Comparison of the Rat Repletion Method with Other Methods of Assaying the Nutritive Value of Proteins In Cottonseed Meals

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The increase in protein quality of cottonseed meal through improvement in current practice in manufacture resulted in a collaborative effort by several agencies. As part of the problem, relative precision of the different methods for measuring protein quality has not been firmly established. Samples, prepared in commercial plants, were studied for the effect of various processing factors. These samples were tested by different methods in different laboratories. The Cannon rat repletion method was adopted by the authors to study twelve of the samples. Comparison of results showing the degree of correlation between methods and known characteristics of the meals indicate that highest protein nutritive value is associated with low processing temperature and low bound and free gossypol content. Because small differences in protein quality are often obscured by experimental error, agreement among the various methods points to factors needing control in meal processing. The rat repletion method is shown to be helpful in measuring protein quality in meals.

A MODIFICATION of the Cannon (*3*) rat repletion method of protein assay has been adapted in this laboratory to the nutritive evaluation of proteins in various feedstuffs. The method used in obtaining these data is similar to the modification of the original Cannon technique which has been used by Frost and Sandy (*4*). This modification was a low-protein basal diet fed ad libitum both during depletion and repletion periods; the protein supplement fed separately from the rest of the diet; and gain in weight as the sole criterion of repletion response. Observations by Cabell and Earle (*2*) showed that thyroprotein, fed to rats on a low-protein diet, hastens the reduction of body reserves of protein without apparently altering the repletion response; therefore, in preparing rats for these repletion tests, 0.1% of Protamone (iodinated casein) was included in the diet during the depletion periods.

The objectives of this report are: to present results obtained by this rat repletion method in estimating the protein nutritive value of 12 cottonseed meals; to compare these results with others obtained by various methods of protein assay on the same meals; and to evaluate the effect of various processing treatments and chemical components of the meals on nutritive value of the proteins.

Because the estimate of nutritive quality of proteins is known to be dependent to some degree on the method of measurement used, the over-all plan was to utilize tests made on the meals by several methods. The various tests for protein assay were carried out as part of a collaborative study. This study was initiated by the Southern Utilization Research Branch, Agricultural Research Service, U.S.D.A., to investigate the effects of various processing methods on the nutritive value of cottonseed meal. The meals, submitted to seven different laboratories, were assayed for protein quality by the current methods in use in the respective laboratories. The data from the other six collaborators have been made available to the authors for purposes of this report.

There is enough agreement by the various methods to increase confidence in the estimate of the effect of certain factors on protein quality and to indicate that the rat repletion method can be of value in this study of cottonseed meal.

The term, quality of protein, is somewhat indefinite in meaning. It is used here, as in many nutritional studies, to express what Sherwood and Weldon (*8*) have called an attempt to express a complex interrelationship between available amino acids, both singly and collectively, and requirements of the animal.

Materials and Methods

Healthy adult male rats weighing between 200 and 300 grams were prepared for test by feeding the low-protein diet containing 0.1% of Protamone for 3 weeks. This diet is given in Table I. The depleted rats were divided into 8 groups of 7 each, according to body weight. The animals of each group were equal in weight within a few grams. They were then randomly assigned to the 7 protein-containing test feeds or supplements. Assignment of litter mates to the same supplement was avoided. Thus, the feeding trails were randomized in complete block designs with 8 replications.

The repletion period was for 10 days and during this time the rats were fed the supplement, in measured daily allowances, sufficient to supply 0.1564 gram of nitrogen. To save time and reduce weighing errors, the total protein for each rat for the 10-day period was weighed and divided into 10 approximately equal feedings, stored in small air-tight cups, and fed from a convenient rack as daily feedings. The supplement was fed at the same time each day, and was consumed completely and immediately. In addition to the supplement, the rats were fed the same basal diet used in the depletion period except that the Protamone was omitted during repletion.

