EVALUATION OF CHITINOUS MATERIALS AS A FEED FOR RUMINANTS

by

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INTRODUCTION

The world's plant protein supply has many demands on it, the most important being a human population growing faster (2% per year) than food production (1.8% per year) (11). The United States population is expected to be 300 million by the year 2000, with world population doubling to 7.5 billion by this date. This is quite obviously a worsening situation and firm measures are needed now.

Insects and ruminants also impose great demands on the plant protein supply. The magnitude of the demands on the plant kingdom by insects is often devastating, and it is readily pointed out that ruminants exist solely on plants and have a low (10%) efficiency in converting grains to meat protein. But a grain producer will send his harvest through ruminants only as long as it is profitable; supply and demand could readily leave the ruminant nothing to eat but grassland forages.

Herein lies the main point supporting the ruminant's importance to man: The ruminant is quite capable of converting grass to meat protein, which represents an excellent use of the 350 million hectares (28) of land in the continental United States unsuitable for crop production but most adequate for grazing.

In addition, the ruminant has been shown to produce meat protein from many products formerly believed to be waste.

Ruminants have been fed newspaper, urea, biuret, chicken feces, seaweed, crude oil, sawdust, and many other compounds of absolutely no food value to humans. All of these were utilized with varying degrees of success.

The ruminant's importance and the understanding of its versatility in the human food chain is increasing constantly, and its ultimate usefulness is limited only by the imagination of the investigators involved. For example, the impetus for this investigation arose from a desire to evaluate the feasability of feeding insects to ruminants.

Insects contain chitin, a poly-N-acetylglucosamine, which is very similar to cellulose, second only to cellulose in abundance as a polysaccharide but noted for its insolubility. Chitin, though not found throughout the entire shell, is the fiberous portion of the shell of most anthropods. Thus, another source of chitinous feedstuffs for ruminants is from the marine seafood industry. Only 15% of a crab is used for human consumption, the remainder being considered as waste and a problem to industry, amounting to 200 million pounds annually (20). The shrimp industry has an equally high waste percentage.

The objectives of this research were: 1) To evaluate the digestibility, by ruminants, of chitinous materials of insect and marine sources, and 2) To evaluate the nutritional benefit ruminants could derive from crabmeal, a dried and ground crab waste product.

The approach to the solution of these objectives was threefold:

1. *In vivo* incubation of various chitinous compounds in fistulated steers
2. Feeding trials of crabmeal with young functional ruminants
3. *In vitro* incubation of chitinous compounds.

The realization of the objectives outlined could have the following benefits:

1. Liberate more plant protein for human consumption while still meeting the nutritional needs of ruminants
2. Advance the cause of ecology
3. Save money spent for insect control.

REVIEW OF LITERATURE

Prospectus

Justification for this research is realized by documentation of the collision course the world population is taking with starvation. The world population is increasing at 2% per year, while the world food supply is increasing at only 1.8% per year (11). If the ever-increasing millions are to be fed, it is imperative that advantage be taken of every available food or potential reserve. One such food reserve might be chitin. Utilization of this compound by ruminants would result in the conversion of large quantities of inedible material into human food.

The Occurrence and Nature of Chitin

Chitin is one of the most abundant polysaccharides in the world, second only to cellulose (47). Insects and crustaceans rely on chitin for structural integrity, as do all fungi and some yeast. Chitin is a poly-N-acetylglucosamine analogous to cellulose except the hydroxyl on the C-2 position of cellulose is replaced by an N-acetylamino group on the chitin molecule (47).

Wigglesworth (48) reported that chitin is insoluble in water, alcohol, ether, other organic solvents, dilute acids, and dilute and concentrated alkalis. Concentrated mineral acid hydrolizes chitin to lower saccharides, glucosamine, and acetic acid. Concentrated basic solutions at high

temperatures cleave the acetyl group selectively, creating a compound called chitosan (48).

It is commonly believed that 5% of the amino groups of chitin are unacetylated (25). Giles et al. (12) presented strong evidence that 12% of the amino groups of chitin are unacetylated. Morgulis (29) in agreement with Giles et al. (12) found 12.5% of the nitrogen of chitin was more resistant to sulfuric acid hydrolysis. A value of 12.5% corresponded with one out of eight or seven acetylated for every unacetylated glucose moiety.

Campbell (3), in 1929, stated the most probable empirical formula for chitin was $(C_{32}H_{54}O_{21}N_4)n$. Wigglesworth (48) reported a formula of $(C_8H_{13}O_5N)n$, while Giles et al. (12) stated that chitin was 82.5% $(C_8H_{13}O_5N)n$, 12.5% chitosan $(C_6H_{11}O_4N)n$ and 5% bound water.

Chitin always occurs in close association with other substances. In crustaceans, calcium carbonate serves as the cementing substance and accounts for some crustacean meals being 18% calcium (20). Fresh water crayfish meal was stated to be 14% chitin (20), while salt water crayfish meal was reported as being 12.3% chitin (1). The chitin content of crabmeal was indicated as being 12.9% (21) while shrimpmeal was related as being 7.6% chitin (2). Crude fiber analysis, as performed in feed evaluation, was believed by several investigators to be a reliable estimate of the chitin content of shellfish meals (1, 20, 21).

Insect cuticle, wherein chitin is intimately associated with protein, is composed of three basic layers: epicuticle, exocuticle, and endocuticle. In the cockroach, _Periplaneta americana_ (L), these layers contain 0, 22, and 60% chitin, respectively (48), with the non-chitin portion of each layer being predominantly protein. In growth and development of insects, the exoskeleton is shed periodically until adulthood is achieved. This periodic loss of integument, called moulting, involves a mechanism of expansion of the cuticle in which replacement of lost epicuticle, exocuticle, and endocuticle occurs. In the elucidation of this mechanism, an insight has been gained into the structure of insect cuticle.

Through the influence of the moulting hormone, ecdysone, in the blood, the epidermal cells are caused to separate from the cuticle (36). Moulting fluid, containing inactive proteinase and chitinase, is secreted into the space between the two layers. Prior to the activation of these enzymes, a new layer of cuticulin (part of the new epicuticle) is secreted, providing protection against digestion of what will become the new cuticle (4). The enzymes proceeded to digest all the old endocuticle but have little effect on the old exocuticle. The products of digestion of the old endocuticle, as well as moulting fluid, are reabsorbed to the extent that 90% of the cuticle may be reclaimed before ecdysis (4). The reabsorbed exoskeleton constituents can serve the insect as a food reserve if necessary but are usually deposited in the new cuticle. Once

the insect sheds the undigested exocuticle, the physiological machinery begins sclerotization, or tanning, of the new cuticle, which results in a hard and usually dark exoskeletor

In the tanning process, water soluble arthropodin was converted to insoluble sclerotin by incorporation of o-quinones into the cuticle (26), which were believed to do the actual tanning. N-acetyldopamine was the immediate precursor for o-quinones (17) and was converted to o-quinone by dipheny oxidase (16), an enzyme first isolated by Schweiger and Karlson (42). Tyrosine was shown to be the precursor of N-acetyldopamine (26, 43) as its rise in hemolymph concentratio before tanning (27) and decrease afterward (4) indicated.

Feeding Chitinous Materials

There is a lack of information available concerning the feeding of chitinous products to ruminants. The nutritional information that is available is derived almost exclusively from poultry research or from evaluation of human foods.

Earlier workers established mineral levels in crabs (31, 32, 45). Essential amino acids in the protein hydolysate of crabmeal were examined by several workers (18, 19, 34). Manning (24) reported shellfish meals contain excellent quality protein even though it is in lower amounts than in fish meals.

A protein digestibility in crabmeal of 80% was observed by Mangold and Hock (23). The protein availability of

shell-free crabmeal was found to be 94% by Schmalfuss and Werner (41) and 81% by Mangold and Damkobler (22).

Lubitz et al. (21) fed crabmeal to rats and observed a crude protein availability of 84% and a biological value of 76, compared to casein at 100; when fed to chickens, a biological value of 59 was observed. Then workers (21) also fed purified chitin to rats and found 24% of its nitrogen was available to these animals but concluded that chitin had no supplementary protein value for rats.

Parkhurst et al. (35) stated that crabmeal satisfactorily replaced fishmeal in chick diets when the calcium to phosphorous ratio was adjusted. Lovell et al. (20) fed a diet containing fresh water crayfish meal to rats and found when corrected for chitinous nitrogen (i.e., assumed chitin indigestible to the rat) crayfish protein was essentially equal in availability to methionine supplemented soy protein. Rutledge (39) pointed out that the high mineral content of shellfish meals limited incorporation into feeds to 10%. At this level, any protein contribution was greatly reduced, so Rutledge presented a method of milling and screening of meals which doubled the protein content, reduced the calcium and chitin content by as much as 68 and 82%, respectively, and left phosphorous only slightly altered.

