). Under these circumstances, stimuli 1 msec in duration were used for direct activation of the muscle. The control muscle in this series of experiments was incubated in a solution with DTC, without stimulation.

Some of these experiments were conducted in the Department of Biochemistry, Moscow University (Head, Academician S. E. Severin), in the winter of 1983 in the course of a joint study to determine carnosine concentrations in resting and working muscles. The remainder of the experiments were conducted in Leningrad University in the spring and summer of 1983. All the incubation media were analyzed for their β-alanine content in Leningrad University, at the Institute of Macromolecular Compounds, and the Institute of Antibiotics and Enzymes (densitometry).

The incubation fluids were preserved by freezing, proteins contained in them were precipitated with TCA, the residue was removed by centrifugation, and the supernatant was dried. In some experiments proteins were not precipitated. Subsequent analysis showed that proteins did not affect the determination of β-alanine. The dried incubation media were treated with 50-200 µl of 0.1 N $NaHCO_3$, then dansylated with 5-dimethylamino-1-naphthalene sulfonylchloride

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TABLE 1. Release of β-Alanine from Working Muscles (in % resting release, M ± m)

Experimental conditions	Ringer's solution	Ringer's solution with D-tubocurarine
Stimulation of nerve	392±213	—
Total stimulation	290±64*	49±42

Legend. *P < 0.05 compared with control.

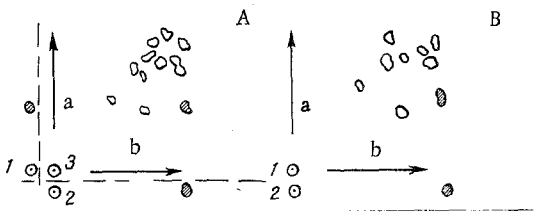

Fig. 1. Thin-layer chromatography of standard β-alanine (A) and test samples of muscle incubation solution (B). A) Identification of position of β-alanine spot (shaded). Position of β-alanine during one-way HETLC in system 1 in direction a, shown on left of vertical broken line (1 denotes start). Position of β-alanine during one-way HETLC in system 2 in direction b shown below horizontal broken line (2 denotes start). Scheme of plate after two-way HETLC of β-alanine against the background of a standard mixture of amino acids shown in center (3 denotes start); B) scheme of plate after two-way HETLC in incubation medium of muscle: 1) start of sample; 2) start of standard β-alanine applied for quantitative determination (subjected to one-way HETLC only). Spots of β-alanine shaded.

(DNS-chloride, from Serva, West Germany) to obtain dansyl derivatives of the amino acids [5]. On UV-irradiation (460 nm) these derivatives fluoresce with a maximum in the 550 nm region. Two-way HETLC was carried out on glass plates measuring 60 mm, covered with silica-gel with particle size of 8-12 μ, with silica-sol as the binder [1] in two systems of eluents: 1) acetone—isopropanol—ammonia (9:7:0.5) twice; 2) chloroform—benzyl alcohol—ethyl acetate—glacial acetic acid (6:4:5:0.2), once. The position of DNS-β-alanine was first identified against the background of DNS-derivatives of a standard mixture of amino acids, obtained by dansylation by the method in [9]. To determine β-alanine quantitatively in the incubation media 2-5 μl of the test sample was applied to the plate. After double dilution in system 1, 1-3 μl (from 2.7 to 60 ng) of the standard β-alanine (from Reanal, Hungary) was applied to the dried plate, 3-4 mm below the starting point of the sample, after which it was fractionated in system 2. The β-alanine spots were subjected to densitometry on the MFTKh-1 microabsorptiometer-fluorometer with integrating system and scanning stage.

EXPERIMENTAL RESULTS

The chromatographic mobility (R_f) of the standard β-alanine was 0.3 in system 1 and 0.7 in system 2 of eluents (Fig. 1A). During chromatography of β-alanine, together with the standard mixture of other amino acids, its spot could be reliably separated from the ohter spots.

A spot was found in the characteristic position for β-alanine on chromotograms of 41 of

the 42 incubation media of the resting and working muscles (Fig. 1B). According to results of 20 determinations the value of R_f was 0.34 ± 0.01 in system 1 and 0.72 ± 0.01 in system 2. Application of the standard β-alanine together with the test sample of incubation medium increased the intensity of fluorescence of this spot. This spot was therefore identified as β-alanine. Besides the β-alanine spot, 12 to 15 other spots, which were not analyzed in this investigation, were constantly present on chromatograms of the incubation media. In Ringer's solution, equal in concentration to the incubation media, no β-alanine was found.

This result is evidence that β-alanine escapes from the muscles into the incubation medium. Release of β-alanine from resting muscles has been reported previously [4]. It was important to discover whether this release changes with activity.