Table I. Basal Low-Protein Diet[a]

Ingredient	Amount, G./Kg.	B-complex Vitamins	Amount, G./Kg.
Dextrinized starch	434	Riboflavin	0.007
Sucrose	434	Thiamin HCl	0.004
Agar	50	Pyridoxine HCl	0.007
Lard	40	Niacin	0.04
Salt mix[b]	40	Ca pantothenate	0.015
Liver concentrate[c]	1	Inositol	0.05
Choline	1	Para amino benzoic acid	0.05
Alpha tocopherol	0.02	Menadione	0.001
Beta carotene	0.02	Folic acid	0.0002
Irradiated yeast (18,600 I.U. vit. D/g.)	0.06	Vitamin B_{12}	0.00003

[a] Protamone (iodinated casein) 1 g./kg. added to diet during depletion period.
[b] Jones and Foster salt mixture no. 12 (*6*).
[c] A powdered 1 to 20 concentrate of liver extract.

The meals were studied in a series of four trials in which the same rats were used each time, being redepleted between trials. After each repletion, the colony diet was fed for 3 days; the depletion diet was then fed for 7 days, and another experimental repletion trial was begun immediately.

The following processing conditions used in the manufacture of the cottonseed meals are given by Altschul (*1*).

Nine of the meals carry the letter *T* in their numbers and are screw press meals made in commercial mills under controlled experimental conditions. All *T*1 meals are made by the usual processing method used in the plant producing the meal. *T*2 meals are low temperature screw press meals which have entered the press at a temperature not exceeding 200° F. Meals 12*T*2 and 12*T*3 were cooked at maximum temperatures of 160° F. and 180° F., respectively. The meals designated as 14*T*3 and 15*T*3 are especially processed meals which were first cooked as for hydraulic press operations and then put through a screw press. In all these meals the free gossypol content is low enough not to constitute a factor in the investigation. The other three cottonseed meals represent special methods of processing. Meal *S*714 is a prepress solvent extracted meal produced in a commercial plant under special conditions which were expected to effect little denaturation of the proteins. Meal 8329 is a meal produced in the pilot plant of the Southern Regional Research Laboratory in which the pigment glands of the cottonseed were ruptured or weakened by rolling prior to cooking. As a result of this operation, the free gossypol content of the meal is low and the nitrogen solubility is still fairly high. Meal *S*676 is a butanone extracted product which is expected to show near maximum quality for cottonseed meal because it has been subjected to a minimum amount of heat, contains a low quantity of free gossypol, has high nitrogen solubility, and high thiamin content.

A sample of soybean meal for comparison was received with the other meals and was designated Standard Soybean Meal. Other products used for comparison purposes were crude casein, a mixture of casein plus 0.31% tryptophan, and 0.84% methionine and defatted whole egg.

Results and Discussion

Results of Rat Repletion Assays Results of the rat repletion tests expressed as mean weight gains for the entire repletion period for each group of rats fed each of the test proteins are given in Table II. Direct comparisons of weights in different trials were not made because the homogeneity test shows a probability of less than 30%. Hence, the experiments were not treated in a combined analysis but separate statistical analyses, run on each of the trials, resulted in calculating the least significant differences and coefficients of variation. The coefficients of variation ranged from 10.9 to 14.0 with the first test giving the largest value. The least significant differences were very similar in the first three tests but a reduced value was obtained in the fourth trial. This also indicates that at least four trials can be run without any loss of precision due to repeated depletion.

Best response was obtained on the proteins of known high quality—for example, whole egg and casein supplemented with amino acids. Among the cottonseed meals the butanone extracted meal *S*676 gave the best response, while poorest results were obtained with meal 8329 in which the pigment glands were ruptured before cooking. When a comparison within a trial in meals that carry the letter *T* in their numbers is made between the plant run or *T*1 meals and the *T*2 or *T*3 meals, responses on the latter are always higher. Although this difference is not always statistically significant, its consistency indicates that the changed procedures resulted in meals with protein quality superior to plant-run products.

If the proteins tested are arranged in decreasing order of response of the depleted rats, they tend to fall as follows: whole egg; casein; standard soybean meal and butanone extracted cottonseed meals; low temperature screw press meals, and screw press meals previously prepared as for hydraulic cooking; usual screw press meals; prepress solvent extracted meal *S*714; and the special experimental meal 8329. The degree of certainty of this ranking of the meals can be estimated by comparing the differences between the mean values and least significant differences, Table II.