Saito and Regier (40) fed a 29% shrimpmeal diet to brook trout and reported superior coloring and flavor over fish fed a commercial diet. Campbell (3) in citing work by

Wester (46) stated that chitin was not attacked by mammalian digestive enzymes. No reports were encountered in the literature of attempts to feed insects to livestock, but it is common knowledge that insects are indispensible in the ecological food chain. Insects are consumed in great quantities by birds and fish and to a notable degree by skunks, bears, anteaters, apes, and other omnivorous mammals. According to Haskell (38), Shulov has observed increased egg production and improved general health when poultry were fed locusts.

Chitin Assay of Insect Skins

Chitosan, the deacetylated form of chitin, gives a violet color when exposed to iodine (49). This reaction was used as an endpoint for chitin detection by both Wigglesworth (48) and Campbell (3). The assay was conducted by exposing the material to be qualitated to saturated potassium hydroxide (KOH) for 20 min at 160C. After the material had been washed, it showed a rose-violet color when flooded with 0.2% iodine in 1.0% sulfuric acid. The color change was observed under a microscope.

Chitosan obtained in the above procedure was soluble in 3% acetic acid or 10% sulfuric acid. As a method to check the purity of chitosan, it was allowed to stand in 10% sulfuric acid at 70C until spherites of chitosan sulfate separated out (48). Chitosan was completely broken down at temperatures beyond 184C (14). Van Wisselingh's original

method employed sealed tubes heated in glycerine. These obviously explosive conditions prompted other workers to modify in the direction of safety, whereby all heating was done in open vessels (3). This resulted in boiling and possible physical damage to the sample, but Campbell showed that this could be avoided by using potassium hydroxide concentrated at room temperature.

The only other natural organic compound known to withstand hot alkali treatment is cellulose. Differentiation between cellulose and chitosan is facilitated by the lack of solubility of cellulose in 3% acetic acid and its non-reactivity to iodine-dilute acid. Furthermore, cellulose turns blue in the presence of 75% sulfuric acid (3).

Chitin Assay of Shellfish Meals

Brown (2) and Rutledge (39) working with shrimp and fresh water crayfish, respectively, followed the procedure of Black and Schwartz(1). The sample was hydrolyzed under both acidic and basic conditions. The material to be assayed was exposed to 1N HCl and heated on a steam bath for 1 hr. The washed pH neutralized sample was then heated in 5% NaOH for 1 hr. At this point, the sample was washed with boiling water and acetone, dried to a constant weight, ashed, and reweighed. Weight loss after ashing was assumed to be chitin.

Lubitz *et al.* (21) hypothesized that the crude fiber fraction of a shellfish meal was equivalent to the chitin content.

Lovell *et al.* (20) tried three different methods of chitin determination. The formic acid procedure involved the exposure of 10g of crabmeal to 90% formic acid for 24 hr. The samples were centrifuged and the precipitate washed and dried. The sample was refluxed in 5% NaOH for 90 min, filtered, washed, and dried. Loss of weight after ashing was assumed to be chitin. The second method employed 1N HCl instead of formic acid, and the samples were heated on a steam bath for one hour rather than room temperature for 24 hr. The third method used cold 5% HCl instead of formic acid. All other steps remained unchanged from the formic acid procedure.

Lovell and coworkers reported that the cold HCl method proved to be the superior procedure. Three criteria were invoked to arrive at this conclusion: 1) 5% acetic acid solubility; deacetylated chitin (chitosan) was soluble in 5% acetic acid (48); 2) Nitrogen content; a value higher than theoretical implied poor protein elimination, a low value implied deamination; 3) Ash content; incomplete decalcification would result in a high ash content. Formic acid treated samples showed a high ash content. The cold HCl treated samples had the lowest acetic acid solubility and the most reliable nitrogen level.

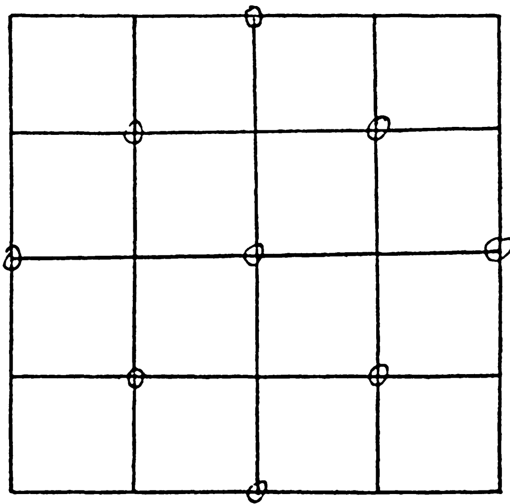
Three methods of decalcifying crustacean shells were compared by Giles *et al.* (12). The first method examined was that of Clark and Smith (5) wherein calcium carbonate was removed with cold dilute nitric acid, and protein was removed by hydrolysis with boiling aqueous sodium hydroxide for 4 hr. The second method studied was first investigated by Thor (37). This procedure was milder in that sodium carbonate was used as the hydrolyzing agent. The third method evaluated as a means of removing protein was pepsin digestion, which hydrolyzed tyrosine, an abundant amino acid in crustacean shells (8). Giles and coworkers chose Thor's method because it was less time-consuming than that of Clark and Smith and required less sample preparation than enzyme digestion.

Giles *et al.* (12) showed that hot solutions of pH less than 2.5 caused deacetylation, loss of nitrogen, and oxidation of chitin.

MATERIALS AND METHODS

In Vivo Digestibility

Three basic techniques were used to investigate the chitinous compounds studied: *In vivo* digestibility with fistulated steers, *in vitro* rumen incubations, and feeding trials with young ruminants. The *in vivo* digestibility procedure involved a variation of the suspended nylon bag technique of Neathery (30). A known amount of dried sample was placed in a tared nylon bag measuring 4 x 12 cm. The nylon bag was then placed in a perforated plastic cylinder (7.5 x 30 cm) and suspended inside the rumen of a fistulated Jersey steer. The steers, 4 years old and fistulated at 18 months of age, were on a high fiber diet. In the evaluation of a given feedstuff, nine treatments with four steers per treatment were utilized. The treatments represented nine points on a lattice of 25 points formed by five different times of incubation and five different amounts of sample (Figure 1). This experimental procedure enabled use of the response surface method of Gardiner *et al.* (10). Once a plastic container was suspended in a rumen, nylon bags were withdrawn at 12 (15 g sample), 24 (10 and 20 g sample), 36 (5, 15, and 25 g sample), 48 (10 and 20 g sample), and 60 (15 g sample) hr. The samples were frozen at -15C until further assay. Upon thawing, soluble material was removed from all samples by washing in a Maytag clothes washer (wringer type) for 15 min in water at 40C. The samples were dried in a forced air oven (60C) for 48 hr



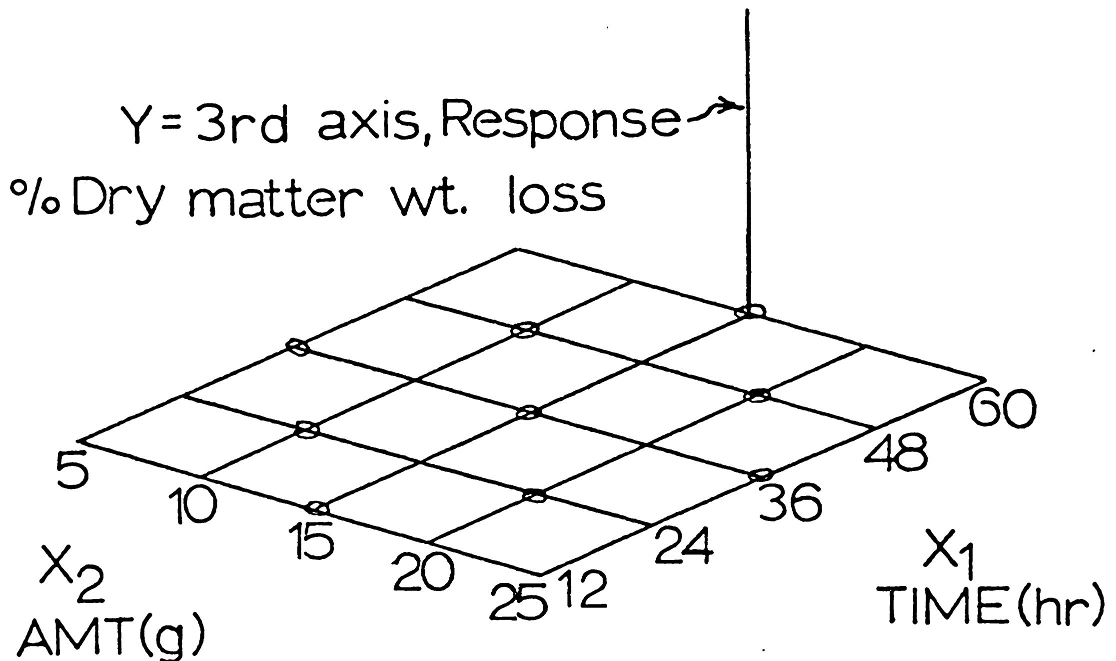


Figure 1. Outline of the sample distribution used in response surface experiment. Circled points represent time and amounts used. A response surface was calculated from the following multiple regression equation:

$$\hat{Y} = b_o + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$$

where; x_1 = time

x_2 = amount

and Y = predicted response.

and weighed to determine weight loss in the rumen. This weight loss, expressed in percent on a dry basis, represented the third dimension of the response surface method (Figure 1). The four replications of a given sample were pooled, divided into two samples, and analyzed for proximate constituents. Dried, unincubated samples were analyzed for control values.

