COTTONSEED MEAL AND GOSSYPOL TOXICITY STUDIES WITH RUMINANTS AND NON-RUMINANTS

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Submitted to the Faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
August, 1965

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Thesis Approved:

Thesis Adviser

ACKNOWLEDGMENTS

The author expresses his appreciation to Dr. A. D. Tillman, Professor of Animal Science, for his counsel and guidance during the course of this study and in the preparation of this thesis.

Appreciation is also extended to Dr. J. V. Whiteman, Professor of Animal Science, Dr. N. N. Durham, Professor of Microbiology, Dr. W. S. Newcomer, Professor of Physiology and Pharmacology, and Dr. J. E. Webster, Professor of Biochemistry, for their suggestions in the preparation of this thesis.

Further acknowledgment is due Mrs. Jane Witte, Laboratory Technician, Mr. Daniel Brittain, Student Animal Husbandman, Dr. R. J. Panciera, Professor of Veterinary Pathology, and Dr. E. C. Nelson, Assistant Professor of Biochemistry, for their assistance during this study.

Special recognition is extended to the wife of the author, Sara, for her understanding and assistance during the course of this study.

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CHAPTER I

INTRODUCTION

Cottonseed meal, and its use as a protein source, has been the subject of study for many years. Each species of animals has its own characteristic response when fed cottonseed meal; in general, the ruminant nutritional problem is concerned with nitrogen solubility of the cottonseed protein, the non-ruminant with gossypol, the toxic, yellow pigment of cottonseed, and the poultry industry is concerned with discoloration of eggs laid by hens fed cottonseed meal.

Reducing the nitrogen solubility will increase nitrogen retention of proteins fed to ruminants; nitrogen solubility can be reduced by applying heat and moisture. Sherrod and Tillman (1962, 1964) found that as the length of autoclaving time of cottonseed meal increased, there were corresponding decreases in nitrogen solubility, protein digestibility, and urinary nitrogen. Nitrogen retention, however, appeared to be maximum when the meal was heated for less than 90 minutes. Thus, two trials were conducted to determine the optimum length of time to autoclave or steam cottonseed meal in order to obtain maximum nitrogen retention in sheep.

Ruminants can be fed large amounts of raw cottonseed meal, which contain high levels of free gossypol, without any apparently adverse effects while non-ruminants fed small amounts of unheated cottonseed meal readily succomb to the toxic effects of the gossypol. The mechanism whereby gossypol is detoxified in the ruminant and that of gossypol action in the non-ruminant remain unknown. Therefore a series of trials was conducted

to determine the physiological and histological effects of injected gossypol in ruminants, the hematological effects of gossypol in rats, and the effect of iron, hexahomoserine, lysine, and heat treatment of cottonseed meal of gossypol toxicity in rats.

CHAPTER II

REVIEW OF LITERATURE

Effect of Nitrogen Solubility on Nitrogen Retention by Sheep

Nitrogen metabolism in the ruminant is dependent primarily on the ability of rumen micro-organisms to enzymatically convert the various protein and non-protein nitrogen (NPN) sources to simple amino acids and ammonia. Some of the important factors regulating the rate and degree of this conversion are nitrogen source such as NPN or protein, protein solubility, and type of protein utilized by the animal. The protein and NPN degradation products can be utilized for synthesis of microbial protein which are later digested by the host animal.

Although Sym (1938) was the first to report that ruminal contents possessed strong proteolytic activity, Pearson and Smith (1943) were the first to show that both breakdown and synthesis of protein occurred in the rumen. McDonald (1948, 1952, 1954) established that ammonia was a major end product of the degradation of proteins and was the main component of the NPN fraction, that the breakdown of protein in the rumen was of quantitative importance to the animal, and that ammonia was available for protein synthesis during bacterial growth. That peptides and amino acids occurred as intermediates in this process was shown by Annison (1956).

The rate of ammonia production in the rumen and the subsequent assimilation of microbial protein exert important influences on the nutritive value of protein supplements for ruminants. Major factors influencing

the balances of these processes appear to be solubility of dietary protein and amount and type of carbohydrate in the ration. The importance of protein solubility in ruminant nitrogen utilization was first shown by Pearson and Smith (1943), who found that proteolysis occurred when highly soluble casein or gelatin was incubated with rumenal contents; however, when insoluble blood meal was used as the protein source, a net protein synthesis was noted. McDonald (1952), Annison et al. (1954), and Annison (1956) noted that proteins such as casein or ground-nut meal yielded high ruminal ammonia levels, whereas the production of ammonia from relatively insoluble proteins such as zein was very low. In extending these studies, Chalmers and Synge (1954) observed that total nitrogen retention favored the less soluble proteins. When carbohydrate supplements were added to casein or casein hydrolysate rations, ammonia concentrations were reduced due to the carbohydrates furnishing carbon fragments necessary for synthesis of microbial protein.

Lewis and McDonald (1958), el-Shazley (1958), and Karr et al. (1963a, 1963b) studied fermentation products of various combinations of carbohydrate and protein feeds. They noted that nitrogen retention increased as ammonia release decreased or as carbohydrate source became more soluble, and concluded that maximum utilization of protein supplements occurred when a carbohydrate material which was fermented at a comparable rate was also fed. El-Shazley (1958) also concluded that ruminal ammonia production was directly related to the nitrogen solubility of the protein supplement, but that solubility should not be used as the sole criterion for protein evaluation, since highly insoluble proteins may have a low nutritive value for ruminants even though ruminal ammonia levels are low. Also, protein digestibility in his trials was a poor criterion for evaluating a protein supplement for ruminants as highly digestible supplements

promoted rapid ammonia production in the rumen.

Using *in vitro* techniques, the effects of carbohydrate solubility on the net changes of ammonia content in rumen liquor were studied by Phillipson *et al.* (1959). During the incubation of rumen fluid from sheep fed grass or hay with protein supplements, total ammonia increased, whereas a decrease was noted when sheep were fed hay with other beet fodder or a mixture of flaked maize and maize gluten meal. Since net protein synthesis occurred when the ammonia production was lowest, the authors concluded that the addition of soluble carbohydrates to high protein rations depressed ammonia nitrogen concentration due to an increase in bacteria capable of assimilating ammonia nitrogen. Little *et al.* (1963) studied effects of nitrogen solubility on rate of *in vitro* ammonia production and found no definite correlation when nitrogen solubility was determined using dilute sodium hydroxide or distilled water; however, the correlation between solubility of nitrogen in rumen fluid and ammonia production was high (0.93). Nitrogen solubility had little effect on protein digestibility or nitrogen retention when rations containing carbohydrate sources of low solubility were fed; however, cellulose digestibility decreased as nitrogen solubility decreased. Thus, they postulated that a readily available nitrogen source stimulated bacterial growth, thereby enhancing rumen function, a requirement for maximal utilization of relatively insoluble protein supplements.

The effects of protein processing conditions on nitrogen metabolism in the rumen as related to the whole animal, commenced with the work of Miller and Morrison (1944). Comparing the nutritive value of ground soybeans, solvent-extracted soybean oil meal, and solvent-extracted soybean oil meal heated for 70 min. at 250° F., they found that protein digestibility, dietary nitrogen retention, and nitrogen retention as a percent

of digestible nitrogen was superior in the heat processed solvent-extracted soybean meal as compared to the non-heated solvent-extracted soybean meal. In all tests the raw ground soybeans yielded the poorest results.

When Cuthbertson and Chalmers (1950) fed casein supplements to ewes, it was noted that those ewes on high roughage rations excreted 60 to 70% of the total intake of nitrogen, whereas those on high levels of concentrates excreted a lesser amount of nitrogen. When casein was heated, Chalmers et al. (1954) found that nitrogen retention was improved even though protein digestibility and ruminal ammonia concentrations were reduced. Thus, a greater efficiency in the conversion of dietary nitrogen to microbial protein occurred. Casein, introduced directly into the duodenum, resulted in better nitrogen utilization. Supplementing a purified lamb ration with either urea, gelatin, casein, soybean protein, or bovine blood fibrin, Ellis et al. (1956), noted that nitrogen digestibilities were not different and ruminal ammonia concentrations varied little, with the exception of urea, which yielded higher values three hours after feeding than did the other supplements. Nitrogen retention from urea was significantly less than from all other sources. Gelatin promoted less nitrogen retention than did casein, which yielded lower retention than soybean protein or bovine blood fibrin. Digestibility, as the sole criterion in protein evaluation, does not reflect its true nutritive value for ruminants.

Whitelaw et al. (1961) fed calves rations containing 16.5% protein prepared from commercial groundnut meal, heat-treated groundnut meal and fish meal. Respective nitrogen solubilities of the three meals were 47.6, 39.6, and 17.6%. Blood urea concentrations were directly related to nitrogen solubilities and confirmed the work of Lewis (1957) and Drori and

Loosli (1961), who showed that the magnitude and direction of postprandial changes in blood (blood urea) were indicative of the changes in levels of rumen ammonia.

Woods et al. (1957, 1958) fed lambs cottonseed meal, sesame meal, and soybean oil meal at protein levels of 4, 6, and 8% of the ration. In all trials, digestibility and nitrogen retention of the sesame meal and soybean oil meal was superior to those of cottonseed meal. These results were explained on the basis of a high crude fiber content of the cottonseed meal rather than nitrogen solubility, although those lambs fed the cottonseed meal rations had a greater rate of gain than those lambs fed either sesame or soybean meal.

Woods et al. (1962) compared two cottonseed meals differing widely in nitrogen solubility to a soybean meal of high nitrogen solubility. Protein digestibility by sheep was directly related to protein solubility and urinary nitrogen was inversely related. Nitrogen retention, however, appeared to be dependent on protein source rather than solubility, although within a given protein source, protein of low solubility gave the best nitrogen retention. Tagari et al. (1962) and Sherrod and Tillman (1962) compared solvent-extracted soybean and cottonseed meals subjected to various heat treatments. Protein solubility was lowered in direct proportion to the amount of heat applied. Protein digestibility and nitrogen retention varied inversely and in a linear fashion; whereas urinary nitrogen, ruminal ammonia levels, and blood urea concentration varied directly with nitrogen solubility. Sherrod and Tillman (1964) autoclaved cottonseed meals for 0, 60, 120, 180, and 240 min. and found that nitrogen solubility and fecal nitrogen loss decreased, and protein digestibility and fecal nitrogen increased in a linear fashion as autoclaving times increased. Total nitrogen retention, however, was maximal when the meal was

autoclaved for 60 min. They postulated that continued heating beyond a given time results in decreased nitrogen retention, because at some point urinary nitrogen loss would not be decreased sufficiently to compensate for increased fecal nitrogen loss.

Chalupa et al. (1964) fed steers a low-nitrogen, semi-purified diet with varying combinations of urea and corn gluten meal to supply either 0, 46, or 92% of the animals' nitrogen intake as urea. They noted that fecal and urinary excretions increased with increasing amounts of urea nitrogen in the diet. Although microbial activity was enhanced in the urea-containing diets, nitrogen retention was negative due to a high ruminal ammonia concentration. They suggest that to obtain maximal protein utilization by ruminants, one must have a caloric density which coincides with nitrogen solubility of the protein supplement.

Physiological and Pathological Effects of Gossypol

The literature dealing with the physiological activity of gossypol must be interpreted with care, since many workers have not considered the effect of dietary protein level, physical state of administered gossypol, variance in the concentration of other toxic materials, and the degree of inactivation of the gossypol (Adams, et al., 1960).

