THE AMINO ACID COMPOSITION OF CHINCHILLA FUR IN RELATION TO THE FUR-CHEWING SYNDROME

by

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I. INTRODUCTION

Background

Removing an animal from its natural habitat and growing it in captivity present many complications including the satisfaction of its nutritional requirements. Among chinchillas, domestication has led to the appearance of the "fur-chewing" syndrome. This eating of their own fur has been suggested to result in part from a nutritional imbalance.

Since commercial fur-producers depend upon the quality of their products, the fur-chewing syndrome becomes a major problem resulting in heavy economic losses. Information on the cause of this bizarre eating habit would be useful to the producer.

Many workers feel that fur chewing results from several causes, among them genetic, environmental, psychological, infectious, and nutritional factors.

A thorough review of the literature revealed a limited number of observations bearing upon the problem. The approach to the fur-chewing program used here was based upon data obtained from the Florida Agricultural Experiment Station (1) report on rabbit fur chewing. The Florida data indicated that the fur-chewing syndrome in the rabbit was corrected by the addition of either sulfate, methionine, or cystine to the ration, indicating a nutritional deficiency in sulfur compounds.

King (2), however, found that the addition of either sulfate or sulfur amino acids to the ration of chinchillas failed to correct the syndrome. Watlington, Baker, and King (3) have established the absence of qualitative differences in the amino acids of normal and chewed furs or quantitative differences in total nitrogen.

Although it was known that no qualitative differences existed between the amino acid composition of the two furs, and that animal hair was composed of amino acids, fatty acid, and carbohydrates, no evidence was available as to the quantitative amino acid composition of the two furs. It was suggested that the presence of quantitative difference between the fur types might indicate an amino acid imbalance in the ration.

Problem

This investigation was undertaken to determine whether analysis of fur from normal and chewed pelts might reveal significant differences in the amino acid composition and suggest an amino acid imbalance in the ration.

Upon observing an apparently well-fed animal consuming foreign substances, it is often assumed that some inadequacy exists in the ration. The usual approach to such a problem has been to find a protective feed, isolate the protective factor, and add it to the ration. However, in the instance of fur chewing, no known protective feedstuff is known, and another approach to the problem was necessitated.

First, it was assumed that when fur is chewed the chinchilla was attempting to obtain a nutrient in which the ration is deficient. The composition of most animal hair suggested that either an amino acid or a fatty acid could be expected as the deficient nutrient.

In this study, the amino acid composition of fur from normal and fur-chewed chinchillas was determined, and the data were compared for quantitative differences. To confirm the data and, at the same time, to evaluate different methods for determining amino acids, the fur hydrolyzates were analyzed using both microbiological assays and two chromatographic methods.

II. EXPERIMENTAL

Source and Preparation of Samples for Analysis

The chinchilla fur for this investigation was obtained from pelts representing normal and fur-chewing individuals, donated by members of the National Chinchilla Breeders of America, Inc. To obtain a pooled sample of each fur type, 10 grams of fur was plucked from the dorsal region of the pelt near the base of the neck from each of 10 pelts. Each sample was then cut into short lengths with stainless steel scissors and pooled with the other samples.

Before analysis for amino acids, the two pooled, fur samples were dried at 95° C for 48 hours to remove moisture and extracted with ethyl ether (4) for 24 hours removing the lipids. Three one-gram samples (moisture and fat-free) of each fur were subjected to acid hydrolysis using 6 N HCl (100 ml. of acid per gram of fur) for 24 hours under reflux, cooled to 25° C, adjusted to pH 4.5, and filtered through Whatman No. 1 filter paper, thus removing the humin and saving the clear filtrate. Total nitrogen content of the fur was obtained by analyzing the humin and the filtrate for nitrogen. By adding the two values, the total nitrogen was obtained.

Each fur sample was prepared in triplicate for analysis and all analyses were determined in duplicate.

Methods

In general, the analytical methods were followed as outlined in the references with only minor changes. The methods included the

microbiological assays of Barton-Wright (5) and the ion-exchange techniques for quantitative amino acid analysis of Moore and Stein (6, 7) using the modified ninhydrin reagent procedure of Moore and Stein (8). Total nitrogen determinations included two procedures, the A.O.A.C. Kjeldahl (9) and Johnson's micro-Kjeldahl (10). Lipids were removed in the Bailey-Walker soxhlet fat extraction unit. Equipment required for the various analyses can be found in Table 1 and chemicals in Table 8.

Nitrogen (Total)

In order to determine the total nitrogen content of the samples after acid hydrolysis of the fur sample, it was necessary to use two methods: The standard A.O.A.C. Kjeldahl method (9) was used to determine the nitrogen content of the humin and Johnson's (10) micro-Kjeldahl proved useful in obtaining the nitrogen content of the hydrolyzed filtrate.

Calculation of Standard Kjeldahl:

$$\frac{(\text{ml. of HCl} \times \text{N of HCl}) - (\text{ml. of NaOH} \times \text{N of NaOH}) \times 0.014 \times 100}{\text{Weight of Sample}} = \%\ \text{Nitrogen}$$

Johnson's Micro-Kjeldahl

A standard curve was established with each group of unknowns analyzed. The colorimeter readings for the unknowns were read on the standard curve giving the micrograms of nitrogen present in the sample and calculated to give the percentage of nitrogen in the original sample.

TABLE 1

MAJOR EQUIPMENT REQUIREMENTS FOR THIS INVESTIGATION

Preparation of Fur Samples for Amino Acid Determinations

Equipment	Use
Soxhlet extractors (ground glass-jointed, 200 ml.)	Extraction of fat from fur
Reflux condenser - Liebig + extraction flask (300 ml.)	Acid hydrolysis of fur
Analytical balance (chainweight)	Weighing of samples and chemicals
Standard Kjeldahl digesting and distilling apparatus	Nitrogen determination
Micro-Kjeldahl digesting unit	Nitrogen determination
Bausch and Lomb photoelectric colorimeter (filter 540)	Nitrogen determination using Nessler's reagent
Beckman pH meter	Adjustment of hydrolysate pH
Bailey-Walker soxhlet extraction unit	Fat extraction of fur

TABLE 1 (Cont.)

Microbiological Assays for Amino Acids

Equipment	Use
Autoclave	Sterilizing
Two incubators, 30° C and 37° C	Growing of cultures and incubation of samples
Cannon dispenser	For pipetting and titrating samples and standards
Wire culture tube racks	For holding 60 tubes

TABLE 1 (Cont.)

Column Chromatographic Equipment for the (1948) Moore and Stein 15-cm. Column for Basic Amino Acids

Equipment*	Use
Column, 25-cm. that could be closed with a stopcock, 0.9 cm. x 25 cm. with porous disc sealed 5 cm. from stopcock	For holding resin in separation of basic amino acids
Four separatory funnels	250 ml. for each buffer used for elusion of amino acids

* Other equipment used will be found under the (1954) Moore and Stein amino acid 100-cm. column.

TABLE 1 (Cont.)

Column Chromatographic Equipment for the (1954) Moore and Stein 100-cm. Amino Acid Column

Equipment	Use
Jacketed column, 0.9 cm. x 150 cm. with porous disc sealed 5 cm. from restricted bottom of column	For holding resin with which to separate amino acids
Water bath (10-gallon tank), two heating elements (immersion), automatic thermostat	For control of column temperature ranging from 30.0 ± 0.5° C to 52.0 ± 0.5° C
Circulating water pump	For circulation of water through jacketed column to control temperature
Automatic fraction collector	For collection of amino acids eluted from column at a constant volume per tube
Automatic hand pipette (two)	For measuring large numbers of small volumes accurately, 1.0 and 4.5 ml.
Bausch and Lomb photoelectric colorimeter (filters 440 mμ and 570 mμ wavelengths	For reading amino acid samples and standards
Test tube racks with covers holding 50 tubes (1.5 cm. x 18 cm. Pyrex)	Tubes for collecting samples in fraction collector and analysis of amino acids. Racks hold tubes in boiling water bath and covered for cleaning
Boiling water bath (dishpan)	Boiling ninhydrin tubes
Dowex-50 cation exchange resin	Column packing

Results

As can be seen from Table 2, there was close agreement (9.7% higher N for chewed-fur) between the two fur types in regard to total nitrogen. A smaller variation (1.2% to 2.9% for three treatments and 1% for the fourth) existed between replicates of single treatment, indicating uniformity of sampling, and reliability of the methods. Although there was no appreciable difference between the total nitrogen of the two fur types, there existed a 21% difference in the humin nitrogen of the two fur types. The chewed-fur humin contained 21% more nitrogen than the normal-fur humin.