Comparison of Rat Repletion Method with Other Methods Table III shows data comparing the rat repletion method with results from six other laboratories using rat

Table II. Results of Rat Repletion Tests

(Gain in weight of groups of rats fed various protein supplements for repletion period of 10 days)

Supplement	Trial 1, G.	Trial 2, G.	Trial 3, G.	Trial 4, G.
Casein	31.9	36.5	36.3	35.0
Casein + amino acids	..	45.9	..	..
Whole egg	..	48.4	..	..
Soybean	27.0	..	..	..
C. S. meal *S*676	27.1	..	30.6	..
8329	13.4	..	13.8	..
*S*714	16.8	..	..	20.8
12*T*1	..	..	21.8	..
12*T*2	..	..	25.4	..
12*T*3	..	..	25.9	..
14*T*1	17.4	..	..	22.3
14*T*2	21.2	..	..	24.8
14*T*3	..	23.8	..	23.9
15*T*1	..	20.8	22.0	..
15*T*2	..	26.1	..	22.1
15*T*3	..	22.4	..	21.8
L.S.D.[a] 0.05	3.3	3.6	3.5	2.7
0.01	4.5	4.8	4.7	3.6
C.V.[b] %	14.0	11.2	13.8	10.9

[a] Least mean difference necessary for significance at probability of 0.05 and 0.01.
[b] Coefficient of variation.

Table III. Comparison of Rat Repletion Method with Other Methods

For estimating the nutritive value of proteins of cottonseed meals

	Index of Protein Quality							Other Constants[a]			
	Rat repletion	Rat growth		Chick growth			Microbiological	Max. cooking temp., °F.	Gossypol		Sol., % nitrogen in 0.5N NaCl
	Lab. 1	Lab. 2[b]	Lab. 3	Lab. 4	Lab. 5	Lab. 6	Lab. 7[b]		Bound, %	Free, %	
C. S. meal[c] S676	100	100	100	100	100	100	100	Not cooked	0.13	0.023	62.7
12T3	85	..	97	105	..	..	..	180	0.59	0.030	53.6
12T2	83	..	107	103	..	79	..	160	0.58	0.050	55.2
14T2	81	85	..	69	86	83	98	200	0.81	0.029	21.6
14T3	79	65	..	53	76	76	89	230	0.92	0.030	18.6
15T2	79	76	90	44	73	65	92	200	0.91	0.031	16.5
15T3	73	63	69	32	67	74	75	240	1.06	0.043	18.4
12T1	72	..	87	98	81	..	..	230	0.77	0.030	23.3
14T1	70	70	..	48	71	82	89	240	0.83	0.043	16.1
15T1	70	47	66	23	63	60	81	250	1.07	0.040	10.8
S714	66	74	102	114	100	85	..	190	0.74	0.019	38.0
8329	47	62	..	41	78	70	72	230	1.31	0.033	...
	Correlation Coefficients										
Laboratory 1	..	0.38	0.42	0.31	−0.10	0.18	0.78[d]	−0.15	−0.85[e]	−0.13	0.46
2	0.38	..	0.89	0.61	0.63	0.66	0.72	−0.26	−0.84[e]	−0.20	0.69
3	0.42	0.89	..	0.84[d]	0.90[d]	0.72	0.84	−0.30	−0.76[d]	−0.08	0.87[e]
4	0.31	0.61	0.84[d]	..	0.90[e]	0.72[d]	0.74	−0.15	−0.75[e]	−0.29	0.77[e]
5	−0.10	0.63	0.90[d]	0.90[e]	..	0.70[d]	0.49	−0.25	−0.71[d]	−0.36	0.86[e]
6	0.18	0.66	0.72	0.72[d]	0.70[d]	..	0.42	−0.14	−0.85[e]	−0.41	0.56
7	0.78[d]	0.72	0.84	0.74	0.49	0.42	..	−0.21	−0.82[d]	−0.20	0.30

Data in this table were supplied by the corresponding laboratory designations.