The active form of gossypol is usually referred to as "free gossypol" or gossypol and gossypol-like pigments extractable from the cotton seed with aqueous acetone followed by spectrophotometric determination of the aniline complex of the extract. The inactive form of gossypol is referred to as "bound gossypol" and is determined by subtracting the value for free gossypol from the value obtained for total gossypol. Total gossypol is that amount of gossypol or gossypol-like pigments obtainable only by acid treatment of the cottonseed product, or by some other treatment harsh

enough to release all the gossypol (Pons et al., 1958; Smith, 1958).

Withers and Carruth (1915) were the first to report that gossypol was the toxic component of cottonseed meal. They separated gossypol from the cotton seed and found it to be toxic to rabbits. A positive correlation between the toxicity of raw cotton seeds and their gossypol content was reported by Schwartze and Alsberg (1923, 1924). Occurrence of toxic symptoms vary with the type of animal and level of gossypol fed (Eagle, 1960). Although cattle can consume large quantities of raw cotton seeds continuously for a long period of time without the slightest indications of any ill effect, animals with simple stomachs will die within a short time if they eat large amounts.

Alsberg and Schwartze (1919) noted that subacute cases of gossypol toxicity result in death from pulmonary edema, while chronic cases produce pronounced cachexia and inanition. Menaul (1922) demonstrated that gossypol toxicity was greatly increased when it was introduced directly into the bloodstream, 0.05 gm. being fatal to a rabbit in 4 min. The intraperitoneal administration of 25-35 mg. per kg. of body weight to rats caused edema and intestinal inflammation. Odell et al. (1964) orally administered formyl-^{14}C labeled gossypol to chickens until total activity was approximately one μc. Total recovery of administered ^{14}C activity in the tissues and feces ranged from 95 to 102%, with approximately 95% of the recovered activity found in the feces. Most of the absorbed portion was found in the liver, with lesser amounts in the muscle, blood, and kidneys.

Gossypol toxicity symptoms in rats are emaciation, diarrhea, and a loss of hair around the head and neck (Gallup, 1927a; Clark, 1929). Chronic gossypol intoxication in cats, pigs, and rabbits results in a loss of appetite, paralysis with nerve degeneration, shortness of breath,

cardiac hypertrophy, edema of the lungs, and an effusion into the serous cavities (Schwartze and Alsberg, 1924; Harms and Holley, 1951).

Smith (1957) and Rigdon et al. (1958, 1959) found that chicks and pigs with inhibited growth rate due to gossypol toxicity have hemolytic anemia, and a ceroid-like pigment in the intestines, spleen, and liver. Later this pigment passes into muscular tissue, where it causes a necrosis of the striated muscle and cardiac tissue. Injured muscle fibers disintegrate and the debris is phagocytized by macrophages. True mechanism of removal from the hepatic cells of the liver is not yet known, although a small portion is excreted via the biliary tract.

A common effect of gossypol toxicity is cardiac irregularity, thus death is generally caused by circulatory failure (Alsberg and Schwartze, 1919). Menaul (1922, 1923) found that gossypol prevented the liberation of oxygen from oxyhemoglobin and had a hemolytic effect on erythrocytes. Harms and Holley (1951) fed gossypol to rabbits and pigs, and noted the development of a hypoprothrombinemic condition. Comparing gossypol to dicumarol as an anticoagulant, the authors concluded that gossypol was not as effective as dicumarol, but that it was more rapid in reacting. Clawson et al. (1962) observed a significant trend toward lowered hemoglobin in the blood of pigs fed rations containing high levels of gossypol. Gossypol toxicity thus places an extreme burden on the respiratory and circulatory organs owing to a reduced oxygen carrying capacity of the blood.

Detoxification of Gossypol and Cottonseed Meal for Non-Ruminants

Many methods of gossypol detoxification have been tried with varying degrees of success. Extraction of gossypol from the meal with suitable organic solvents has been used since Carruth (1918) first used ether.

Since then, many other solvents including acetone, petroleum ether, butanone, and chloroform have been used. Many laboratory procedures utilizing organic solvents will extract most of the free gossypol, but will not remove any bound gossypol (King et al., 1957). Commercial procedures for reducing the free gossypol content in the meal are limited, because all procedures require large amounts of the solvents and the application of mechanical action for the purpose of rupturing the pigment glands (King et al., 1961).

Many authorities consider that the reduction of free gossypol to 0.04% of the meal provides one which does not cause toxicity when incoporated into a poultry or swine ration. Hence, a "degossypolized cottonseed meal" refers to a meal containing 0.04% or less of the pigments measured as free gossypol (Swenson et al., 1942; Couch et al., 1955; Heywang and Bird, 1955). Commercial preparation of such meals is accomplished by prepressing, followed by solvent extraction (King et al., 1961).

Among the earliest and most successful methods of gossypol inactivation or destruction, have been autoclaving and steaming either the raw cotton seeds, or the resultant meal after pressing out the oil. Withers and Carruth (1918) noted that the toxic property of cotton seeds was reduced by steam cooking. Dowell and Menaul (1923) autoclaved cottonseed meal at 15 lb. of steam pressure for 20 min. and fed it to pigs in such a manner that each consumed cottonseed meal equivalent to 1.33% of its body weight daily. Pigs fed non-autoclaved cottonseed meal died, whereas those fed autoclaved meal survived. Gallup (1926, 1927b) found that dry-heating cotton seeds caused a change in the form of gossypol (as determined by solubility in ether), but only slightly reduced toxicity. Heating moist seeds in an autoclave rapidly destroyed the gossypol and produced a non-toxic product. When fed to rats, the raw meal gave poor results (Gallup,

1927a) while the autoclaved meal gave good results. In experiments with pigs, Lyman et al. (1944) found that autoclaving cottonseed meal for short periods of time rendered it safe if the level in the total diet did not exceed 25%. These workers suggested that gossypol combined with the free amino groups of cottonseed protein, an idea first advanced by Carruth (1918).

Baliga and Lyman (1957) studied the nutritive significance of bound gossypol in cottonseed meal and found that, with the formation of a gossypol-protein complex, prepared by reacting pure gossypol with purified proteins, lysine availability decreased to about one-half of the original value (82.9 to 48.7%). When the bound gossypol was removed, lysine availability improved, indicating that the nutritive value of cottonseed proteins can be reduced by reaction with gossypol.

Baliga et al. (1959) and Conkerton and Frampton (1959) developed a procedure by which they could determine the free ε-amino groups of lysine in the various proteins. Their results indicated that when proteins were reacted with gossypol, the number of free ε-amino groups was significantly reduced. It was also noted that when the pH of the reaction mixture was increased, there was a reduction in the free ε-amino groups of lysine. Lyman et al. (1959) prepared protein-gossypol complexes and noted that the maximum number of free lysine ε-amino groups disappearing per mole of gossypol was two, indicating that both carbonyl groups of gossypol are linked to lysine. When gossypol combined with cottonseed protein with or without heat, peptic and tryptic digestion decreased markedly, an observation first noted by Jones and Waterman (1923) and Meinke (1952). Autoclaving purified cottonseed protein, free of carbohydrates, gossypol, or phytin, did not reduce the in vitro digestibility with pepsin or with trypsin. These results help to explain the findings of Lyman et al. (1953),

Wallace et al. (1955) and Hale and Lyman (1961) who found that lysine supplementation improved gains and feed efficiency of non-ruminants. Hale and Lyman (1962) and Clawson et al. (1961) noted that even though lysine improved growth rate, it did not protect against gossypol toxicity. Martinez et al. (1961) reported that the nutritive index of cottonseed meal was highly correlated with its free epsilon-amino groups of lysine, but poorly correlated with its total lysine content. Cabell and Earle (1956) and Kornegay et al. (1961) concluded that protein sources containing high levels of lysine, detoxified gossypol better than low lysine containing protein sources. According to Cabell and Earle (1956), and Hale and Lyman (1957, 1962) animals were able to tolerate higher levels of free gossypol as total protein content of the ration increased. These results indicate that as cottonseed meal is steamed or autoclaved, free gossypol combines with the ε-amino group of lysine, yielding a non-toxic meal, but one lower in protein quality.

Dietary iron was first noted to alter the toxic effects of ingested gossypol by Withers and Ray (1912). By adding ferrous sulfate to a cottonseed meal ration for rabbits, the toxic effects of cottonseed meal were counteracted. Later, Withers and Carruth (1917) fed various iron compounds such as iron citrate, ferric chloride, ferric oxide, or ferrous ammonium sulfate, and concluded that iron was the beneficial agent. Deaths were either postponed or averted in all treatments and the animals made better gains. Gallup (1928, 1931a) and Stevenson et al. (1965) noted that due to its insolubility, addition of ferric oxide to cottonseed meal diets was of no value. All other ferrous or ferric compounds allowed the animals to make nearly normal growth, and gains among iron sources were not significantly different. Hale and Lyman (1962) noted that iron salts at 500 ppm supplemented the protective action of animal proteins, however, at higher

levels of iron supplementation, a poorer growth rate was noted. Clawson et al. (1962) found that as the gossypol content of the pig rations increased, liver gossypol content increased and liver iron content decreased proportionally. When gossypol was injected into rats, dietary iron compounds were found to be without beneficial effect. Iron-dextran injected into the peritoneal cavity simultaneously with corn oil containing gossypol, was partially effective in preventing the growth depression resulting from injected gossypol but was not completely effective in overcoming the death losses which resulted. Since Gallup (1931a) had shown the reaction between gossypol and iron to be of a quantitative nature, these investigators were of the opinion that an insoluble iron-gossypol complex was formed, thereby preventing the absorption of gossypol from the gastrointestinal tract.

The inclusion of sodium bicarbonate and calcium carbonate in the diet of experimental animals has been reported by Gallup and Reder (1934, 1935, 1936) to lower the toxicity of the meal. These salts probably formed an alkaline medium in which gossypol was unstable.

Gossypol Toxicity in Ruminants

Cottonseed meal has been utilized as a protein feed for livestock since the early part of the 19th century. Success as a feed, however, was not immediate, for as early as 1845 a report by Voelker indicated that cottonseed meal had toxic properties. Other reports of cottonseed meal toxicity in livestock soon followed (Emery, 1894; Dinwiddle and Short, 1912; Moore, 1914; and Combs and Curtis, 1921). In contrast, Bennett and Menke, (1890) and Curtis (1895) did not find cottonseed meal to produce detrimental effects when fed to ruminants. Nameche (1900) concluded that the harmful effects on ruminants which were noted when cottonseed meal was

fed were due to carlessness of feeders, ignorance on the use of cottonseed meal or a lack of cleanliness. Gallup (1931b) found that cottonseed meal, injury was caused by a nutrient deficiency; the nutrient being vitamin A. Consequently, when a source vitamin A was added to cottonseed meal rations good results were obtained.

The mechanism whereby gossypol is detoxified by the ruminant is unknown. No one has isolated microorganisms which will utilize gossypol. Reiser and Fu (1962) found that the total gossypol content did not decrease when rumen liquor was incubated with rumen microorganisms either aerobically or anaerobically, and concluded that gossypol was detoxified by some other means. As the free gossypol content decreased, along with a corresponding disappearance of free lysine, they concluded that ruminants detoxified gossypol by binding it, in the rumen, to soluble proteins; this bond remaining permanent during digestion. This hypothesis has support from Tillman and Kruse (1962), who, using in vivo techniques with sheep, noted that heating alone or the addition of 1.0% gossypol-acetic acid to protein had no significant effect on the digestibility of the protein. However, the combination of adding gossypol, then heating the protein, significantly lowered protein digestibility.