For each feedstuff suspended in the rumen, a study on its solubility in water was performed. Ten g of dried sample were placed into four tared nylon bags and suspended in a water bath at 37C for 48 hr. After hydrolysis, the samples were washed and dried in a manner outlined above for rumen incubated samples. After determination of weight loss, the samples were pooled, divided into two samples, and analyzed for proximate analysis. This procedure (determination of rumen and water solubility) was used on cellulose, alfalfa hay, crabmeal, shrimpshells, cockroaches (P. americana), grasshoppers (Melanoplus packardi S), and purified chitin, reagent grade (obtained from Sigma Chemical Company, St. Louis, Missouri).

Feeding Trial I

Of the several chitinous feedstuffs investigated, only crabmeal was available in sufficient quantity to enable a feeding trial. Crabmeal was obtained from the Hunt Crab Meal Company of Hampton, Virginia, in 45.4 kg (100 lb) bags. Two feeding trials were undertaken; the first involved six

animals, the second involved ten. The first trial was initiated in the early spring. The six male Holstein-Fresian animals weighed an average of 74 kg at trial initiation. Prior to initiation of the experiment, they were raised in normal fashion receiving milk replacer soon after birth followed by calf-starter and hay. At the time of trial initiation, they were 12 weeks old. Three diets were fed in the first trial: a base ration, a 10% and 20% crabmeal ration (Table 1 and Table 9, page 50). The crabmeal rations were achieved by substituting 10% and 20% by weight, respectively, of the base ration with crabmeal. The base ration was formulated to meet 100% of published National Research Council (33) maintenance and growth requirements of the experimental animals.

A 3 x 3 Latin square design was employed by using three of the six animals and the three diets over three 4-week periods. A second replication was performed using the other three calves with identical feeds and time periods. Each time period of each replication was divided into one-week adjustment and a three-week full-ration period. During the third week each calf was placed in a metabolism stall. After being in the stall two days, collection of feces and urine was begun and continued for five days. Urine output was measured daily; for every .45 kg of urine excreted, 10 ml were pooled and frozen. Urine samples were assayed for total nitrogen via the Kjeldahl method. Feces were collected daily

Table 1. Rations utilized in feeding Trials I and II

Ingredients[1]	Base	10% Crab	20% Crab
	(kg/100 kg)		
Hay	45	40.5	36.0
Corn	30	27.0	24.0
Soybean meal	15	13.5	12.0
Molasses	7	6.3	5.6
Deflourinated phosphate	2	1.8	1.6
Salt	1	.9	.8
Crabmeal[2]	0	10	20
Dry Matter Basis			
Crude Protein	16.03	17.94	20.16
Crude Fiber	18.75	17.64	17.17

[1]Each ration was fortified with 1,630 I.U./kg of Vitamin A and 2,000 I.U./kg of Vitamin D.

[2]Crabmeal had a guaranteed analysis of 31.0% crude protein, 1.0% crude fat, and 14.0% crude fiber.
Crabmeal ground to pass through a sieve of pore diameter = 2 mm.

and the total amount for the five days was mixed, weighed, and a sample frozen until subsequent analysis. The feces dry matter was determined by observing the weight loss of a 200 g fecal sample placed in a forced air oven at 60C for 48 hr. A portion of all fecal samples was analyzed for proximate constituents. Daily feeding was ad libitum throughout the trial and total feed consumption was obtained by summation of daily intake. Proximate analysis was obtained on the three different feeds presented to the animals. All animals were weighed weekly during the trial.

Feeding Trial II

The second feeding trial was begun in early fall and continued until January. The ten male Holstein-Fresian calves utilized in this trial weighed an average 149 kg. Two diets were fed: a basal ration, and a 20% crabmeal diet, both identical to the diets of Trial I. The ten calves were divided into two groups and each group fed one of the diets. Two animals from each group were selected to be placed in metabolism crates, selection being based on nearness of weights to the group average. Using the two diets, a pair of calves from each group, and two time periods, a 2 x 2 Latin square experimental design was followed. The time periods were of six weeks duration. During the first two weeks the animals were gradually adjusted to the respective diets. During the first time period, the animals were placed in the

metabolism crates after only one week on the full diet. During the second time period, the animals were placed into the crates after two weeks on the diet. Sample collection, feeding, and weighing were performed in a manner identical to Trial I.

Neutron Activation Analysis

The determination of trace elements in crabmeal feeds was accomplished using Neutron Activation Analysis (9). This service was provided by the Department of Physics at the Virginia Polytechnic Institute and State University. A known amount of sample was placed in a 1 x 2 cm polyethylene vial with a snap-shut lid. Gloves were worn to avoid contamination due to NaCl from perspiration. Duplicate samples, as well as blank controls, were thus analyzed in parts per million for bromine, calcium, copper, iodine, potassium, sodium, and magnesium.

In this analysis, samples of crabmeal, basal diet, 10% crabmeal diet, and 20% crabmeal diet were activated in the as-fed state.

Calcium Digestion

The quantitation of feed and fecal calcium was accomplished using a Perkin Elmer Model 403 Atomic Absorption Spectrophotometer. Sample preparation was as follows: Two g of feces (or feed) were placed in a crucible

and ashed in a muffle furnace for 24 hours at 500C. The ashed sample was dissolved in 3 ml of 50% HCl (v/v) and then taken up to a volume of 25 ml with distilled water. From this 25 ml volume, 100 µl were withdrawn and mixed with 10 ml of distilled water. From this mixture, 100 µl were withdrawn and mixed with 16 ml of 0.5% lanthium oxide. This solution, which represented a dilution of 1 to 16,000 of the original 25 ml solution, was read on the spectrophotometer, yielding the concentration of calcium. Knowing feed and fecal calcium concentration, digestibility estimates of this mineral were possible.

Chitin Assay Principles

A uniqueness to the problem undertaken herein causes conventional chitin assays to be only partly applicable. Specifically, all methods reviewed have been for extracting chitin from its natural embedding compounds, calcium carbonate or sclerotin. Once a chitinous compound is passed through a ruminant digestive tract, it unavoidably is mixed with plant and feed particles, and one is faced with the problem of separating several fibrous compounds which differ only slightly in molecular structure. The situation is further confounded by the abundant acetic acid of the rumen environment being confused with acetate which may have been derived from deacetylated chitin.

The basic hypothesis first established was that the amount of acetate recovered from chitin, by heating in KOH, with subsequent acidifying and steam distillation, was directly proportional to the amount of chitin, and the amount of chitin only. It was imperative that this hold true regardless of the source of the chitinous substance involved. With these considerations in mind, the following plan of study was pursued.

The first task undertaken was the establishment of a dose-response relationship between chitin and acetate. Once this was accomplished, standard curves were run to further clarify this basic premise. The effect of the length of time of heating in KOH was investigated, followed by an elucidation of the degree of resolution of the assay.

Endeavors to this point produced a reliable assay for chitin of insect origin. Efforts were then turned to assaying for chitin of marine derivation. The neutral detergent fiber analysis (44) was useful for dividing a plant's feed components into categories of nutritionally available or partially available (and then only via microbial fermentation) (44). Acid detergent fiber analysis represented the sum of cellulose and lignin (44) and thereby included chitin.