Laboratory 2 Madelyn Womack (*5*), Human Nutrition Research Branch, Agricultural Research Service, Agricultural Research Center, U.S.D.A.

Laboratory 3 C. M. Lyman (*7*), Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station.

Laboratory 4 J. R. Couch (*7*), Department of Poultry Husbandry, Texas Agricultural Experiment Station.

Laboratory 5 C. R. Grau, Poultry Husbandry Department, University of California.

Laboratory 6 A. B. Watts, Poultry Industry Department, Louisiana State University.

Laboratory 7 Millard J. Horn (*5*), Human Nutrition Research Branch, Agricultural Research Service, Agricultural Research Center, U.S.D.A.

[a] Determined at Southern Utilization Research Branch, Agricultural Research Service, U.S.D.A.

[b] These data were modified to agree with corrected data (*5*).

[c] Data reported at conference of cooperators on cottonseed meal nutrition.

[d] Probability of 0.05.

[e] Probability of 0.01.

growth, chick growth, and microbiological methods. The table includes the maximum cooking temperatures and other constants which have been determined on the meals.

In order to simplify direct comparison of results of protein quality measurement from the different laboratories, an index of protein quality has been calculated. This value, based on meal *S*676 as a standard, is determined by dividing the response on each meal by the corresponding response on meal *S*676, and multiplying by 100. Some modification was necessary in calculating the values in the second column of Table III from the responses in Table II because meal *S*676 was fed in only two of the four trials. However, casein was fed in all four trials and the response ratio between casein and meal *S*676 was 85% in two trials; hence, a value of 85% of the casein response has been assigned to meal *S*676 in all four trials and the values in column two of Table III calculated in this manner. When the meals were fed in more than one trial an average was taken. All of the values for index of protein quality in Table III are expressed as ratios based on the same standard.

In comparing these protein quality values on the different meals the previously mentioned relationship between the meals, which carry the letter *T* in their numbers, again shows that the plant-run (*T*1) meals gave less response than the *T*2 and *T*3 samples. Although this is true to some extent with the other methods, there are exceptions in all of the other methods. The notable exceptions are meal 12*T*1, which three other methods rate higher than some of the *T*2 and *T*3 meals, and meal 15*T*3, which most of the other methods rate lower than some of the *T*1 meals. However, the major disagreement by the rat repletion method with others is the low value for meal *S*714.

In order to further study the data comparing the rat repletion method with others and other constants that had been determined on the meals, correlation coefficients were calculated and are included in Table III. The correlation coefficient for any desired comparison may be selected accordingly—e.g., to compare rat repletion in laboratory 1 with chick growth in laboratory 4, follow down column 2 to the value in line 4 of the correlation table, or down column 5 to line 1 of the correlation table. Coefficients for the other constants are found in the same way, but only single entries are available as compared to the double entries for the index of protein quality. Although in some cases the number of pairs of values were not large, these coefficients show some interesting relationships.

Rat repletion was positively correlated with all methods except one, but significantly correlated only with the microbiological method. Most significant correlation among the methods appears to be between those using chick growth. The coefficient of 0.89 for the two rat growth methods is high, but values were too few for significance. The most significant effect appears to be the negative correlation of all the methods with bound gossypol. This is in agreement with the conclusion of Lyman, *et al.* (*7*), who showed high negative correlation of nutritive value and total gossypol on these same meals. The uniform negative correlation of all the methods with free gossypol, though not statistically significant for any one method, is of considerable interest, especially because the free gossypol was thought to be low enough not to constitute a factor in nutritive value of the meals.

Negative correlation of all methods with cooking temperature, though not significant for any one method, probably means that better protein quality is

produced at lower temperatures provided other factors are favorable. A contributing factor is that higher coefficients would have been obtained had a temperature value been available for meal 5676.

The relatively high positive correlation of most of the methods with solubility of nitrogen in dilute sodium chloride indicates that this constant is of value in measuring protein quality. However, Lyman (*7*) found certain meals had extremely low protein solubility, and yet had excellent protein quality as determined by both chick and rat feeding tests.

