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Measurement of Lysine Damage in Proteins Heated with Gossypol

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Experiments were conducted to investigate which analytical method for lysine determination best predicted the amount of lysine available to the rat from proteins that had been heated with gossypol. Glanded commercial cottonseed flour (CF) was analyzed without further treatment, while samples of glandless CF, egg white, and ovalbumin were studied either as is or after being heated with gossypol acetic acid. Relative to the level of bound gossypol and to the fall in lysine potency estimates determined from a rat growth assay, there were only small reductions in the lysine content as measured by amino acid analysis following sodium borohydride treatment or reaction with fluorodinitrobenzene, but the dye binding procedure with acid orange 12 gave closer agreement. Lysine released after digestion with Pronase underestimated the rat assay response. The reduction of in vivo nitrogen digestibility due to the gossypol treatment served to explain the differences observed between the values for reactive lysine determined from chemical procedures and those from rat growth.

The heating of protein foods during processing can result in a reduction in protein quality through destruction, or a reduction in availability, of susceptible amino acids, particularly lysine. Most attention has been given to the damage to lysine that can occur from reaction with reducing sugars through Maillard reactions as in the drying of milk (Mottu and Mauron, 1967). There have been several studies to determine the analytical method that best predicts the physiologically useful lysine in materials where protein has reacted with reducing sugars (Hurrell and Carpenter, 1974; Hurrell et al., 1983) and also with formaldehyde (Hurrell and Carpenter, 1978) or with a polyphenol, such as caffeic acid (Hurrell et al., 1982). Here we extend the study to complexes of protein with gossypol both in cottonseed flours (CF), where the dialdehyde gossypol occurs naturally and can bind to the protein during processing, and in a purified protein–gossypol mixture. The low value of protein–gossypol complexes as a source of lysine has already been shown in studies of animal growth (Baliga and Lyman, 1957; Smith et al., 1958; Frampton, 1965; Major and Batterham, 1981) and digestibility, both in vitro (Ingram et al., 1950; Horn et al., 1952; Cater and Lyman, 1970) and in vivo (Kuiken, 1952; Craig

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and Broderick, 1981; Tanksley et al., 1981). However, it has not been clear which of the possible chemical procedures is to be preferred for estimating the biologically available lysine.

MATERIALS AND METHODS

Test Materials. Glanded commercial CF (Traders Millers Co., Fort Worth, TX), glandless CF (hexane extracted, Rogers GL-6 variety), and egg white (spray dried, ICN, Cleveland, OH) were studied without further processing and also after being heated with gossypol acetic acid following the method of Smith (1972). Fifty grams of samples was slurried with 12.5 mL of H_2O and 100 mL of diethyl ether–95% ethanol (1:1) containing either 0.0 or 2.0 g of gossypol acetic acid in the case of egg white and 1.10 g with the glandless CF. After evaporation of the ether, the samples were stoppered and heated at 75 °C for 4 h. then they were washed with ether and filtered to remove any residual-free gossypol. The samples were dried, ground, and stored at 4 °C. Three further materials were formed from lyophilized ovalbumin (Grade III, Sigma Chemical Co., St. Louis, MO) by the treatment stated above using either 0.0, 1.5, or 11.5 g of gossypol acetic acid. The higher level of gossypol was chosen to be more than adequate for all lysine amino groups to be bound.