Microbiological Assay

The microbiological assay was based upon the use of a basal medium containing an ample supply of all the nutrients required for the growth of the organism used in the assay, except the component (amino acid) to be determined. An outline of special equipment will be found in Table 1, page 11.

Organisms:

Of the many organisms available for amino acid assays, only three were selected, *Lactobacillus arabinosus* 17/5, *Leuconostoc mesenteroides* P 60, and *Streptococcus faecalis*. The stock cultures were acquired from the American Type Culture Collection*. The organisms and amino acids determined by them are described in Table 3.

---

* 2029 M Street, N. W., Washington 6, D. C.

TABLE 2

NITROGEN CONTENT OF FUR SAMPLES

Type Fur	Sample No.*	Per Cent N in Humin**	Per Cent N in Fur Hydrolysate (Filtrate)***	Per Cent N Average Total
Chewed	1	6.02	12.80	
	2	6.00	11.48	
	3	6.16	12.29	
	Average	6.06	12.19	18.25
Normal	1	4.90	13.13	
	2	4.76	12.90	
	3	4.76	12.87	
	Average	4.80	12.97	17.77

* One-gram sample used.
** Determined by the A.O.A.C. standard Kjeldahl method.
*** Determined by Johnson's micro-Kjeldahl method.

TABLE 3

ORGANISMS USED TO ASSAY AMINO ACIDS

Organisms	Amino Acids Assayed
Lactobacillus arabinosus 17/5	Leucine Isoleucine Valine **Glutamic Acid *Lysine
Leuconostoc mesenteroides P 60	*Lysine Phenylalanine **Glutamic Acid Glycine Proline Histidine Serine Aspartic Methionine Cystine
Streptococcus faecalis	Arginine ***Threonine

Alanine was not run as another organism was required. Tyrosine and tryptophan were run on an alkaline hydrolysate.

* The use of *Lactobacillus arabinosus* 17/5 and *Leuconostoc mesenteroides* P 60 for lysine gave the same type of results for the two fur types.
** *Lactobacillus arabinosus* 17/5 proved to be the most effective organism for glutamic acid.
*** Same medium for threonine as was used for lysine.

Modifications of Microbiological Assay and Precautions:

The modifications from the Barton-Wright outline include the following: the maintenance of the organisms, several techniques which were found useful in obtaining reproducible results, reduction of the over-all assay time, and reduction in assay volumes.

Although Barton-Wright (5) recommend one medium for maintaining all three microorganisms, only Streptococcus faecalis remained in a vigorous state of maximum growth. Hence, it was necessary to acquire different media for the other two organisms. A search of the literature revealed media suitable for growth of vigorous microorganisms shown in Table 4.

All cultures were incubated at their optimum growth temperature for 24 hours and then refrigerated until the next transfer. They were transferred every fourth day.

It soon became apparent that the use of a 10-ml. sample for each tube would be both costly and time consuming. After many preliminary tests, the final volume of the assay sample was reduced from 10 ml. to 2 ml. as used by Henderson and Snell (11). The samples or standards were added in volumes from 0.2 to 1.0 ml., made up to 1 ml. with triple distilled water, and made to the 2 ml. with 1 ml. of the double-strength basal medium.

For each determination made, three sets of tubes at five levels (0.2, 0.4, 0.6, 0.8, and 1 ml.) were used, making a total of 15 tubes for each analyses.

TABLE 4

STOCK MEDIA AND TEMPERATURE REQUIREMENTS FOR MAINTENANCE OF ORGANISMS

Microorganism:	*Streptococcus faecalis* (lactis Rogers)
Growth temperature:	34° C
Storage temperature:	10° C
Grown:	As slants cultures
Medium:	Liver - Tryptone Agar
	Distilled water - - - - - - - - - - 500 ml.
	Glucose - - - - - - - - - - - - - 1.0 gm.
	Difco tryptone - - - - - - - - - - 1.0 gm.
	K_2HPO_4 - - - - - - - - - - - - - 0.2 gm.
	$CaCO_3$ - - - - - - - - - - - - - - 0.3 gm.
	Liver extract - - - - - - - - - - -10.0 gm.
	Inorganic salt solution A* - - - - 0.5 ml.
	Inorganic salt solution B** - - - 0.5 ml.
	Agar - - - - - - - - - - - - - - - 1.0 gm.
Microorganism:	*Lactobacillus arabinosus* 17/5
Growth temperature:	37° C
Storage temperature:	10° C
Grown:	As stab cultures
Medium:	Agar Medium
	Difco bacto yeast extract - - - - 5.0 gm.
	Anhydrous glucose - - - - - - - - - 1.0 gm.
	Anhydrous sodium acetate - - - - - 1.0 gm.
	Agar - - - - - - - - - - - - - - - - 3.0 gm.
	Distilled water - - - - - - - - - -200 ml.
Microorganism:	*Leuconostoc mesenteroides* P 60 #8042
Growth temperature:	30° C
Storage temperature:	10° C
Grown:	As stab cultures
Medium:	Tomato Juice Agar Stabs
	Distilled water - - - - - - - - - - 800 ml.
	Tryptone - - - - - - - - - - - - - 10 gm.
	Yeast extract - - - - - - - - - - - 10 gm.
	Agar - - - - - - - - - - - - - - - 13 gm.
	Tomato juice (pH 7.0) - - - - - - 200 ml.

* Contents: 25 grams of K_2HPO_4, plus 25 grams of KH_2PO_4 dissolved in water and made up to a 250-ml. volume.

** Contents: 10 grams $MgSO_4 \cdot 7H_2O$; 0.5 grams $MnSO_4 \cdot 4H_2O$, and 0.1 grams of $FeCl_3$ (anhydrous), dissolved in 250 ml. of water, plus 5 drops of conc. HCl acid (to prevent any precipitation).

Preparation of all samples for microbiological assays that required an increase in the pH of a solution were changed using KOH in place of NaOH.

For convenience and to enable one person to handle as many samples as possible, the test tubes were placed in a wire rack holding 60 tubes and were filled with a Cannon dispenser (12). The samples, water, and medium were all pipetted in a very short period using the Cannon dispenser. In place of using the conventional screw caps, a wire frame, padded with cotton, was adapted to cover all 60 culture tubes in the rack.

After incubation for 72 hours at 37° C, the lactic acid produced was titrated directly in the assay tube with 0.02 N NaOH. The end-point was determined by titrating the samples to a pH of 7.0, electrometrically using the technique described by Cannon (12). Using quinhydrone to conduct the electric current through the sample, the NaOH was added and measured until the galvanometer reached the deflection obtained using a standard pH buffer.

