and necrosis of the adrenals. Animals which receive, concomitantly, thyroxin injections or thyroid powder become granulocytopenic and leucopenic, while the incidence of anemia and of adrenal hemorrhage and necrosis is greatly reduced. The granulocytopenia and leucopenia of these rats may be corrected by treatment with *L. casei* factor.

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Microbiological Determination of Amino Acids. IV. Lysine, Histidine, Arginine, and Valine

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Microbiological assays for amino acids have been reported in a series of papers from this laboratory.[1-3] A considerable portion of our more recent work has been anticipated by published results from other laboratories,[4-6] and we are therefore presenting only in abbreviated form additional methods for lysine, histidine, arginine, and valine and results of their application to 6 purified proteins: casein, gelatin, ovalbumin, β-lactoglobulin, horse hemoglobin, and silk fibroin.

Assay Procedures. Samples for assay were prepared by autoclaving (at 15 lb. steam pressure) 100 mg of the protein with 2 cc of 10% hydrochloric acid in a sealed tube for 24 hours. All assays were carried out as described by McMahan and Snell.[1]

Leuconostoc mesenteroides was employed for the lysine and histidine assays. The medium of McMahan and Snell[1] was modified for *L. mesenteroides* by increasing the phosphate content (Salts A) 8-fold and the arginine content 3-fold, and by altering the vitamin supplement (Solution 2) as described by Hac *et al.*[2] †

The lysine assay was incubated 63 to 65 hours at 32 to 34° C. The standard curve was constructed from response of the test organism to graded amounts of *l* (+)-lysine in the range 5-50 γ per 2.5 cc of medium. Growth response was measured turbidimetrically after dilution of the contents of each tube with 5 cc of a saturated aqueous solution of chlorothymol. Growth response may also be measured acidimetrically‡ using the micro quinhydrone-calomel-electrode technic or with the glass-electrode assembly described by McQuarrie and Jones.[7] When the assay tubes were to be titrated, 2% glucose was used instead of 1%, to permit formation of larger quantities of acid.

For the histidine assay, graded amounts of *l*(+)-histidine in the range 1-12 γ made up the standard curve. The culture tubes were incubated at 32 to 34° C for 72 hours. Shorter periods of incubation resulted in poor recoveries and drifting assay values.

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[1] McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, 1944, **152**, 83.

[2] Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1945, **159**, 273.

[3] Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, **159**, 291.

[4] Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 715.

[5] Dunn, M. S., Camien, M. N., Shankman, S., and Rockland, L. B., *J. Biol. Chem.*, 1945, **159**, 653.

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

† The folic acid requirement is based on a concentrate with a potency of 40,000.

‡ The values obtained for the lysine content (uncorrected for moisture) of a casein hydrolyzate assayed turbidimetrically and acidimetrically were 7.0 and 7.1%, respectively.

[7] McQuarrie, E. B., and Konen, H. J., *Ind. Eng. Chem.*, Anal. Ed., 1944, **16**, 205.

TABLE I
Recovery of Amino Acids from Protein Hydrolyzates

Protein Hydrolyzate	Amino Acid	Amino Acid Content Found %	Amino Acid Added %	Total Content Found %	% Recovery
Casein	lysine*	7.3	7.0	14.3	100
"	"*	7.8	7.0	14.6	97.4
"	"*	7.34	7.0	13.9	94.0
"	"†	7.13	8.0	14.63	93
β-Lactoglobulin	"†	9.87	9.83	19.71	100
Casein	histidine*	3.25	3.0	6.15	97
"	"*	3.11	3.0	6.17	101.9
Gliadin	"*	1.99	2.0	3.89	95
Horse hemoglobin	"*	7.63	7.6	15.3	99.1
" "	"*	7.72	7.6	15.4	101
Casein	arginine†	3.74	4.0	7.94	105
β-Lactoglobulin	"†	2.78	2.81	5.57	99.3
Casein	valine†	6.95	6.0	12.95	100
β-Lactoglobulin	"†	5.5	5.8	11.0	94.6
Gelatin	"†	2.4	2.4	4.85	102

Percentages are uncorrected for moisture.
* Determined with *L. mesenteroides*.
† Determined with *S. faecalis*.

TABLE II.
Lysine Content* of Several Proteins.

Sample	Lysine determined by *Streptococcus faecalis* %	Avg.	*Leuconostoc mesenteroides* %	Avg.	Other methods
Casein (Labco)	7.83, 7.39, 7.34, 7.76	7.6	7.50, 7.54, 8.0, 7.48, 7.70, 6.70	7.5	8.34 7.76
Gelatin (Knox)	5.2, 5.2	5.2	3.98, 3.75	3.87	5.86
Ovalbumin†	6.04, 6.1, 5.85	6.0	5.6	5.6	6.66
β-Lactoglobulin†	10.4, 10.0, 10.4, 10.4, 10.6	10.4	10.5	10.5	11.16
Horse hemoglobin†	8.66	8.66	8.4	8.4	
Silk fibroin†	0.90, 0.90	0.90	0.58	0.58	0.604 0.726

* Percentages are expressed on the moisture-free basis.
† Kindly supplied by the late Dr. Max Bergmann.

