Carnosine levels in blood[1]

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Summary. Carnosine levels were determined in chick erythrocytes (2510 nmoles/g cells) and plasma from chick (27 nmoles/ml), rat and rabbit. Carnosine was also measured in rabbit reticulocyte-rich blood (105 nmoles/g cells), normal blood (18 nmoles/g cells) and in bone marrow.

In 1974 van Balgooy and co-workers found that carnosine (β-alanylhistidine) was present in nucleated erythrocytes of chick and frog[4]. They were unable to detect carnosine in the anucleate erythrocytes of mouse. Why carnosine should be present in large amounts in nucleated erythrocytes remains an enigma. Carnosine-synthetase activity has been detected in chick erythrocytes[5], so it is likely that carnosine is synthesized in these cells rather than accumulated from some other source.

In this paper the levels of carnosine in chick erythrocytes and plasma were determined. Levels of carnosine in rat and rabbit blood were also determined using a quantitative technique somewhat more sensitive than that used by van Balgooy[4].

Since carnosine is present in nucleated erythrocytes of chick and frog[4], it was of interest to determine carnosine levels in mammalian reticulocytes which contain some ER and mitochondria. Studies of this nature hopefully can give insight into the physiological role of carnosine in nucleated erythrocytes.

Materials and methods. All blood used in these studies was purchased from Pel-Freeze Biologicals (Rogers, Arkansas). A mixture of blood from both the male and female of each species was obtained. Chick blood was from 8–9-week-old Cornish Rock chickens. Rat blood was from sexually mature Sprague-Dawley and Wistar rats. Rabbit blood was from 8–10-week-old New Zealand albino rabbits. Reticulocyte-rich whole blood and bone marrow were from old New Zealand albino rabbits that had been injected daily with 0.7 ml of a 0.23 M phenylhydrazine solution for a period of 6 days. A 90% reticulocyte count was obtained by this method. Blood was shipped wet-iced, with heparin added as an anticoagulant. Blood was used immediately upon arrival.

3 samples were taken from each batch of blood and treated separately. Each blood sample was centrifuged at $900 \times g$ for 20 min to separate plasma from the red cells. The plasma was removed and saved. The red cell pellet was washed with 2 volumes of a wash solution containing 0.1 $MgCl_2$ and 0.4 M sucrose in a 0.01 M Tris-HCl buffer (pH 7.5) and recentrifuged at $900 \times g$ for 10 min. Plasma and the washed erythrocytes were then homogenized in 4 volumes of 0.9 M perchloric acid with a Tekmar Tissuemixer homogenizer, and 500,000 cpm of (^{3}H)carnosine was added to each sample to serve as a marker and internal standard. The homogenate was centrifuged at $7000 \times g$ for 10 min and the pellet discarded. The supernatant was heated to 90 °C in a water bath for 12 min, and then cooled to 0 °C on ice. Solid potassium carbonate was added to each sample until the pH was approximately 5.5. The precipitated potassium perchlorate was removed by centrifugation at $3500 \times g$ for 5 min and the supernatant was stored frozen until further use. All of the above steps were carried out at 0–5 °C.

The perchloric acid extract described above was evaporated to dryness in a Buchler flash evaporator, and reconstituted in 3.0 ml deionized water. The sample was applied to a Dowex AG 50WX8 column (1×60 cm) and eluted with 50 ml of water followed by several hundred ml of 0.15 M 2.6 lutidine. Carnosine was eluted from the column with the lutidine wash. The (^{3}H)carnosine added previously served as a marker for the carnosine containing fractions. Carnosine fractions were pooled (50–80 ml), evaporated to dryness on a Buchler flash evaporator, and reconstituted in 8.0 ml deionized water. All samples except chick red cells were further concentrated to 0.3 ml total volume. 30-µl aliquots of each sample were spotted in triplicate on CM-82 cellulose papers along with 5, 10, and 20 nmoles of carnosine standards. Papers were eluted with 0.15 M lutidine for 2 h, and dried in an oven at 100 °C. Carnosine was visualized and quantitated by the use of sulfanilic acid-sodium nitrite reagent as described by van Balgooy and Roberts[6]. Samples containing only radioactive carnosine gave no detectable color and blanks were prepared from areas of the paper containing no carnosine. Samples were corrected for 70% recovery.

Carnosine was identified as such from each blood source by the following criteria 1. Co-elution from Dowex AG 50WX4 column with authentic (^{3}H)carnosine standard. 2. Diazo-positive as well as ninhydrin-positive. 3. Co-migration with authentic carnosine standards on CM-82 cellulose paper with 0.15 M lutidine and on Silica gel 60 TLC sheets eluted with absolute ethanol:14.8 M ammonia:water (70:5:20). 4. Disappearance of original material and liberation of histidine and β-alanine following 24 h hydrolysis in 5.7 N HCl at 100 °C.

Results and discussion. As seen in the table, carnosine is present in chick, rat, and rabbit erythrocytes. Plasma levels of canosine were considerably lower than red cell levels. Carnosine levels were much higher in chick erythrocytes than in the mammalian erythrocytes investigated. The concentration of carnosine in chick erythrocytes reported here is roughly the same as in chick skeletal muscle[7].

It was of interest to determine carnosine levels in mammalian reticulocytes which have some characteristics of a nucleated cell. Although carnosine levels were not as high in reticulocytes as in chick erythrocytes, rabbit reticulocytes contained nearly 6 times as much carnosine as normal rabbit erythrocytes. Bone marrow from phenylhydrazine-treated rabbits had only a small amount of carnosine. It was thought that since carnosine was present in relatively high amounts in reticulocytes, it might be present in similar amounts in bone marrow cells. Possibly immature erythrocytes acquire carnosine only during the reticulocyte stage.

