inverse relationship appears to exist between the per cent recovery of quinic acid and the amount of this acid in the extract—for example, the total per cent recovery from Sweet Italian prunes is low. By fractionating solutions containing various amounts of quinic acid alone, and quinic, malic, and other acids together, it was found that, with quinic acid at a concentration approximating that in Sweet Italian prunes, 80% or less of the quinic acid is recovered. This low rate of recovery is likewise evident in French prunes, where the quinic acid content is slightly higher than in Italian prunes.

The predominant acid of Italian and French prunes is malic acid. Many studies of plums have shown this to be so. However, in the Sweet Italian prune there is almost as much quinic as malic acid. Quantities of phosphoric and other acids are about the same in all three varieties. Citric acid in small amounts has been found in plums (*3, 17*), but Dickinson and Gawler (*4*) found none in the Victoria plum. Some workers (*8, 13, 14*) have detected tartaric acid, but others (*4, 15*) failed to do so. Reports of the presence of oxalic acid (*3, 8*), glyoxalic acid (*2*), malonic acid (*8*), and salicylic acid (*19*) are to be found in the literature, but these acids were not detected in studies on the Victoria plum (*4, 15*). Differences in variety, stage of maturity, and methods of analysis must be borne in mind when these reports are considered.

No definite conclusions regarding the internal browning of prunes can be drawn from this study, but the differences in levels of quinic acid between the Sweet Italian and French varieties is striking. Internal browning has not been reported in Sweet Italian prunes. Both the Italian (*20*) and French varieties (*7*) are susceptible to internal browning; however, the disorder was not observed in any of our samples. Interest in chlorogenic acid has centered on its function as a substrate for polyphenolases; the darkening of fruits on injury may be due to the oxidation of chlorogenic acid. It is not known whether the internal browning of prunes can be explained in this way, but in a study now under way of Italian prune fruits at progressive stages of development, quinic, malic, phosphoric, and traces of citric and benzoic acids, but no chlorogenic acid, have been detected.

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AMINO ACID DETERMINATION

Microdetermination of Lysine in Protein Hydrolyzates

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A simple colorimetric procedure for the direct microdetermination of lysine in protein hydrolyzates is based primarily on quantitative conversion of the amino acids into their copper salts, followed by treatment of the latter with 1-fluoro-2,4-dinitrobenzene. Under these conditions, lysine will be the only amino acid which yields a colored dinitrophenyl derivative, and it can be estimated by measuring the absorbance of the copper-free and ether-extracted mixture. The accuracy and specificity of the method have been demonstrated. The procedure has been applied to micro quantities of proteins, and the results are comparable with those previously reported. The present method is direct and economical in time and materials, and does not require special equipment.

THE early chemical methods for determination of lysine in protein hydrolyzates are indirect, tedious, and nonspecific. These difficulties are partly overcome by the microbiological and enzymic assays, which, however, are time-consuming. The defects inherent in the older methods are avoided by the use of ion exchange chromatography. The new procedures are far from simple, and at each step require careful attention to specific conditions.

A satisfactory chemical procedure which may be applied specifically for the estimation of lysine in protein hydrolyzates has not been developed previously. The determination of a specific amino acid such as lysine is of value in nutrition and metabolic studies. The present investigation has been undertaken to develop a simple chemical micromethod

offering a direct and rapid means for estimating the lysine content of proteins.

The new technique is based on three essential factors.

First, in a mixture of copper complexes of amino acids, the α-amino and α-carboxyl groups are bound in a stable chelate structure to copper, whereas the ε-amino group of lysine remains free and reactive. On treatment of the copper salts with 1-fluoro-2,4-dinitrobenzene, only the ε-amino group of lysine, the phenolic group of tyrosine, the sulfhydryl group of cysteine, and possibly the imidazole group of histidine combine with the reagent. Second, after removal of the copper, the ε-DNP-lysine will be the only colored substituted amino acid derivative present in the mixture. Third, extraction of the acidified and copper-free solution with ether removes the excess reagent and the colored artifacts—dinitrophenol and dinitroaniline—leaving the completely ether-insoluble ε-DNP-lysine in the aqueous layer. The yellow color of the latter is contributed solely by the lysine derivative, and its absorbance can be measured spectrophotometrically at 390 mμ.