The medium of McMahan and Snell[1] was modified for *Streptococcus faecalis* by doubling the phosphate concentration and by altering the vitamin supplement in the manner described by Hac *et al.*[2] The medium is very similar to that used by Stokes *et al.*[6] The assay was carried out on a 2.5 cc scale, incubated 16 hours at 30° C, and growth measured turbidimetrically after dilution of each culture with 5 cc of saturated aqueous chlorothymol. Stokes *et al.*[6] use 10 cc cultures, and follow growth titrimetrically after an incubation period of 40 hours. The determination of arginine, valine, and lysine was investigated using *S. faecalis* as test organism. Growth response increased with increasing concentration over the following ranges for the various amino acids: 8-50 γ *l*(+)-arginine monohydrochloride, 10-100 γ *dl*-valine, and 20-100 γ *l*(+)-lysine monohydrochloride per 2.5 cc of medium.

Results. Recoveries of amino acids added to protein hydrolyzates were good (Table I). There were no drifts in assay values as dosage levels of samples were increased; and assay values of protein samples tested at different times were reproducible (Tables II and III).

In Table II values for the lysine content of various proteins as determined with both *S. faecalis* and *L. mesenteroides* are given. Values for the histidine, arginine, and valine content of proteins are given in Table III. Values obtained by other investigators are

TABLE III.
Histidine, Arginine, and Valine Content* of Several Proteins as Determined Microbiologically.

Sample	Histidine†		Arginine		Valine		Values cited from the literature	
	%	Avg.	%	Avg.	%	Avg.		
Casein (Labco)	3.3, 3.2, 3.2, 3.2, 3.2	3.2	3.82, 3.85, 3.53, 3.58, 3.60, 3.84	3.71	7.0, 6.5, 7.09, 7.09, 6.88	6.91	Histidine Arginine Valine	3.15; 2.86 3.96 6.76
Gelatin (Knox)	0.83, 0.83	0.83	8.73, 8.8	8.77	2.6, 2.6	2.6	Histidine Arginine Valine	0.586 9.16 2.76
Ovalbumin	2.28, 2.4, 2.35, 2.45	2.4	5.72, 5.79, 5.83, 5.50	5.7	7.07, 7.66	7.36	Histidine Arginine Valine	2.36 5.96 7.06
β-Lactoglobulin	1.72, 1.8, 1.67	1.73	2.98, 2.95, 2.70	2.88	6.07, 5.75	5.91	Histidine Arginine Valine	1.56 2.86 5.56
Horse hemoglobin	8.2, 8.16, 8.13, 8.2, 8.06	8.2	2.8	2.8	9.0, 9.8	9.4		
Silk fibroin	0.41, 0.4	0.41	0.99	0.99	3.36, 3.46	3.41	Histidine Arginine Valine	0.345; 0.416 1.116 3.56

Histidine was determined with *L. mesenteroides*, arginine and valine were determined with *S. faecalis*.

* Values are expressed on the moisture-free basis.

† Employing the organism *L. delbruckii* 3 and a slightly different medium the values obtained for the histidine content of casein and gelatin—3.2 and 0.84, respectively—are in excellent agreement with those above.

also given for comparison.

Discussion. Values for the lysine content of proteins obtained with *S. faecalis* and *L. mesenteroides* are in good agreement with each other and with values cited from the literature, except in the cases of gelatin and silk fibroin. The value obtained with *L. mesenteroides* for gelatin appears to be low, and that for silk fibroin with *S. faecalis* appears to be high when compared with the data cited. The value obtained for the lysine content of horse hemoglobin is in excellent agreement with that obtained by the isotope dilution method.[8]

The single value (2.8%) for the arginine content of horse hemoglobin probably should be disregarded since it is much lower than the values 3.4, reported by McMahan and Snell,[1] and 3.7, reported by Foster.[8] The value (9.4%) for the valine content of horse hemoglobin is somewhat high as compared with the value, 8.8%, reported by McMahan and Snell.[1] Recent values for the histidine content of horse hemoglobin are unavailable. The agreement among other values reported in Table III is excellent.

[8] Foster, G. L., *J. Biol. Chem.*, 1945, **159**, 431.

Summary. A method of assay for histidine and lysine employing *Leuconostoc mesenteroides* as test organism has been developed. This method is compared with those of Dunn *et al.* [4,5] A method of assay similar to that of Stokes *et al.*[6] for lysine, arginine, and valine using *Streptococcus faecalis* as test organism has been independently developed and studied.

With few exceptions, the data reported agree closely with those obtained by previous investigators with microbiological or isotope dilution methods.

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The Nature of Circulating Estrogen.*

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In the course of investigations on the physico-chemical nature of circulating estrogen, we have found a large and constant portion of the latter to be closely associated with the blood proteins. Coincidentally, we have observed that blood estrogens will dialyze quantitatively past a collodion membrane.

Methods. The methods used for the extraction of blood estrogens will be published in detail elsewhere. In brief, the procedure generally consisted of precipitation of the blood proteins with acetone. The supernatant fraction was combined with the acetone-ether washings of the precipitate, after which the two fractions were treated separately. In all instances, precipitation and washing of the protein was done in the cold.

After evaporation of acetone and ether from the supernatant fraction, the latter was hydrolyzed with 4% H_2SO_4. The freed estrogen was then extracted with ether, and the water-washed extract was made up in a small amount of olive oil for subsequent assay.

The previously undenatured protein precipitate was subjected to partial alkaline hydrolysis with 0.1 N NaOH. After neutralization with CO_2, this material was extracted with ether and made up in olive oil.

Other methods of estrogen extraction included alcohol-ether precipitation, and ammonium sulfate precipitation followed by prolonged hot alcohol-ether Soxhlet extraction of the protein.

Portions of the same or similar bloods, as well as pure solutions of various natural estrogens, were also subjected to dialysis against distilled water through sausage casing (Visking, 25/32″ diam.) The dialysate

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