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ΧΗΜΕΙΑ.—The amino acid composition of the hemoglobin of the turtle Emys Caspica[1], *by* ***John G. Georgatsos****. Ἀνεκοινώθη ὑπὸ τοῦ κ. Γεωργ. Ἰωακείμογλου.

Our knowledge of the amino acid composition of the hemoglobins in cold blooded animals is comparatively poor. This paper deals with the amino acid composition of hemoglobin in the fresh water turtle *Emys caspica*.

EXPERIMENTAL

Preparation of globin. The turtles were decapitated, and the blood was collected in one tenth volume 0.2 M sodium citrate. The red cells were separated by centrifugation, washed three times with physiological saline, and hemolyzed by twice their volume with distilled water. The nuclear material was separated out by centrifugation, and the supernatant from the hemolysis was cooled at O°C. The globin was precipitated by adding the cooled hemoglobin solution into twenty volumes of cold acid acetone according to the method of ROSSI - FANELLI et al (1).

Hydrolysis. The globin was hydrolyzed with 6 N hydrochloric acid either under refluxing for twelve hours, or in a sealed tube at 105 - 110° C for 24 hours.

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* ΙΩΑΝΝ. ΓΕΩΡΓΑΤΣΟΥ, Ἀνάλυσις τῶν ἀμινοξέων τῆς αἱμοσφαιρίνης τῆς νεροχελώνης (Emys Caspica).

Chromatography. Two dimensional ascending paper chromatography on Whatman No. 1 filter paper was used for the identification and quantitative estimation of the amino acid content of the hydrolysate. The solvents used were water-saturated phenol in one direction and a mixture of pyridine-amyl alcohol-ethyl alcohol-water (80:70:35:60) in the second direction. During the phenol run potassium cyanide and ammonia were placed in two separate containers in the cabinet. A frame for running several chromatograms simultaneously has been employed throughout this work (2). The paper was developed by spraying it with a 0.2% solution of ninhydrin in a 1:1 mixture of butanol and acetone. The color development was allowed to take place at room temperature overnight.

Quantitative estimation of the individual amino acids. The chromatograms used for the quantitative estimation of the amino acids, were sprayed with a 1.0% ninhydrin solution. In parallel with the globin hydrolysate a known amount of a pure amino acid was chromatographed on a different sheet of paper, and treated in exactly the same manner as the hydrolysate. The spots were cut out of the paper 24-36 hours after the application of the ninhydrin reagent, and were extracted in the dark with 5 cc of methyl alcohol for 24 hours. The optical density of the extracts was measured in a Bausch and Lomb Spectronic photometer at 570 mμ. A piece of filter paper from the spot-free area of the chromatogram about the size of an average spot was also extracted, and was used as a blank for setting the photometer at O density.

Tests for individual amino acids.

Methionine. Since valine and methionine have almost the same Rf values in the system used, the presence of methionine was proved by means of Mc Carthy and Sullivan's sodium nitroprusside test, on the hydrolysate. Tryptophan, which interferes with this reaction is destroyed during the course of acid hydrolysis, while histidine, which also gives a methionine-like colour, was removed from the hydrolysate by precipitation with phosphotungstic acid. The amount of methionine was calculated by measuring the size of the methionine-valine spot on the chromatogram and subtracting from it the quantity of valine after oxidizing the methionine with hydrogen peroxide (see below).

Valine. After the establishment of the presence of methionine, the amount of valine was determined by oxidizing methionine with hydrogen peroxide and ammonium molybdate (3), and measuring the remaining ninhydrin reacting spot in the usual way.

Basic amino acids. The presence of the three basic amino acids was confirmed by precipitating these acids as the phosphotungstates, and rechromatographing the supernatant solution, after removal of the excess phosphotungstic acid with ether, whereby the usual place these basic amino acids occupy was completely empty, and also by removing the phosphotungstic acid from the precipitate with a 1 : 1 mixture of amyl alcohol and ether in the presence of 5N hydrochloric acid (4), and rechromatographing the water solution of the amino acids All three basic amino acids reappeared in their usual places on the chromatogram.