Standard Curves:

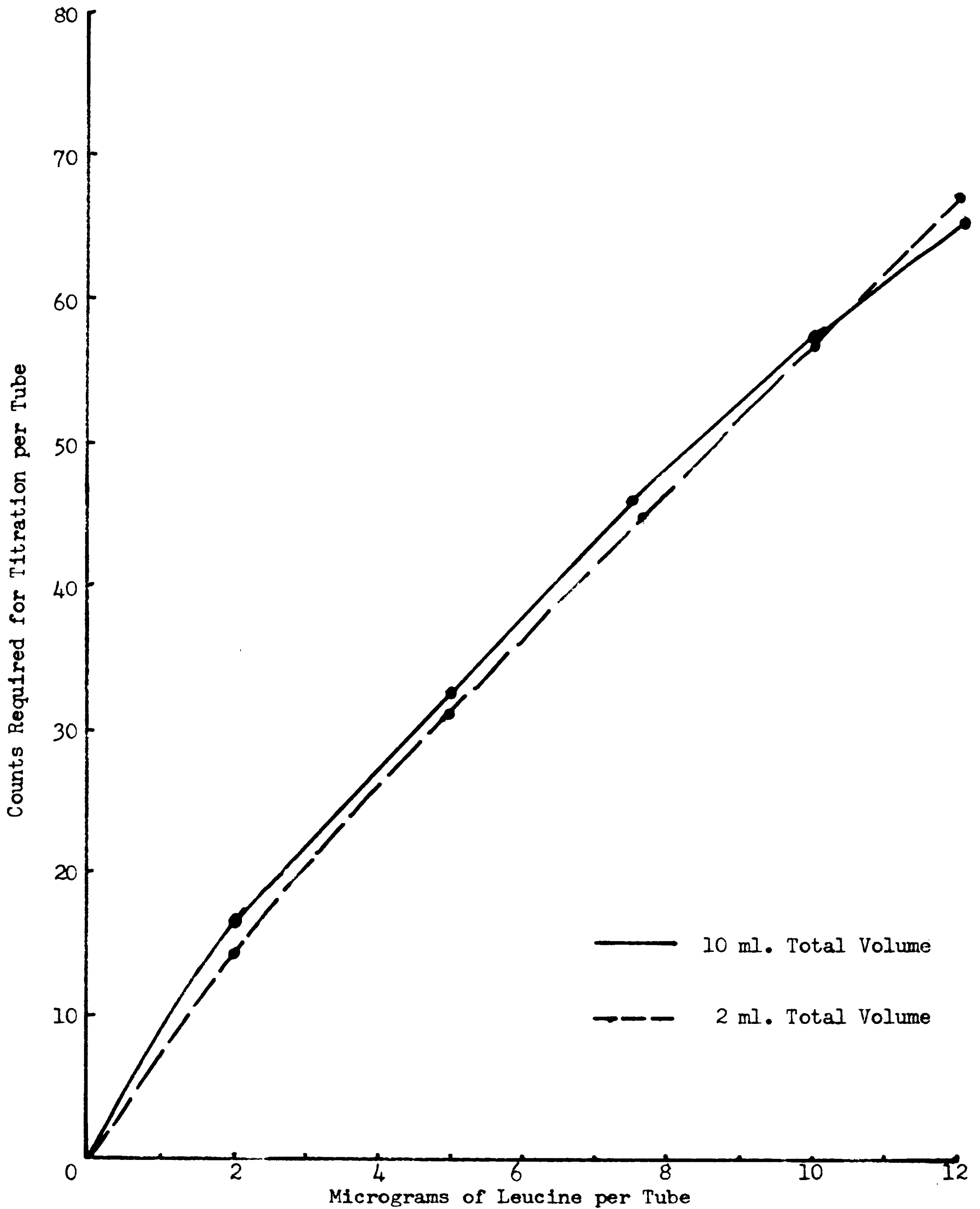
In the course of perfecting each amino acid assay, it became apparent that at the higher recommended levels of standard, the acid production began to level off. To overcome the resulting lack of accuracy at high amino acid levels, a standard assay was run to determine the concentration of standard where the curve began to flatten. Then a new standard was run covering only the range of linear response to amino acid levels.

Results:

Preliminary studies using total volumes of 10 ml. and 2 ml. for the microbiological assays produced similar standard curves (Figure 1). Testing the applicability of the smaller volume and the assay method required determination of a series of parallel assays for two unknown amino acids prepared by the major professor using the two volumes indicated above. The data shown in Table 5 indicated excellent recoveries, thereby indicating the reliability of the assay method using the 2 ml. total volume.

The data here presented in Table 6 (expressed as per cent nitrogen) indicate that chewed fur contained more amino acid nitrogen than the normal fur. The data show that the amino acids (phenylalanine, 1.7% N, chewed, 2.5% N, normal; lysine, 3.5% N, chewed, 6.2% N, normal; and arginine, 2.5% N, chewed, 5.4% N, normal) were noticeably lower in the chewed fur than in the normal fur, while the other amino acids, with the exception of leucine (9.9% N, chewed, 6.7% N, normal), were only slightly higher percentagewise in nitrogen. The data for each amino acid represent the average of three to four determinations.

As a means of testing the behavior of the microbiological assays, an acid-hydrolyzed casein (prepared in our laboratory) was assayed with each unknown as a check on the method for each of 15 amino acids shown in Table 7. With the exception of aspartic acid, glycine, phenylalanine, and arginine, there was general agreement between amino



Leucine Assay: Comparison of Standard Curves Using 10-ml. and 2-ml. Volumes

Figure 1

TABLE 5

LEUCINE ASSAY: COMPARISON OF RECOVERIES USING 10-ML. AND 2-ML. ASSAY VOLUMES

No. for Unknown Sample	Volume Added ml.	Leucine in Unknown γ/ml.	10-ml. Volume		Volume Added ml.	2-ml. Volume	
			Recovery γ	Recovery/ml. γ		Recovery γ	Recovery/ml. γ
1	1	13	13.0	13.0	0.5	6.6	13.2
	3		37.0	12.3	0.7	9.1	12.9
	4		52.0	13.0	1.0	13.0	13.0
2	0.5	37	18.5	37.0	0.3	11.3	37.6
	1.0		37.0	37.0	0.6	21.8	36.2
	1.5		55.5	37.0	1.0	37.0	37.0
3	0.3	58	17.4	57.9	0.3	17.4	57.9
	0.5		29.0	58.0	0.5	29.0	58.0
	1.0		58.0	58.0	1.0	58.0	58.0

TABLE 6

AMINO ACID COMPOSITION OF NORMAL AND CHEWED FUR MICROBIOLOGICAL ASSAY
(FIGURES ARE AVERAGE OF 3 TO 6 ASSAYS)*

Amino Acid	Chewed Fur	Normal Fur
Aspartic acid	9.2	10.3
Threonine	6.0	5.7
Serine	9.4	8.3
Proline	5.4	5.5
Glutamic acid	10.9	9.3
Glycine	5.0	4.1
Alanine	- -	- -
Valine	3.8	2.7
Cystine	11.0	10.5
Isoleucine	3.4	3.2
Leucine	9.9	6.7
Tyrosine	- -	- -
Phenylalanine	1.7	2.5
Lysine	3.5	6.2
Histidine	3.5	2.8
Arginine	2.5	5.4
Per Cent of Total Nitrogen Accounted For	85.2	83.2

* Amino acid nitrogen as per cent of total nitrogen.

TABLE 7

COMPARISON OF AMINO ACIDS FOUND IN HYDROLYZED CASEIN BETWEEN THESE ASSAYS AND THOSE OF DUNN (13) (FIGURES ARE AVERAGE OF 3 TO 6 ASSAYS)

Amino Acids	% Amino Acids	% Amino Acids as Reported by Dunn (unpub.) (13)
Aspartic acid	2.4	6.4
Threonine	4.6	3.6
Serine	6.6	7.5
Proline	10.4	12.0
Glutamic acid	24.2	23.0
Glycine	4.2	2.1
Valine	6.7	7.0
Cystine	0.8	0.8
Isoleucine	6.1	6.2
Leucine	9.3	9.7
Phenylalanine	2.0	6.0
Lysine	7.6	8.3
Histidine	2.2	3.0
Arginine	6.0	3.8

acid results of Dunn's (13) and our laboratory. The differences may be due to the source of casein used in the two laboratories, thus one would expect some differences.

Since the data obtained could have demonstrated an imperfection in the microbiological assay for the four amino acids (phenylalanine, lysine, arginine, and leucine), new samples were prepared from the fur pelts, determined microbiologically, and analyzed using the column chromatography technique.

Calculation:

To obtain a clear understanding of the calculations involved, the data collected from the assay of valine, in triplicate, will be illustrated.