CHAPTER III

EXPERIMENT I

Previous ruminant studies in this laboratory (Sherrod and Tillman, 1962, 1964) have shown that as nitrogen solubility of cottonseed meal was decreased by autoclaving, fecal nitrogen increased and urinary nitrogen decreased in a linear fashion. Total nitrogen retention, however, was greatest when the meal was heated for periods of time less than 90 minutes. The purpose of the first trial was to determine the optimal length of autoclaving time required to obtain a nitrogen solubility giving maximal nitrogen retention when cottonseed meal is fed to sheep. After attaining the optimum autoclaving time in the first trial, a second trial was initiated to determine if steaming cottonseed meal at atmospheric pressure would reduce nitrogen solubility in the same manner as autoclaving and produce comparable nitrogen retention data when total heat inputs of the two methods are comparable.

Experimental Procedure

Trial 1.

A cottonseed meal was prepared by extraction with cold hexane with no heat being applied at any time during extraction. Portions of the meal were then subjected to autoclaving conditions of 121° C. and 1.05 gm./sq. cm. of pressure for 30, 45, 60, 75, and 90 min. To insure uniform heating, the meal was placed at a depth of 1½ cm. in metal pans lined with heavy paper. After autoclaving, the meals were dried, reground to their

original particle size and compared in semi-purified, iso-nitrogenous, and iso-caloric rations whose compositions are shown in Table I.

TABLE I

AVERAGE PHYSICAL AND CHEMICAL COMPOSITION OF RATIONS USED IN SHEEP DIGESTION TRIALS 1 AND 2

	Trial 1	Trial 2
Ingredients	%	%
Autoclaved cottonseed meal[a]	22.0	-----
Steamed cottonseed meal[a]	-----	22.0
Dextrose	17.8	17.8
Starch	17.8	17.8
Cottonseed hulls	8.9	8.9
Corn oil	2.0	2.0
Mineral mix[b]	5.0	5.0
Cellulose	26.4	26.4
Choline chloride	0.10	0.10
Vitamins A and D mixture[c]	0.03	0.03
Proximate composition, dry matter basis, %		
Organic matter	93.75	93.83
Ash	6.25	6.17
Crude protein	14.11	11.96
Ether extract	2.74	2.28
Crude fiber	24.98	27.02
NFE	51.92	52.57

[a]See text for description of meals.
[b]Oltjen et al. (1962).
[c]Mixture contained 20,000 I.U. and 2,500 U.S.P. units/gm. of vitamins A and D, respectively.

Fifteen crossbred wethers averaging about 36 kg. were used in a completely randomized design with three animals per treatment. After the first collection period was completed, the animals were given a standard ration during a 30-day rest period. The experiment was then repeated, the sheep being rerandomized to treatments with the restriction that no animal was placed on the treatment it had received during the first collection period. For both trials, the animals were placed in metabolism stalls (Briggs and Gallup, 1949) for a 7-day adjustment period followed by

successive 10-day preliminary and 10-day collection periods.

Each lamb was fed twice daily a ration equal to two times maintenance as calculated from the equation, TDN in lbs. = $0.0436\ W_{Kg.}^{0.73}$ (Maynard and Loosli, 1956). Standard procedures for the collection of feces and urine as outlined by Tillman and Swift (1953) were used, with the exception that the wet weight of the feces was recorded daily and a 10% aliquot was frozen for analysis. Proximate analyses of feed, feces, and urine were determined by the methods of A. O. A. C. (1960). Nitrogen solubility of the cottonseed meal was determined by the method as described by Lyman et al. (1953).

Data were analyzed statistically by analysis of variance and the multiple range test (Duncan, 1955) and tested for linearity using the coefficients of orthogonol polynomials.

Trial 2.

Portions of dehulled cotton seeds were subjected to live steam at atmospheric pressure (95° C.) for 10, 20, 40, 60, 80, and 100 min., then extracted with cold hexane. A control or zero time sample of cottonseed meal was prepared by extracting the seed with cold hexane with no heat being applied at any time prior, during, or after extraction. All cotton seeds were then dried and ground to the desired particle size. Nitrogen solubilities of the cottonseed meals were 98.6, 83.3, 81.6, 80.5, 78.3, 74.8, and 73.5% respectively, for the 0, 10, 20, 40, 60, 80, and 100 min. of steaming. Semi-purified, and approximately iso-caloric and iso-nitrogenous rations were prepared for each of the meals for each of three collection periods. Physical and chemical compositions of the rations are shown in Table I.

Fourteen crossbred wethers averaging 34 kg. were used in a completely randomized design with two animals per treatment per collection period

until a total of three collection periods was completed. At the completion of each collection period, sheep were rerandomized to treatments with no animal receiving the same treatment it had previously received. After the sheep were placed in metabolism stalls for a 7-day adjustment period, three successive 10-day preliminary and 7-day collection periods followed, with neither rest nor adjustment periods following the first and second collection periods.

Level and method of feeding, feces and urine collection procedures, and chemical and statistical methods of analysis were the same as those outlined in Trial 1.

Results and Discussion

Trial 1.

Digestibilities of the proximate analysis components by sheep fed the autoclaved cottonseed meal rations are shown in Table II. All components of the various treatments were identical, with the exception of the cottonseed meals. No zero time cottonseed meal was used since Sherrod and Tillman (1962, 1964) found that autoclaving raw cottonseed meal improved nitrogen retention and the present arrangement of treatments also allowed more comparisons of autoclaved meals.

Digestibility of organic matter, crude fiber, and nitrogen-free extract was not significantly affected by increasing the autoclaving time. Since the major portion of the organic matter, ether extract, crude fiber, and nitrogen-free extract was added to the rations in the same physical form these findings are not unusual. Heating effect on cottonseed meal expressed as percent protein digestibility, was expressed by a quadratic equation ($P < .01$). The protein which was autoclaved 45 min. was significantly more digestible than the 30 ($P < .05$), 60 ($P < .05$), and 90 ($P < .01$)

TABLE II

EFFECT OF DIFFERENT AUTOCLAVING TIMES ON NITROGEN SOLUBILITY AND DIGESTIBILITY OF COTTONSEED MEAL BY SHEEP (TRIAL 1)

Component	Rations (length of time cottonseed meal autoclaved, min.) 30	45	60	75	90	Standard error of treatment means
	%	%	%	%	%	
N solubility	40.0	35.4	34.3	33.3	33.1	----
Organic matter	68.7	71.6	69.0	67.2	65.6	2.03
Protein[a]	51.2	60.1	53.4	56.0	47.8	2.16
Ether Extract[b]	78.2	86.4	81.2	78.4	70.8	3.98
Crude fiber	48.3	51.8	52.6	46.4	43.8	5.54
NFE	80.3	81.0	78.3	77.8	79.0	1.16

[a] $45 \geq 90$ ($P < .01$), $45 > 30$, $60 (P < .05)$, $75 > 90$ ($P < .05$). Orthoganol polynomial displayed significant ($P < .01$) quadratic effect.

[b] $45 > 90$ ($P < .05$).

min. autoclaved meals and the 75 min. autoclaved meal more digestible than the 90 min. heated meal ($P < .05$). These data indicate that autoclaving cottonseed meal for 45 min. allowed maximal protein digestibility and that autoclaving for 90 min. exerted a detrimental effect.

Nitrogen balance data are shown in Table III. Fecal nitrogen, expressed as a percent of nitrogen intake, decreased significantly ($P < .05$) as the meal was autoclaved from 30 to 45 min. Thereafter, autoclaving for longer times caused a significant increase in fecal nitrogen which reached the highest value (52.2%) at 90 min., and resulted in a significant quadratic effect ($P < .01$). This quadratic effect is in contrast to the results of Sherrod and Tillman (1962, 1964), which indicated that increased autoclaving time reduced protein digestibility and increased fecal nitrogen in a linear fashion. A possible explanation of the apparently divergent results involves a consideration of differences in intervals between autoclaving times: Sherrod and Tillman had long intervals of time,

45 min. in 1962 and 60 min. in 1964, whereas in the present trial, time intervals were only 15 min. Urinary nitrogen, expressed as a percent of nitrogen intake, was relatively constant for all treatments, indicating that heat treatment had little effect on urinary nitrogen excretion. Nitrogen retention, expressed as a percent of intake, showed that as heating time was increased from 30 to 45 min., retention increased from 25.0 to 32.5% but increasing the time of heating from 45 to 60 or 75 min. resulted in no further change. As time of heating was increased from 75 to 90 min., a significant ($P < .01$) decrease was noted. This quadratic effect ($P \ll .01$) indicates that for highest nitrogen retention, cottonseed meal should be autoclaved for at least 45 min. but not longer than 75 min.

TABLE III

EFFECT OF FIVE AUTOCLAVING TIMES ON NITROGEN UTILIZATION OF COTTONSEED MEAL BY SHEEP (TRIAL 1)

	Rations (length of time cottonseed meal autoclaved, min.)					Standard error of treatment means
	30	45	60	75	90	
Daily N intake, gm.	15.3	15.1	16.1	16.0	15.7	----
Fecal N, % of intake[a]	48.8	39.9	46.6	44.0	52.2	2.10
Urinary N, % of intake	25.7	27.6	25.7	24.0	28.8	2.61
N retained, % of intake[b]	25.0	32.5	27.9	31.9	19.0	2.87
N retained, % of absorbed[c]	47.8	54.1	49.3	56.6	38.5	4.94

[a] $45 < 90$ ($P < .01$), $45 < 30, 60$ ($P < .05$), $75 < 90$ ($P < .05$).

[b] $45, 75 > 90$ ($P \ll .01$), $60 > 90$ ($P < .05$). Orthoganol polynomial displayed significant ($P < .01$) quadratic effect.

[c] Orthoganol polynomial displayed significant ($P < .10$) quadratic effect.

Trial 2.

Results of this trial are shown in Table IV. The unheated meal had

the highest ($P < .05$) protein digestibility with no significant differences being noted between the heated meals; however, the effect of steaming on protein digestibility is expressed by a quadratic equation ($P < .05$). Figure I illustrates this effect graphically, where fecal nitrogen, as a percent of nitrogen intake, is plotted as a function of steaming time. When only the heated meals are considered, it can be seen that successive increases in steaming time resulted in an apparent decrease in fecal nitrogen loss, reaching its lowest point at 100 min., however, differences between the meals heated 10 or 100 min. were not significantly different ($P > .05$). The fact that there was no overlapping of values as heating time increased would further indicate that the mild heating obtained by steaming improved digestibility of the cottonseed meal protein. Urinary nitrogen loss was highest ($P < .10$) in the unheated meal. When considering only the heated meals, differences are not significant; however, it appears that there is an association of urinary nitrogen loss with time of heating, since these data are expressed by a quadratic equation ($P < .07$).