Tryptophan could not be detected chromatographically, since it is destroyed during acid hydrolysis. The presence, however of this amino acid was established by means of the Hopkins - Cole reaction on the intact globin.

Proline was determined by the Troll and Lindsley photometric method (5), since the methanol extract of the yellow proline spot showed no deflection on the photometer either at 570 m μ or at 440 m μ.

DISCUSSION

The nitrogen content of the turtle globin preparation was found to be 15.5%, and the amide ammonia 1.0% (calculated on a dry basis). Table 1 shows the percentage composition of the turtle globin molecule in amino acids. One may note a high glutamic acid content, and a comparatively small amounts of histidine and proline. The nitrogen recovery of 103.0% strengthens ones belief that the method does not lack in sensitivity (A similar determination using methionine as a standard showed a nitrogen recovery of 98 8%).

Tryptic digestion of the globin preparation was not of any help in determining the amount of tryptophan.

There is work under way at present in this laboratory to establish the N- and C- terminal amino acids of the turtle globin molecule.

TABLE I

The amino acid composition of hemoglobin of the turtle *Emys caspica*.
(The sample applied on the chromatogram contained 33.6 γ nitrogen)*

Amino acid	Mg of amino acid per 100 mg of protein	Nitrogen contributed by each amino acid in the sample used (γ)
Arginine	4.7	4.3
Histidine	3.9	2.5
Lysine	9.0	4.2
Tyrosine	3.5	0.7
Threonine	5.5	1.6
Leucines	18.2	4.7
Aspartic acid	6.4	1.6
Glycine	3.5	1.5
Serine	7.4	2.4
Alanine	8.2	3.1
Proline	1.0	0.3
Glutamic acid	16.4	3.8
Valine	7.5	2.2
Methionine	1.1	0.4
Phenylalanine	4.9	1.0
Amide - NH_3	1.0	0.3
Total	102.2	34.6
Nitrogen recovery		103.0%

* Aspartic acid was used as a reference standard.

SUMMARY

The qualitative and quantitative amino acid composition of the turtle *Emys caspica* hemoglobin was established, mainly by means of two dimensional paper chromatohraphy. A comparatively high percentage of glutamic acid, as well as a low percentage of histidine were noted, the rest of the amino acids being present in analogous amounts as in mammalian hemoglobin.

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ΠΕΡΙΛΗΨΙΣ

Ἐγένετο ποιοτικὴ καὶ ποσοτικὴ ἀνάλυσις τῶν ἀμινοξέων τῆς αἱμοσφαιρίνης τῆς νεροχελώνης Emys Caspica. Ἡ ἀκολουθηθεῖσα μέθοδος ἔχει ὡς ἑξῆς.