First, the micrograms of nitrogen per ml. of fur hydrolysate was established (Table 2), 784.5 γ of N/ml., diluted 5 to 200 (19.61 γ of N/ml.) for the assay. Then the per cent nitrogen for the amino acid being concerned was calculated as given below:

$$\frac{\text{(Atomic wt. of nitrogen) (Atoms of N/molecule)}}{\text{Molecular wt. of amino acid (valine)}} \times 100 = \%\text{ N in amino acid (valine)}$$

$$\frac{(14)(1)}{117.1} \times 100 = 11.9\%\text{ N}$$

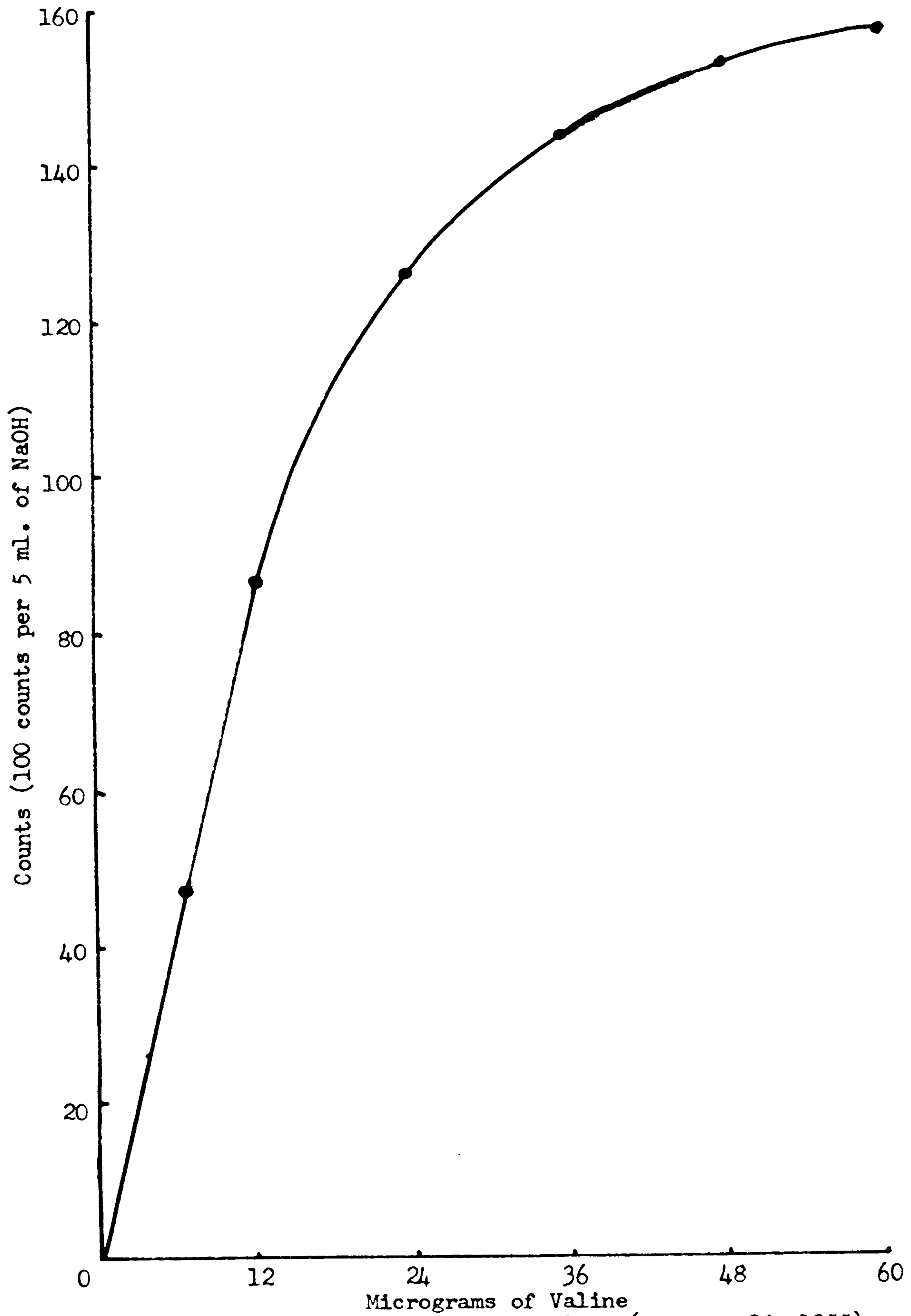
The unknown sample, a standard of the amino acid under consideration, and casein were assayed in triplicate at 5 concentrations. The standard was run to establish a standard curve for calculating the amount of

amino acid present in the sample (Figure 1), and as a further means of verifying the assays, casein was determined with each run as a check on the stability of the assays.

Next, the standard valine curve (Figure 2) was produced from which the micrograms of valine were obtained and treated in the following manner, thus obtaining the per cent valine nitrogen in the fur sample.

The micrograms of valine (from curve) times the per cent N in valine, times 100 equals the micrograms of valine nitrogen in the unknown. It was then necessary to establish the relationship of valine N in the unknown to the total nitrogen in the sample.

$$\frac{\gamma \text{ of valine N in unknown}}{\gamma \text{ of total amino acid N in unknown}} \times 100 = \% \text{ of valine in total N in fur sample}$$



Typical Valine Curve Using 2-ml. Volume (January 28, 1955)
Figure 2

Ion-Exchange Technique

The development of sulfonated polystyrene resins, such as Dowex-50 x 4 and others having different cross-linkages (x 3, x 5, x 8, etc.) has made it possible to obtain quantitative separation of the amino acids from a wider variety of substrates (for example, fluids containing as high a salt content as blood) than was possible with the starch column. Also, duplication of results has been improved. As demonstrated in Tables 1 and 8, quite an impressive list of equipment and chemicals were required for the operation of the ion-exchange column for amino acids. When one considers the description of the Moore and Stein techniques, the equipment, and the chemicals, it must be kept in mind that once the procedures have been established, any deviation from the method used can be changed in only such a way as to equal the original conditions.

Selection of Amino Acid Column:

The improved version of the Moore and Stein (6) column technique was used successfully (Table 13), except with respect to the basic amino acids. Although every precaution was used in analyzing the basic amino acids on the 100-cm. column using a mixture of Dowex-50 x 4 and x 5, it soon became apparent that another method would have to be adopted.

Procedure Including Establishing Modifications and Precautions for the 100-cm. (Long) Column:

The long column consisted of a tube 0.9 cm. x 170 cm., sealed at the bottom with a porous alundum plate, enclosed in a water jacket

TABLE 8

CHEMICALS AND SOLUTIONS REQUIRED FOR MICROBIOLOGICAL ASSAYS AND COLUMN CHROMATOGRAPHY OF THE AMINO ACIDS

Reagent Requirements	Use and Source (if special)
Ethyl ether, C.P.	Extraction of lipids from fur
Hydrochloric acid, C.P.	Hydrolyses of fur
Sulfuric acid, C.P. Copper sulfate, C.P. Sodium sulfate, C.P. Sodium hydroxide, 40% Sodium hydroxide, 0.1 N Hydrochloric acid, 0.1 N Methyl red	Kjeldahl nitrogen
Nessler's reagent Sodium hydroxide, 3.3 N Selenious acid Ammonium sulfate, C.P.	Micro-Kjeldahl nitrogen
Amino acids, L and DL forms Adenine Guanine Uracil Xanthine Thiamine Calcium d-pantothenate Pyridoxine Pyridoxal Nicotinic acid Riboflavin p-Aminobenzoic acid Biotin Folic acid	For microbiological assay (Obtained in purest form possible) (Whenever possible, these reagents were obtained from Mann Research Laboratories, Inc.)
Potassium phosphate, dibasic (K_2HPO_4) Potassium phosphate, monobasic (KH_2PO_4) Manganese sulfate ($4H_2O$) Magnesium sulfate ($7H_2O$) Ferric chloride	Used for making salt solutions

TABLE 8 (Cont.)