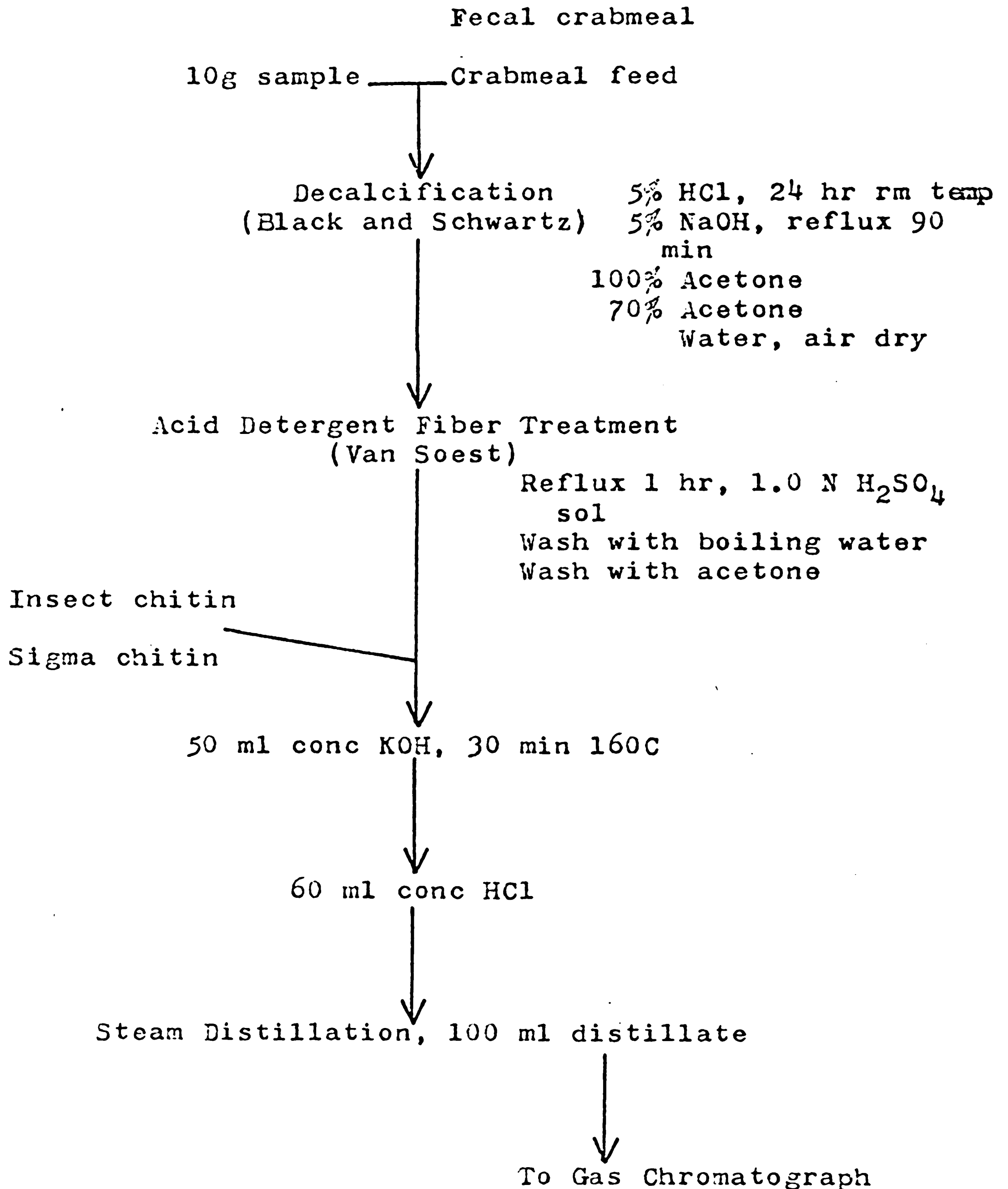
In assaying for chitin of marine derivation, the decalcification of Black and Schwartz (1) was first performed. This was followed by an acid-detergent fiber

treatment which prepared the sample for potassium hydroxide hydrolysis (48, 3, 49). That this was the most efficacious order of treatments was established by a test of all combinations of treatment sequences. As a final step, steam distillation enabled quantitation of acetate, which served as an endpoint for chitin quantitation for insect as well as crab chitin. These basic concepts are outlined in Figure 2, which is a flow chart of the chitin assay procedure. With the foregoing as an introduction, the specifics of the methods used and the elucidation of the various experiments follow.

Chitin Assay Procedures

The basic chitin assay was as follows: Dried samples (either pure chitin, insect skins, or decalcified marine samples) were placed in 125 ml flasks and 50 ml of KOH, saturated at room temperature, were added. The flasks were then heated in cooking oil for 30 min at 160C. After cooling, 10 ml of water, followed by 60 ml of 12N HCl were slowly added to the flasks, and the entire contents transferred to 500 ml roundbottom flasks and steam distilled until 15 ml of distillate had been obtained. It was later found that it was necessary to collect 100 ml of distillate to obtain acceptable efficiency levels. Five microliters of distillate were injected into Beckman Model GC-2A gas chromatograph for acetate quantitation.

Figure 2. Chitin Assay Flow Chart



Pilot Studies on Chitin-Acetate Dose-Response

Purified chitin obtained from the Sigma Chemical Company was exposed to the chitin assay procedure in duplicate in .75 and 1.00 g and then .10, .25, .50, .75, and 1.0 g amounts to ascertain the degree of acetate quantitated from the amounts of chitin. As a further attempt to standardize results, the effect of time of heating in KOH on acetate recovery was investigated. One gram samples of chitin, in duplicate, were heated for 30 min, 45 min, 60 min and 90 min in 14N KOH.

Insect chitin was obtained by picking cast skins from cockroach batteries, washing twice in chloroform-methanol (2:1, v:v) to remove lipids, grinding by hand and drying. After a pilot check, a dose-response curve was executed using .25, .50, .75, and 1.0 g of cast skins, in duplicate. Also, an internal standard and an unknown were run.

In an effort to determine the resolving power of the insect chitin assay, a dose-response curve was run using 350, 400, and 450 mg insect skin samples in duplicate, which were quantitated for acetate.

Assaying for Chitin of Marine Derivation

The procedure outlined below, decalcification followed by acid detergent fiber digestion, was carried out in duplicate on samples of crabmeal and purified chitin. The reverse process, acid detergent fiber digestion followed by

decalcification, was also carried out in duplicate on samples of chitin and crabmeal. To complete the pilot study, crabmeal and chitin, in duplicate, were exposed to each process alone. The results indicated the following procedure was most efficient for recovering acetate.

Ten grams of sample to be assayed, feces or feed from the crabmeal feeding trial, were placed in a 125 ml flask. To this flask 100 ml of 5% HCl were added. The flask was agitated at room temperature for 24 hr, the contents placed in a 250 ml centrifuge bottle, and spun at 3000 x g for 15 min. The supernatant was siphoned off, the bottle refilled with 100% acetone, agitated for one minute, then recentrifuged as above. The process was repeated using 70% acetone, after which the sample was placed in a 600 ml beaker and refluxed for 90 min in 100 ml of 5% NaOH. The refluxed samples were centrifuged at 3000 x g for 15 min, the supernatant siphoned off, the the precipitate resuspended in water. After two additional centrifugations in water, the decalcification procedure was finished. The precipitated samples were then placed in a 600 ml beaker with 100 ml of acid detergent fiber solution (44) and 2 ml of decalin to serve as an anti-foaming agent. The samples were refluxed in acid detergent solution for one hour then filtered by vacuum through a sintered glass crucible. The retained material was washed twice with boiling water and twice with acetone while it remained in the crucible under suction. Samples were then dried

in an oven at 100C for 24 hr. These dried samples were introduced into the chitin assay procedure outlined previously at the point of heating in KOH.

In Vitro Incubation

In vitro incubation of purified chitin was undertaken in the following manner. To six 500 ml flasks, 400 mg of chitin were added, along with 100 ml of fresh rumen fluid, strained through cheesecloth. Rumen fluid was also added to six flasks without chitin. Of these 12 flasks, three containing chitin plus rumen fluid and three containing only rumen fluid were incubated for 3 hr at 37C. The incubation flasks were flushed with nitrogen to remove oxygen and stoppered with a rubber cork fashioned with a balloon to allow for gas expansion. The remaining six flasks, three containing rumen fluid and chitin and three containing only rumen fluid, were subject to the addition of 20 ml of 6N H_2SO_4 to stop microbial fermentation, as were the incubated flasks after the 3 hr incubation. Once acid was added, all 12 flasks were treated in the following manner: The contents were transferred to a 250 ml centrifuge bottle and spun for ten minutes at 3000 x g. The supernatant was decanted and the process repeated twice. After the third centrifugation the entire bottle contents was filtered through Whatman #3 filter paper, the residue washed into a 125 ml flask and dried on a steam bath. The dried samples were treated as outlined above in the chitin assay beginning with the addition of 14N KOH.