Chemical Analyses. Total lysine was measured after ion-exchange chromatography using a 0.9 cm × 13 cm column of W-1 resin (Beckman 120C amino acid analyzer, Palo Alto, CA) of acid hydrolysates with and without prior treatment with sodium borohydride (Hurrell and Carpenter, 1974). FDNB-reactive lysine was estimated after reaction with 1-fluoro-2,4-dinitrobenzene (FDNB), then hydrolysis, and measurement of (dinitrophenyl)lysine spectrophotometrically (Booth, 1971). Correction factors of 1.05, 1.09, and 1.20 were used for analyses of materials containing ovalbumin, egg white, and CF, respectively. The Rayner and Fox (1976) method of in vitro digestion with Pronase (Calbiochem-Behring, San Diego, CA) followed by amino acid analysis was modified by replacement of picric acid with 5% sulfosalicylic acid for precipitation of the protein. The determination of dye-bound lysine (DBL) was by the difference in binding of acid orange 12 dye without (A reading) and with (B reading) prior treatment with propionic anhydride (Hurrell et al., 1979). The quantities used were calculated in the same way as in the published procedure, but the subsequent method was modified to use equipment obtained from UD Corp., Fort Collins, CO (Udy, 1971). All reactions were carried out in 50-mL Nalgene bottles containing three stainless steel ball bearings. Samples were first shaken with 1.0 mL of 2-propanol for 10 min for thorough wetting. Then 4.3 and 4.0 mL of 5% sodium acetate trihydrate were added to the A and B bottles, respectively. After a 5-min shake, 0.3 mL of propionic anhydride was added to the B bottles. After a further 15-min shaking, 40 mL of dye solution (3.77 mmol/L) was added to both bottles and shaking continued for 60 min. Filter-aid was then added to each bottle, and caps fitted with glass fiber filters were attached; the filtrate was squeezed out and diluted 1:50, and the absorbance was measured at 475 nm.

Free gossypol and total gossypol were measured by AOCS (1975) methods Ba 7-58 and Ba 8-78, respectively. The nitrogen (N) content of dietary ingredients and feces were determined by the macro-Kjeldahl method (AOAC, 1975). Chromic oxide was measured by adapting the method of Varnish and Carpenter (1975) for determination of smaller amounts (1–4 mg). All reagents except the digestion mixture were reduced to one-fifth the levels given in the earlier paper, and the final solution was clarified by centrifugation instead of filtration.

Table I. Composition of the Lysine-Deficient Basal Diet[a]

ingredient[a]	%
wheat gluten (78.6% CP)[b]	6.5
skim milk powder (35.4% CP)[b]	6.0
zein (88.0% CP)[c]	10.0
sesame seed meal[d]	9.0
L-tryptophan	0.1
L-methionine	0.3
L-histidine	0.25
L-threonine	0.3
L-isoleucine	0.3
L-valine	0.4
corn oil	5.0
mineral mixture (Briggs-Williams)[e]	4.0
vitamin mixture (AIN-76)[f]	1.0
choline chloride	0.1
cornstarch	10.0
cellulose	5.0
sucrose	to 100

[a] Cr_2O_3 bread (15% Cr_2O_3) was incorporated in the diet at the 1% level in experiment 2. [b] ICN Biochemical Co., Cleveland, OH. [c] Sigma Chemical Co., St. Louis, MO. [d] Substituted in experiment 2 with the following: 3.8% gelatin (ICN Biochemical Co., Cleveland, OH), 0.07% L-tryptophan, 0.1% L-methionine, 0.05% L-cystine, 0.07% L-histidine, 0.15% L-isoleucine, 0.13% L-valine, 0.19% L-leucine, 0.18% L-phenylalanine, 0.08% L-threonine, 0.14% L-tyrosine, and 0.18% L-arginine hydrochloride. [e] Grams per kilogram of salt mixture: $CaCO_3$, 207; $CaHPO_4$, 323; Na_2HPO_4, 186; KCl, 208.6; $MgSO_4$, 65.7; $MnSO_4H_2O$, 4.4; $CuSO_4$, 0.37; ferric citrate, 4.3; $ZnCO_3$, 0.6; KIO_3, 0.03. [f] Vitamins per kilogram of diet: thiamin hydrochloride, 6.0 mg; riboflavin, 6.0 mg; pyridoxine hydrochloride, 7.0 mg; nicotinic acid, 30.0 mg; calcium(II) pantothenate, 16.0 mg; folic acid, 2.0 mg; biotin, 0.2 mg; cyanocobalamin, 10.0 μg; vitamin A, 4000 IU; vitamin D, 1000 IU; vitamin E, 50 IU; menadione, 50 μg.