Reagent Requirements	Use and Source (if special)
Glucose (anhydrous), C.P. Sodium acetate (hydrated), C.P. Sodium chloride, C.P.	For microbiological assay
Resin: Dowex-50 x 4 (90%) Dowex-50 x 5 (10%) (200 to 400 mesh)	Used to separate the amino acids on the column (Dow Chemical Company, Midland, Michigan)
Citric acid, monohydrate Sodium acetate, trihydrate Acetic acid, glacial Sodium phosphate, monobasic (H_2O) Sodium phosphate, dibasic Disodium versenate (Bersworth Chemical Co., Framingham, Mass.) "BRIJ-35" (a detergent) (Atlas Powder Co., Wilmington, Del.)	Required for preparation of buffers
Ninhydrin, C.P. Ascorbic acid 4N Sodium acetate buffer (pH 5.5) (sodium acetate trihydrate) (acetic acid) Methyl cellosolve (monomethyl ether of ethylene glycol) Methyl alcohol	For making hydrindantin

patterned after a West condenser. The jacketed column permitted temperature control by circulating water through the jacket from a constant temperature bath with a circulating pump.

Resin Requirements and Preparation of Column:

Satisfactory resins were obtained using the minus-400 mesh resin containing 50 per cent minus-325 resin, when screened as the sodium salt. The resin in the sodium form was driven through a 200-mesh sieve using a strong jet of tap water, resulting in a 50 per cent yield. It is interesting to note that, at this point, only 25 per cent of the original resin remained. Particle size was further selected by shaking the remaining resin several times with water and decanting after an hour, removing the colloidal resin.

Once the resin had been sized, it could be used over again and again as long as it had been cleaned and regenerated. Cleaning and regeneration of the resin consisted of shaking the resin with a generous quantity of 0.4 N HCl (6 to 8 times the volume of the resin), decanting after one hour, and repeating using 2 N NaOH in place of the 0.4 N HCl. This cycle was repeated twice, followed with a distilled water rinse, and decantation after one-half hour. The resin was then equilibrated with the 2 N, pH 5.5 buffer (Table 9), diluted 1 to 10, adjusted as the slurry to a pH 3.1 with 4 N HCl, decanted, washed with distilled water, and decanted.

A suspension consisting of one part resin to two parts 0.2 N buffer, pH 3, without detergent (Table 9) was prepared and poured

TABLE 9

BUFFERS REQUIRED FOR 100-CENTIMETER ION-EXCHANGE COLUMN

Buffer	Citric acid·H_2O gm.	Acetic acid gm.	NaOH (97 per cent) gm.	Sodium acetate-3 H_2O gm.	HCl (conc.) ml.	Volume (Final) liters
pH 2.2 ± 0.03*, 0.2 N Na citrate	105		42		80	5
pH 3.1 ± 0.03*, 0.2 N Na citrate	105		41.5		57.8	5***
pH 5.1 ± 0.02**, 2.0 N Na citrate-acetate	525	107.35	235.5	680.9		5****

* Before using, add 5 ml. of BRIJ-35 solution per liter.

** Before using, add 1 ml. of BRIJ-35 solution per liter.

*** For adjustment of pH, 5 ml. per liter of conc. HCl corresponds to about 0.03 pH units.

**** "Caution": Dissolve the citric acid in 2 liters of water and add the NaOH in small portions to avoid overheating. In adjustment of the pH, 70 ml. of glacial acetic acid or 40 gm. of NaOH corresponds to about 0.03 pH unit.

into a large glass funnel connected to the top of the column. After the resin had settled (about 2 hours) the glass funnel was removed, and a porous 1/16-inch alundum disc was floated onto the surface of the resin. Then a 300-ml. separatory funnel containing 200 ml. of 0.2 N NaOH (plus BRIJ-35) (Table 10) was mounted on the column allowing at least 100 ml. of the buffer to run through the column (overnight). Having cleaned the column, it was ready to be regenerated with the first buffer. The 0.2 N NaOH (plus BRIJ-35) solution was replaced by 0.2 N buffer, pH 3.1, containing plus BRIJ-35 (Table 10). About 10 hours was required to replace the 0.2 N NaOH.

Operation of Long Column (100 cm.):

The following precautions concerning the operation of the column, including time periods and temperatures, proved helpful in reproducing the results, and insured ample time for completion of results in a timely manner. It should also be noted that the only force required for movement of the sample and buffers through the column was gravity.

Having prepared the column, the hydrolyzed fur sample was placed upon the column, as given in the following details:

Upon removal of the buffer above the alundum disc with a pipette, 0.5 to 3.0 ml. of sample containing 2.5×10^{-3} to 50×10^{-3} micromoles of total amino acids was pipetted onto the column. Because the sample must have a lower pH than the buffer used to elute the first amino acid off the column, the samples were adjusted to pH 2.2. After

TABLE 10

BUFFERS REQUIRED FOR 15-CENTIMETER ION-EXCHANGE COLUMN

Buffer	Composition*
5.0 ± 0.1	500 ml. pH 5 sodium citrate**
	500 ml. H_2O
	0.1 gm. disodium versenate***
	1.5 ml. benzyl alcohol per 100 ml.****
6.8 ± 0.03	500 ml. 0.1 M Na_2HPO_4
	450 ml. 0.1 M $Na_2H_2PO_4 \cdot H_2O$
	0.1 gm. disodium versenate
	1.5 ml. benzyl alcohol per 100 ml.
	(0.05 ml. of 0.5 N HCl required to bring 1 ml. of buffer to pH 5)
6.5 ± 0.05	42 gm. of citric acid monohydrate
	580 ml. 1.0 N NaOH to volume of 1 liter
	0.1 gm. disodium versenate
	1.5 ml. benzyl alcohol per 100 ml.

* Add to all buffers, 1 ml. of BRIJ-35 solution per 100 ml.

** Buffer employed for the ninhydrin method (8).

*** Analytical grade.

**** Used to improve the resolution of tyrosine and phenylalanine.

pipetting the sample onto the column, the column was allowed to run almost to dryness followed with several small portions of buffer to wash the sample into the resin. The column was then filled with the same buffer, pH 3.1, and mounted over the fraction collector, and the reservoir (a serum bottle, which rested on a magnetic stirrer) containing the buffer was connected to the column. Then the water jacket about the resin column was connected to the pump in the constant temperature bath and all tubing connections were wired. Next, the fraction collector and water pump were started. The fraction collector was adjusted to collect 1.5 ml. per fraction. About 8 minutes were required to fill each tube. Circulation of the water from the constant temperature bath enabled the operator to maintain the proper temperatures throughout the run.

The determination was started at 30° ± 0.5° C (82° F) and increased to 50° C (122° F) after serine emerged from the column (about 110 tubes). Other changes taking place in the eluting solvents were the pH and normality of the buffers. The 0.2 N buffer at pH 3.1 passed through the column until alanine emergence (about 155 tubes) when a gradual increase in the ionic strength and pH of the eluting buffer was initiated. As described by Moore and Stein (6), after refilling the reservoir with the 0.2 N buffer, pH 3.1, a second reservoir, consisting of a 500-ml. separatory funnel, containing 2.0 N buffer (pH 5.1), was placed tightly into the top of the original reservoir. A capillary tube attached below the stopcock of the separatory funnel minimized

the flow of buffer and thus the rate of pH change as the magnetic stirrer mixed the two buffers.

The analysis ended when arginine emerged from the column, usually requiring about 500 fractions over a 72-hour period for the run. Since more than one buffer and temperature were required, removal of the resin for cleaning and regeneration was necessary.

Short Column (15 cm.) and Operations:

In this investigation, all attempts to obtain duplication of the basic amino acid data with the 100-cm. Moore and Stein column (6) ended in failure, requiring another approach. The Moore and Stein short (15 cm.) chromatographic column (7) was selected, using the same resin employed in the 100-cm. column (6). The 15-cm. column not only required the use of a different type column, but the operational requirements were different, including temperature, sample pH, buffers, and means of applying the buffers to the column.

The column consisted of a Pyrex tube (0.9 cm. x 25 cm.) having a porous disc sealed near the beveled delivery tip. The resin was prepared and poured as for the 100-cm. column; however, it was not essential that the resin be removed after each run because there was no change in the temperature (25° C) or radical change in the ionic strength of the buffers.

The resin was regenerated after each run by running approximately 200 ml. of 0.2 N NaOH for cleaning, followed with 100 ml. of buffer containing BRIJ-35. All samples placed on the column were adjusted to pH 5.0 in this investigation.