The applicability of the procedure has been demonstrated by analyzing five proteins, both lysine-rich and lysine-poor. The method is sufficiently sensitive to permit the analysis of 1 to 2 mg. of proteins and determination of as little as 0.1 μmole of lysine.

Table I. Lysine Content of Proteins

(Grams/16 grams N)

Protein	Present Procedure: Analytical values			Av.	Other Methods	References
Egg albumin	6.50	6.49	6.55	6.5	6.5	(1)
Serum albumin (bovine)	8.68	8.70	8.74	8.7	8.6	(3)
Hair (human)	3.20	3.10	3.12	3.1	2.8	(5)
Edestin	2.83	2.80	2.80	2.8	2.9	(4)
Gluten (wheat)	1.80	1.85	1.82	1.8	1.7	(6)

Methods and Materials

All reagents were of the highest purity obtainable commercially. The nitrogen content of proteins was determined by the standard micro-Kjeldahl procedure.

ε-2,4-Dinitrophenyl-L-lysine hydrochloride was prepared from L-lysine monohydrochloride according to the method of Porter and Sanger (7). The product melted at 187° C. (uncorr.) [literature 186° C. (8)], and analysis showed that it was pure. Absorbance of a 23.2μ*M* solution of the ε-DNP-lysine in 1*N* HCl was 0.234 at 390 mμ, using a Unicam spectrophotometer, Model S.P. 500. This gives a molar extinction coefficient which agrees with that reported by Sanger (9).

Copper phosphate suspension in borate buffer (pH 9) was prepared as described by Spies and Chambers (11), but the precipitated copper phosphate was washed with the borate buffer three times instead of twice and the addition of NaCl to the final suspension was omitted. Fresh suspension was prepared every week.

Borate Buffer (pH 9). Sodium borate ($Na_2B_4O_7.10H_2O$, 76.4 grams) was dissolved in 4 liters of distilled water and filtered.

Egg albumin, E. Merck, Darmstadt. Nitrogen, 15.01% of moisture- and ash-free protein; moisture, 12.01%; ash, 4.3%.

Blood albumin (bovine), British Drug Houses. Nitrogen, 15.15% of moisture- and ash - free protein; moisture, 10.95%; ash, 8.8%.

Edestin, British Drug Houses. Nitrogen, 14.68% of moisture- and ash-free protein; moisture, 11.2%; ash, 6.1%.

Wheat gluten, British Drug Houses. Nitrogen, 15.4% of moisture- and ash-free protein; moisture, 13.35%; ash, 5.84%.

Human hair (washed and air-dried). Nitrogen, 15.8% of moisture- and ash-free protein; moisture, 4.2%; ash, 1.83%.

Procedure

Duplicate 1- to 2-mg. samples of the protein were weighed into a 2-inch length of glass tubing (6 mm. in inside diameter) sealed at one end. One-tenth milliliter of 6*N* HCl was pipetted in, and the tubes were sealed off and incubated in an oven at 110° C. for 24 hours. The seal was opened, and the acid was removed by evaporating once to dryness on the steam bath. The dried residues were dissolved in 0.6 ml. of the borate buffer.

Preparation of Amino Acid–Copper Complexes. One milliliter of the copper phosphate suspension was pipetted into the buffered protein hydrolyzate, and the mixture was well mixed by a glass rod. The contents of the tube were transferred to a 12-ml. capped centrifuge tube and shaken mechanically for 5 minutes, followed by centrifugation for 5 minutes.

Dinitrophenylation of Lysine-Copper Complex. A 0.5- to 1-ml. aliquot of the clear centrifugate (equivalent to 0.1 to 0.5 μmole of lysine) was transferred to a stoppered borosilicate glass test tube, and the volume was adjusted to 1 ml. by addition of the requisite amount of the borate buffer. To this was added 2.5 mg. of 1-fluoro-2,4-dinitrobenzene (FDNB) in 0.02 ml. of methanol, and the mixture was shaken for 1 hour at 40° C. The tubes were immersed in a water bath maintained at 40° C. and shaken mechanically. This process and, whenever possible, subsequent operations were carried out in darkness by covering the equipment with a black cloth. Subdued light was used in steps requiring visual manipulations.