Rat Assays. Weanling male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were used in both experiments. On arrival, they were caged individually and fed the basal diet for 2 days prior to randomization to the experimental diets. This diet (Table I) was based on one previously found to be limiting only in lysine (Bjarnason and Carpenter, 1969). Four rats were then allotted to each treatment, and body weight gain and food consumption were measured over the entire period. Experiment 1 lasted 16 days and experiment 2, 15 days. For the standards, the regression of growth, "weight gain/food intake", against the level of supplementary lysine in the diet was calculated, and the response to each test material was compared with the response to lysine by using the slope–ratio method (Finney, 1964).

In experiment 1, lysine monohydrochloride was added in increasing amounts to supply 0, 1.4, 2.8, and 4.2 g of lysine/kg to the basal diet to generate a standard growth–response curve. Since the test materials contained free gossypol, a second standard curve was also run containing 0.005% gossypol acetic acid, an amount equal to the highest level contributed by a test material. Free gossypol was then equalized across all other test material diets. The test materials in this first experiment were based on the CF's or egg white. They were each fed at the same three levels of crude protein. In further treatments supplementary lysine was added to the highest level of each material to check that lysine was the factor limiting growth. Due to an error, zein was replaced gram per gram rather than on an isonitrogenous basis, resulting in changes in the total crude protein in the diets from 16.4% to 20.3%. At the end of the experimental period, feces were collected from rats fed the 4.2 g of supplementary lysine/kg diets (with and without gossypol) and from those fed the highest level of each test material without added lysine. The feces

Table II. Gossypol (GSP) Content (g/16 g of N) and Lysine Analysis (g/16 g of N) of the Test Materials

test materials	crude protein, %	free GSP	bound GSP	binding ratio[a]	total lysine	total lysine after $NaBH_4$	FDNB-reactive lysine	DBL[b]	Pronase-released lysine
glandless CF	54.6	0.106	0.09	0.011	4.6	n.d.[c]	4.7	5.3	3.9[d]
glandless CF + GSP	55.2	0.058	2.81	0.377	4.2	n.d.	3.9	3.3	1.9[d]
glanded commercial CF	58.3	0.057	1.87	0.245	4.3	n.d.	4.0	3.2	2.0[d]
egg white									
untreated	80.2				7.2	n.d.	6.6	6.8	6.1
treated (-GSP)	82.1				7.2	n.d.	7.1	7.0	6.0
treated + GSP	74.9	0.003	3.12	0.241	7.3	n.d.	5.9	3.8	2.8[d]
ovalbumin									
untreated	86.6				6.8	n.d.	6.5	6.1	5.8
treated	86.4				6.7	6.5	6.6	6.1	5.7
treated + low GSP	84.5	0.007	2.53	0.216	6.6	5.9	6.3	4.8	3.3
treated + high GSP	75.3	1.023	16.78	1.48	6.4	5.5	5.5	1.3	0.03
pooled SEM[e]					0.16	0.14	0.05	0.07	0.10

[a] Moles of bound gossypol aldehyde groups:moles of total lysine. [b] DBL = dye-bound lysine. [c] n.d. = not determined. [d] Based on one determination and pooled SEM does not apply. [e] SEM = standard error of treatment means.

were freeze-dried, ground, and analyzed for N.

Experiment 2 was conducted similarly, but sesame seed meal was not available, so gelatin and an amino acid mixture were substituted in the basal diet. The ovalbumin-based materials were incorporated isonitrogenously at the expense of zein and sucrose into the basal diet at three crude protein levels. The standard response curve was generated with dietary levels of 0, 1.0, 2.0, and 3.0 g of lysine/kg. Lysine was again added to each of the diets containing the largest amount of test material, and free gossypol acetic acid (0.055%) was standardized across all treatments. Chromium bread (Kane et al., 1950) containing 15% Cr_2O_3 was included in all diets as a digestibility marker. Feces were collected for the last 5 days of the experiment. After freeze-drying and grinding, they were analyzed for N and Cr_2O_3.