A similar *in vitro* incubation was carried out using the cast skins of cockroaches. Cast skins, in 400 mg amounts, were placed into each of four 500 ml flasks. To these flasks, plus four additional flasks, 100 ml of strained rumen fluid were added. Two flasks containing chitin, and two without, were incubated in a water bath for 3 hr at 37C. These flasks were evacuated of oxygen and stoppered as above in the purified chitin incubation. Microbial activity was stopped with 20 ml of 6N H_2SO_4 and chitin quantitation carried out according to the assay procedure outlined.

RESULTS AND DISCUSSION

In Vivo Incubation

In vivo solubilities of five chitinous compounds and two sources of cellulose were investigated using the suspended nylon bag technique (30). The actual data of the percent weight loss from nylon bags of cellulose, alfalfa hay, crabmeal, shrimpmeal, cockroaches, grasshoppers, and purified chitin are seen in Table 2. Multiple regression analysis of the data resulted in the equations seen in Table 3. These equations were utilized to create the response surfaces depicted in Figures 3-9. These response surfaces are depicted with percent dry matter weight loss plotted along the vertical axis. Although the surfaces would appear differently had g dry matter weight loss been employed, the present system relates more easily to *in vivo* studies and facilitates comparison between sample types.

Cellulose (Figure 3) solubility was effected both by time of incubation (P .01) and amount of sample (P .01) with interaction being nonsignificant. The solubility of cellulose dry matter exhibited the greatest response to treatment of all materials examined in that values ranged from 6 to 73%. Alfalfa hay (Figure 4) responded in like manner, with both time and amount (P .01) but not interaction significant. A point readily apparent from Figure 4 is that according to the data presented, alfalfa is digested more at 36 hr than at 60 hr. The differences observed are not compatible with any known nutritional axioms but are probably explained by the fact that

Table 2. Dry matter weight loss from cellulose reference samples and chitinous samples placed in nylon bags and suspended in the rumen of fistulated steers for varying time lengths.

Time of Incubation (hr)	12	24	24	36	36	36	48	48	60
Amount of Sample (g)	15	10	20	5	15	25	10	20	15
	(Weight Loss; % of initial)								
Reference Material									
Cellulose	4.9	50.0	32.6	57.5	34.0	35.7	40.3	34.6	73.0
Alfalfa	27.0	53.5	52.2	55.1	43.6	41.2	50.6	42.7	48.5
Chitinous Sources									
Crabmeal	26.7	31.4	34.9	39.1	33.2	36.3	42.0	40.3	38.3
Shrimpmeal[1]	11.9	15.5	13.3	20.4	17.0	17.1	21.6	20.6	22.3
Cockroaches	61.0	63.9	66.2	68.0	63.7	67.8	68.7	69.8	70.9
Grasshoppers[1]	30.2	29.9	31.9	32.4	31.4	31.9	35.3	33.2	34.58
Purified Chitin	19.6	21.7	17.8	24.1	23.2	22.1	22.5	22.3	26.0

[1] Due to a scarcity of grasshoppers, sample amounts of 2, 4, 6, 8, and 10 g were used, as was the case for shrimpmeal.

Table 3. Regression coefficients, standard error of means, and coefficients of variation describing the effects of incubation time and amount of sample on the solubility of various chitin sources in the rumen.

Chitinous Source	S.E. of the Mean	C.V.[2]	Composition of Total S.S. (%)					
			Treatment	Linear x_1	Linear x_2	Quad.	Inter	Lack Fit
Cellulose[1]	3.04	15.12	91.9**	44.1**	11.9**	4.0**	1.1	30.7**
Alfalfa[1]	2.04	8.87	84.3**	10.9**	14.5**	19.5**	1.5	15.7**
Crabmeal	1.52	8.46	74.9**	51.2**	.45	11.0**	2.8	9.3*
Shrimpshells	.65	7.34	90.6**	79.7**	6.6**	2.6*	.3	1.3
Cockroaches	.88	2.63	80.1**	63.3**	.79	7.6*	.4	8.0*
Grasshoppers	.89	5.51	55.0**	40.9**	.16	.7	9.2*	3.9
Chitin	.89	8.04	68.1**	40.6**	8.5*	1.7	5.0*	12.1*

[1]Reference Materials
[2]Coefficient of Variation = $100 \times \sqrt{EMS} / \bar{x}$
* $p < .05$
** $p < .01$

Table 3, Continued. Regression coefficients, standard error of means, and coefficients of variation describing the effects of incubation time and amount of sample on the solubility of various chitin sources in the rumen.

Chitinous Source	Regression Coefficient[3]					
	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}
Cellulose	34.891	10.705	-5.569	1.076	2.987	2.948
Alfalfa	49.368	2.558	-3.102	-2.537	.054	-1.166
Crabmeal	35.899	3.251	-.307	-.676	.634	-1.317
Shrimpshells	17.287	2.863	-.825	-.017	.392	.392
Cockroaches	65.364	2.351	.267	.254	.75	-.334
Grasshoppers	32.083	1.275	.080	.126	.061	-1.051
Chitin	21.436	1.510	-.694	.232	.303	.923

[3]Regression equation: $\hat{Y} = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$; where $\hat{Y}$ = predicted dry matter solubility, x_1 = hours of sample incubation, and x_2 = grams of sample incubated.

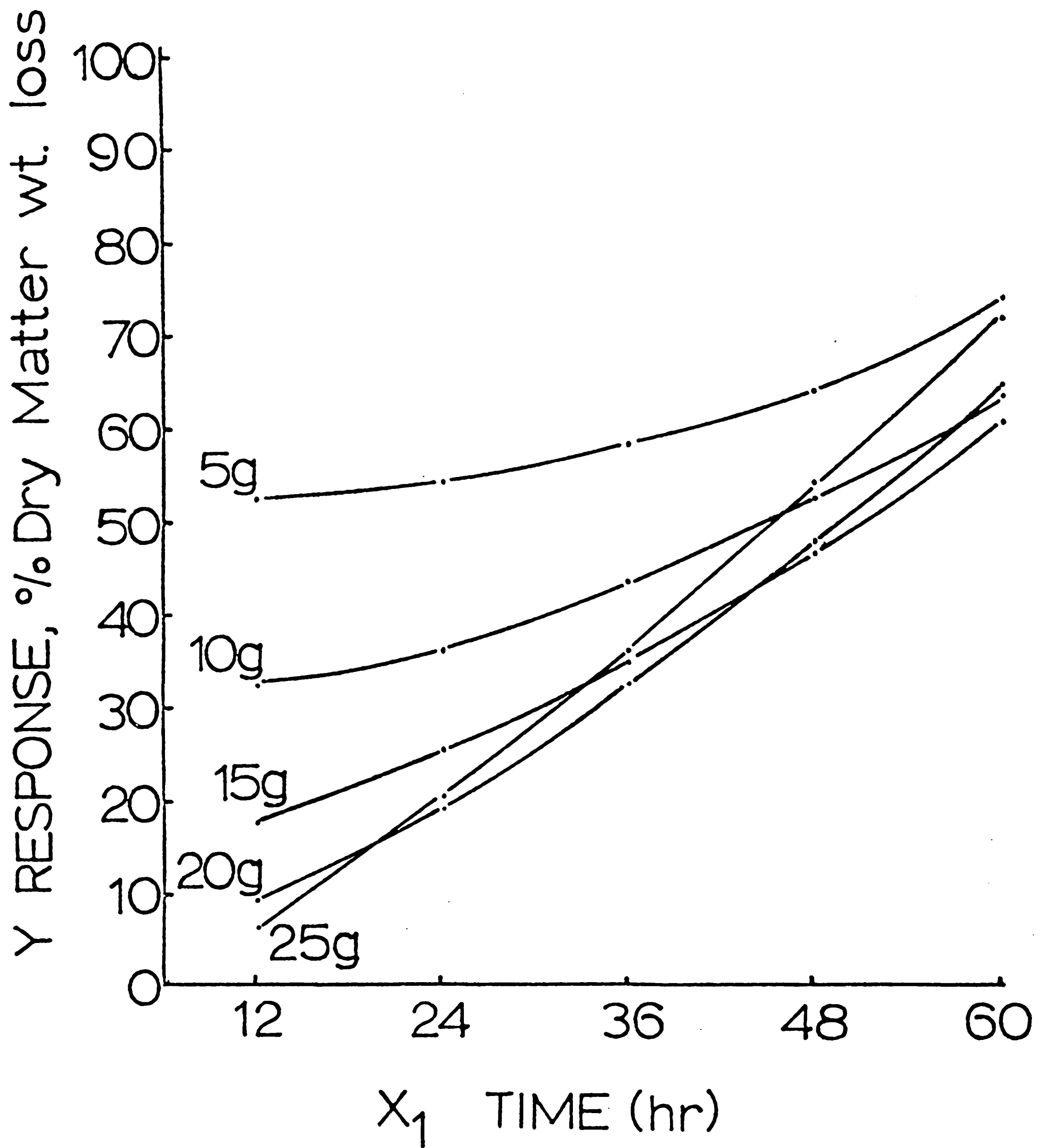


Figure 3. Response for cellulose solubility; effect of time of incubation (hr) and amount of sample (g) on dry matter weight loss.

