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Some Amino Acids of Chicken Erythrocytes.*

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The chicken erythrocyte is a cell which is readily available and ideal for various cytochemical and physiological studies. Furthermore, nuclei practically free of cytoplasm can be prepared in quantities sufficient for analysis by relatively simple procedures such as hemolyzing washed erythrocytes with saponin[1] or lysolecithin.[2] Morphological, chemical, and physiological studies on these erythrocytes and isolated nuclei from them allow for comparisons of nuclear and cytoplasmic functions which would be much more difficult to obtain on other types of cells. This report deals with some of the amino acids contained in the whole erythrocyte as determined by microbiological assays of hydrolyzates. Similar studies on nuclei are in progress.

Preparation of Erythrocytes. Blood samples were collected from healthy hens (White Rocks) by cutting the jugular vein and collecting the blood in an Erlenmeyer flask containing sodium citrate (5 mg per ml of blood) as an anticoagulant. The blood was transferred to 50 ml centrifuge tubes and centrifuged for 2 or 3 minutes at 3000 revolutions per minute. The supernatant plasma containing some erythrocytes and leucocytes was aspirated from the tubes. Ten ml of physiological saline (0.9% sodium chloride adjusted to pH 7.0 with M/15 phosphate buffer) was added to the tubes and the erythrocytes were resuspended. The tubes were centrifuged and the supernatant fluid and the layer of leucocytes which formed above the erythrocytes were removed by aspiration. Washing was repeated 5 additional times in order to free the erythrocytes of plasma proteins, as well as aid in removing the remaining leucocytes. After the last washing, a smear was prepared and stained with Wright's stain to check the absence of leucocytes in the preparation. The erythrocytes were transferred to evaporating dishes and dried over night at 105°C. The dried material was pulverized in an agate mortar and stored in weighing bottles. Five samples of dried erythrocytes, each weighing about 2.5 g, were prepared for this work.

Preparation of Hydrolyzates. One-gram samples of the dried erythrocytes were transferred to vials prepared by drawing out 150 x 22 mm Pyrex test tubes. To each vial 10 ml of 10% (by volume) hydrochloric acid was added. The vials were sealed and autoclaved for 10 hours at 15 lbs pressure. After cooling, the ampules were broken and the hydrolyzate washed into a beaker with a small amount of water. The hydrolyzate was neutralized with 5 N sodium hydroxide, the pH adjusted to 6.8, filtered, and diluted to a final volume of 50 ml with water. The hydrolyzates were stored under toluene in a refrigerator and, as a rule, were diluted with distilled water 1:25 ml or 1:50 ml before use.

Preparation of Amino Acid Standards.‖ Solutions of the *l* isomers of the various amino acids to be determined were prepared

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1 Dounce, A. L., and Lan, T. H., *Science*, 1943, **97**, 584.

2 Laskowski, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 354.

‖ Amino acids used as standards were furnished by Dr. Madelyn Womack, Department of Chemistry, University of Illinois.

† These organisms were obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D.C., where *Streptococcus faecalis* is listed as No. 9790, *Lactobacillus arabinosus* 17-5 as No. 8014, and *Lactobacillus delbruckii* LD5 as No. 9595. Folic acid used in the assay media was furnished by Dr. Beverly Guirard, University of Texas. The author is grateful for the suggestions of Dr. F. M. Clark, Department of Bacteriology, University of Illinois.

TABLE I.
Milligrams of Amino Acid per Gram of Dried Erythrocytes.

Sample	1	2	3	4	5	Average	%
Amino Acid							
Histidine	37.50	38.75	37.50	36.25	37.50	37.50	3.75
Arginine	42.50	42.50	45.00	39.00	48.60	43.52	4.35
Lysine	59.38	68.75	68.75	65.63	78.13	68.13	6.81
Leucine	87.50	85.00	92.50	92.50	102.50	92.00	9.20
Isoleucine	31.76	32.19	30.78	31.25	32.83	31.96	3.20
Valine	67.50	75.00	72.50	67.50	70.00	70.50	7.05
Methionine	11.25	13.75	11.88	13.13	12.50	12.50	1.25
Threonine	49.25	50.75	50.75	46.25	49.25	49.25	4.93
Tryptophane	12.80	12.60	13.00	13.00	12.80	12.84	1.28
Phenylalanine	41.25	42.50	45.00	41.25	42.50	42.50	4.25
Tyrosine	24.00	29.00	23.00	28.00	26.00	26.00	2.60

and stored under toluene in a refrigerator.