Digestibility of Test Materials. *Experiment 1.* The apparent N indigestibility of the diets was calculated from the fecal N expressed as a percentage of the N intake. Since the diets were not isonitrogenous and contained varying levels of zein, we assumed that the unprocessed starting materials had the following N digestibility values: glandless CF, 92% (Tanksley et al., 1981); egg white, 99% (Boctor and Harper, 1968). Using an average value of 75% digestibility for zein [obtained in this laboratory on two other lots of zein (Jenkins et al., 1984)], we then estimated the change in digestibility between the basal diet and the test diet from the differences in the apparent N digestibility.

Experiment 2. The apparent percentage N indigestibility was calculated as grams of N per gram of Cr_2O_3 in fecal samples expressed as a percentage of gram of N per gram of Cr_2O_3 in diet samples. The difference in indigestibility of N between the test diet and the basal diet was calculated to be the amount contributed by the indigestible test material minus the amount from the replaced zein. We made two estimates for each test material, one based on the average of 75% for zein and the other on the highest value of 85% obtained with one batch of zein. The *differences* in estimated digestibility of the materials are independent of which value is used for zein.

RESULTS

Chemical Analyses. These results are summarized in Table II. The analyses of the glandless CF showed the presence of some gossypol, though less than 1/10th that in the glanded sample: this was presumably due to contamination during production. The procedure used for reacting the lower quantity of gossypol with the test materials resulted in levels of bound gossypol slightly higher than that found in the commercial CF. Heating ovalbumin with excess gossypol caused a 7-fold rise in bound gossypol, increasing the molar binding ratio of gossypol aldehyde:lysine to greater than 1.0.

The total lysine values were similar to those expected for the three starting materials, i.e., CF (Tanksley et al., 1981), egg white, and ovalbumin (Osuga and Feeney, 1977), and were little changed by the processing conditions, with or without gossypol. The procedure measuring total lysine, after reductive treatment of the samples with sodium borohydride, was only applied to the ovalbumin series. The values found were about 90% of the ordinary total lysine values in each case.

Turning to the FDNB results for reactive lysine, the untreated glandless CF and ovalbumin gave values not significantly different from those for total lysine. However, the untreated egg white gave a lower FDNB value, which was increased by the processing without gossypol and was then not different from the corresponding total value. This result is not unexpected since the denaturation caused by treatment with alcohol can expose previously hindered lysine ϵ-amino groups for reaction (Porter, 1948). Treatment with gossypol caused a significant fall in value in each series.

The measurement of reactive lysine by dye binding gave lower than total values for untreated egg white and ovalbumin and a higher value for the glandless CF. Within each series the treatments with gossypol caused large falls in DBL values, considerably greater than those seen with FDNB.

The free lysine measured after enzymic treatment with Pronase corresponded to 85% of the total lysine value for each of the three starting materials. Processing again caused dramatic falls, with the egg white treated with excess gossypol giving almost a zero value.

Rat Assays. The results of experiment 1 are listed in Table III. The response of the rats to increasing levels of supplemental lysine was linear up to 2.8 g/kg and was not changed by the addition of 0.005% free gossypol acetic acid to each diet. With the five test materials, supplementary lysine in each case gave a large further response, indicating that lysine was still the first factor limiting growth in the presence of the test materials.

The responses to the different levels of each sample also showed no obvious departure from linearity. Analysis of variance of the food efficienty data indicated that the results did not differ significantly from Finney's slope-ratio model, so that we could obtain valid estimates of potency and standard error. Processing the glandless CF with