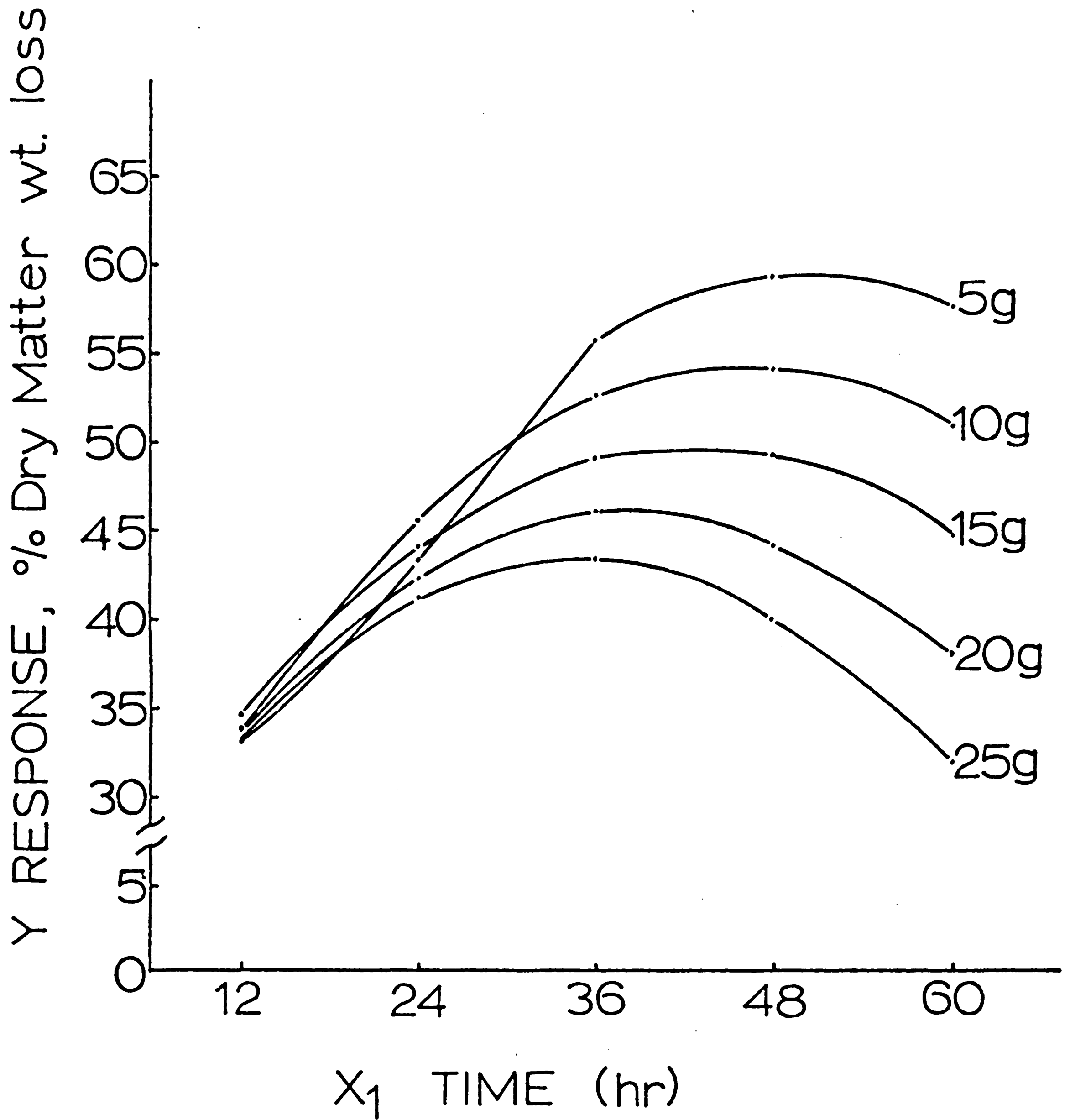


Figure 4. Response for alfalfa solubility; effect of time incubation (hr) and amount of sample (g) on dry matter weight loss.

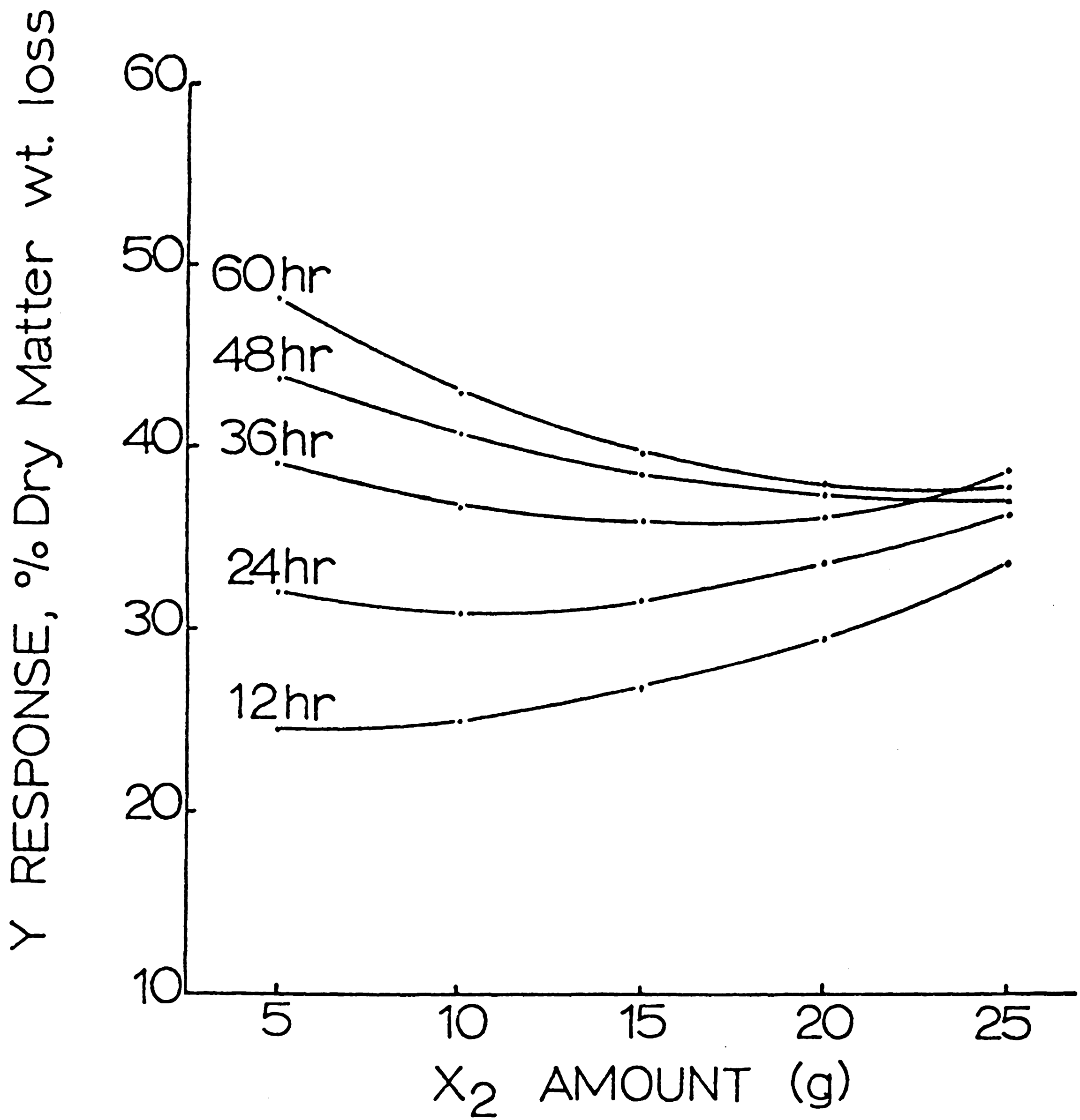


Figure 5. Response for crabmeal solubility; effect of time of incubation (hr) and amount of sample (g) on dry matter weight loss.

