A Comparison of Chemical Methods for the Determination of Available Lysine in Various Proteins

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The available lysine in bovine serum albumin, glandless cottonseed meal, solvent extracted cottonseed meal, gossypol complexed bovine serum albumin, and glanded cottonseed meal and glandless cottonseed meal has been determined by the dinitrofluorobenzene (DNFB), sodium borohydride ($NaBH_4$), Carpenter, and trinitrobenzenesulfonic acid (TNBS) methods. The DNFB and $NaBH_4$ methods form an acid-resistant bond between the ϵ-amino groups of lysine and the blocking compound is formed so that this lysine is not released by acid hydrolysis. The unavailable lysine was determined after hydrolysis with the Beckman amino acid analyzer. The DNFB, $NaBH_4$, and Carpenter methods produced consistent comparable available lysine values in proteins for bovine serum albumin. Available lysine values for cottonseed meals were significantly lower when determined by the Carpenter method. Available lysine values obtained with the TNBS method were consistently lower than those obtained by the other three methods.

The processing of foods and feeds has a direct effect on the nutritive value of the protein and the biological availability of the amino acids, especially lysine. Lysine reacts with glucose monohydrate in the presence of heat (Stevens and McGinnis, 1947). Overheating casein lowers the biological availability of lysine (Greaves et al., 1938). A reaction between glucose and the free amino groups in casein has been reported (Lea and Hanna, 1949, 1950). Gossypol reacts with the free amino group of lysine in albumin or cottonseed protein (Lyman et al., 1959).

The reagents 1-chloro-2,4-dinitrobenzene and 1-fluoro-2,4-dinitrobenzene were used by Sanger (1945) to react with free amino groups which formed dinitrophenylamino acids with a yellow color that could be estimated colorimetrically. Carpenter and Ellinger (1955) used 1-fluoro-2,4-dinitrobenzene (DNFB) to react with the free ϵ-amino group of lysine in 15 samples of animal by-products and showed a highly significant correlation between results obtained and a biological assay of protein quality with chicks. This reagent (DNFB) has been used to evaluate fish protein (Bruno and Carpenter, 1957). The method was modified to remove interfering substances (Carpenter, 1960). Roach et al. (1967) developed the "difference procedure" for determining available lysine as outlined. Paper chromatography has been used to facilitate the separation of ϵ-dinitrophenyllysine from interfering substances in estimating the protein quality of cottonseed meal (Baliga et al., 1959). Conkerton and Frampton (1959) exposed protein to gossypol prior to treatment with DNFB and noted a reduction in the number of ϵ-amino groups that would react with DNFB.

Ion-exchange column chromatography has been used to isolate the ϵ-dinitrophenyllysine in determining the available lysine in feed ingredients (Nielsen and Weidner, 1966). There was good agreement in the available lysine figures reported by these workers and values obtained in feeding trials with pigs as the test animal.

Comparative studies of several chemical methods to determine the available lysine in native proteins in foods and feeds have been reported by Blom et al. (1967). The most common chemical methods for measuring availability are based on the Sanger reaction (1945) which is based on the reaction of DNFB with the free ϵ-amino group of lysine in the protein molecule. Variations in the various methods are found in the isolation of the ϵ-dinitrophenyllysine from the hydrolyzed sample and the measurement of this derivative.

Kakade and Liener (1969) used 2,4,6-trinitrobenzenesulfonic acid (TNBS) as a labeling agent for the free ϵ-amino groups of lysine in protein.

The suggestion for using $NaBH_4$ to bind free ϵ-amino groups in protein is traceable to the reports of Grazi et al. (1962), Fischer et al. (1958), and Thomas (1970).

The purpose of the present investigation was to determine the effectiveness of DNFB, TNBS, and $NaBH_4$ as reagents for binding ϵ-amino groups of lysine in proteins that were not previously bound and which might serve as a measure of the biological availability of this amino acid.