Evaluation of Rat Repletion Method

The principal criticism of the rat repletion method, based on these experiments, is failure to give the desired precision. This, of course, is a general fault with protein quality measurement. Another objection is the necessary use of much arbitrary technique because optimum points in the method have not been fully established—for example, degree of depletion of the rats, length of depletion period, and daily level of nitrogen. Frost and Sandy (*4*) found that more significant differences could be obtained with a level of 0.24 gram of nitrogen than with 0.12 gram, but this did not establish an optimum level. It is possible that precision could be improved with a higher level of nitrogen than used here with these meals.

Because the test products and the reference protein are fed at the same nitrogen level, determination of nitrogen in both the test products and the reference protein is necessary. The method has the following marked advantages.

1. Effects of palatability of diet are eliminated. 2. Effects of unequal protein intake are avoided. 3. Advantages of economy of time—rats may be prepared for assay usually in three weeks, the actual assay requiring only 10 days. Because it is not necessary to use rats of exact weight and age, the work can be made to fit into other schedules. 4. A relatively small amount of sample is required, equivalent to approximately 12.5 grams of nitrogen. 5. The same rats may be used several times with no more than a 10-day interval between tests.

Summary

Data are reported from the application of a rat repletion method to estimation of protein quality in 12 cottonseed meals used in a collaborative study of the effects of processing methods on the nutritive properties of cottonseed meal. Mean gain in body weight by groups of protein depleted rats during a 10-day repletion period was used as the criterion of response for estimating protein quality. The meals were tested in four feeding trials and mean gains on the 12 meal samples ranged from 13.6 to 28.8 grams with calculated least significant mean differences (P = 0.05) ranging from 2.7 to 3.6 grams. Results with the rat repletion method are compared with those obtained on the same samples by other laboratories using other biological methods.

Data are presented to show correlation existing between the rat repletion method and other measurements of protein quality. Significant negative correlations were found between the rat repletion method and bound gossypol content of the meals. Negative correlation between all protein quality methods and free gossypol, though not statistically significant for any one method, indicates that free gossypol in amounts below 0.05% has an adverse effect on growth.

Acknowledgment

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FERMENTATION ACCELERATOR

Dried Activated Sludge as a Fermentation Accelerator

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IT HAS BEEN FOUND that dried activated sludge and several preparations derived from this material are activators in the fermentation of various sugars to ethyl alcohol by yeast. (The material studied in detail here was the dried activated sludge produced by the Sewerage Commission of the City of Milwaukee at its plant in Milwaukee, Wis., and sold under the trade name of Milorganite.) The addition of comparatively small amounts of these activators results in a considerable decrease in the fermentation time and, in many cases, in an increase in the amount of carbon dioxide evolved. It is this acceleration of the rate of fermentation which is designated as "activation."

Dried activated sludge has not heretofore been used as a constituent of fermentation media either as a source of nutrients or activators for fermentations by yeast. There have been a number of preparations described previously for promoting or activating a yeast fermentation. Typical materials discussed are an aqueous extract of soybeans (*14*), acid or alkaline hydrolyzates of scleroproteins (*6*), corn and wheat proteins (*7*), alkaline earth hydroxides (*11*), phosphoric acid hydrolyzates of protein (*8*), acid hydrolyzates of animal protein (*2*), bran infusion (*4*), activated char containing protein material in its pores (*9*), activated carbon (*15*), amino acids and proteins (*13, 27*), ethanolamine (*26*), sodium salts of the fatty acids (*10*), an aqueous corn extract (*25*), an aqueous extract of liver (*24*), and a cozymase derivative (*12*). These activating substances have been known to the fermentation industry for some time, yet none of these has found application in industrial practice. This is probably due to the fact that many are inactive in small amounts, and the large amounts necessary to give the desired effect introduce a prohibitive cost. Also many of these substances are activators when the yeast is fermenting pure sugar solutions, and they no longer show activation if introduced into grain or molasses mashes used industrially. In

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