It is possible that an explanation of these results can be obtained by considering the relationship between heat and protein denaturation. Individual proteins may show differences in sensitivity or chemical changes to different methods of denaturation. Heat or high pressures, or a combination of both, will cause protein denaturation altering its solubility properties. Successive increases in autoclaving and steaming times decreased nitrogen solubility in a linear fashion; however, autoclaving reduced nitrogen solubility to 40% in 30 min., whereas steaming for 100 min. reduced solubility to only 73.5%. Cottonseed proteins apparently did not respond to the two types of heat treatments to the same degree. It is possible that the degrees of disruption and unfolding of the protein

TABLE IV

NITROGEN BALANCE DATA FOR SHEEP FED SEVEN STEAM HEATED COTTONSEED MEAL RATIONS

	Rations (Length of steaming, min.)							Standard error of treatment means
	0	10	20	40	60	80	100	
Daily D M intake, gm.	690.8	691.8	641.9	687.3	675.4	688.0	685.3	----
Daily N intake, gm.	13.6	12.7	12.5	12.4	13.0	13.1	12.8	----
Protein digestibility, %[a]	60.4	49.7	53.5	52.2	53.0	53.5	55.8	2.92
Fecal N, % of intake[b]	39.6	49.1	48.7	47.8	47.0	46.1	44.2	2.85
Urinary N, % of intake[c]	45.3	34.8	36.9	34.2	40.2	43.1	41.1	3.52
N retention, % of intake	15.1	16.1	16.9	18.0	12.8	10.9	14.7	4.10
N retention, % of absorbed	36.8	50.9	48.3	47.7	43.2	46.1	42.0	6.88

[a] 0 > all rest ($P < .05$). Orthoganol polynomial expressed by quadratic equation ($P < .05$).

[b] 0 < all rest ($P < .05$). Orthoganol polynomial expressed by quadratic equation ($P < .05$).

[c] 0 > all rest ($P < .10$). Orthoganol polynomial expressed by quadratic equation ($P < .07$).

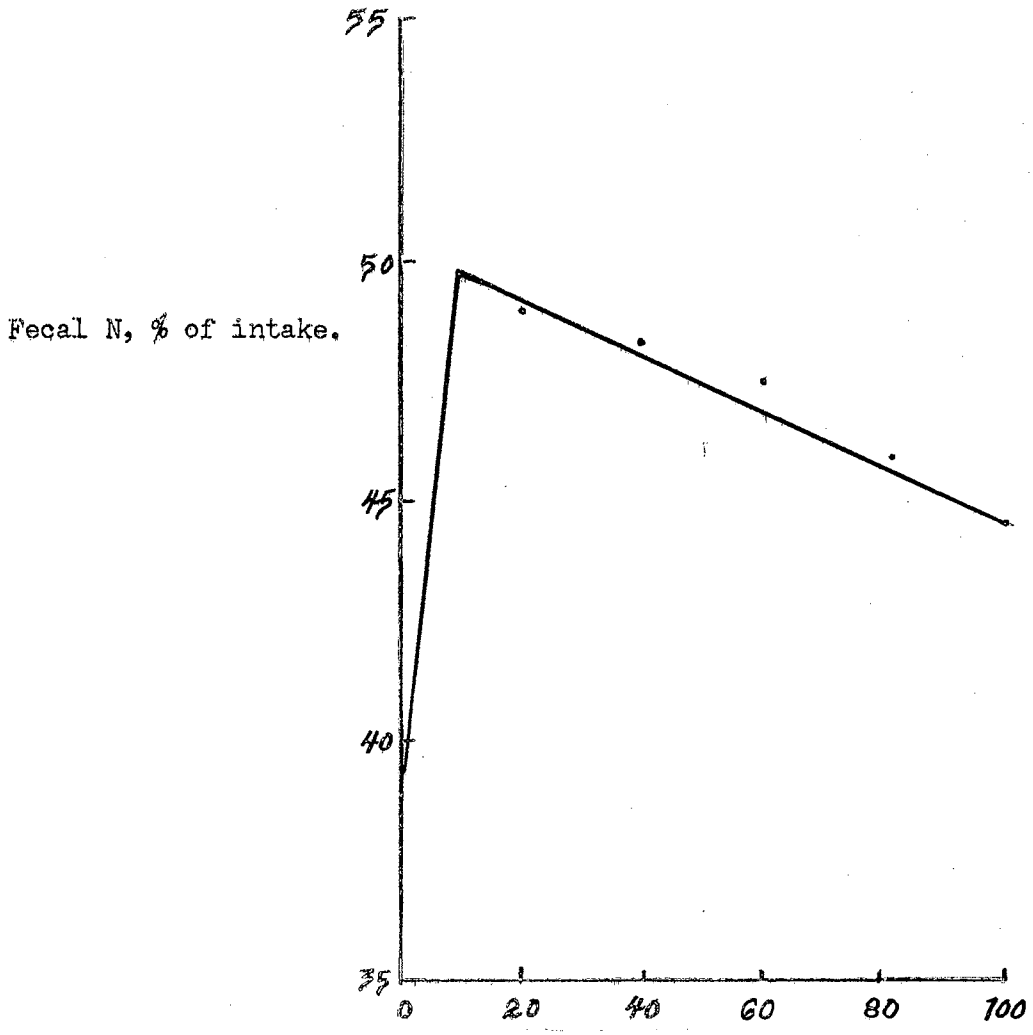

Figure 1

Effect of Steaming Cottonseed Meal on Fecal Nitrogen

molecules were not the same. Autoclaving for 75 min. apparently completely disrupted the tertiary structure of the protein molecule, leaving only insoluble peptide chains. As autoclaving time increased beyond 90 min., another step in denaturation evidently occurred, that being the breakdown of the secondary structure of the peptide, or cleavage of the hydrogen bonding, thus allowing the weaker bonds holding the peptide chains together to be broken (Fruton and Simmonds, 1958). As this occurs, nitrogen solubility would be expected to increase. Data indicating that such an explanation is true has been reported by Sherrod and Tillman (1964).

Protein denaturation also provides a possible explanation of the urinary nitrogen excretion pattern of the two trials. Denatured protein is usually more susceptible to attack by proteolytic enzymes than native protein; however, bacterial amino acid deaminases do not act on amino acid residues of peptides caused by protein denaturation, but will act on native protein (Fruton and Simmonds, 1958). Presumably, the amino acids of denatured protein are gradually released by bacterial peptidases and utilized for protein synthesis. Also, the possibility exists that certain peptides may be utilized by some organisms without prior hydrolytic cleavage of the peptide bonds. Thus, the native protein of non-heated cottonseed meals would be rapidly deaminated, while rate and degree of deamination would decrease in the heated meals. This would offer a possible explanation for the results of the two trials; in Trial 1, all meals were heated, therefore no significant differences were found, whereas in Trial 2, urinary nitrogen excretion by animals fed the non-heated meal was significantly ($P < .05$) greater than excretion by animals fed the heated meals. This explanation is in accord with results of Sherrod and Tillman (1962), where ruminal ammonia levels and plasma non-protein nitrogen levels were reduced in sheep fed autoclaved cottonseed meal as compared to

non-heated meals.

It is also postulated that the apparently divergent protein digestibility and fecal nitrogen patterns between the two trials can be explained by protein denaturation. Highly soluble, non-heated meals are rapidly deaminated, the resulting ammonia absorbed, and the excess excreted via the urine, thereby causing high apparent digestibility. On heating the meals, the initial denaturation would cause a decreased rate and magnitude of deamination, resulting in a lower apparent digestibility (Trial 2, Table IV). Thereafter, until the secondary structure of protein begins breaking down, as length of the heating time increases, the degree of denaturation and subsequent disruption of the protein molecule and release of peptides would also increase, thus increasing the ease whereby protein is utilized, and in this manner increasing apparent digestibility. When disruption of the secondary structure of protein begins, digestibility would become more difficult, hence total digestibility would decrease (Trial 1, Table III). Thus, in the protein denaturation process, a change in the direction of digestibility apparently occurs as each structural configuration becomes denatured. Maximum nitrogen retention then, occurs at that point in the curve where deamination is minimum and utilization of amino acids and peptides is maximum.

Summary

Thirty sheep were used in a digestibility and nitrogen retention trial to determine the optimum autoclaving time of cold-hexane extracted cottonseed meal required for maximum nitrogen retention. Portions of the meal were autoclaved under 1.05 kg./sq. cm. pressure for time periods consisting of 30, 45, 60, 75, and 90 min. It was found that between 45 and 75 min. of autoclaving time were required to produce a meal in which

maximum nitrogen retention occurred.

In Trial 2, 42 sheep were used to study the effect of reduced nitrogen solubility, as produced by steaming, on nitrogen digestibility and retention. Portions of cold-hexane extracted cottonseed meal were subjected to live steam and atmospheric pressure (95° C.) for 0, 10, 20, 40, 60, 80, and 100 min. Nitrogen solubility decreased only slightly, but in a linear fashion, as steaming time was increased. The unheated meal had the highest protein digestibility and urinary nitrogen excretion and lowest fecal nitrogen. The effect of heating on the three criteria is expressed by quadratic equations.

CHAPTER IV

EXPERIMENT II

The adverse physiological effects of gossypol on non-ruminants can be overcome by addition of a number of compounds. Among these are calcium and sodium salts (Gallup and Reder, 1935), soluble iron salts (Gallup, 1928; Stevenson et al., 1965), lysine (Lyman et al., 1953), and amines (Castillon and Altschul, 1950). Other successful methods which alleviate gossypol toxicity when feeding cottonseed meal to non-ruminants are autoclaving (Gallup, 1927a) and increasing the level of protein in the diet (Hale and Lyman, 1957). Menaul (1922, 1923) found that gossypol prevents liberation of oxygen from oxyhemoglobin and has a hemolytic effect on erythrocytes. Harms and Holley (1951) noted the development of a hypoprothrombinemic condition and Clawson et al. (1962) observed a significant trend toward lowered hemoglobin in the blood of pigs fed rations containing high levels of gossypol. In this series of trials, the effects of free gossypol and supplemental iron on gossypol toxicity and blood constituents of rats were studied.

Experimental Procedure

Trial 1.

Portions of dehulled cotton seeds were subjected to live steam at atmospheric pressure (95° C.) for 0, 10, 20, 40, and 80 min., then extracted with cold hexane in such a manner that no heat was applied to the meal during or after the extraction process. Total, free, and bound gossypol, nitrogen solubility, and ε-amino group data for the meals are shown in

Table V. A second factor, ferrous sulfate, was incorporated into the treatments at a 0.20% level, making a 5 X 2 factorial arrangement of treatments. Compositions of the rations used in this trial are shown in Table VI. The reported iron levels in the rations are calculated values, taking into consideration the amount of iron in the cottonseed meal, the amount added by addition of Salts XIV as the mineral source, and the amount added as ferrous sulfate.

Sixty weanling male rats of the Holtzman strain (62 to 79 gm.) were allotted to the 10 treatments in a completely randomized design. Rats were weighed initially, at 3-day intervals and at the completion of the 28-day trial. All animals were individually-fed ad libitum, and water was available at all times. On completion of the feeding trial, all rats were exsanguinated and the blood samples preserved using sodium citrate as the anticoagulant. Livers were excised and frozen. Response criteria were gain and feed efficiency, red blood cell count, hemoglobin content, percent packed cells, blood iron, liver iron, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and volume index. Statistical analysis was conducted using analysis of variance and orthoganol comparisons.

Total gossypol was determined by the method of Pons et al. (1958) and free gossypol by the method of Pons and Hoffpauir (1957). Nitrogen solubility was determined as described by Lyman et al. (1953). Epsilon-amino groups of lysine were determined by the method of Baliga et al. (1959)[a]. Blood and liver iron were determined by the method as described by Sandell (1959).

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[a]These samples were analyzed independently by Barrow-Agee Laboratories, Inc., Memphis, Tenn., B. C. White, chemist, and by C. Wamble, Cottonseed Products Research Laboratory, College Station, Texas.