MATERIALS AND METHODS

Preparation of Proteins. Glandless cottonseed meal (CSM) and bovine serum albumin were two of the proteins used in these investigations. The cottonseed meal was hexane extracted without heat. Particles of hulls and lint were removed by passing the meal through a 35-mesh sieve. The glandless CSM was ground in a ball mill for 1 h and sifted through an 80-mesh sieve.

Determination of Amino Acids. The samples (50 to 200 mg) were weighed into a 20 × 150 mm Pyrex test tube, 5 ml of deionized water was added, and the test tube was placed in a crushed ice bath. After 15 min in the ice bath, the sample was subjected to sonification using the Branson Ultrasonic Sonifier at 3 A for 1 to 3 min and transferred to a 300-ml round-bottomed flask.

The samples were boiled in 150 ml of 6 N HCl under reflux condensers fitted with capillary tubing 1 in. above the hydrolyzing solution for 24 h. The system was flushed with nitrogen to remove the oxygen and a constant flow of nitrogen into the hydrolyzing flask was maintained for the 24-h period. The hydrolyzed sample was filtered through glass filter paper and the filter paper and flask were rinsed three times with deionized water. The volume was reduced to approximately 30 ml on a rotary evaporator under vacuum with a water bath temperature of 45 °C. The sample was rinsed three times with 50-ml portions of ethyl ether or until there was no color in the ether layer

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and was evaporated to dryness on the rotary evaporator. Ten milliliters of deionized water was added to the flask and evaporated to dryness. This was repeated twice to remove all of the acid. Diluting buffer pH 2.2 was used to transfer the sample to a volumetric flask, the size of which depended upon the concentration of amino acids or lysine in the sample. The sample was filtered through Whatman No. 1 filter paper and stored in the dark under refrigeration.

Amino acid determinations were made on a Model 120-C Beckman automatic amino acid analyzer. Beckman Custom Research Resin types PA-28 and PA-35 were used in the columns. The determinations for total lysine were made according to the procedure of Moore et al. (1958) using resin type PA-35 in a short column to a depth of 9 cm.

Reaction with 2,4-Dinitrofluorobenzene. The general procedure was that described by Baliga et al. (1959), except that the reaction was completed after 4 h at 39 °C. The sample was made acid by adding 2.5 ml of 6 N HCl and evaporated to dryness on a vacuum rotary evaporator at a temperature of 40 °C. The sample was then extracted four times with 50-ml portions of anhydrous ethyl ether and brought to dryness.

Reaction of Sodium Borohydride with Protein. Sample preparation was the same as that described above. After sonification the test tube was packed in crushed ice for 15 min. Crystallized sodium borohydride was added slowly (5 mg every 5 min until 25 mg had been added). A glass rod was used to stir the solution after each addition. After 1 h the samples were removed from the ice bath for 30 min and then washed into a 300-ml round-bottomed flask with 150 ml of 6 N HCl and treated as indicated above for the determination of unavailable lysine.

Carpenter's Method. Samples were prepared according to the method of Carpenter (1960). The available lysine was calculated from a standard curve prepared from pure dinitrophenyllysine.

Trinitrobenzenesulfonic Acid Method. Samples were reacted with 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the method of Kakade and Liener (1969). The TNBS treated samples were hydrolyzed in 8 N HCl for 1 h in an autoclave at 15 lb of pressure. The hydrolyzed sample was extracted several times with ethyl ether to remove substances that would interfere with spectrophotometric determination of the trinitrophenyllysine. A blank was prepared by preventing the TNBS from complexing with the lysine and carried through the same steps as the sample. The molar extinction coefficient was determined at 346 mμ, and the trinitrophenyllysine concentration was calculated from a standard curve.