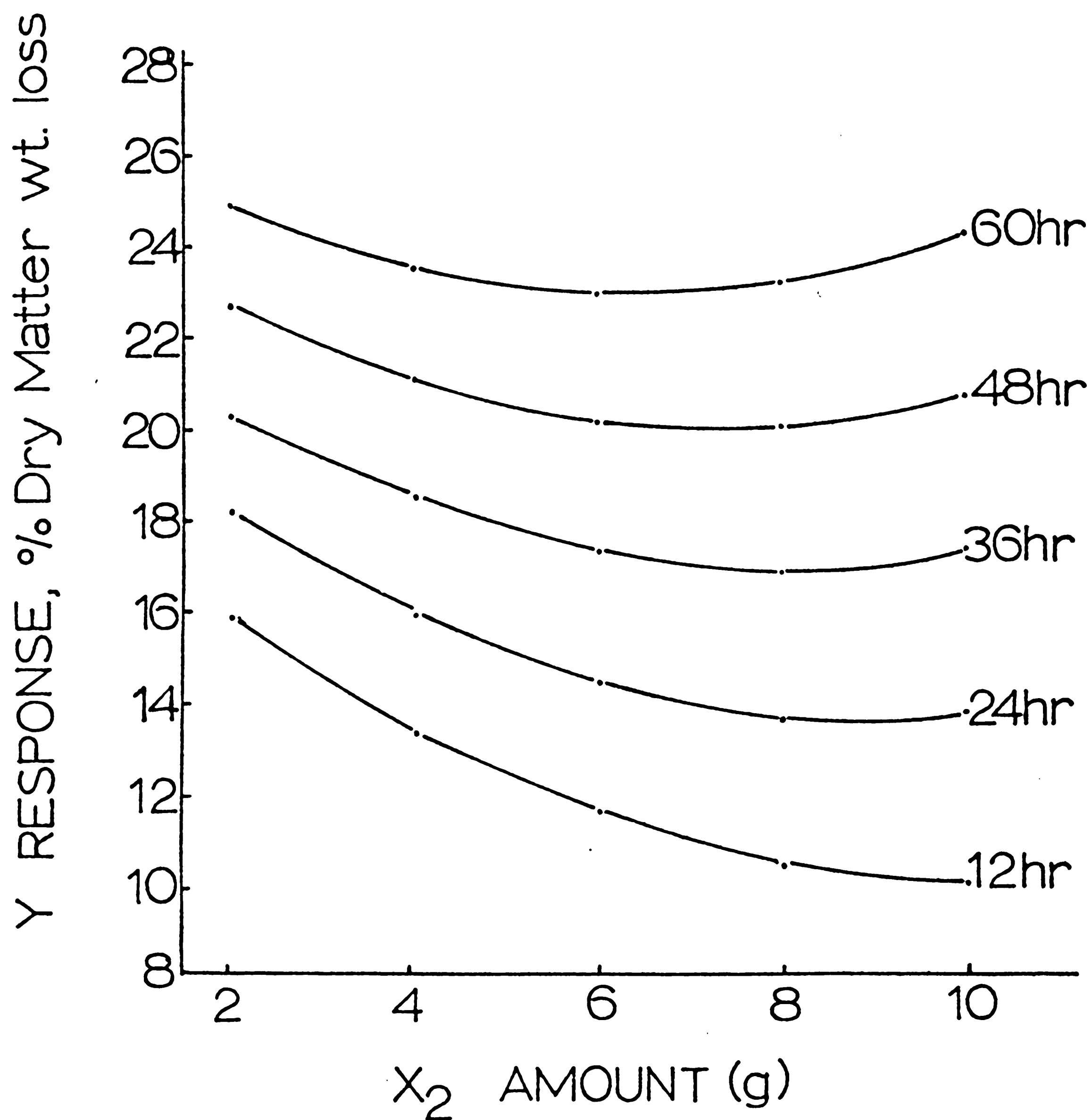


Figure 6. Response for shrimpmeal solubility; effect of time of incubation (hr) and amount of sample (g) on dry matter weight loss.

the alfalfa data conformed poorly to the hypothesized model. This is evident from Table 3 where lack of fit for alfalfa accounted for almost 45% of the total sum of squares. Solubilities ranged from 31 to 59% for alfalfa hay. Crabmeal solubility (Figure 5) ranged from 25 to 48% but was effected only by time of incubation (P .01).

Shrimpmeal solubility increased linearly as a function of time (P .01) (Figure 6). The amount of sample incubated resulted in a slight but significant (P .01) linear decrease in dry matter solubility. Solubilities ranged from 10 to 25%.

Cockroaches (Figure 7) seemed to be the most uniformly highly soluble of the samples investigated with observed solubilities ranging from 61.6 to 73.2%. Dry matter solubility increased significantly (P .01) in a linear fashion with incubation time, but amount of sample and interaction were non-significant.

Grasshoppers (Figure 8) were considerably less soluble than cockroaches, ranging from 29 to 35%. This was possibly due to the method of preserving the grasshoppers; a mixture of acetic acid and formaldehyde was used. Formaldehyde treatment has been used to protect protein from microbial degradation in the rumen through a reduction of solubility in the rumen (7). Its use for the preservation of grasshoppers could explain lowered rumen solubility as compared to cockroaches. Grasshoppers showed a significant (P .01) linear increase in solubility with time. Amount of sample resulted in no effect but there was a significant interaction (P .05) between time-amount.

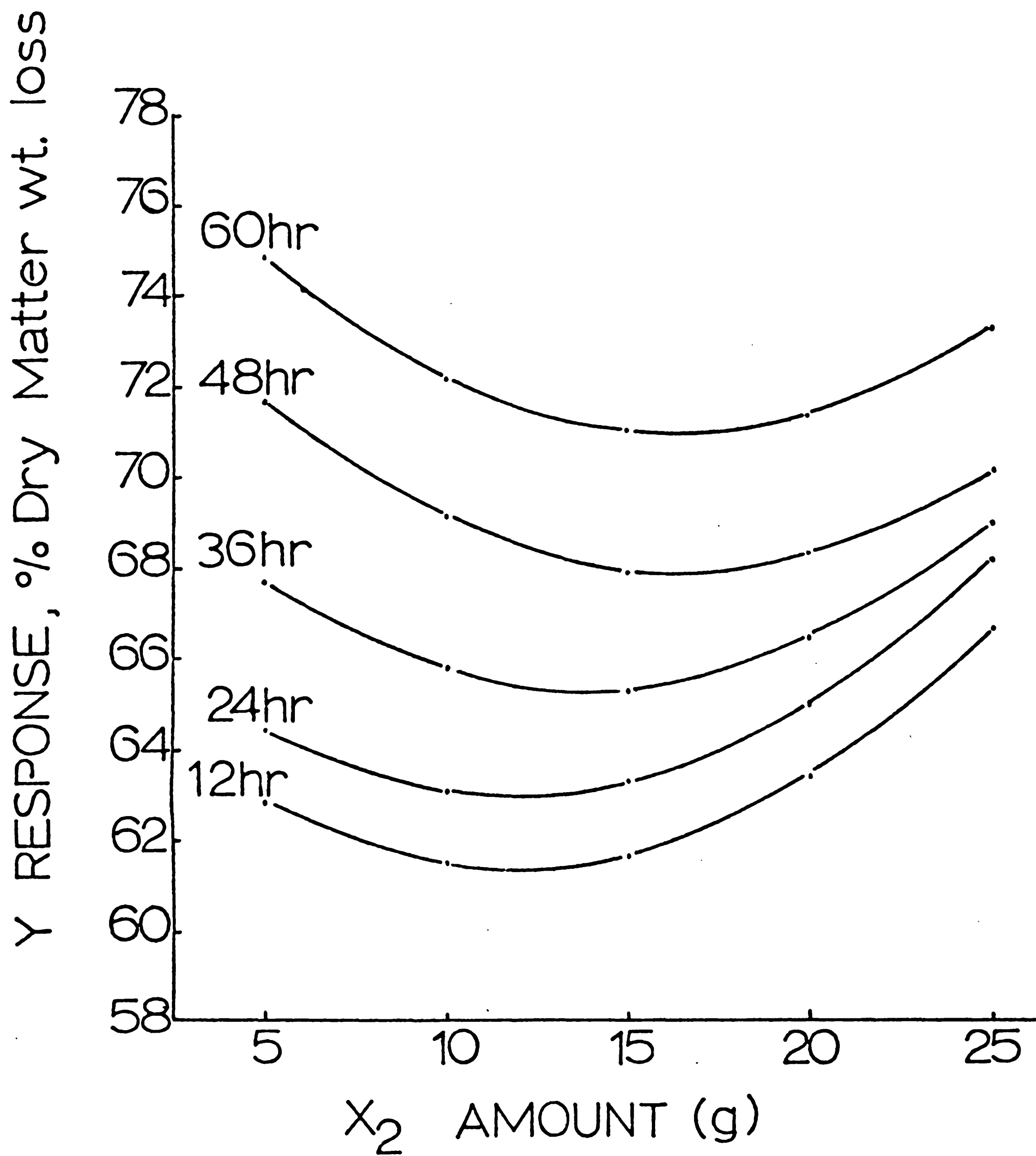


Figure 7. Response for cockroach solubility; effect of time of incubation (hr) and amount of sample (g) on dry matter weight loss.