Table III. Response of Rats to Dietary Lysine or Test Protein Supplementation (Experiment 1)[a]

test material	g of CP[b]/kg of diet	supplemental lysine, g/kg	weight gain, g[c]	food intake, g[c]	food efficiency[c]	lysine potentcy, g/16 of N	app. indig. of diet N, %	N dig. of test material, %
standard curve		0	30.5; 29.5	122; 116	0.250; 0.254			
		1.4	60.8; 59.2	165; 160	0.368; 0.374			
		2.8	89.3; 91.5	195; 199	0.457; 0.460			
		4.2	102.8; 104.8	195; 203	0.528; 0.517			18 (17)
glandless CF	17.7	0	45.3	139	0.326			
	35.3	0	64.3	165	0.389	5.0	11	92
	53.0	0	76.0	178	0.428			
	53.0	4.6	(113.5)	(204)	(0.558)			
glandless CF + gossypol	17.7	0	41.3[d]	131[d]	0.316[d]			
	35.3	0	48.8	145	0.337	3.3	13	84
	53.0	0	61.8	163	0.379			
	53.0	4.9	(112.5)	(209)	(0.538)			
commercial CF	17.7	0	38.3	130	0.295			
	35.3	0	52.5	153	0.342	3.1	15	76
	53.0	0	60.5	162	0.374			
	53.0	5.0	(108.5)	(195)	(0.560)			
egg white (untreated)	17.7	0	50.8	152	0.335			
	35.3	0	80.3	185	0.434	7.2	10.5	99
	53.0	0	95.3	187	0.509			
	53.0	3.1	(103.5)	(184)	(0.562)			
egg white + gossypol	17.7	0	40.5	139	0.292			
	35.3	0	45.8	140	0.327	2.4	30	31
	53.0	0	56.8	163	0.348			
	53.0	3.8	(99.5)	(201)	(0.500)			
pooled SEM[e]			4.1	10.4	0.011	0.29	0.9	

[a] Mean of four rats for 16 days. [b] CV = crude protein. [c] Numbers following a semicolon are responses without added gossypol. [d] Mean of three rats only. [e] SEM = standard error of treatment means.

Table IV. Response of Rats to Dietary Lysine or Test Protein Supplementation (Experiment 2)[a]

test material	g of CP[b]/kg of diet	supplemental lysine, g/kg	weight gain, g	food intake, g	food efficiency	lysine potency, g/16 g of N	app. indig. of diet N, %	N dig. of test material, %[c]
standard curve		0	18.7[d]	111[d]	0.168[d]			
		1.0	37.1	138	0.269		12.5	
		2.0	49.8	147	0.334		13.8	
		3.0	64.5	149	0.431			
ovalbumin (treated without gossypol)	16.4	0	30.3	124	0.243			
	32.8	0	38.3[d]	141[d]	0.272[d]	4.2	15.2	54; 63
	49.2	0	54.9	154	0.358		18.3	
	49.2	1.2	(68.4)	(172)	(0.397)			
ovalbumin + low gossypol	16.4	0	27.1	122	0.223			
	32.8	0	28.3	120	0.236	2.4	18.2	37; 47
	49.2	0	39.0	144	0.271		21.8	
	49.2	1.9	(58.8)	(155)	(0.376)			
ovalbumin + high gossypol	16.4	0	29.4	149	0.199			
	32.8	0	29.8	140	0.211	1.8	22.4	9; 18
	49.2	0	46.5	180	0.258		29.5	
	49.2	3.6	(91.3)	(201)	(0.454)			
pooled SEM[e]			4.5	10.0	0.019	0.48	2.16	

[a] Mean of four rats for 15 days. [b] CP = crude protein. [c] Based on zein digestibilities of 75% and 85%. [d] Mean of three rats. [e] SEM = standard error of treatment means.

gossypol significantly reduced the lysine potency to a value similar to that for commercial CF, while treating the egg white with gossypol reduced the potency from a value identical with its total lysine content to one-third of that value.

The replacement of zein by the highest level of either CF or untreated egg white led to improved overall digestibility. Adding the "egg white plus gossypol" led to considerably more indigestible N; i.e., it was less digestible than the zein it replaced. In fact, when the N digestibilities were calculated (assuming the assigned value of 92% for glandless CF and 99% for egg white), the egg white–gossypol complex was found to be only 30% digestible, while the other gossypol-containing test materials had digestibility estimates of 84% (glandless CF plus gossypole and 76% (commercial CF).