Bacteriological and Assay Procedures. Stab cultures† of *Streptococcus faecalis, Lactobacillus arabinosus* 17-5, and *Lactobacillus delbrückii* LD5 were maintained on the following medium: Bacto-tryptone 5 g, Bacto-yeast extract 3 g, dextrose 1 g, agar 15 g and water to 1 liter. Cultures were stored in a refrigerator and subcultured each month. Inoculum for each of the assays was produced by transferring a small amount of growth from a stab culture to a tube containing basal medium to which had been added the amino acid which was to be determined by the particular assay. This culture was incubated at 37.5°C for 24 hours, centrifuged, washed twice with 10 ml portions of sterile water, and suspended in 90 ml of sterile water. The tubes of the assay were inoculated with one drop of this bacterial suspension using a sterile hypodermic syringe as recommended by Black and Arnold.[3]

Medium for the determination of amino acids as developed by Stokes, Gunness, Dwyer, and Caswell[4] using *Streptococcus faecalis* was employed to determine histidine, arginine, lysine, leucine, isoleucine, and tryptophane. Valine and threonine were determined with *Lactobacillus arabinosus* 17-5 according to Hier, Graham, Freides, and Klein.[5] The medium of Schweigert, McIntire, Elvehjem and Strong[6] was used for methionine and phenylalanine and *Lactobacillus arabinosus* 17-5 was the assay organism. Tyrosine was determined according to the method of Gunness, Dwyer and Stokes[7] using *Lactobacillus delbrückii* LD5.

For the various assays used in this work 5 ml of basal medium, lacking the particular amino acid being determined, was placed in lipless tubes (180 x 22 mm) arranged in a metal rack. A standard series was prepared by adding various amounts, ranging from 0 to 5 ml, of the standard amino acid solution of the *l* isomer to tubes containing 5 ml of basal medium. To another series of tubes, each containing 5 ml of the basal medium, was added in duplicate 1.0, 2.0 and 3.0 ml of diluted hydrolyzate. The volume in each of the assay tubes was adjusted to 10 ml, if necessary, by adding water. All tubes were plugged and autoclaved 13 minutes at 15 lbs pressure. After inoculation, the tubes were incubated at 37.5° for 72 hours. Titration of the acid produced was carried out with 0.05 N sodium hydroxide with bromthymol-blue as an indicator. A curve was prepared from the titration data of each standard series by plotting the ml of 0.05 N sodium hydroxide against γ of the *l* isomer per tube. The concentration of each amino acid present in the hydrolyzate was estimated from these standard curves.

Results and Discussion. The results ob-

[3] Black, T. L., and Arnold, A., *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 344.

[4] Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

[5] Hier, S. W., Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, 1945, **161**, 705.

[6] Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, 1944, **155**, 183.

[7] Gunness, M., Dwyer, I. M., and Stokes, J. L., *J. Biol. Chem.*, 1946, **163**, 159.

tained from assays on 5 samples of dried chicken erythrocytes are presented in Table I. The values represent milligrams of amino acids per gram of dried erythrocytes.

A survey of the literature indicates rather meager information on the amino acids contained in erythrocytes of various species of animals. However, analyses have been reported for several hemoglobins and stroma.[8] It is of interest to note the similarity in the results presented in Table I with those reported by Stokes, Gunness, Dwyer, and Caswell[4] for "blood meal." The chicken erythrocytes are somewhat higher in arginine and threonine and lower in histidine, lysine, leucine, and phenylalanine.

Summary. The following amino acids contained in the chicken erythrocyte have been determined by means of microbiological assays and are expressed as percentages of the dry weight: histidine 3.75, arginine 4.35, lysine 6.81, leucine 9.20, isoleucine 3.20, valine 7.05, methionine 1.25, threonine 4.93, tryptophane 1.28, phenylalanine 4.25, and tyrosine 2.60.

8 Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods*, 1945, Springfield.

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Renal Clearance of Essential Amino Acids: Threonine and Phenylalanine.

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Protein hydrolysates or amino acid mixtures are being employed widely today in clinical medicine. Present practices of administering these preparations either orally or parenterally in relatively large amounts have emphasized the need for additional information concerning the extent to which the various amino acids are reabsorbed by the kidney tubules at elevated plasma levels.

Previous publications from this laboratory in which microbiological methods of assay for the specific determination of amino acids in blood and urine were employed have been concerned with the renal clearances in dogs of the amino acids leucine, isoleucine, valine and tryptophane[1] and arginine, histidine, lysine and methionine.[2] This paper describes the renal clearances of threonine and phenylalanine, the remaining 2 amino acids that are essential dietary components for one or more mammalian species.

Methods. The physiological procedures employed in these experiments have been described in previous publications.[1,2] Creatinine clearances were used as a measure of glomerular filtration rate while clearances of para-aminohippuric acid determined at low plasma levels were employed as a measure of minimal renal plasma flow.

The experiments involving threonine and phenylalanine were designed to determine the clearances of the individual amino acids at normal postabsorptive blood levels followed by similar clearance periods at elevated plasma levels. Increased plasma levels were obtained by the use of priming doses and constant intravenous infusions of the compounds.

Microbiological determinations of the amino acids in plasma were carried out on protein-free filtrates prepared according to the method of Dunn *et al.*[3] Determinations

1 Beyer, K. H., Wright, L. D., Russo, H. F., Skeggs, H. R., and Patch, E. A., *Am. J. Physiol.*, 1946, **146**, 330.

2 Wright, L. D., Russo, H. F., Skeggs, H. R., Patch, E. A., and Beyer, K. H., *Am. J. Physiol.*, 1947, **149**, 130.