# ACTION OF ANSERINE AND CARNOSINE IN THE PECTORAL MUSCLE OF CHICKENS

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The spectrum of action of the muscle dipeptides, anserine and carnosine, is wide. Their influence extends to such vitally important processes as glycolysis, oxidative phosphorylation, and muscle contraction [6, 8, 9]. It has often been shown that the working capacity of a muscle and the dipeptide content are interrelated: muscles carrying heavy loads as a rule contain more dipeptides, and the working capacity of isolated muscle preparations increases in the presence of these compounds [2, 12]. In the case of muscle contraction three directions can be postulated in which the action of dipeptides ultimately leads to an increase in working capacity of the muscles. First, this action of dipeptides may be due to maintenance, with their aid, of processes supplying energy to the muscle tissue (glycolysis and oxidative phosphorylation). Second, the direct participation of these compounds in the act of contraction and their linkage with the actual contractile structures of muscle tissue may be assumed. Third, dipeptides may take part in the transmission of nervous impulses from nerve to muscle [1, 10, 11]. On the basis of these hypotheses, a more or less uniform distribution of dipeptides may be expected in such intracellular structures as myofibrils, or in the region of neuromuscular synapses. Data relating to the content of anserine and carnosine in the structural elements of the cell obtained by differential centrifugation (nuclei, mitochondria, microsomes) are contradictory and give evidence of a rather uniform distribution of dipeptides in the cytoplasm [18, 19]. Unconvincing data have been obtained by the use of methods such as electrophoresis in the aqueous phase and ultrafiltration, in an attempt to discover a link between dipeptides and the proteins of the muscle plasma [5, 7]. A clearer picture of the intracellular localization of dipeptides would give valuable evidence enabling the role of these compounds and their mechanism of action to be interpreted.

The object of the present investigation was to continue the study of the intracellular distribution of anserine and carnosine in the pectoral muscle of chickens by the method of histoautoradiography, using carbon-labeled $\beta$-alanine-3-$C^{14}$, and to determine the linkage of these peptides with the proteins of muscle plasma by electrophoresis on paper.

## EXPERIMENTAL METHOD

The pectoral muscle of chickens aged 15-20 days, containing 600-800 mg % anserine and 20-80 mg % carnosine, was used in the experiments. To determine the linkage between dipeptides and muscle proteins, the muscle plasma was subjected to electrophoresis in phosphate buffers of different ionic strength and pH for 4-18 h ("Leningrad fast" paper, potential gradient 5-6 v/cm). After electrophoresis, the paper was dried and stained with 0.5% ninhydrin solution in acetone, and then heated to 120° for 5 min to reveal dipeptides, or with 0.02% solution of amido black to reveal protein fractions. Muscle plasma was obtained by centrifugation (at 18,000 rpm for 20 min) of frozen (with a mixture of dry ice and acetone) pectoral muscle chopped up with a scalpel. From 10 g of such a mince about 3 ml plasma was obtained; 0.03 ml plasma was applied to the electrophoresis paper.

Labeled $\beta$-alanine was synthesized by the following scheme:

$$Cl\cdot CH_2\cdot COOH \xrightarrow{KC^{14}N} N\vdots C^{14}\cdot CH_2\cdot COOH \xrightarrow{[H]/Ni-Renay} NH_2\cdot C^{14}H_2\cdot CH_2\cdot COOH;$$

the initial labeled material was $KC^{14}N$ with a specific activity of 70 $\mu$ Ci/g. The synthesis of cyanacetic acid was carried out by the method suggested by Mandl and Brown [16], and it was converted into $\beta$-

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TABLE 1. Effect of Histological Treatment on Content of Dipeptides in Tissue

| Sample no. | Histological treatment of tissue | Anserine | | Carnosine | |
|---|---|---|---|---|---|
| | | in mg/g tissue | in percent of untreated tissue | in mg/g tissue | in percent of untreated tissue |
| 1 | Untreated | 9.15 | – | 1.76 | – |
| 2 | Usual treatment for time x with volume of reagents y | 6.75 | 73 | 1.5 | 85 |
| 3 | Treatment during time x with volume of reagents 2y | 5.9 | 64 | 1.3 | 74 |
| 4 | Treatment during time 2x with volume of reagents 2 y | 4.7 | 51 | 1.4 | 80 |