In experiment 2, the response to supplemental lysine was again linear to the top level of 3.0 g/kg of diet (Table IV); the test materials all showed a response to extra lysine, and the analysis of variance showed no significant deviation from the model. The estimates of potency of the samples containing gossypol were significantly lower than that of the sample processed without gossypol, which itself was much lower than expected. Even this sample showed a low digestibility (54 or 63%, according to the assumed digestibility of zein), and the sample heated with a high level of gossypol was apparently no more than 20% digestible.

DISCUSSION

The primary aim of this work has been to investigate which laboratory method for lysine determination is most

Table V. Lysine Values from Each Method Expressed as a Percentage of the Total Lysine of the Starting Material within Each Sample Series and Digestibility Data

test material	total lysine[a]	total lysine after $NaBH_4$	FDNB-reactive lysine	DBL[b]	Pronase-released lysine	rat assay	N digestibility, %[c]	FDNB × digestibility[d]
glandless CF	100 (4.6)		102	115	85	109	92	94
glandless CF + GSP[e]	91		85	72	41	72	84	71
commercial CF	94		87	70	44	67	76	66
egg white								
untreated	100 (7.2)		92	94	85	100	99	91
treated (-GSP)	100		99	97	83			
treated + GSP	101		82	53	39	33	31	25
ovalbumin								
untreated	100 (6.8)		96	90	85			
treated (-GSP)	99	96	97	90	84	62	54; 63	52; 61
treated + low GSP	97	87	93	71	49	35	37; 47	34; 44
treated + high GSP	94	81	81	19	<1	26	9; 18	7; 15

[a] Absolute values (g/16 g of N) are given in parentheses. [b] DBL = dye-bound lysine. [c] Absolute values based on zein digestibility of 75% (CF and egg white series) and 75 and 85% (ovalbumin series). [d] FDNB-reactive lysine expressed as percentage of total lysine (column 3) × N digestibility. [e] GSP = gossypol.

sensitive as an indicator of nutritional damage to the lysine in food proteins as a result of their reaction with gossypol processing. Our results on cottonseed, as summarized in Table V, are in general agreement with published work in showing little or no change in total lysine and significant falls in FDNB-reactive lysine as a result of either commercial processing or reaction with gossypol under laboratory conditions (Lyman et al., 1959; Conkerton and Frampton, 1959). Martinez et al. (1961), Couch and Thomas (1976), and Craig and Broderick (1981) have also reported falls in total lysine with processing at elevated temperatures.

It would also appear from our results that the reductions in FDNB-reactive lysine do not represent the full change in biologically available lysine. At the other extreme the procedure using in vitro digestion with Pronase underestimated the values of the materials containing bound gossypol. It has been a common finding that in vitro enzymic digestion is less efficient than in vitro vertebrate digestion with relatively resistant materials (Szmelcman and Guggenheim, 1967; Mottu and Mauron, 1967) but in general the bacterial enzyme Pronase has been more active than mammalian enzymes used in vitro and a useful predictor of rat available lysine with animal proteins processed under various conditions (Ford and Salter, 1966). However, we have found (unpublished results) that addition of free gossypol to the reaction medium intereferes with Pronase digestion, decreasing the amount of lysine released. Inhibition of enzyme activity by gossypol has been observed by others (Tanksley et al., 1970), but most importantly, in our study the in vitro digestibility results do correlate well with digestibility changes observed in rats.

The differential dye binding procedure (DBL) gave results closest to the rat potencies in the cottonseed series, although the value for the control material was higher than we can explain. Since the DBL procedure is preferable to the FDNB procedure on grounds of speed and convenience in the laboratory, it appears promising as a method for the quality control of cottonseed products. It is interesting that it should apparently be a *more* sensitive indicator than FDNB for these materials while the opposite has been found to be the case for relatively mild damage occurring during the processing or storage of milk powders (Hurrell et al., 1983).