Release of β-alanine at rest in normal solution was 19.3 ± 4.0 nmoles/100 mg wet weight of muscle (nine experiments) and 13.8 ± 8.4 nmoles/100 g in the solution with DTC (four experiments). The difference between these values is not statistically significant. In winter frogs (females) resting release was 19.3 ± 5.6 nmoles/100 mg (seven experiments, in normal solution and with DTC), and in spring-summer frogs (males) it was 14.8 ± 3.7 nmoles/100 mg (five experiments), i.e., the difference was not statistically significant. The working capacity of the frogs' muscles did not differ significantly in the winter and spring-summer period.

In the experiments in the spring and summer, β-alanine release from the working muscles did not differ significantly from resting release, either in the case of nerve stimulation (15.9 ± 6.4 and 19.8 ± 3.4 nmoles/100 mg, three pairs of muscles) or in the case of direct stimulation of the muscles in the presence of DTC (8.4 ± 2.0 and 7.3 ± 1.8 nmoles/100 mg respectively, two pairs of muscles).

In winter frogs, however, release of β-alanine from working muscles in response to nerve and total stimulation was significantly higher than release at rest (Table 1). In the nerve stimulation experiments this excess was about 30%, but it was not statistically significant because of the small series of cases (four pairs of muscles) and the great variability of the data. In total stimulation experiments (four pairs of muscles) the excess was rather smaller (200%) but it was statistically significant ($P < 0.05$). Since activation of intramuscular nerve branches, more readily excitable by short electrical stimuli than muscle fibers, predominated in the case of total stimulation, these two series of experiments were essentially of the same type and their results could be pooled. After pooling in this way the mean release of β-alanine from the working muscles was 334 ± 90% of the resting release ($P < 0.05$, seven pairs of muscle).

Meanwhile in winter frogs contractions of the muscles in response to direct stimulation in solution with DTC were not accompanied by an increase in β-alanine release compared with the resting value (Table 1); indeed, a tendency for release from working muscles to be reduced could be distinguished.

The increased release of β-alanine from working muscles observed in experiments on winter frogs was thus connected with activation of the cholinergic mechanism of the neuromuscular synapse and not with muscle contraction and not with nerve excitation as such. Release of β-alanine from muscles may conjecturally be linked with the breakdown of some intramuscular carnosine. A more detailed description of the mechanism determining this release at rest and its intensification during synaptic activation, which occurred in winter forgs, must await further research.

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SPIN PROBE STUDY OF STRUCTURAL CHANGES IN ERYTHROCYTE MEMBRANES DURING COOLING

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The writers showed previously that disturbance of the barrier function of erythrocyte membranes during cooling takes place within the temperature range corresponding to the freezing out of spatial water [10]. The results formed a basis for a dehydration hypothesis, according to which the freezing out of water initiates structural transformations in lipid—lipid and lipid—protein complexes, leading to disturbance of the integrity of the bilayer.

According to data in the literature [3], freezing affects the structure of the lipid complexes first, and these may undergo thermotropic and lyotropic mesomorphism.

During freezing, however, the structure of the membrane protein also is significantly affected [2], and like phase transitions of lipids, this also leads to disturbance of protein—lipid interactions, which are evidently particularly sensitive to the action of low temperatures.

In this investigation the physical state of the lipids and of protein—lipid interactions were studied in erythrocyte membranes during cooling.

EXPERIMENTAL METHOD

Cells were washed free from plasma by centrifugation in 5 volumes of solution A: 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, at 3000-4000g in the cold. The procedure was repeated two or three times. White ghosts were obtained as described previously [14]. After separation the membranes were suspended in solution A. Lipids were extracted from the white ghosts by the method in [4]. The solvent was evaporated on a rotary evaporator, and the resulting lipids were treated with 1 ml of solution A, and liposomes were obtained by mechanical shaking [7].

The spin probe was the stearic acid derivative 5-doxylstearate (Fig. 1), from Syva (USA), and was added to a suspension of erythrocytes, white ghosts, and liposomes in the form of an alcoholic solution (final concentration in the sample 5×10^{-5} M). The final ethanol concentration was 2%. To remove the residue of ethanol and of probe not incorporated by the membranes, the test samples of erythrocytes, white ghosts, and liposomes were washed twice by centrifugation in the cold at 3000, 9000, and 18,000g respectively.

EPR spectra were recorded on an E-109 radiospectrometer (from Varian, USA), with a working frequency of 9 GHz, and with thermostatic control of the samples, within the temperature range from -50 to 20°C. The parameters of recording the EPR spectra were chosen, depending on the temperature conditions of the experiment, to abolish distortion of the spectrum due to saturation of superhighfrequency irradiation or rapid scanning. During lowering of the temperature of the sample the power of superhigh-frequency irradiation varied from 3 to 15 mW, the modulation frequency was 100 kHz, the amplitude 2 G, the range of scanning of the magnetic field 20 G, recording speed from 4 min to 1 h, and the filter 0.0128-1 sec. To characterize the orderliness of anisotropic movements of the hydrocarbon chains of the phospholipids in the membranes, the parameter $2A_{max}$ was used; this reflects the distance between the outer extrema

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