Note: List of reagents used, order and duration of their application during histological treatment of material:

| | | |
|---|---|---|
| Carnoy's mixture | 40 min at | 5° |
| Absolute alcohol | 30 " " | 5° |
| " " | 30 " " | 5° |
| Absolute alcohol: chloroform = 1:1 | 30 " " | 20° |
| Chloroform | 18 h " | 20° |
| Xylene | 20 min " | 20° |
| " | 20 " " | 20° |
| Absolute alcohol | 10 " " | 20° |

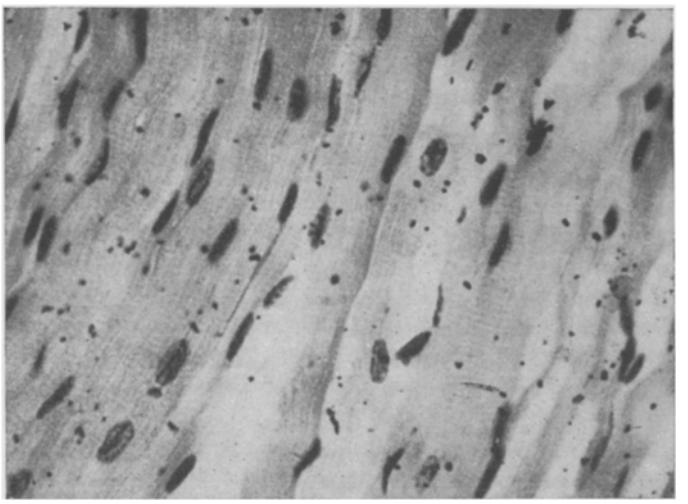

Fig. 1. Autoradiograph of pectoral muscle of a chicken. Exposure with emulsion for 15 days. 400×.

alanine by the method suggested by Fritzson [17]: hydrogenation under a pressure of 15-20 atm hydrogen in the presence of Renay nickel [13]. The preparations of β-alanine-3-$C^{14}$ thus obtained (specific activity 1.5 and 3.5 μCi/g) were identified chromatographically. The yield by activity was 33 %.

A solution of β-alanine-3-$C^{14}$ (dose 100 μCi) was injected once into the leg muscle of a chicken in 0.5 ml 0.9% NaCl solution. Histoautoradiography of the muscle tissue was carried out by the method described by Zhinkin [4]. The histological treatment included fixation of the tissue in Carnoy's mixture, dehydration with absolute ethanol, and embedding in paraffin wax through chloroform. Type "R" liquid

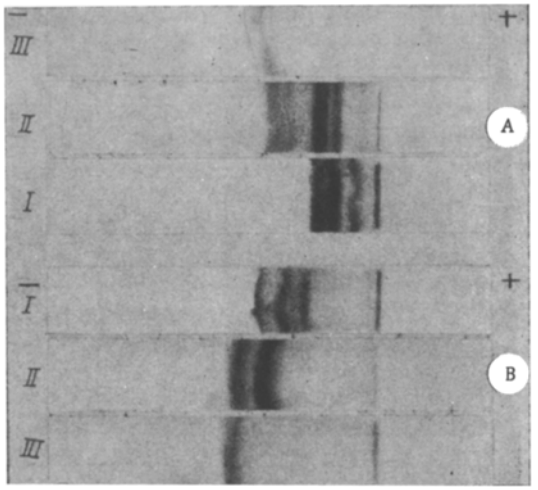


Fig. 2. Electrophoresis of muscle plasma: A) phosphate buffer, pH 7.0, $\mu$ = 0.09; B) phosphate buffer, pH 7.0, $\mu$ = 0.3. I—Plasma stained with amido black; II) plasma stained with ninhydrin; III) standard solution of anserine and carnosine, stained with ninhydrin.

photographic emulsion was used for autoradiography and was applied in a thin layer over the fixed muscle sections (thickness of section 7$\mu$). The duration of exposure of the preparations with the emulsion was 15-30 days. After development of the photographic emulsion the preparations were stained with hematoxylin-eosin.