Preparation of Gossypol–Protein Complexes. *Glandless Cottonseed Meal.* The cottonseed protein (glandless CSM) was suspended and the gossypol was dissolved in methanol in a container covered with foil to exclude light and the mixture was stirred with a magnetic stirrer at 4 °C for 72 h. The protein–gossypol complex was removed by filtering through Whatman No. 42 filter paper on a Buchner funnel and washed three times with ether to remove unreacted gossypol and dried in a desiccator to remove remaining ether.

Bovine Serum Albumin. Bovine serum albumin was dissolved in 15 ml of H_2O. Gossypol (200 mg) was dissolved in 15 ml of acetone and diluted to 500 ml with methyl Cellosolve. The two solutions were combined and stirred for 48 h in a cold room at the end of which time the mixture was extracted three times with 3 volumes of ether to remove the free gossypol. The aqueous solution was lyophilized, placed in a brown bottle, wrapped in foil, and stored in a desiccator.

Determination of Gossypol. Free gossypol on the glandless cottonseed protein–gossypol complex and the gossypol–lysine albumin complex was determined by the method of Pons and Guthrie (1949) with the exception that 80% 2-propanol was used as the diluting agent instead of 95% ethyl alcohol. Total gossypol was determined by the method of Pons et al. (1950).

Statistical Treatment of Results. Available lysine levels of the various proteins and protein–gossypol complexes were subjected to the analysis of variance as outlined by Steel and Torrie (1960) for a completely random design with equal replications. Treatment means were separated by Duncan's multiple range test (1955). The standard error of the mean was calculated from three replications for all means reported.

RESULTS AND DISCUSSION

Determination of Available Lysine in Proteins by Four Methods. *Bovine Serum Albumin.* Bovine serum albumin was found to contain 11.99% lysine when using the regular short column procedure for basic amino acid determination on triplicate hydrolyzed samples as described by Moore et al. (1958) (Table I). This protein has previously been reported to contain 12% lysine by molecular weight and sequence studies (Dayoff and Eck, 1967).

The reagent (DNFB) combines with protein by bonding with free ε-amino groups in the protein. The free ε-amino groups of lysine combined with DNFB and formed a stable DNB–ε-lysine complex which resists acid hydrolysis under normal conditions. When the DNB reacted sample was acid hydrolyzed and analyzed for lysine by the method of Moore et al. (1958), the lysine, which was bound and not available for combination with DNFB, was measured. This was considered to be unavailable lysine. The available lysine as measured by the DNFB procedure was 11.56% and the unavailable lysine 0.43% (Table I).

Sodium borohydride ($NaBH_4$) forms a bond between the ε-amino acid group of lysine and the blocking compound by reduction so that this lysine is not released by acid hydrolysis. The $NaBH_4$ samples were acid hydrolyzed and the lysine determined according to the method of Moore et al. (1958). Almost identical results for available lysine were obtained with the $NaBH_4$ method and the DNFB methods.

In the method of Carpenter (1960), the sample is treated with DNFB followed by acid hydrolysis and the ε-dinitrophenyllysine is measured photometrically (Table I). There was no significant difference between the available lysine values in bovine serum albumin as determined by the Carpenter method (1960) and the DNFB and $NaBH_4$ methods.

The reagent trinitrobenzenesulfonic acid (TNBS) was used to complex the free ε-amino groups of lysine and the resulting ε-trinitrophenyllysine was determined spectrophotometrically after 1 h of acid hydrolysis in an autoclave. This method gave a statistically significantly ($P < 0.01$) lower figure for available lysine than that obtained with the other three methods.

Glandless Cottonseed Meal. The four methods as described above were used to determine the available and unavailable lysine in glandless cottonseed meal (CSM) (Table I). There was no significant difference in the total available lysine in glandless CSM for the DNFB, $NaBH_4$, and Carpenter methods (Table I), respectively. The TNBS method produced a statistically lower value for total available lysine.