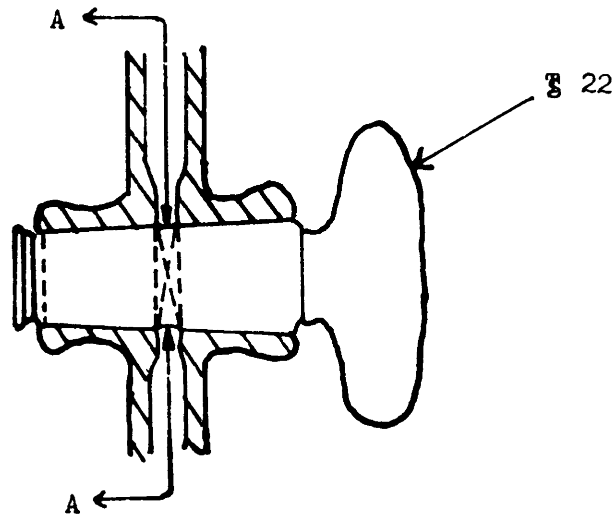
For convenience, the 3 buffers (Table 10) used for eluting the samples from the column were placed in separate separatory funnels to speed the change of buffers. One-ml. samples were collected by the fraction collector for amino acid analysis.

Since two of the buffers used were at an undesirable pH for the maximum ninhydrin color reaction (pH 5.0), it was necessary to adjust those fractions to pH 5.0 by adding 0.05 ml. of 0.5 N HCl per ml. of buffer at pH 6.8, and 0.05 ml. of 2.0 N HCl per ml. of buffer at pH 6.5.

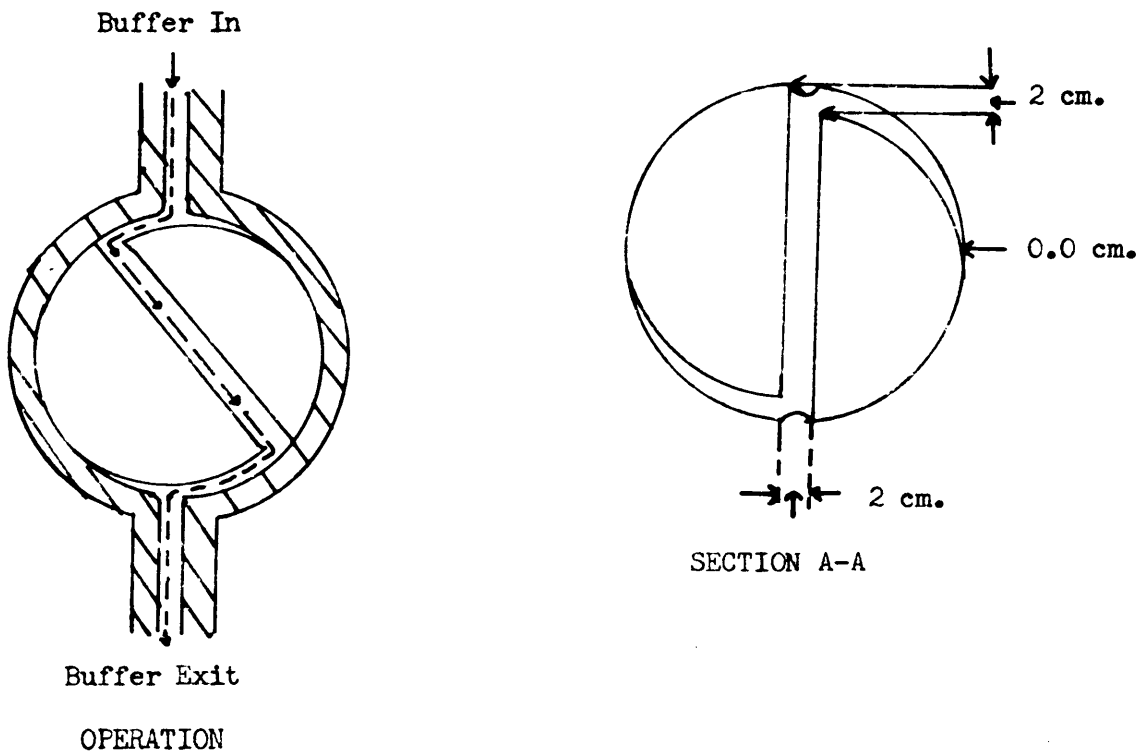
The retention volume of this column was 6 ml.

The shortness of the 15-cm. column presented another problem not found in the 100-cm. column. The buffer flow through the 15-cm. column was decreased to a minimum of 1 ml. per 8 minutes to obtain sharp separation of the amino acids.

This was accomplished by two means: adjusting the length of the separatory funnel to reduce or increase the pressure head on the column, and by filing slits halfway around the stopcock from the opening (as diagram, Figure 3), making it possible to reduce the flow of buffer from 50 ml. per hour to 4 ml. per hour. After pipetting a 2-ml. sample (pH 4.0, containing about 6 mg. of amino acids) onto the column, the amino acids were eluted from the column using 3 buffers (Table 10). The acid amino acids were eluted from the column in the first 25 1-ml. fractions with the first buffer, pH 5.0, forming two peaks. The first peak contained all of the amino acids with the exception of tyrosine and phenylalanine, which came off on the second peak.



SIDE VIEW



OPERATION

SECTION A-A

By filing the groove in the stopcock, as shown in Section A-A, it was possible to control the flow of buffer from the 15-cm. column, as shown left.

Adjustment of Stopcock

Figure 3

The next amino acids eluted from the column included tryptophan, histidine, and lysine, followed by ammonia, which were eluted with approximately 75 fractions using the phosphate buffer pH 6.8, leaving only arginine to be eluted with the citrate buffer at pH 6.5.

Analysis of Fractions Eluted from Chromatography Columns:

The analysis of fractions from both columns was similar, except with regard to the size of sample and the adjustment of the pH of all fractions collected from the 15-cm. column with the exception of those fractions eluted with the first buffer.

A 1.5-ml. fraction was obtained from the 100-cm. column and 1.0-ml. fraction from the 15-cm. column.

The analysis of the fractions was carried out in the following manner:

Whenever possible, as soon as 50 tubes were filled, they were placed in a wire rack and adjusted to the proper pH if necessary, which was followed by the addition of 1.0 ml. of ninhydrin solution to each tube. The rack (holding the 50 tubes) was shaken (about 10 seconds), heated for exactly 15 minutes in a boiling water bath, cooled in a cold water bath, and diluted to 7.0 ml. with one to one methanol-water mixture. After each tube was shaken, the optical density was determined on a "Spectronic 20" colorimeter, using two wavelengths (440 mμ for proline and 570 mμ for the other amino acids).

TABLE 11

STANDARD COLOR YIELDS OF AMINO ACIDS (AVERAGE OF 3 DETERMINATIONS)*

Amino Acid	Micromoles per O. D. Unit	
	This report	Gill (14)
Aspartic acid	0.159	0.159
Threonine	0.189	0.173
Serine	0.150	0.164
Proline	0.912	0.865
Glutamic acid	0.209	0.169
Glycine	0.121	0.175
Alanine	0.143	0.164
Valine	0.192	0.188
Cystine	0.384	0.378
Isoleucine	0.208	0.190
Leucine	0.239	0.190
Tyrosine	0.303	0.129
Phenylalanine	0.282	0.155
Lysine	0.235	0.165
Histidine	0.278	0.188
Arginine	0.323	0.139
NH_3	0.055	- - -

* Micromoles of amino acid per optical density unit (100-cm. column).

TABLE 12

STANDARD COLOR YIELDS OF AMINO ACIDS (AVERAGE OF 3 DETERMINATIONS)*

Amino Acid	Micromoles per O. D. Unit
Histidine	0.436
Lysine	0.386
Arginine	0.467
Ammonia	0.055

* Micromoles of amino acid per optical density unit (15-cm. column).

Upon reacting with ninhydrin, all of the amino acids gave a blue color, with the exception of proline which gave a yellowish-brown color. All reagents were added to the tubes with a hand-operated "continuous pipette" (Table 1, p. 13). As a means of checking the factors (micromoles of amino acid per O. D. unit) obtained from the standard solution, Gill's factors are used in Table 11 for comparison. The only differences between the runs were the buffers and the addition of Dowex-50 x 5 to resin used by Gill (14).