TABLE V

GOSSYPOL, NITROGEN SOLUBILITY, AND ε-AMINO LYSINE DATA OF STEAM HEATED COTTONSEED MEAL USED IN RATIONS FOR RAT TRIALS 1 AND 2

Heat treatment, min.[a]	0	10	20	40	80
Particular components					
Total gossypol, %	1.51	1.56	1.32	1.31	1.44
Free gossypol, %	1.15	0.44	0.33	0.12	0.04
Bound gossypol, %	0.36	1.12	0.99	1.19	1.40
Nitrogen soluble in 0.02 N. NaOH, % of total	97.35	81.62	79.65	81.48	69.70
ε-NH_2 lysine, gm./16 gm.N	4.15	4.06	4.04	3.94	3.78

[a]Minutes steam heated (see text for details of processing).

TABLE VI

COMPOSITION OF BASAL RATIONS USED IN RAT TRIALS 1 AND 2

	Trial 1	Trial 2
Ingredients	%	%
Cottonseed meal[a]	10.00	20.00
Purified soybean protein	15.00	10.00
Corn oil	10.00	10.00
Salts XIV mineral mix	4.00	4.00
Corn starch[b]	33.20	30.20
Purified cellulose	2.00	2.00
Corn dextrose	25.00	23.00
B vitamins[c]	0.05	0.05
Choline chloride	0.20	0.20
Vitamins A and D mixture[d]	0.05	0.05
NaCl	0.50	0.50

[a]See text for details of processing conditions and lengths of heating time of the cottonseed meals that were incorporated into each of the rations.

[b]When used, $FeSO_4$ (0.20%) replaced an equal weight of corn starch.

[c]Oltjen et al. (1962).

[d]Mixture contained 20,000 I. U. and 2,500 USP units/gm. of vitamin A and D, respectively.

Trial 2.

Results from Trial 1 indicated performance and blood constituent interactions between heat and supplemental iron. This trial was conducted to study further the observed interactions. The steam heated cottonseed meals used in this trial (0 and 40 min.) were the same as those described in Trial 1. Total, free, and bound gossypol, nitrogen solubility, and epsilon-amino groups for the treatments are shown in Table V. Again, iron sulfate (0.20%) was used as a second factor making this experiment a 2 X 2 factorial arrangement of treatments. Composition of the basal ration is shown in Table VI.

Forty-eight male weanling rats of the Holtzman strain (53 to 75 gm.) were allotted to the four treatments in a completely random design. The rest of the trial was conducted the same as in Trial 1, with the exception that this trial was terminated at the end of 14 days rather than 28 days.

Results and Discussion

In Trial 1, there were no significant ($P < .05$) differences between the heated meals and level of iron; therefore, the effects of heating were obtained by pooling all heated treatments having the same level of iron.

Gains, feed consumption, and feed efficiencies of Trials 1 and 2 are shown in Tables VII and VIII, respectively. In both trials, the addition of either ferrous sulfate or the steaming of cottonseed meal resulted in significantly ($P < .01$) improved gains and feed efficiencies. The combination of iron and steaming did not further improve gains over either single factor. The heat by iron interaction which was found, was also present for feed consumption in Trial 2.

Data on blood constituents and iron levels in blood and liver are

TABLE VII

EFFECT OF FIVE HEAT TREATMENTS AND TWO IRON LEVELS ON PERFORMANCE OF RATS
(TRIAL 1)

Min. steamed	0		10		20		40		80		Standard error of treatment means
PPM Iron	175	900	175	900	175	900	175	900	175	900	
Init. weight, gm.	68.3	67.5	67.8	67.5	67.7	67.4	68.9	69.8	70.8	70.0	1.80
Av. total gain, gm.[a]	112.3	150.0	153.3	148.7	142.2	148.6	153.5	139.5	161.1	145.3	7.96
Av. Feed consumed, gm.[b]	468.2	514.3	547.8	530.2	523.8	546.7	540.8	534.0	559.0	531.3	19.70
Feed/gain, gm.[c]	4.2	3.4	3.6	3.6	3.7	3.8	3.6	3.9	3.5	3.7	0.09

[a]Av. total gain. Significant ($P < .01$) heat by iron interaction. Significant simple effects: 0 heat - Fe_{175} < heat - Fe_{175} ($P < .01$), 0 heat - Fe_{175} < 0 heat - Fe_{900} ($P < .01$).

	0 heat	Heat
Fe_{175}	112.3 **	** 152.5
Fe_{900}	150.0	145.5

[b]Feed consumption. Significant main effect. 0 heat < heat ($P < .01$).

[c]Feed efficiency. Significant ($P < .01$) heat by iron interaction. Significant simple effects: 0 heat - Fe_{175} > heat - Fe_{175} ($P < .01$), 0 heat - Fe_{900} < heat - Fe_{900} ($P < .05$); 0 heat - Fe_{175} > 0 heat - Fe_{900} ($P < .01$).

	0 heat	Heat
Fe_{175}	4.2 *	* 3.6
Fe_{900}	3.4 *	* 3.7

TABLE VIII

EFFECT OF TWO HEAT TREATMENTS AND TWO IRON LEVELS ON PERFORMANCE OF RATS (TRIAL 2)

	0		40		Standard error of treatment means
	215 (A)	900 (B)	215 (C)	900 (D)	
No. of rats	12	12	12	12	
Init. weight, gms.	61.8	62.1	63.0	62.4	0.87
Av. total gain, gms.[a]	6.9	51.8	58.6	61.7	4.54
Av. total feed consumed, gms.[a]	94.0	145.2	150.3	161.4	8.06
Feed efficiency, feed/gain gm.[a]	12.8	2.9	2.7	2.9	2.39

[a]Significant ($P < .01$) interaction. Simple effects: A vs. B ($P < .01$), A vs. C ($P < .01$), B vs. D (N.S.), C vs. D (N.S.).

shown in Tables IX and X, respectively. In Trial 1, hemoglobin and mean corpuscular hemoglobin were reduced ($P < .05$) by the non-heated meals. Percent packed cells, mean corpuscular volume, and volume index were lowered ($P < .05$) by the non-heated and non-supplemented meal. Iron or heat, alone or combined, in Trial 2, increased ($P < .05$) hematocrit, mean corpuscular volume, and volume index. Mean corpuscular hemoglobin concentration was greatest ($P < .10$) in rats fed the non-heated, non-supplemented rations. In both trials, supplemental iron increased ($P < .01$) liver iron.

Menaul (1923) postulated that gossypol caused death of non-ruminant animals by reducing the oxygen-carrying capacity of blood (anemia), and causing a hemolytic effect on erythrocytes, resulting in an increased burden on the respiratory and circulatory organs. The erythrocytes from those animals fed the untreated diet in Trial 1 were microcytic and hypochromic in nature, indicative of an iron deficiency; the result of defective iron absorption, of long continued use of a diet poor in iron, or due to excessive demands for iron (Wintrobe, 1961). Frequently, all these

TABLE IX

EFFECTS OF FIVE HEAT TREATMENTS AND TWO IRON LEVELS ON BLOOD CONSTITUENTS AND IRON LEVELS IN BLOOD AND LIVER (TRIAL 1)

Min. steamed	0		10		20		40		80		Standard error of treatment means
PPM Iron	175	900	175	900	175	900	175	900	175	900	
RBC $X10^6/mm^3$.	6.1	5.9	6.1	5.9	6.3	6.0	5.9	6.1	6.2	6.3	0.14
Hb., gm./100 ml.[a]	14.0	13.9	15.1	15.1	15.2	14.9	14.4	16.6	14.8	15.7	0.68
Packed cells, %[b]	31.2	33.6	33.7	33.6	34.8	33.8	33.8	35.0	34.1	35.3	0.57
Mean corp. vol., μ^3.[b]	51.0	56.9	55.1	57.1	55.9	56.9	57.9	57.6	55.9	56.0	1.19
Vol. Index[c]	90.4	103.4	97.7	102.7	96.3	104.0	104.3	102.8	99.8	96.0	3.73
Mean corp. Hb, $\mu\mu$ gm.[a]	22.9	23.6	24.7	25.6	25.9	25.0	24.5	27.3	24.2	24.8	1.02
Mean corp. Hb. conc.,%	44.9	41.5	44.8	44.8	46.3	44.0	42.4	47.4	43.5	44.2	1.62
Mg. Fe/100 ml. Blood	44.4	45.4	37.6	36.0	34.4	52.5	36.9	58.0	38.7	41.0	6.75
Liver iron, ppm[d]	352	621	594	648	532	624	589	723	557	556	82

[a]Hemoglobin and Mean corpuscular hemoglobin. Significant main effect: No heat < heat ($P < .05$).

[b]Packed cells and mean corpuscular volume. Significant ($P < .05$) heat by iron interaction. Significant simple effects: No heat - Fe_{175} < Heat - Fe_{175} ($P < .01$), No heat - Fe_{176} < No heat - Fe_{900} ($P < .01$).

[c]Volume index. Significant ($P < .05$) heat by iron interaction. Significant simple effects: No heat-Fe_{175} < Heat-Fe_{175} ($P < .05$), No heat-Fe_{175} < No heat-Fe_{900} ($P < .05$).

[d]Liver iron. Significant main effect: No iron < iron ($P < .05$).

	No heat	Heat
Fe_{175}	31.22 *	34.10
Fe_{900}	33.57 **	34.43

	No heat	Heat
Fe_{175}	50.96 *	56.19
Fe_{900}	56.87 **	56.91

	No heat	Heat
Fe_{175}	90.44 *	99.50
Fe_{900}	103.39 **	101.83

TABLE X

EFFECTS OF TWO HEAT TREATMENTS AND TWO IRON LEVELS ON BLOOD CONSTITUENTS AND IRON LEVELS IN BLOOD AND LIVER (TRIAL 2)

Length of steam heating, min.	0		40		Standard error of treatment means
Iron level, ppm.	215	900	215	900	
Red blood cells $X10^6/mm^3$.	4.8	4.8	4.7	4.5	0.19
Hemoglobin, gm./100 ml.	12.3	11.9	11.3	12.1	0.41
Packed cells, %[a]	25.4	27.4	27.6	28.8	0.68
Mean corpuscular volume, u^3.[b]	53.6	58.0	59.3	67.9	2.08
Volume index[c]	124.0	133.1	140.6	170.6	10.96
Mean corpuscular hemoglobin, uu gm.	25.9	25.7	24.6	28.6	1.24
Mean corpuscular hemoglobin conc., %[d]	48.4	44.1	41.2	42.1	1.55
Whole blood iron, mg./100 ml.	41.7	36.4	38.8	36.2	4.90
Liver iron, ppm.[e]	495	640	390	636	35

[a]Packed cells. Significant main effects: No Heat < Heat ($P < .01$), No iron < iron ($P < .05$).

[b]Mean corpuscular volume. Significant main effects: No Heat < Heat ($P < .01$), No iron < iron ($P < .01$).

[c]Volume index. Significant main effects: No Heat < Heat ($P < .05$), No iron < iron ($P < .10$).

[d]Mean corpuscular hemoglobin concentration. Significant ($P < .10$) interaction. Significant simple effects: No Heat - Fe215 > Heat - Fe215 ($P < .01$), No Heat - Fe215 > No Heat - Fe900 ($P < .05$).