## EXPERIMENTAL RESULTS

Histoautoradiography.* Since carnosine and anserine are synthesized from components and are dipeptides $\beta$-alanyl-histidine and $\beta$-alanyl-1-methyl-histidine [6], administration of $\beta$-alanine-3-$C^{14}$ facilitates incorporation of the label into the molecules of both anserine and carnosine. Incorporation of labeled $\beta$-alanine into other compounds (pantothenic acid, coenzyme A, fatty acids) was slight and could not distort the picture of the autograph, as was also the case with the presence of free $\beta$-alanine-3-$C^{14}$, which, as experimental results showed, is rapidly excreted and practically absent from the muscles 4 h after injection [3, 14]. Hence, the tracks in the preparation examined, obtained in the photographic emulsion from the $\beta$ radiation from carbon, must correspond only to anserine and carnosine molecules. As the autograph illustrated in Fig. 1 shows, the distribution of the label was diffuse in character, with no marked localization. Preparations of chicken pectoral muscle were obtained which were taken 4, 20, and 42 h after injection of the labeled material. Subsequent experiments showed that the first period (4 h) was marked by very high specific activity of carnosine, while later periods showed transfer of the label into the anserine molecules. In both cases the character of distribution of the tracks in the preparations was identical. Some variations in the method of preparing the autographs—application of photographic emulsion on a gelatin backing or without it, fixation of the tissue sections to the slide by a dry method or with the use of water—revealed no appreciable changes in the pattern observed.

Application of the histoautoradiography method to compounds such as anserine and carnosine, with their low molecular weight and high solubility in water and in aqueous alcohol, is complicated by the fact that considerable elution is possible during histological treatment of the tissue. Experiments showed that the method of histological treatment usually used by the author enabled 70-80% of the initial content of dipeptides to be kept in the tissue (see Table 1). When the duration of treatment or volume of the reagents was increased, so also was the percentage of dipeptides eluted. In this respect, prolonged contact between the tissue and alcohol is particularly undesirable, causing a decrease in the content of dipeptides in the treated material to 20-40%. Elution and the associated redistribution of anserine and carnosine during histological treatment were minimized as far as possible by carrying out the fixation and dehydration of the tissue with absolute alcohol in the cold. Nevertheless, the probability of secondary redistribution of the dipeptides was not ruled out, and allowance was made for it during analysis of the autographs obtained. Another factor which could not be forgotten was that the $\beta$ particles of the carbon isotope, with comparatively high emission energy (50,000 eV) may give a strong scatter of tracks visible in the photographic emulsion. It has been shown [15] that the weak $\beta$-ray emission from tritium (5700 eV) enables autographs with much higher resolution to be obtained than is possible with $C^{14}$. The use of tritium as label might therefore enable the localization of dipeptides relative to the structures of the muscle cell to be detected where only a diffuse picture could be obtained by means of $C^{14}$.

Electrophoresis. In this series of experiments another attempt was made to discover the link between the dipeptides and proteins of the muscle plasma by electrophoresis on paper. If such a link is present it may be expected that the dipeptides will migrate in an electric field together with a particular protein frac-

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tion. Phosphate buffers pH 5.5, 7.0, and 8.4 and with ionic strength 0.3 and 0.09 were used for electrophoresis. In all conditions suggested, carnosine and anserine migrated during electrophoresis as a common band independently of the protein fractions (Fig. 2). The rate of migration of the dipeptides during fractionation of the muscle plasma corresponded strictly to the rate of movement of standard anserine and carnosine solutions applied to a parallel strip of paper. Staining the paper with amido black, which gives no reaction with dipeptides, revealed no traces of protein in the zone of anserine and carnosine. The results obtained demonstrate that no link between the greater part of the dipeptides and the proteins of muscle plasma can be detected by electrophoresis on paper; at the same time, these results do not rule out its possibility completely. It may be that in muscle plasma obtained by thawing frozen and minced muscle some of the dipeptides are in a free form and the rest bound to proteins. However, it is also possible that the presence of bound dipeptides, which must migrate in an electric field together with a particular protein and lie in its zone, could not be found by the methods used because of masking of the dipeptide stain by the color reaction of the proteins with ninhydrin.

It is evident that migration of imidasol-containing dipeptides, bound to proteins of the muscle plasma during electrophoresis, may be demonstrated more accurately only if labeled material is used and if the bond is not broken by application of the electric field.

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