Carnosine levels in blood cells, plasma, and bone marrow

Blood	Cells*	Plasma**
Chick	2510±70	27±3
Rabbit	18±2	Not detected
Rat	Trace***	Trace
Rabbit (reticulocyte-rich)	105±11	3.5±0.7
Stimulated bone marrow	Trace	–

Results are the mean±SD for 3 carnosine samples. * nmoles carnosine/g cells; **nmoles carnosine/ml plasma; *** carnosine detected, but present at less than 5.0 nmoles/g.

Carnosine-synthetase activity has been shown to be present in chick erythrocytes[5]; however, no synthetase activity was detected in rabbit reticulocytes or erythrocytes under our conditions. It may be that the enzyme lost activity prior to the time of assay, since there is a loss in enzyme activity from rat skeletal muscle shipped in this manner.

It was interesting to find higher carnosine levels in rabbit reticulocytes than in rabbit erythrocytes. Mammalian reticulocytes differ from mammalian erythrocytes in many respects[8,9]. Reticulocytes have the ability to synthesize globin, whereas mature erythrocytes have lost all of their ability to synthesize protein[9-11]. It may be that carnosine is somehow involved in RNA and/or protein synthesis in reticulocytes. Studies with inhibitors of carnosine synthesis may be useful in elucidating the physiological function of carnosine in mammalian reticulocytes.

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Hydroxydihydroergosine, a new ergot alkaloid analogue from directed biosynthesis by *Sphacelia sorghi*[1]

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Summary. A new ergot alkaloid, 9′-hydroxydihydroergosine, has been produced by axenic cultures of *Sphacelia sorghi* to which 4-hydroxy-proline was added when biomass accumulation was complete. Evidence for a lack of biosynthetic specificity for the first, second and third amino acid in cyclic tripeptide alkaloids has now been found amongst ergot fungi.

As an ergot fungus parasitic on sorghum *Sphacelia sorghi* produces a group of ergoline alkaloids in the sclerotia[2]. The principal alkaloid is dihydroergosine (DHES), a cyclic tripeptide derivative of dihydrolysergic acid, the tripeptide being composed of proline, leucine and alanine. Other alkaloids, most of which may be regarded as biosynthetic intermediates, such as chanoclavine, festuclavine, pyroclavine and dihydroelymoclavine also occur as minor components. However, when an isolate of *S. sorghi* capable of alkaloid synthesis in vitro is grown in surface liquid culture several other indole alkaloids are evident in trace amounts on chromatograms of culture filtrate extracts[3,4]. Extended attempts to characterise these alkaloids have failed but there is evidence that at least some are artefacts of the cultural and/or work-up conditions.

Laboratory culture of *S. sorghi* for the production of dihydrogenated ergot alkaloids is best achieved using a sucrose/asparagine medium but the fungus in current use gave optimum alkaloid yields, and accumulated the least glucan, after modification of the medium formerly preferred[5]. Therefore cultures were both maintained and grown for alkaloid production at 27 °C on a medium containing sucrose, 100 g/l; asparagine, 10 g/l; $Ca(NO_3)_2 \cdot 4\,H_2O$, 1 g/l; KH_2PO_4, 0.25 g/l; $MgSO_4 \cdot 7\,H_2O$, 0.25 g/l; KCl, 0.125 g/l; $FeSO_4 \cdot 7\,H_2O$, 0.033 g/l; $ZnSO_4 \cdot 7\,H_2O$, 0.027 g/l; yeast extract, 0.1 g/l; L-cysteine hydrochloride, 0.01 g/l; distilled water; presterilisation pH 5.2 adjusted with NaOH; sterilised at 15 psi for 15 min. Erlenmeyer flasks (50 ml or 500 ml) contained 10 ml or 100 ml liquid medium respectively. Solid media contained 15 g/l agar. Surface liquid cultures were established using tissue (circa 0.5 cm^2), from the center of a 3-week agar slant culture, macerated into the production medium using an inoculating spatula.

Autoradiography of the alkaloid produced by cultures given radiolabelled (^{14}C) proline, leucine or alanine showed their incorporation principally into DHES, but at least 2 other alkaloid components were labelled by all 3 amino acids. Following feeding of a 14-day *S. sorghi* culture with the proline analogue allo-4-OH-proline (4 mg ml^{-1}) and incubating until alkaloid biosynthesis was complete (21 days, 0.5 mg alkaloid ml^{-1}), the proportion of one of these minor alkaloids [R_f 0.15 on silica gel chromatograms developed in chloroform/methanol/ammonium hydroxide

Incorporation of amino-acid analogues into ergot alkaloids

Ergot alkaloid	Ergot fungus	Amino acid sequence of peptide side chain	Amino acid analogue incorporated	Reference
Dihydroergosine	*S. sorghi*	*Pro*-Leu-Ala	4-Hydroxy-proline	Present paper
Ergotamine		Pro-*Phe*-Ala	p-Fluorophenylalanine	Beacco et al.[9]
Ergocryptine		Pro-*Leu*-Val	L-Norleucine	Beacco et al.[9]
Ergocornine	*C. purpurea*	Pro-*Val*-*Val*	L-Norvaline	Beacco et al.[9]
Ergotamine		Pro-Phe-*Ala*	α-Aminobutyric acid	Belzecki et al.[10]