[e]Liver iron. Significant main effect: No iron < iron ($P < .01$).

factors play a role in reducing the iron stores (Wintrobe, 1961). It is possible that the free gossypol in the unheated rations chelated with the iron in the intestinal tract; thus reducing iron absorption. Only ionic iron is absorbed from the intestinal tract (Wintrobe, 1961). Underwood (1962) reported that chelating agents in the blood can increase urinary iron losses 100-fold. Even though only 5% of ingested free gossypol is absorbed in rations containing normal iron levels (Odell et al., 1964), it would be expected that rats on high levels of free gossypol would have lower iron storage than those on lower free gossypol levels (heated meals). Supplemental iron in these trials overcame the effect of gossypol. It should be noted, however, that in both trials, liver iron stores were more dependent on iron level than on free gossypol content of the diet. Unless the iron in the unsupplemented rations was unavailable, the amount present (175 ppm) should have been sufficient to meet normal rat requirements (Cuthbertson, 1957). Thus, widely different liver iron levels between the two iron treatments would not be expected if the iron mucosal block theory were true. It has been observed that alterations of the diet and administration of large amounts of iron will result in an increased iron absorption (Wintrobe, 1962). These data would tend to invalidate the mucosal block theory (Underwood, 1962).

Results of Trial 2 indicate a second factor may be present in the gossypol-erythrocyte complex. In this trial, the microcytic condition was present in those rats fed the untreated ration, but the erythrocytes were normochromic. Trial 2 was a 14-day trial instead of 28 days as in Trial 1, thus, time might be the factor responsible for these divergent results. Mean corpuscular hemoglobin concentration was actually increased in those animals fed the untreated ration, indicative only of spherocyte formation. There are two types of spherocytes, namely, the hereditary

form and the type found in acquired hemolytic anemias (Wintrobe, 1961). Chemical agents such as napthalene, benzene, saponin, etc., will induce hemolytic anemia; the severity being related to the quantity of the chemical absorbed (Wintrobe, 1961). In most of these cases, however, normocytic or macrocytic cells were produced, instead of the microcytic cells observed in this trial. A microcytic, hemolytic anemia is produced in a hereditary condition known as Thalassemia, an abnormality with impaired hemoglobin formation (Wintrobe, 1961). The symptoms of Thalassemia are pallor, cardiac dilation, and in advanced stages, edema and effusion into serous cavities; these conditions are associated with gossypol toxicity.

Summary

One hundred and eight weanling male rats were used in two trials to study the effect of gossypol, iron, and heat treatment of cottonseed meal on the blood constituents and liver iron levels. High levels of free gossypol resulted in microcytic-hypochromic anemia in animals fed for 28 days, while those fed for 14 days had the microcytic-normochromic form. Either supplemental iron or steaming the meal, which reduced the free gossypol level, alleviated these conditions.

CHAPTER V

EXPERIMENT III

Among the earliest and most successful methods of gossypol inactivation or destruction have been autoclaving and steaming either the raw cotton seeds or the resultant meal after pressing out the oil (Withers and Carruth, 1918; Dowell and Menaul, 1923). Gallup (1926, 1927b) found that dry-heating cotton seeds caused a change in the form of gossypol (as determined by solubility in ether), but only slightly reduced toxicity. Heating moist seeds in an autoclave under pressure rapidly destroyed the gossypol and produced a non-toxic product. When fed to rats, the raw meal gave poor results (1927a) while the autoclaved meal gave good results. Lyman et al. (1944) found that autoclaving cottonseed meal for short periods of time rendered it safe if the level in the total diet did not exceed 25%. The method of cottonseed meal preparation appears to alter the form of gossypol and its toxicity. This trial was conducted to determine the effect of different types of heat treatment on the chemical change of cottonseed meals and their effect on blood constituents when fed to rats.

Experimental Procedure

Four types of treatments were used in preparing the cottonseed meals for this study: (1) extraction with cold hexane with no heat being applied at any time during the extraction process, (2) extraction as in treatment 1, and then heated in an oven at 100° C. for 100 min. (dry heat),

(3) dehulled cotton seeds were steamed at 95° C. at atmospheric pressure for 100 min., then extracted with cold hexane and ground, and (4) extraction with cold hexane, then autoclaved at 121° C. and 1.05 kg./sq. cm. of pressure for 100 min. Gossypol content and nitrogen solubility data resulting from the four treatments are listed in Table XI. Composition of the basal ration is shown in Table XII.

TABLE XI

CHEMICAL ANALYSIS OF COTTONSEED MEALS HEATED FOR 100 MINUTES BY FOUR DIFFERENT METHODS

Treatment of Cottonseed Meal[a]	No heat	Dry heat	Steam heat	Autoclave
Particular components	%	%	%	%
Total gossypol	1.51	1.50	1.55	0.07
Free gossypol	1.15	1.01	0.09	0.00
Bound gossypol	0.36	0.49	1.46	0.07
Nitrogen soluble in 0.02 N NaOH, % of total	97.35	92.63	69.70	32.62

[a]See text for details of processing conditions for each of the four cottonseed meals.

TABLE XII

COMPOSITION OF BASAL RATION

Ingredients	%
Cottonseed meal[a]	10.00
Purified soybean protein	15.00
Corn oil	10.00
Salts XIV mineral mix	4.00
Corn starch	33.20
Purified cellulose	2.00
Corn dextrose	25.00
B vitamins[b]	0.05
Choline chloride	0.20
Vitamins A and D mixture[c]	0.05
NaCl	0.50

[a]See text for details of processing.

[b]Oltjen et al. (1962).

[c]Mixture contained 20,000 I. U. and 2,500 USP units/gm. of vitamin A and D, respectively.

Thirty-two weanling male rats of the Holtzman strain (70 to 100 gm.) were randomly allotted in equal numbers to the four treatments. Rats were weighed initially, at 3-day intervals, and at the completion of the 28-day trial. All animals were individually-caged and fed ad libitum, water being available at all times. On completion of the trial, all rats were exsanguinated and the blood samples preserved using sodium citrate as the anticoagulant. Livers were excised and frozen. Response criteria were gain and feed efficiencies, red blood cell count, hemoglobin content, percent packed cells, blood iron, liver iron, mean corpuscular hemoglobin concentration, and volume index. Statistical analyses were conducted using analysis of variance and orthoganol comparisons.

Total gossypol was determined by the method of Pons et al. (1958) and free gossypol by the method of Pons and Hoffpauir (1957). Nitrogen solubility was determined as described by Lyman et al. (1953). Blood and liver iron was determined by the method as described by Sandell (1959).

Results and Discussion

Table XIII shows the performance data on rats fed the different cottonseed meals. Processing the raw meal with dry heat improved ($P < .05$) weight gain and feed efficiency. Steam heat improved gains and feed efficiency over that obtained when no heat was applied, however, steam heating did not further improve gains or feed efficiency ($P > .05$) over that obtained with the dry heat treatment. Autoclaving, when compared to steaming, lowered gains ($P < .05$) and feed efficiency ($P < .07$);

differences between autoclaving, dry heat, and no heat treatment were not significant ($P > .05$).

The blood and liver analyses are shown in Table XIV. Mean corpuscular volume and the volume index, which is indicative of increased erythrocyte size, was largest ($P < .01$) for those rats fed the steam heated meal; however, mean corpuscular hemoglobin concentration was lower ($P < .05$) in the rats fed steam heated meal when compared to those fed dry-heated meal. Red cell count was greater ($P < .05$) in rats fed the autoclaved meal than in those fed the non-heated meal. It is possible that these results can be explained by considering the amount of available protein in the diets and the effect of free gossypol on the non-ruminant. Growth rate is decreased by either high levels of free gossypol or by reducing the amount of available protein in the diet. High levels of free gossypol limit growth of the non-ruminant. The non-heated and dry-heated meals contained high levels of free gossypol while that of the autoclaved meal was destroyed. Nitrogen solubility, an indicator of available protein, was greatly reduced by autoclaving and only slightly by the dry-heat process. When the level of free gossypol and nitrogen solubility are chemical criteria, it appears that the most favorable meal for utilization by the non-ruminant animal would be the steam heated meal; steaming the meal appeared to effectively bind free gossypol with little protein destruction (Table XI).

The performance and blood data support the above hypothesis. Mean corpuscular volume was largest and mean corpuscular hemoglobin concentration was lowest in rats fed the steam-heated meal, indicating a higher proportion of young red blood cells. This would be expected in rapidly growing animals as new erythrocyte formation is required to compensate for additional growth as well as for normal blood turnover. Also, red blood

TABLE XIII

PERFORMANCE OF RATS FED FOUR HEAT TREATED COTTONSEED MEALS

Heat treatment	No heat	Dry heat	Steam heat	Autoclave	Standard error of treatment means
Number of rats	8	8	8	8	
Initial weight, gm.	85.5	86.4	83.8	84.8	1.24
Av. total gain, gm.[a]	80.9	100.8	115.1	85.7	6.46
Av. total feed consumed, gm.[b]	381.1	406.5	445.6	378.8	17.07
Feed eff., feed/gain, gm.[c]	4.8	4.1	3.9	4.5	0.20

[a]Steam heat > No heat, autoclave ($P < .01$); Dry heat > No heat ($P < .05$).

[b]Steam heat > No heat, autoclave ($P < .01$).

[c]Steam heat < No heat ($P < .01$); Dry heat < No heat ($P < .05$).

TABLE XIV

EFFECTS OF FOUR HEAT TREATED COTTONSEED MEALS ON BLOOD CONSTITUENTS AND IRON LEVELS IN BLOOD AND LIVER

Cottonseed meal treatment	No heat	Dry heat	Steam heat	Autoclave	Standard error of treatment means
Red blood cells $X10^6/mm^3$.[a]	5.6	5.9	5.7	6.1	0.14
Hemoglobin, gm./100 ml.	13.0	13.8	13.3	13.6	0.25
Packed cells, %[b]	30.3	30.8	32.4	31.6	0.63
Mean corpuscular volume, μ^3.[c]	54.1	52.5	56.8	52.1	0.75
Mean corpuscular Hb, $\mu\mu$ gm.	23.3	23.5	23.5	22.3	0.56
Mean corpuscular Hb conc., %[d]	43.1	44.8	41.3	42.6	0.99
Volume index, %[e]	90.0	87.9	95.2	88.0	1.28
Whole blood iron, mg.Fe/100 ml.	35.0	34.5	35.0	37.8	3.26
Liver iron, ppm/gm. dry liver	484	475	486	487	46

[a]Autoclave > No heat ($P < .05$).

[b]Steam heat > No heat ($P < .05$).

[c]Steam heat > No heat ($P < .05$), autoclave, dry heat ($P < .01$).

[d]Steam heat < Dry heat ($P < .05$).

[e]Steam heat > No heat, autoclave, dry heat ($P < .01$).

cells decrease in size and increase in hemoglobin concentration as they increase in maturity. Decreasing the growth rate, by feeding a protein-deficient ration (Whipple, 1942) would tend to suppress the rate of red blood cell formation, thereby, offering a possible explanation for the smaller red blood cell as well as the slightly increased hemoglobin concentration that was noted in the autoclaved meal. A lowered number of erythrocytes was found in rats fed the rations containing high levels of free gossypol as compared to those fed the autoclaved meal. This was possibly caused by the free gossypol and may have been due to a hemolytic effect or an inhibition in red blood cell biosynthesis. An acquired hemolytic effect tends to produce macrocytic, hyperchromic cells (Wintrobe, 1961); however, these cells were noted to be slightly microcytic. Inhibition of red blood cells biosynthesis would tend to produce a lowered cell count and, as the cells mature, a smaller corpuscular volume and an increased hemoglobin concentration (Wintrobe, 1961). These effects were noted when comparing the high free gossypol containing rations to the steam heated ration.