Table I. Determination of Total Available and Unavailable Lysine in Bovine Serum Albumin, Bovine Serum Albumin-Gossypol Complex, Glandless Cottonseed Meal, Glandless Cottonseed Meal-Gossypol Complex, and Glanded Cottonseed Meal Solvent Extracted by Different Methods

	% lysine for method								
		DNFB		$NaBH_4$		Carpenter's method (1960)		TNBS	
Protein	Total	Unavailable	Available[a]	Unavailable	Available	Unavailable	Available	Unavailable	Available
Bovine serum albumin	11.99[b] ± 0.015	0.43 ± 0.020	11.56[a] ± 0.017	0.43[c] ± 0.014	11.56[a] ± 0.017	0.51 ± 0.003	11.48[a] ± 0.043	1.96 ± 0.020	10.03 ± 0.020
Bovine serum albumin-gossypol complex	10.50 ± 0.023	1.09 ± 0.014	9.41[a] ± 0.008	1.01 ± 0.025	9.49[a] ± 0.045	1.10 ± 0.003	9.40[a] ± 0.014	1.77 ± 0.075	8.73[b] ± 0.064
Glandless cottonseed meal	4.21 ± 0.020	0.33 ± 0.029	3.88[a] ± 0.008	0.26 ± 0.003	3.96[a] ± 0.023	0.45 ± 0.035	3.76[b] ± 0.023	0.54 ± 0.031	3.68[c] ± 0.014
Glandless cottonseed meal-gossypol complex	4.12 ± 0.005	0.67 ± 0.011	3.45[a] ± 0.005	0.65 ± 0.025	3.47[a] ± 0.020	0.89 ± 0.015	3.23[b] ± 0.020	1.10 ± 0.017	3.02[c] ± 0.023
Glanded cottonseed meal solvent extracted	4.25 ± 0.007	0.73 ± 0.023	3.52[a] ± 0.034	0.69 ± 0.012	3.56[a] ± 0.020	0.94 ± 0.011	3.31[b] ± 0.018	1.21 ± 0.032	3.04[c] ± 0.043

[a] Statistical analyses were completed on available lysine levels. [b] Standard error of the mean was calculated from three replications for all means reported. [c] Means within the same protein or protein complex bearing the same roman superscript do not differ significantly ($P < 0.01$).

Table II. Gossypol Content of Protein-Gossypol Complexes and Solvent Extracted Glanded Cottonseed Meal

	% gossypol		
Protein source	Total	Free	Bound
Bovine serum albumin-gossypol complex	1.78	0.15	1.63
Glandless cottonseed meal-gossypol complex	1.26	0.32	0.94
Glanded cottonseed meal solvent extracted	1.00	0.18	0.82

Glanded Cottonseed Meal. The total available lysine in glanded CSM was found to be 3.52 and 3.56% by the DNFB and $NaBH_4$ methods (Table I), respectively. The total available lysine as determined by the Carpenter (1960) method was 3.31% and for the TNBS method, 3.04%. The available lysine value as determined by the TNBS method was significantly lower than that of the other three methods and that of the Carpenter (1960) method was significantly decreased below that of the DNFB and $NaBH_4$ methods.

Bovine Serum Albumin–Gossypol Complex. Samples of bovine serum albumin and glandless CSM were reacted with gossypol as previously described. The gossypol combined with a small portion of the free ϵ-amino groups of lysine. The purpose of this treatment was to obtain similar levels of gossypol bound with free ϵ-amino groups of lysine as that found in solvent extracted glanded CSM. The total gossypol in the bovine serum albumin–gossypol complex, the glandless CSM complex, the glanded CSM solvent extracted, and glanded CSM ranged from 1.0 to 1.78% (Table II). The bound gossypol (gossypol:ϵ-amino group of lysine) content ranged from 0.82 to 1.63% (Table II). It is evident from the bound gossypol figures (Table II) that the available lysine content of the protein sources is decreased through binding of gossypol with ϵ-amino groups.