We hoped to study whether this was due to the different characteristics of gossypol, in contrast to those of reducing sugars in their reaction with protein, by evaluating the products formed by reacting gossypol with an animal protein. We began by using commercial egg white since we needed to purchase sufficient for animal feeding. Then, because of the possibility that some of the unexpected findings were to be explained by the presence of traces of glucose, we changed to a more refined grade of ovalbumin. In fact, the pattern of results with the two materials was very similar.

When either egg white or purified ovalbumin was heated with gossypol, the DBL values again fell to a greater extent than did the FDNB values. This was seen most clearly when ovalbumin was heated with an excess of gossypol. DBL fell from 90% to 19% of the total lysine value in untreated ovalbumin while the FDNB value fell only from 96% to 81%. From the quantity of gossypol bound we thought it likely that virtually all the reactive lysine had been bound to gossypol. Certainly the moles of bound gossypol were greatly in excess of the "bound" lysine as measured with FDNB. Similarly, Hurrell et al. (1982), in a study of reactions between protein and the polyphenol caffeic acid, found that under their conditions "between 4 and 9 moles of caffeic acid have been incorporated for each mole of lysine rendered unreactive to FDNB".

It seems likely that most of the gossypol is attached to other groups in the protein or has formed large gossypol polymers, binding to very few lysine groups, as indicated by the following evidence. First, we did not find a significant release of gossypol prior to the acid hydrolysis step in the FDNB procedure. Both gossypol–ovalbumin samples were treated with the same solvents as in the initial stages of the FDNB method but without FDNB. After removal of ether, lyophilization, and measurement of both free and total gossypol, we found that although some bound gossypol was released, it was not enough to account for the high level of FDNB-reactive lysine. Another possibility would be that FDNB could bind to the lysine–gossypol Schiff's base, which is thought to form by reaction of gossypol with the ϵ-amino group of lysine, and serve to displace gossypol during acid hydrolysis. This could result in falsely high DNP-lysine values and has been suggested in the caffeic acid–protein binding studies conducted by Hurrell et al. (1982). However, treatment of the samples with $NaBH_4$, which would stabilize these Schiff's bases so that FDNB could not bind and so gossypol could not be removed during the acid hydrolysis step, resulted in lysine values similar to the FDNB-reactive lysine.

In relating the chemical analyses to the results of the feeding experiments another problem arises. Egg albumin had already been found to be especially liable to a drastic

fall in digestibility as a result of processing (Valle-Riestra and Barnes, 1970; Knipfel et al., 1975). This is again apparent from the results of our feeding trials even though they were not designed primarily to measure protein digestibility accurately. The sample of ovalbumin treated with alcohol and ether in the absence of gossypol has an estimated digestibility of only 54 or 63% and an "available lysine" potency equivalent to approximately 62% of its total lysine content. Therefore, if the portion of the protein that was digested had the same lysine content as the total sample, then the lysine of that portion can be said to be essentially fully available. Extending the same argument to the samples treated with gossypol, we again find that the product of "digestibility" and "FDNB-reactive lysine" gives values that are similar to the estimates of "rat potency", as seen in Table V. It could therefore be that under our particular conditions gossypol is reducing the digestibility of the protein as a whole (for example, by cross-linkages involving sulfhydryl and hydroxyl groups besides the amino groups) to a greater extent that it is binding specifically and irreversibly to lysine units. It is, of course, only to be expected that when the overall digestibility of a protein is seriously changed, one cannot fully predict biologically "available" amino acid values from chemical procedures (Boctor and Harper, 1968; Carpenter, 1973).

Although the digestibility of cottonseed is less influenced by processing than is egg, the changes are enough in our study that the product of "FDNB values" × "digestibility" again shows good agreement with rat assay values. If FDNB truly measures the unbound lysine units in protein–gossypol complexes, which is in agreement with the $NaBH_4$ results, we can only explain the lower DBL values by suggesting that the relatively large dye molecule is hindered from reacting with some of the free ε-amino lysine groups within the complex, in a manner analogous to the inability of digestive enzymes to reach the peptide bonds that they would otherwise be able to split.

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