The mixture was acidified by the addition of 2 ml. of 2*N* HCl, and H_2S was then bubbled through for 2 minutes. The mixture was transferred quantitatively to a 12-ml. centrifuge tube, and the copper sulfide precipitate was centrifuged off and washed twice with 1*N* HCl. H_2S was driven from the combined centrifugate and washings by gentle aeration for 1 minute. The solution was taken to a 10-ml. volumetric flask and diluted to volume with 1*N* HCl. The mixture was freed from the excess FDNB and from the artifacts, dinitrophenol and dinitroaniline, by treatment with peroxide-free ether, by transferring the mixture to a dry 60-ml. separatory funnel and extracting it with 10 ml. of ether. The extraction was repeated twice with 5-ml. portions of ether. The aqueous layer was then freed from the dissolved ether by gentle bubbling of air for 3 minutes.

Estimation of ε-DNP-Lysine. The absorbance of the final yellow solution was read at 390 mμ in a Unicam spectrophotometer (Model SP 500) against a 1*N* HCl blank. The concentration of lysine was calculated from the observed absorbance by matching the readings against those of standard 1*N* HCl solutions of the pure crystalline ε-DNP-lysine.

Results and Discussion

The validity of the method was determined by analyzing triplicate microsamples of the hydrolyzates of various proteins. The lysine values of these proteins, as determined by the present work, are given in Table I, together with those recently obtained by other methods. The table shows that the results secured by the present method are reproducible and comparable with those previously reported.

Table II. Recovery of Lysine Added to Aliquots of Protein Hydrolyzates Containing 0.1 to 0.5 μmole of Lysine

Protein	Lysine, μmole: Added	Recovered	Recovery, %
Egg albumin	0.150	0.149	99
	0.400	0.402	100
Serum albumin	0.100	0.101	101
	0.200	0.198	99
Hair	0.300	0.297	99
	0.450	0.448	100
Edestin	0.250	0.251	100
	0.450	0.453	101
Gluten	0.200	0.201	101
	0.500	0.497	99

Table III. Specificity of Method for Determination of Lysine

Amino Acid, 0.5 μmole	Absorbance at 390 Mμ
Lysine	0.500
Alanine	0.000
Arginine	0.005
Cysteine	0.002
Glycine	0.000
Histidine	0.000
Methionine	0.000
Proline	0.000
Serine	0.000
Threonine	0.000
Tryptophan	0.000
Tyrosine	0.004
Valine	0.000

The precision of the method was demonstrated by simultaneously running recovery experiments with the unknowns. Known amounts of lysine, in two concentration ranges, were added to each hydrolyzate preparation before its conversion into the amino acid–copper complexes. Analyses were then made for the recovery of the added lysine. The recovery of this amino acid was high and, as indicated in Table II, averaged 99 to 101%.

The optimal conditions for the quantitative and rapid conversion of the amino acids in a mixture into their copper salts were thoroughly investigated. Preliminary work indicated that these can be achieved by following the procedure of Spies (*11*). Spier and Pascher (*10*) stressed the necessity of the presence of K ions in the copper phosphate reagent to obtain complete reaction. However, under the conditions of the present work, no differences in results were detected when 3 grams of KCl were dissolved in 100 ml. of the prepared copper phosphate suspension. The effect of varying the quantities of copper phosphate suspension and the amino acids per test was examined. Theoretical values may be obtained by treating aliquots of the amino acid mixture, containing from 5 to 40 μmoles per ml., with 1 to 2 volumes of the copper phosphate suspension. The volume of the reaction mixture was found to be without effect on the results. In most analyses, reaction volumes from 1 to 2 ml. were employed.

The optimal conditions required for quantitative dinitrophenylation of the lysine-copper complex were determined. Standard solutions of the latter were subjected to phenylation under different conditions of FDNB concentrations, solvents, buffers, pH, reaction volume, temperature, and time. The observations showed that rapid and quantitative coupling reaction between the lysine copper and FDNB can be achieved by working in aqueous medium at 40° C. in the presence of a slight excess of the reagent and using a borate buffer of pH 9. Better results were secured by employing a solution of FDNB in methanol than by using the reagent as it is or dissolved in ethanol. The presence of a relatively large excess of the reagent in the reaction mixture resulted in decreased values. The use of 2.5 mg. of FDNB in 0.02 ml. of methanol, in a reaction volume of 1 ml., yielded satisfactory results. Doubling this quantity had no essential effect on the results, whereas somewhat lower values were obtained when it was halved.