The total gossypol content of the serum albumin gossypol complex was 1.78% and the free gossypol content was 0.15%. Subtraction of the free gossypol content illustrates that 1.63% of the sample was bound gossypol (Table II). The molecular weight of gossypol is 437 and of lysine is 146 which makes a molecular weight ratio of 3:1. The gossypol molecule has two aldehyde groups each capable of complexing with a free ϵ-amino group. If it is assumed that the gossypol–lysine complex combines mole/mole, this would suggest that 0.54% of the sample was bound to gossypol and was unavailable. A similar calculation for the gossypol glandless CSM complex gives a figure of 0.31% for the unavailable lysine in this protein because of the reaction of the gossypol.

The lysine content of the bovine serum albumin (BSA)–gossypol complex was reduced approximately 1.5% below that of the original BSA–gossypol (Table I). The available lysine content of the BSA–gossypol ranged from 9.41 to 9.49% by the DNFB, $NaBH_4$, and Carpenter (1960) methods, which indicates good agreement between the three methods for the determination of available lysine in BSA–gossypol. A significantly lower value was obtained with the TNBS method (8.73% available lysine). If it is assumed that 0.51% of the lysine was complexed with gossypol and that 0.43% of the lysine was unavailable in the bovine serum albumin (Table II), this makes a total of 0.94% lysine which can be accounted for through the original unavailable lysine and the estimated gossypol complex lysine. This calculation accounts for most of the unavailable lysine which was found in the BSA–gossypol (Table II).

The lysine content of the glandless CSM was decreased 0.09% by complexing this protein with gossypol (Table II). The available lysine contents of the glandless CSM–gossypol complex were 3.45 and 3.47% as determined by the DNFB and $NaBH_4$ methods, respectively (Table II). The available lysine content was lowered to 3.23% as determined by the Carpenter (1960) method and to 3.02%

with the TNBS method. The total gossypol content of the glandless CSM–gossypol complex was 1.26% and the free gossypol content 0.32% (Table II). This leaves a value of 0.94% bound gossypol if the molar weight complexing ratio is 3:1. Then, 0.31% of the glandless CSM–gossypol complex of the samples was unavailable lysine because of the complexing with gossypol. The original glandless CSM contained 0.33% unavailable lysine (DNFB method, Table II). If the figure 0.31 is added to the figure 0.33, it may be assumed that the total calculated unavailable lysine in glandless CSM–gossypol complex would be 0.64%, which is in close agreement with the unavailable lysine as determined by the DNFB method.

Available lysine in different proteins and protein–gossypol complexes has been determined by four different methods. The available lysine in cottonseed meal samples was significantly decreased as determined by the Carpenter (1960) method as compared with the values obtained with the DNFB and $NaBH_4$ methods. This might be traceable in part to the carbohydrate and other interfering substances in the CSM. The method of Carpenter (1960) has been widely used as a measure of lysine availability and as an evaluation of protein quality. The major criticism of this method has been that a correction factor is required for samples that contain appreciable quantities of carbohydrates. The TNBS method (Kakade and Liener, 1969) calls for acid hydrolysis for only 1 h with all steps carried out with test tubes in small volumes. The ϵ-trinitrophenyllysine is not as stable in boiling 6 N HCl as is the ϵ-dinitrophenyllysine (Okuyama and Satake, 1960; Kotaki and Sataki, 1964). Carbohydrates and other reducing agents can reduce the NO_2 groups during acid hydrolysis which results in a decrease in intensity in the yellow and the light absorption in the spectrophotometer (Sanger, 1945). This has been shown to occur in ϵ-dinitrophenyllysine standard recovery trials (Blom et al., 1967; Mann et al., 1962; Matheson, 1968). There is less of a problem of color fading with increasing concentrations of carbohydrates and with increasing time of hydrolysis with the DNFB method than with the method of Carpenter (1960) and the TNBS method. The $NaBH_4$ method has the advantage of eliminating two hydrolyses and two lysine determinations on the amino acid analyzer in order to obtain the available lysine data.

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