Μετὰ ἀποκεφαλισμὸν τῆς χελώνης λαμβάνεται τὸ αἷμα ἐντὸς σωληναρίων περιεχόντων κιτρικὸν νάτριον. Μετὰ τὸν διαχωρισμὸν τῶν ἐμμόρφων συστατικῶν ἐκ τοῦ πλάσματος διὰ φυγοκεντρήσεως ἐκπλύνονται τὰ αἱμοσφαίρια τρὶς διὰ φυσιολογικοῦ ὀροῦ καὶ ἐν συνεχείᾳ αἱμολύονται δι' ἀπεσταγμένου ὕδατος. Τὰ πυρηνικὰ συστατικὰ διαχωρίζονται διὰ φυγοκεντρήσεως καὶ ἡ σφαιρίνη ἀπομονοῦται ἐκ τοῦ ὑπερκειμένου διὰ καθιζήσεως μετ' ὀξίνου ἀκετόνης κατὰ τὴν μέθοδον τοῦ Rossi - Fanelli καὶ συνεργατῶν αὐτοῦ (1). Ἐν συνεχείᾳ ἡ σφαιρίνη ὑδρολύεται μετὰ διακοσιαπλασίας ποσότητος 6N HCl (βάρος/ὄγκον) καὶ τὸ ὑδρόλυμα χρωματογραφεῖται διὰ τῆς μεθόδου τῆς δυσδιαστάτου ἀνιούσης χρωματογραφίας. Κατὰ τὴν πρώτην φάσιν τῆς χρωματογραφίας χρησιμοποιεῖται φαινόλη κεκορεσμένη δι' ὕδατος, κατὰ δὲ τὴν δευτέραν μεῖγμα πυριδίνης - ἀμυλικῆς ἀλκοόλης - αἰθυλικῆς ἀλκοόλης - ὕδατος (80 : 70 : 35 : 60). Αἱ κηλῖδες τῶν ἀμινοξέων ἀναγνωρίζονται διὰ ραντισμοῦ τῶν χρωματογραφημάτων διὰ 0,2% διαλύματος νινυδρίνης ἐντὸς μείγματος ἀκετόνης - βουτανόλης (1 : 1),

Διὰ τὸν ποσοτικὸν προσδιορισμὸν τῶν ἀμινοξέων ἀκολουθεῖται ἡ αὐτὴ διαδικασία μὲ τὴν διαφορὰν ὅτι ὁ ραντισμὸς τῶν χρωματογραφημάτων ἐκτελεῖται διὰ 1% νινυδρίνης, εἶτα δὲ μετὰ 36 ὥρας αἱ κηλῖδες ἀποκόπτονται καὶ ἐκχειλίζονται διὰ μεθανόλης εἰς τὸ σκότος ἐπὶ 24 ὥρας. Ταυτοχρόνως χρωματογραφεῖται μία γνωστὴ ποσότης ἑνὸς ἀμινοξέος, τὸ ὁποῖον καὶ ἐπεξεργάζεται, ὡς καὶ αἱ κηλῖδες τῶν ἀμινοξέων τοῦ ὑδρολύματος. Ἡ ὀπτικὴ πυκνότης τῶν ἐκχυλισμάτων προσδιορίζεται φασματοφωτομετρικῶς εἰς 570 mμ. καὶ ὑπολογίζεται τὸ ποσὸν τῶν ἀγνώστων ἀμινοξέων διὰ τῆς ἐφαρμογῆς τοῦ νόμου τοῦ Beer· θεωροῦνται ὡς γνωστὰ αἱ ὀπτικαὶ πυκνότητες τῶν τε ἀγνώστων ἀμινοξέων καὶ τοῦ γνωστοῦ καθὼς καὶ ἡ ἐν τῷ διαλύματι πυκνότης τοῦ γνωστοῦ εἰς γραμμομόρια, ὡς ἄγνωστος δὲ ἡ πυκνότης τῶν ἀγνώστων ἀμινοξέων εἰς γραμμομόρια. Διὰ τῆς μεθόδου ταύτης ἡ τρυπτοφάνη δὲν δύναται νὰ προσδιορισθῇ, διότι καταστρέφεται κατὰ τὴν ὑδρόλυσιν ἀλλ' ἁπλῶς ἀνιχνεύεται ἐπὶ τῆς μὴ ὑδρολυθείσης σφαιρίνης διὰ τῆς ἀντιδράσεως Hopkins - Cole. Ἐπίσης ἡ προλίνη, ἥτις δίδει κιτρίνην κηλῖδα μετὰ νινυδρίνης, προσδιορίζεται διὰ τῆς χρωματομετρικῆς μεθόδου τῶν Troll καὶ Lindsley (5).

Εἰς τὸν παρατιθέμενον ἀνωτέρω πίνακα ἀναγράφονται αἱ εὑρεθεῖσαι τιμαὶ δι' ἓν ἕκαστον ἐκ τῶν ἀμινοξέων κεχωρισμένως.

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