The pH as well as the nature of the buffer employed seemed to affect greatly the condensation of the lysine copper with FDNB. When the pH of the reaction mixture was less than 9, decreased values for ε-DNP-lysine were obtained. Also, the presence of $NaHCO_3$ in the mixture interfered significantly with the dinitrophenylation process. The time needed for the quantitative reaction does not exceed 1 hour. Incomplete phenylation resulted when the reaction was performed at temperatures below 40° C., while raising the temperature to 50° C. did not change the results. Three ether extractions of the ε-DNP-lysine solutions removed all the colored artifacts mentioned above. The ether layer in the second extraction was colorless. A series of solutions of ε-DNP-lysine in 1.N HCl of increasing concentrations was prepared from standard pure lysine monohydrochloride solutions in a manner identical to that described above, and their absorbances were measured. Theoretical values revealed that both the conversion of the amino acid to its copper complex and its dinitrophenylation were quantitative. The standard curve obtained showed that Beer's law is obeyed in solutions of concentrations ranging from 5 to 100μ*M*.

To determine the specificity of the present method for estimating lysine in protein hydrolyzates, the possibility of interference from the other amine acids, particularly those which possess free reactive groups in their copper complexes, was studied. Accordingly, lysine, histidine, cysteine, tyrosine, arginine, tryptophan, serine, threonine, methionine, proline, valine, alanine, and glycine were quantitatively converted into their copper salts and then subjected to dinitrophenylation as described above. With the exception of lysine, all the amino acids examined yielded solutions which do not absorb to any noteworthy degree at wave length 390 mμ (Table III). The presence of δ-hydroxylysine in the amino acid mixture would interfere with the quantitative assay for lysine. Nevertheless, this does not present difficulty, since the very limited distribution of hydroxylysine in proteins greatly minimizes the incidence of this interference.

The effect of the presence of the other amino acids on the quantitative dinitrophenylation of lysine was investigated. Thus, two different mixtures (Table IV) were prepared, each from known quantities of lysine monohydrochloride, and four other amino acids. Aliquots were taken from them and converted into the copper salts as described previously. One milliliter from each amino acid–copper complex mixture was subjected to phenylation as before. As demonstrated in Table IV the results of these analyses are no different from those obtained by analyzing pure lysine solutions under the same conditions.

The work of Sanger (*9*) and other investigators shows that the colorless *o*-DNP-tyrosine does not absorb at any wave length longer than 390 mμ. This was also confirmed in the present study. For this reason, the absorbances of ε-DNP-lysine solutions were read at 390 mμ, although the maximum absorption of this derivative is at 350 mμ, to avoid possible interference from *o*-DNP-tyrosine. The present method is economical in time, as only about 2 hours are required for analyzing two samples of protein hydrolyzates and about 3 hours for six samples. The new procedure is free from the troublesome factors and

Table IV. Effect of Presence of Other Amino Acids on Lysine Determination

Material Analyzed	Amino Acid Composition of Known Mixtures, μmole/Ml. of Copper Complex Solution									Total Amino Acid, μmole/Ml.	Lysine Found, μmole/Ml.
	Lysine	Histidine	Tyrosine	Cysteine	Arginine	Alanine	Valine	Glycine	Proline		
Mixture 1	0.300	0.200	0.100	...	...	0.300	...	...	0.100	1.000	0.300
Mixture 2	0.500	...	...	0.200	0.100	...	0.200	0.200	...	1.200	0.503
Pure lysine solution	0.300	...	...	...	...	...	...	...	...	0.300	0.300
	0.500	...	...	...	...	...	...	...	...	0.500	0.499

defects inherent in the dinitrophenylation technique employed for the determination of lysine in intact proteins. These drawbacks have been discussed in detail by Greenstein and Winitz (*2*).

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GENETIC VARIATION

Varietal Differences in Physicochemical Properties of Rice Starch and Its Fractions

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Granular starches were isolated from milled rice of waxy and nonwaxy varieties which differed in gelatinization temperature. The starches were fractionated into their amylose (Fraction A) and amylopectin (Fraction B) components. The resultant components were characterized and their properties compared. These physicochemical properties generally differed among the varieties and were not correlated with gelatinization temperature.