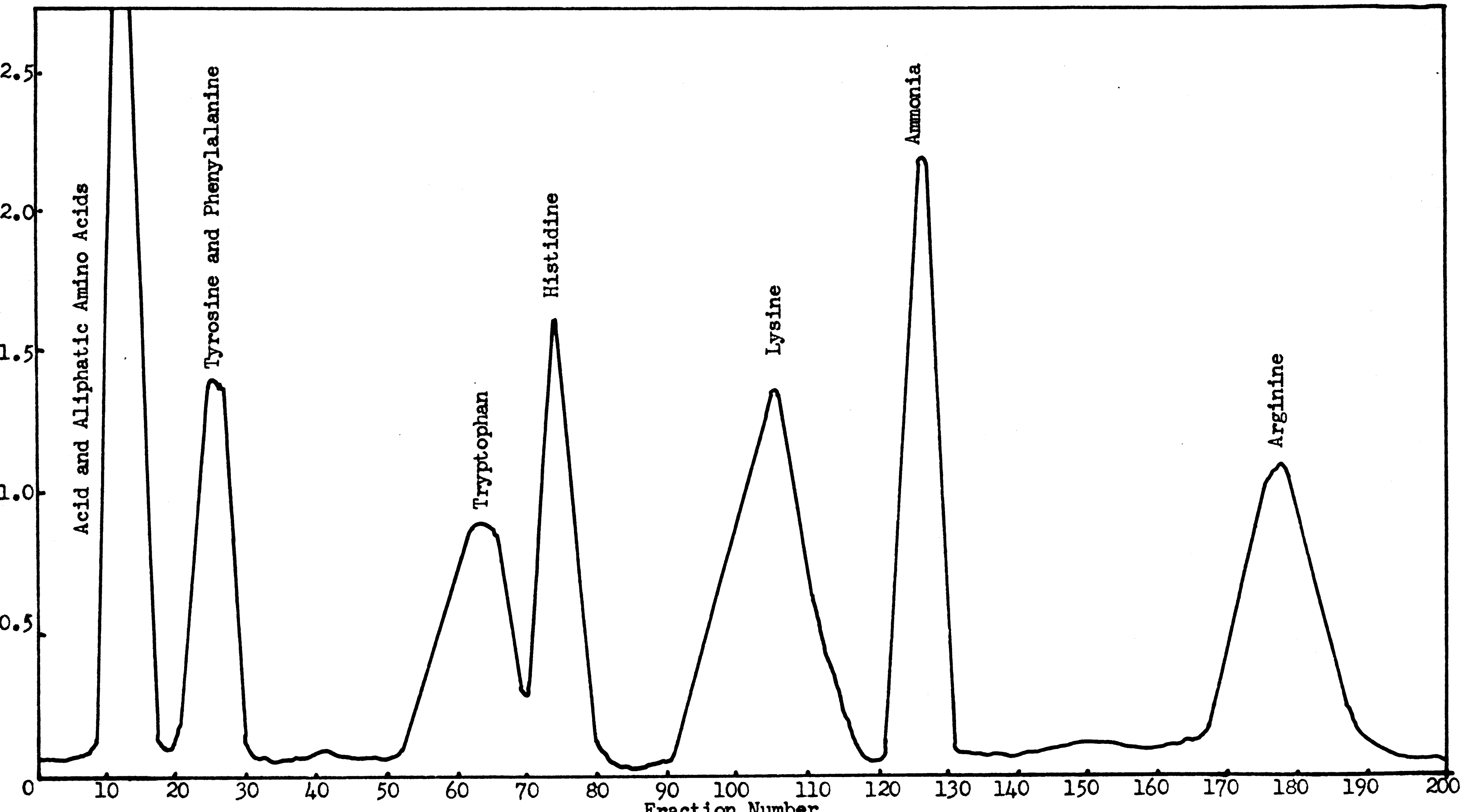
Calculation:

Calculation of the column chromatography data required the development of standard fractions (Figures 4 and 5) to represent each amino acid as micromoles of amino acid per optical density unit (shown in Tables 11 and 12). Development of the standard factors involved running a known amino acid solution through the column, totaling the optical densities for each peak, subtracting the blank O. D. readings, and dividing the figure (O. D.'s) obtained into the micromoles of amino acid in the standard sample analyzed on the chromatography column illustrated below:

$$\frac{\text{Micrograms of amino acid in standard sample}}{\text{Total O. D. reading - Total O. D. blank reading}} = \text{Micrograms of amino acid per O. D. unit}$$

The unknown calculations included totaling of O. D. reading (Figure 6), subtraction of the blank O. D.'s (number of tubes in peak x the O. D. readings in the immediate area of the peak), which was multiplied by the amino acid factors, giving the micrograms of amino acid present.

Typical Elution of Known Amino Acid Mixture (100-cm. Column)



Typical Elution of Known Amino Acid Mixture (15-cm. column for basic amino acids)
Figure 5

1.5
1.0
0.5
0

Aspartic acid
Threonine
Serine
440 mμ
Proline
Glutamic acid
Glycine
Alanine
Valine
Cystine
Isoleucine
Leucine

70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270

Fraction Number

1.0
0.5
0

Tyrosine
Phenylalanine
Ammonia
Lysine
Histidine
Arginine

270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470

Typical Elution of Fur Hydrolysate (100-cm. Column)

Total O. D. Reading - Total O. D. Blank Reading = Total O. D. Reading for Peak x Factor (From Standard) = Micrograms of Amino Acid Present

Then, multiply the micrograms of amino acid obtained above by the per cent nitrogen of the amino acid being considered, giving the micrograms of amino acid nitrogen which in turn is divided by the total nitrogen in the sample x 100, thus converting the results to per cent nitrogen.

Mgm. amino acid x per cent N in amino acid = mgm. amino acid N

$$100 \times \frac{\text{Mgm. amino acid nitrogen}}{\text{Total nitrogen}} = \text{Per cent N as amino acid}$$

Results:

The data obtained from the Stein and Moore chromatographic techniques (Table 13) gave a more complete insight into the amino acid content of the chinchilla fur than the data from the microbiological assays which did not include all of the amino acids.

The total nitrogen accounted for in the chewed fur was 93.09% and in the normal fur 90.40%.

The only differences in the amino acid content of the two furs (with reference to the 100-cm. column) were found in the basic amino acids, as indicated in Table 13. Two amino acids (lysine, 2.48% N, chewed; 6.73% N, normal; and arginine, 2.55% N, chewed; 3.11% N, normal) were lower in the chewed fur than in the normal fur; however, one amino acid (histidine, 2.69% N, chewed; 1.94% N, normal) was higher in the chewed fur than in the normal fur.

TABLE 13

AMINO ACID COMPOSITION OF NORMAL AND CHEWED FUR: CHROMATOGRAPHIC ANALYSIS (FIGURES FOR ALL COLUMN ANALYSES ARE AVERAGE OF 3 ANALYSES)*

Amino Acid	Chewed Fur		Normal Fur	
	100-cm. column	15-cm. column	100-cm. column	15-cm. column
Aspartic acid	3.22		3.13	
Threonine	4.14		4.08	
Serine	7.52		7.10	
Proline	5.17		5.67	
Glutamic acid	8.99		8.58	
Glycine	5.49		5.25	
Alanine	3.26		3.27	
Valine	3.23		2.73	
Cystine	10.71		10.50	
Isoleucine	2.24		1.91	
Leucine	6.14		5.55	
Tyrosine	2.08		2.25	
Phenylalanine	1.63		1.71	
Lysine	2.48	3.70	6.73	4.50
Histidine	2.69	3.40	1.94	2.60
Arginine	2.55	1.90	3.11	2.00
Ammonia	22.09	20.27	21.57	19.57
Per Cent of Total Nitrogen Accounted For	93.63	93.09	95.08	90.40

* Amino acid nitrogen as per cent of total nitrogen.

Since the basic amino acid peaks obtained in the 100-cm. column were rather flat, the 15-cm. column, designed for the determination of basic amino acids, was employed.