Summary

Thirty-two male weanling rats were used to study the effect of different types of heat treatment on the chemical changes in cottonseed meals and the effect of such changes on the blood constituents of rats. Heat treatments were: (1) extraction of the cottonseed meal with cold hexane with no heat being applied at any time during the extraction process, (2) extracted as in treatment 1, and then heated in an oven at 100° C. for 100 min. (dry heat), (3) dehulled cotton seeds were steamed at 95° C. at atmospheric pressure for 100 min., then extracted with cold hexane and ground, and (4) cottonseed meal was extracted with cold hexane,

then autoclaved at 121° C. and 1.05 kg./sq. cm. of pressure for 100 min.

It was found that steaming the meal effectively bound free gossypol to the extent that gossypol toxicity, as determined by performance and red blood cell data, was lower than that of the non-heated or dry-heated meal rations.

CHAPTER VI

EXPERIMENT IV

Efforts to elucidate the mechanism of action by which gossypol exerts its toxic effect upon non-ruminant animals have met with limited success. It has been shown (Lyman et al., 1959; Conkerton and Frampton, 1959) that gossypol can react with purified proteins by combining with the free epsilon-amino group of lysine, thus forming a gossypol-protein complex. This reaction decreases the number of lysine moieties having free epsilon-amino groups and suggests that the depression in growth noted in non-ruminants is due to a simple lysine deficiency.

Athens et al. (1958), feeding a lysine-deficient diet with hexahomoserine to swine, noted the development of an anemic condition followed by a loss in body weight. Hexahomoserine has been shown to inhibit the incorporation of lysine into the protein molecules, thereby preventing normal erythropoiesis and growth (Gaudry, 1955). Thus, it appeared desirable to study the effect of gossypol, hexahomoserine, or both, on the growth of rats fed purified diets with and without lysine.

Experimental Procedure

Sixty-four weanling rats of the Holtzman strain (51 to 58 gm.) were weighed to the nearest gram and separated by weight into eight treatment groups. Eight rats were assigned by a randomized block design to a 2^3 factorial arrangement of treatments, in which 0.85% lysine, 0.15% gossypol, and 0.20% hexahomoserine were added to a basal ration (Table XV.)

Animals were individually caged and fed ad libitum. Initial and final weights were used in determining weight response during the 14-day trial. In addition, interim weights were taken at 3-day intervals. Gross and histological examinations were made on rats at the completion of the trial. Statistical analyses of the data were conducted using analysis of variance.

TABLE XV

COMPOSITION OF BASAL RATION

Ingredients	%
Amino acids, levorotatory[a]	10.00
Starch	46.00
Sucrose	22.00
Corn oil	10.00
Vitaminized glucose[b]	4.95
NaCl	1.00
Mineral mix[c]	4.00
Cellulose[d]	2.00
Vitamin A and D[e]	0.05

[a]Composition of the amino acids simulates casein as given by Block and Bolling (1951), lysine being omitted.

[b]Composition shown by Metta and Mitchell (1954).

[c]Salts mixture USP XIV.

[d]Solka floc, BW20, Brown Company, Berlin, New Hampshire.

[e]Quadrex 20, containing 20,000 and 2,500 USP units/gm. of vitamins A and D, respectively. Courtesy NOPCO Chemical Company, Harrison, New Jersey.

Results

Table XVI exhibits the results, and it can be seen that animals receiving the basal diet, which contained no lysine, lost weight. The addition of gossypol, hexahomoserine, or the combination of these, to the basal diet resulted in no further depression in growth.

Negative values for feed efficiencies are found for diets not supplemented with lysine (1, 2, 3 and 4). When compared to the basal diet, the addition of gossypol (diet 3) resulted in no further depression in feed efficiency; however, the addition of hexahomoserine (diet 2) or the combination of gossypol and hexahomoserine (diet 4) caused a significant ($P < .01$) depression in feed efficiency.

Rats receiving diets containing lysine gained significantly ($P < .01$) faster and more efficiently than those receiving no lysine. The addition of either gossypol or hexahomoserine to the lysine-containing rations caused significant ($P < .01$) reductions in feed intake, gains and feed efficiency. The addition of gossypol to the diet containing lysine resulted in gains which were only 38% as great as those obtained with animals consuming the basal plus lysine diet, while the addition of hexahomoserine resulted in gains 72% as great. Feed efficiency was also lowered by gossypol ($P < .01$) or hexahomoserine ($P < .05$). The combination of gossypol and hexahomoserine, when added to the lysine-containing diet, resulted in a significant depression in growth and feed efficiency when compared to the lysine-containing diet (diet 5) or the diet containing lysine plus hexahomoserine (diet 6); however, the gains were not significantly different from those obtained with the diet containing lysine plus gossypol.

There was a significant ($P < .05$) interaction between the effects of herahomoserine and gossypol upon gains. Gossypol exerted a less adverse effect than did hexahomoserine when lysine-free diets were fed; however, the reverse was true when the diet contained lysine.

Discussion

Hexahomoserine is a competitive inhibitor (Gaudry, 1955) for the

TABLE XVI

EFFECTS OF LYSINE, GOSSYPOL, AND HEXAHOMOSERINE ON PERFORMANCE OF RATS

	No Lysine				Lysine[a]				Standard error of treatment means.
	No Gossypol		Gossypol[b]		No Gossypol		Gossypol		
	No HHS	HHS[c]	No HHS	HHS	No HHS	HHS	No HHS	HHS	
Diet	1	2	3	4	5	6	7	8	
Number of rats.	8	8	8	8	8	8	8	8	
Av. gain or loss, gm.[d]	-4.2	-9.1	-5.3	-8.4	37.8	27.4	14.4	15.6	1.81
Av. total feed consumed gm.[e]	74.7	57.6	64.1	53.8	133.4	115.3	102.0	85.9	5.16
Feed efficiency, gain/feed, gm.[f]	-0.1	-0.2	-0.1	-0.2	0.3	0.2	0.1	0.2	0.02

[a]Lysine (L) fed at 0.85% of diet.

[b]Gossypol (G) fed at 0.15% of diet.

[c]Hexahomoserine (HHS) fed at 0.20% of diet.

[d]Average gain or loss. Significant L level by G level ($P < .01$) and HHS level by G level ($P < .05$) interactions. Significant Simple effects:

No G-No L < No G-L ($P < .01$)
G-No L < G-L ($P < .01$)
No G-L > G-L ($P < .01$)

	No L	L
No G	-6.69 **	32.61 **
G	-6.85 **	14.99 **

No G-No HHS > No G-HHS ($P < .01$)
No G-No HHS > G-No HHS ($P < .01$)
No G-HHS > G-HHS ($P < .01$)

	No HHS	HHS
No G	16.78 **	9.14 **
G	4.52	3.63

[e]Feed consumption. Significant main effect: No HHS > HHS ($P < .01$). Significant L level by G level ($P < .01$) interaction.

No G-No L < No G-L ($P < .01$)
G-No L < G-L ($P < .01$)
No G-L > G-L ($P < .01$)

	No L	L
No G	66.22 **	124.36 **
G	58.97 **	94.45 **

[f]Feed efficiency. Significant L level by G level ($P < .01$) and L level by HHS level interactions. Significant simple effects:

No G-No L < No G-L ($P < .01$)
G-No L < G-L ($P < .01$)
No G-L > G-L ($P < .01$)

	No L	L
No G	-0.12 **	0.26 **
G	-0.13 **	0.16

No HHS-No L < No HHS-L ($P < .01$)
HHS-No L < HHS-L ($P < .01$)
No HHS-No L < HHS-No L ($P < .01$)

	No L	L
No HHS	-0.07 *	0.21 *
HHS	-0.17 *	0.21 *

incorporation of lysine into the protein moiety; thus, the level of lysine used in the present experiment could be expected to partially overcome the adverse effects of the inhibitor (Mertz et al., 1950; Page et al., 1947). Since gains on the diet containing lysine plus hexahomoserine were only 72% of those obtained with the lysine-containing diet (diet 5), these data support this hypothesis.

Gossypol can react with soluble proteins, amino acids, amines, and other substances (Castillon and Altschul, 1950), forming in most cases combination products which are not toxic to non-ruminants. If certain of these substances are present along with gossypol in the diet, the reaction takes place within the gastrointestinal tract (Odell et al., 1964), and the absorption of gossypol is greatly depressed. When gossypol is absorbed or injected into the body, toxicity occurs in rats (Eagle and Bialek, 1950).

Hexahomoserine caused a greater detrimental effect than gossypol upon gains and feed utilization when the lysine-free basal diet was fed; however, the reverse was true when lysine was added to the basal diet. Hexahomoserine is readily absorbed into the body (Mertz et al., 1950) and thus would be available to inhibit the formation of new protein tissue. When the lysine-free diet was fed, it is conceivable that hexahomoserine partially blocked the synthesis of indispensable protein tissue from dispensable protein tissue (Whipple, 1942). At the same time some of the gossypol was detoxified in the gastrointestinal tract, and that which was not detoxified was combined with the soluble protein of the blood (Lyman et al., 1959; Odell et al., 1964). Feed intakes were low in rats consuming the lysine-free diet which contained either gossypol or hexahomoserine.

When lysine was added to the basal diet, gossypol exerted a more

detrimental effect upon gains and feed conversion than did hexahomoserine. A competitive inhibitor would be expected to depress growth rate and the efficiency of feed utilization in proportion to its level in the ration. In the present experiment the expected depression in gains and feed efficiency due to hexahomoserine was about 24%. A 28% depression in gains and a 17% reduction in feed efficiency was obtained. In the case of gossypol only about 11% of the lysine would be expected to be tied up in the gossypol-lysine combination; thus, on the basis of lysine availability, gains would have been depressed by an equal amount. Instead, gossypol depressed gains by 62% and feed efficiency by 50%. The results of Hale and Lyman (1962), in which they found that dietary lysine was not as effective as dietary soluble protein (fish meal) in preventing gossypol toxicity in swine, bear upon this point.

Gross examination of the animals receiving gossypol revealed a loss of hair around the head and neck and an appearance of unthriftiness. Pathological examination revealed subcutaneous edema in the thoracic and cervical regions, pulmonary edema, dilated heart, and a pale yellow-brown colored liver. These symptoms are the same as those reported by Eagle (1960) and El-Nockrashy et al. (1963) when they administered gossypol to chickens, dogs, mice, or rats. In the present experiment, gossypol toxicity symptoms were found in rats receiving gossypol alone or in combination with lysine. These results support the idea that gossypol reduces growth rate in non-ruminants because of its toxicity rather than by causing a deficiency of lysine.

Summary

Sixty-four weanling rats were used in a 2^3 factorial arrangement of treatments. The treatments imposed upon the rats receiving the basal

ration, which contained no lysine, were 0.85% lysine, 0.15% gossypol, or 0.20% hexahomoserine. Lysine increased gain, feed consumption, and feed efficiency, whereas gossypol and hexahomoserine decreased these three criteria.

Gossypol toxicity symptoms were noted. The results are discussed in regard to the mechanism of action of gossypol in causing growth depression in non-ruminants.

CHAPTER VII

EXPERIMENT V

Cottonseed meals containing levels of free gossypol sufficient to cause toxicity in non-ruminants can be fed in large amounts to ruminants without apparent ill effects. Possible explanations concern the detoxification of gossypol in the rumen (Reiser and Fu, 1962) or to the fact that ruminant animals are not subject to the adverse effects of gossypol (Gallup, 1927). The purpose of this experiment was to determine if gossypol is toxic when injected intravenously into adult sheep.

Experimental Procedure

Trial 1.