Rice varieties of different cooking and eating qualities differ in starch composition (amylose and amylopectin content) and starch gelatinization temperature (*13, 15*). Some high-amylose varieties from Thailand have low gelatinization temperatures (*14*). The United States variety Century Patna 231 has a high gelatinization temperature but a low amylose content (*11, 13*). Since these two properties are not linearly correlated (*13, 14*), differences in cooking properties of rice samples of similar amylose and protein contents must result from differences in physicochemical properties of the starch granule other than composition. Stansel and coworkers (*32*) found waxy rice starches of different gelatinization temperatures to differ in molecular weights.

Gelatinization is a complex phenomenon which may depend on granule size, amylose content, molecular weight of its components, and the micellar organization of the granule (*9, 31*). This study was undertaken to obtain data on the physicochemical properties of rice starch and starch fractions from varieties with different gelatinization temperatures.

Materials and Methods

Rough rice samples of the varieties Malagkit Sungsong Puti, Kung-Shan Wu-Hsiang-Keng, Early Prolific, Century Patna 231, Taichung 65, Taichung (Native) 1, and Peta were grown at the institute in 1963. The sample of the Bengawan variety was obtained from Indonesia, and those of Niaw Sanpatong, Khao Dawk Mali 105, Nahng Mon S-4, and Leuang Yai 34, from Thailand. Milled rice samples of the waxy × nonwaxy hybrids, Waxy Century Patna 231 and Crowley 2585-6-1, were obtained from Beaumont, Tex. All samples used were nonwaxy, except Malagkit Sungsong Puti, Niaw Sanpatong, Waxy Century Patna 231, and Crowley 2585-6-1.

Rough rice samples (1 kg.) were dehulled with a McGill sheller and milled and polished with a McGill miller No. 3 (*35*). Contaminant translucent kernels were sorted by hand from Malagkit Sungsong Puti milled rice. The milled rice was soaked in water, disintegrated in a Waring Blendor at medium speed for 5 minutes, and passed through a 180-mesh sieve. Rice starch was prepared from this homogenate by extracting contaminant protein by shaking it five times with a 3% aqueous solution of Santomerse No. 1 (sodium dodecyl benzene sulfonate, 40% active ingredient) which had 0.12% sodium sulfite (*12*). The purified starch was washed free of detergent by being suspended in distilled water, collected in a Sharples supercentrifuge at 20,000 r.p.m., and washed thoroughly with distilled water. The washed starch was air-dried at 35° C., ground to a fine powder with a mortar and pestle, and defatted for 24 hours with refluxing 95% ethanol in a Soxhlet extractor (*39*). The average recovery was 85% of total starch. Protein content was calculated from Kjeldahl nitrogen (*2*) determinations using the factor 5.95. Each starch gave 99% of glucose on hydrolysis with Taka-diastase and hydrochloric acid (*2*) and after correcting for protein content.

The purified starches were fractionated by the method of Wilson and coworkers (*39*) as employed by Tsai *et al.* (*34*). However, Pentasol 27 (mixed amyl alcohols, from the Pennsalt Chemicals Corp.) was used instead of isoamyl alcohol. Amylopectin (Fraction B) was isolated from the mother liquor. Amylose (Fraction A) was recrystallized once in boiling water saturated with 1-butanol following the procedure of Tsai and coworkers (*34*) and dried in a vacuum desiccator over phosphorus pentoxide. Amylose recoveries as a percentage of total amylose were: 43% for Taichung (Native) 1; 54 to 64% for Peta, Nahng Mon S-4, and Leuang Yai; and 75 to 84% for the rest. Amylopectin was precipitated by pouring the mother liquor from amylose crystallization into 5 volumes of 95% ethanol. After 2 days at room temperature, the precipitate was finely divided with a spatula, washed thoroughly with methanol, and dried in a vacuum desiccator over phosphorus pentoxide. Recoveries as a percentage of total amylopectin were 60 to 78% for the nonwaxy samples and 95% for the waxy samples.

The moisture content of starch and starch fractions was determined from the loss in weight of 50-mg. samples at 56° C. over phosphorus pentoxide for 18 hours in vacuo in a vacuum desiccator or an Abderhalden tube.

Phosphorus content was determined by wet oxidation with hydrogen peroxide,