The data obtained from the 15-cm. column show similar results to those from the 100-cm. column, showing the lysine (3.70% N, chewed; 4.50% N, normal) and arginine (1.90% N, chewed; 2.00% N, normal) content lower in the chewed fur, while histidine (3.40% N, chewed; 2.60% N, normal) was higher in the chewed fur.

Since the basic amino acids appeared to vary between the two fur types (chewed and normal), a number of analyses were determined using the 15-cm. column, indicating that lysine varied more than the other basic amino acids. The analyses were tested statistically using the "t" test (15). The data from three determinations for each fur type were collected and analyzed statistically with 5 degrees of freedom. The per cent lysine nitrogen in the chewed fur analyses was 3.72%, 3.55%, and 3.90%, while the per cent lysine nitrogen in the normal fur was 4.85%, 4.50%, and 4.27%, respectively.

The chewed fur proved to be significantly lower in the lysine at the 2.5% level. Other differences in amino acid composition were not evaluated statistically.

III. DISCUSSION

The observation from the Florida Experiment Station (1) involving the fur-chewing syndrome of the rabbit, which was associated with a deficiency of sulfate or sulfur amino acids, was ruled out as a means of correcting the chinchilla syndrome by King's (2) investigation.

The data presented in Table 14 summarize the primary effort of the investigation: whether the two fur types were composed of different amounts of amino acids.

The results indicated that the chewed fur contained only about half as much lysine as the normal fur; arginine was only slightly lower in the chewed fur; however, histidine was higher in the chewed fur than in the normal fur. Average differences in the amounts of valine, leucine, isoleucine, and tyrosine (Table 13) did not appear to be significant.

Not only were the data for the basic amino acids substantiated by all three methods used for determining the amino acids, but the lysine level was shown to be significantly lower in the chewed fur by the "t" test at the 2.5% level. In general, the microbiological data indicated a higher nitrogen level for each amino acid than was obtained from the column fractions. If the same number of amino acids had been determined with the microbiological assays as by column fractions, the total per cent of nitrogen probably would have been well over 100 per cent. Also, there was a respectable degree of consistency within fur types with reference to method changes. For example, in the chewed fur, serine was 7.52% nitrogen by column analysis and 9.4% nitrogen by microbiological assay, while the normal fur serine content was 7.10% nitrogen by column

TABLE 14

SUMMARY OF AMINO ACID COMPOSITION OF NORMAL AND CHEWED CHINCHILLA FUR*

Amino Acid	Chromatography Column				Microbiological Assay	
	Chewed Fur		Normal Fur		Chewed Fur	Normal Fur
	100-cm. column	15-cm. column	100-cm. column	15-cm. column		
Aspartic acid	3.22		3.13		9.2	10.3
Threonine	4.14		4.08		6.0	5.7
Serine	7.52		7.10		9.4	8.3
Proline	5.17		5.67		5.4	5.5
Glutamic acid	8.99		8.58		10.9	9.3
Glycine	5.49		5.25		5.0	4.1
Alanine	3.26		3.27		- -	- -
Valine	3.23		2.73		3.8	2.7
Cystine	10.71		10.50		11.0	10.5
Isoleucine	2.24		1.91		3.4	3.2
Leucine	6.14		5.55		7.9	6.7
Tyrosine	2.08		2.25		- -	- -
Phenylalanine	1.63		1.71		1.7	2.5
Lysine	2.48	3.70	6.73	4.50	3.5	6.2
Histidine	2.69	3.40	1.94	2.60	3.5	2.8
Arginine	2.55	1.90	3.11	2.00	2.5	5.4
Ammonia	22.09	20.27	21.57	19.57	- -	- -
Per Cent of Total Nitrogen Accounted For	93.63	93.09	95.08	90.40	83.2	83.2

* Amino acid nitrogen as per cent of total nitrogen.

analysis and 8.30% nitrogen by microbiological assay, indicating the results between the methods were only relatively comparable.

For some unknown reason, the microbiological assays for aspartic acid were threefold greater than those obtained from the column technique.

As the investigation proceeded, a number of problems arose concerning the methods.

The organisms used for the microbiological assays became sluggish, failing to respond rapidly, even when transferred to a new medium, until new stock media were used for maintaining the stock culture.

Another modification involved the use of a minimum amount of sample (2 ml.) resulting in an appreciable reduction in the cost of the experimental operation. Also, the 2-ml. sample reduced the time required for preparation of the sample by one-fifth, using the Cannon dispenser. The difficulty encountered titrating colored solutions was overcome, using the Cannon dispenser as an electrometric titrator, in the manner described by Henderson and Snell (11).

Although the basic outline of the investigation included the use of the chromatographic technique, the difficulty of reproducing the results within 20% plus or minus, microbiologically, made it imperative that another method be adopted to verify the lysine, as well as the other amino acid data. For example, the data from three determinations of lysine (5.1% N, 7.3% N, and 6.4% N) and proline (6.6% N, 7.6% N, and 7.9% N) indicate the wide variation between microbiological assay runs.

As the investigation proceeded, a number of problems were encountered concerning the chromatographic techniques. To obtain a complete separation, as well as sharper peaks, of tyrosine and phenylalanine, Dowex-50 x 5 was added at the rate of 1 to 10. The significance of producing buffers meeting the rigid requirements of the established methods was discovered in the 100-cm. column procedure when valine or cystine seemed to disappear. From Moore and Stein (6), it was learned that a pH change in the first decimal place below pH 3.1 may elute cystine before valine and that cystine will emerge from the column after valine with a pH increase of one-tenth from 3.1. Adjustment of the buffer of pH 3.1 indicated that cystine had merely overlapped the valine peak, hence neither had disappeared.

Although the original procedure required the use of pressure for the 100-cm. column and vacuum for operation of the 15-cm. column to control the flow rate of the eluting buffers, neither were used. It was not necessary to adjust the flow of the 100-cm. column, but the 15-cm. column flow rate was controlled by the use of a filed stopcock. It was also noted that incomplete breaking of the resin used in the 100-cm. column resulted in an abnormally fast flow of the column. Adjustment of the sample pH below that of the first eluting buffer was essential to hold the sample intact near the surface of the resin until the entire sample was washed into the resin, thereby obtaining sharper peaks. A pH meter (Beckman) was used in place of the "Hydrion" paper advocated by Moore and Stein (7), improving the duplication of runs with both column methods.

In general, the lower the pH and molarity of the buffer, the slower the amino acids were eluted and the higher the pH and molarity, the faster they were eluted from the column. Also, by increasing the molarity, slower flow of buffers was obtained without an increase in pH. Other means of increasing flow rates without broadening peaks include the use of a detergent (BRIJ-35) and an increase in temperature in the 100-cm. column after the emergence of serine.

Since several runs were required to obtain basic amino acid data with the microbiological assays and the 100-cm. column, further evidence was obtained using the 15-cm. column to confirm the basic amino acid data. The results obtained from the 15-cm. column not only completed the basic amino acid data but also confirmed the lysine difference in the two furs which was suggested in the other two methods (Table 14).

Another interesting phenomenon was the per cent of nitrogen found in the humin (Table 1) obtained from the hydrolysis of the fur samples. The humin from the chewed fur samples contained a consistently higher percentage of the total nitrogen than the humin of normal fur hydrolyzate. Whether this is a reflection of differences in tryptophan levels is not certain.

IV. SUMMARY

Amino acids were determined in two types of chinchilla fur (chewed and normal), using three methods for analysis. The methods included microbiological assays of Barton-Wright (5) and two column chromatography techniques of Moore and Stein (7, 8).

The data obtained from this investigation indicate that of the 16 amino acids analyzed, only one (lysine) was found to be significantly lower in the chewed fur, as indicated by the "t" test. This difference between the normal and chewed fur was significant at the 2.5% level. Also, arginine was lower and histidine was higher in the chewed fur than in the normal fur, but these differences were not as conspicuous.

In general, the microbiological analyses were more time consuming and less reproducible than the chromatographic analysis. With the exception of proline, glycine, and leucine, higher values were obtained for the amino acids using the microbiological methods than with the column fractions. The greatest difficulty was encountered in analyzing the basic amino acids.

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