Eight crossbred wethers were selected and randomly assigned to one of four treatment levels of gossypol. Gossypol-acetic acid was weighed and dissolved in 3 mls. of ethanol, then suspended in 12 mls. of physiologic saline solution and injected into the jugular vein of the animal. Daily levels of injected gossypol were: 0.0, 4.5, 9.0, and 18.0 mg./kg. of body weight. Feed and water were available to the sheep at all times. All animals were autopsied at time of death or at the end of six weeks and examined for physiological and histological changes. On the completion of the first experiment, 15 crossbred wethers were randomly allotted and injected with gossypol at levels of 0.0, 12.5, and 25.0 mg./kg. of body weight using the techniques as previously outlined. A third study was initiated in which eight wethers were equally divided, one lot serving as a control, the other injected with 15.0 mg. gossypol/kg. body

weight. The techniques used in this trial were as previously outlined, with the exception that the gossypol was dissolved in 95% ethanol (15 mg. gossypol/ml. ethanol) and injected as such.

Trial 2.

Sherrod and Tillman (1964) obtained good performance when sheep were fed raw cottonseed meal containing a high level of free gossypol in a ration containing roughage. In contrast, sheep consuming the same level of raw cottonseed meal in an "all-concentrate" ration performed poorly. They postulated that the faster rate of passage and lower ruminal pH obtained in the "all-concentrate" diet might constitute conditions which would allow gossypol to pass through the rumen undetoxified. The purpose of this trial was to test this idea. Three rations were used and their compositions are shown in Table XVII. The concentrate to roughage ratios (10:1 vs. 1:10) were similar to those used by Sherrod and Tillman (1964). One percent of ferrous sulfate was added to one of the 10:1 ratio diets for the purpose of detoxifying free gossypol which might escape the rumen. No ferrous sulfate was added to the 1:10 ratio diet. The cottonseed meal, especially prepared by cold-hexane extraction with no heat being applied at any time during extraction, contained 0.628% total gossypol and 0.312% free gossypol. Each of the rations contained 0.163% total gossypol and 0.081% free gossypol.

Nine crossbred wethers, averaging 26.1 kg., were randomly allotted to the three rations. All animals were individually fed ad libitum for 68 days. Feed and water were removed during a 16-hour shrink period, which preceded the initial and final weighing. Weight gains, feed consumption, and feed efficiency were the response criteria. On completion of the trial, all animals were autopsied and observed for symptoms of gossypol toxicity. Statistical analyses were conducted using analysis of

variance and orthoganol comparisons.

TABLE XVII

COMPOSITION OF RATIONS USED IN TRIAL 2

Concentrate to roughage ratio	10:1		1:10
Ration number	1	2	3
Ingredients	%	%	%
Cottonseed meal[a]	26.0	26.0	26.0
Corn dextrose	32.0	32.5	2.25
Corn starch	32.0	32.5	2.25
Purified cellulose	----	----	33.0
Cottonseed hulls	4.5	4.5	32.0
Minerals[b]	2.0	2.0	2.0
Corn oil	1.5	1.5	1.5
Vitamin A and D mixture[c]	0.1	0.1	0.1
Dicalcium phosphate	0.6	0.6	0.6
Calcium carbonate	0.3	0.3	0.3
$FeSO_4$	1.0	----	----
Total	100.0	100.0	100.0

[a]See text for description of meal.

[b]Oltjen et al. (1962).

[c]Mixture contained 20,000 I.U. and 2,500 USP units/gm. of vitamin A and D, respectively.

Trial 3.

A second growth trial was conducted for the purpose of studying further the possibility of gossypol toxicity in ruminants. Widely different gossypol-containing cottonseed meals were used in semi-purified diets: one contained a high level of free and bound gossypol while the second was exhaustively extracted with a hexane-acetone-water azeotrope (51:46:3) solvent until no free or bound gossypol remained. The compositions of the two rations are shown in Table XVIII. The raw cottonseed meal in the high gossypol ration was the same as used in Trial 2. In the low gossypol ration, the cottonseed meal was extracted with a hexane-acetone-water azeotrope (51:46:3) (King et al., 1961) until no yellow color remained in

the effluent. After extraction, the meal, which contained 0.073% total and no free gossypol, was air dried and reground to original particle size in a hammer mill.

Eight sheep, averaging 19.7 kgs. were allotted to the two treatments and individually fed ad libitum for 70 days. Initial and final weighings were preceded by a 16-hour shrink period during which time feed and water were removed. Weight gains, feed consumption, and feed efficiency were criteria of response. At the termination of the trial, all sheep were autopsied for symptoms of gossypol toxicity.

TABLE XVIII

COMPOSITION OF RATIONS USED IN TRIAL 3

Ration	High Gossypol	Low Gossypol
Ingredients	%	%
Cottonseed meal[a]	30.0	30.0
Corn starch	27.5	27.5
Corn dextrose	27.5	27.5
Cottonseed hulls	10.0	10.0
Minerals[b]	2.0	2.0
Corn oil	2.0	2.0
Vitamins A and D mixture[c]	0.1	0.1
Dicalcium phosphate	0.9	0.9
Total	100.0	100.0

[a]See text for description of meals.

[b]Oltjen et al. (1962).

[c]Mixture contained 20,000 I.U. and 2,500 USP units/gm. of vitamin A and D, respectively.

Results and Discussion

Trial 1.

A summary of weight gains and death in the sheep, is shown in Table XIX. Animals injected with 4.5 mg./kg. body weight gained weight, and at

no time during the trial did they appear to be ill. All animals injected with the 9.0 or 18.0 mg./kg. body weight of gossypol were observed to eat less feed and they lost weight. It also appeared that the 18.0 mg./kg. level was more toxic than the 9.0 mg. level.

TABLE XIX

EFFECTS OF INJECTED GOSSYPOL ON WEIGHT RESPONSE AND TOXICITY IN SHEEP

	Mg. gossypol injected/kg. body weight			
	0.0	4.5	9.0	18.0
Number of animals	2	2	2	2
Av. initial weight, kg.	24.5	27.7	25.6	31.7
Change in weight per day, kg.	0.21	0.15	-0.005	-0.004
Number of sheep which died	0	1[a]	1[b]	2[c]

[a]Died during the 14th injection. Symptoms resembled anaphylactic shock.

[b]Died 36 days after start of trial.

[c]Died 23 and 31 days after start of trial.

External appearances of gossypol toxicity included a lack of appetite, followed by drooping of the head and ears. A general unthriftiness was noted within five days after the first injection and persisted until death occurred. Respiration rate increased temporarily immediately after each injection, but this could be attributed to the ethanol in which the gossypol was dissolved. Those animals which died did so quite suddenly, without any apparently worsening of condition.

Autopsy of the animals revealed an accumulation of edematous fluid in the subcutaneous tissue, especially in the thoracic region. A lesser amount was present in the abdominal cavity. Subcutaneous tissue was slightly yellow-green in color. Superficial lymph nodes were moderately enlarged, and firm on palpation. A hard yellow-green substance was present

in the iliac lymph nodes. Livers were slightly swollen, enlarged and gave a typical nutmeg pattern with alternating areas in a lobular distribution. They gave a fine sprinkling of dark brown and gray appearance and on incision of the liver, this nutmeg pattern was more pronounced. The lungs were slightly pale in color with various dark red areas. On palpation, the lighter part was gritty throughout, whereas the dark red portion was firm and consolidated. Foamy exudate was present in the large bronchi and bronchioles. In the smaller bronchioles a small amount of pus or purulent exudate was noted. Many of the arterioles contained plugs of gossypol causing an arteriol necrosis. The hearts were slightly enlarged and swollen edges of the hearts rounded. The myocardium was pale and mottled in appearance. The left ventricles and valves appeared to be distended and dilated. On microscopic observation, a shrinkage of the muscle fibers was noted.

The general emaciated and unthrifty condition noted was essentially the same as that reported by Gallup (1927b) for rats suffering from gossypol toxicity. Gross internal observations were the same as those reported by Schwartze and Alsberg (1924) who studied chronic gossypol intoxication in cats, pigs, and rabbits. Smith (1957) and Rigdon et al. (1958, 1959) studied pathological effects of gossypol toxicity in chicks and pigs. Their reported results coincide closely with those effects of gossypol that were observed in the sheep.

In the studies following the initial trial, three sheep receiving the 25 mg. gossypol/kg. body weight treatment and one receiving the 12.5 mg. gossypol/kg. body weight treatment, died within a week after initial injection. These data, combined with those of the first study, indicate that as gossypol level increases, death due to gossypol toxicity occurs more rapidly. A predisposing factor to true gossypol toxicity was that of

the insolubility of gossypol in the solution of ethanol and physiological saline solution. By first mixing the gossypol with ethanol, then adding the physiological saline solution, an injectable suspension was formed. The micrograins of non-soluble, injected gossypol would lodge in the arterioles where arterial necroses of the lungs would occur. This suspension was eliminated by dissolving the gossypol in sufficient ethanol to have a true solution, however, the amount of ethanol required for injecting the 15 mg. gossypol/kg. body weight was too great and bloat occurred in four of the eight animals within three days after initial injection, therefore injections were discontinued. These data indicate a major problem concerned with the study of gossypol by injection, namely, its insolubility in liquids physiologically acceptable to the animal.

Trials 2 and 3.

Results of Trial 2 are shown in Table XX. Lambs fed the low concentrate ration are significantly ($P < .05$) more than did lambs fed high concentrate rations. Average daily gains and feed efficiency were not affected by treatments. Lambs on the low concentrate rations consumed a greater total amount of both free and total gossypol, however, this increased consumption apparently did not affect growth rate or efficiency of feed conversion. Results of Trial 3 (Table XX) show no apparent differences between the treatments with regard to average daily gain, feed consumption, or feed efficiency. Autopsy of all animals for both trials revealed no gossypol toxicity symptoms.

These results confirm those of Tillman and Kruse (1962) which indicate that orally fed free gossypol has no effect of performance of sheep. The feed consumption and performance data also supports that of Sherrod and Tillman (1964); autopsy data do not support their postulation that gossypol is absorbed from the lower digestive tract thus serving as an appetite depressant. Even though total feed consumption was depressed

TABLE XX

DAILY GAIN AND FEED CONVERSION OF LAMBS FED DIFFERENT CONCENTRATE: ROUGHAGE RATIOS (TRIAL 2)

Concentrate to roughage ratio	10:1		1:10	Standard error of treatment means
	Iron	No Iron	No Iron	
Av. daily gain, gms.	164.8	180.2	189.3	14.42
Av. daily feed consumption, kgs.[a]	1.2	1.2	1.7	0.09
Feed efficiency, feed/gain, kgs.	7.1	7.2	8.8	0.53

[a]1:10 No iron 10:1 Iron and 10:1 No iron; (P .05).

TABLE XXI

DAILY GAIN AND FEED CONVERSION BY LAMBS FED HIGH AND LOW GOSSYPOL CONTAINING RATIONS

	Ration	
	High Gossypol	Low Gossypol
Av. daily gain, gms.	231.5	213.4
Av. daily feed consumption, kgs.	1.1	1.0
Feed efficiency, feed/gain, kgs.	4.7	4.7

($P < .05$) in those animals fed the 10:1 ratio diets, total energy intake for all treatments was approximately the same as is indicated by gain and feed efficiency data. These results would indicate that energy level limited feed intake, and that the free gossypol is detoxified before it reaches the small intestine.

Summary

Gossypol injection studies, involving 31 crossbred wethers, indicate that sheep display the same physiological and histological toxicity symptoms to gossypol as do the non-ruminants. Results of two feeding

trials indicate that gossypol, when fed in highly concentrated diets exerts no adverse effect on the sheep, thereby refuting earlier works which indicated that gossypol reduced feed intake in such rations.

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