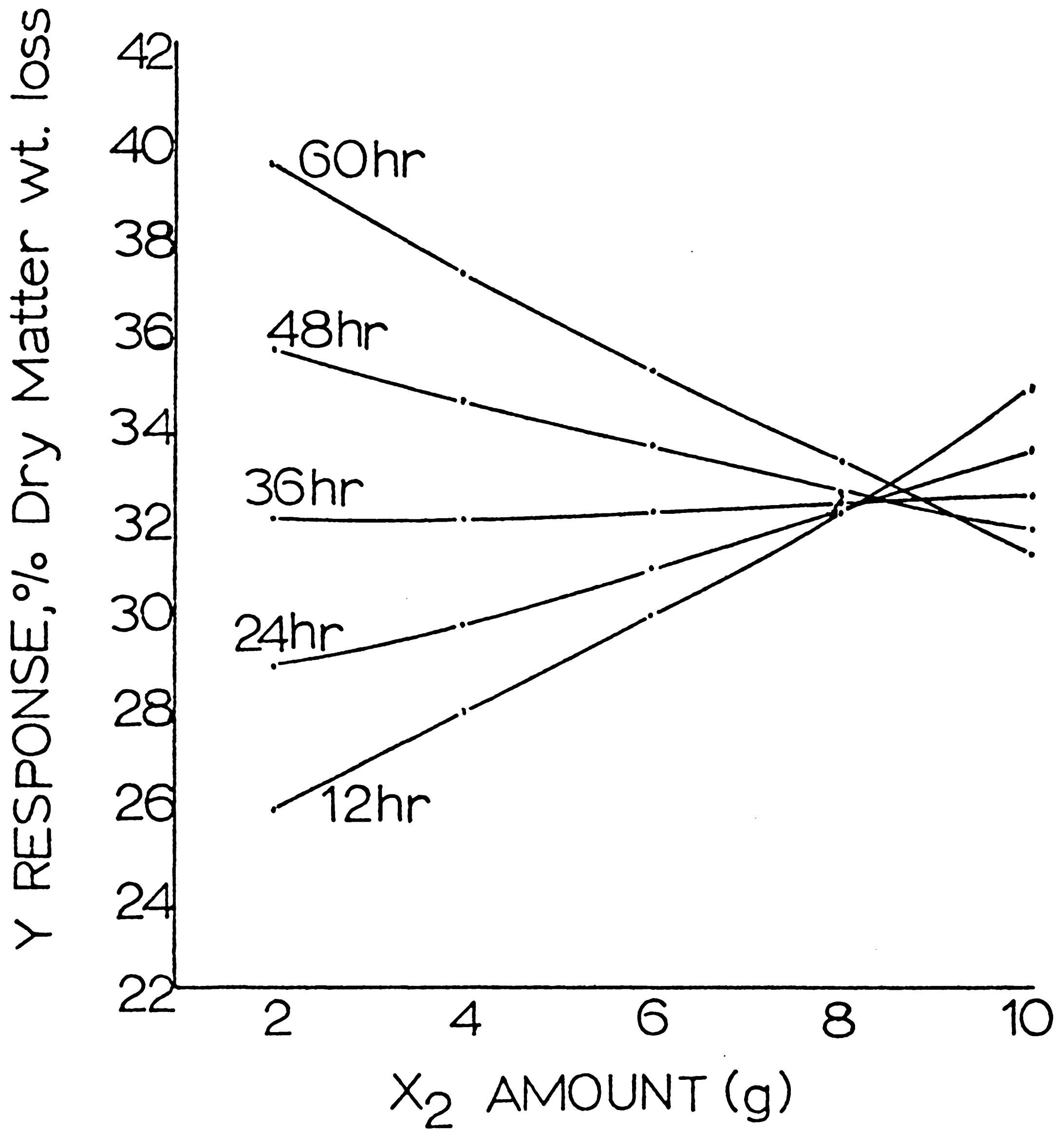


Figure 8. Response for grasshoppers; effect of time of incubation (hr) and amount of sample (g) on dry matter weight loss.

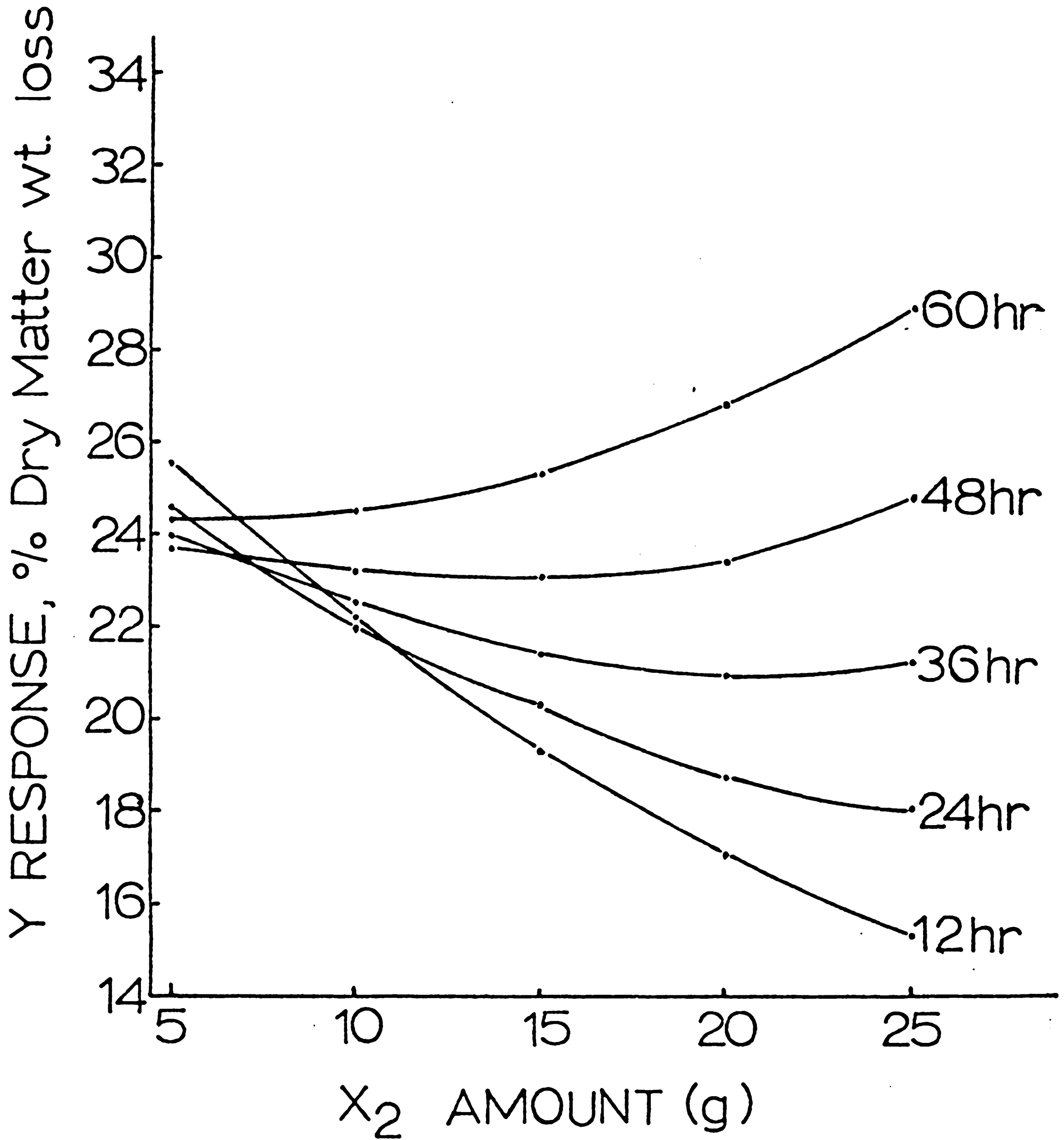


Figure 9. Response for purified chitin solubility; effect of time of incubation (hr) and amount of sample (g) on dry matter weight loss.

Purified chitin (Figure 9) solubility ranged from 15 to 29%. The solubility increased (P .01) with time and decreased (P .05) with increasing amounts of sample. Interaction between incubation time and amount of sample was significant (P .05).

The statistical summary of the response of all samples treated in the response surface experiments are seen in Table 3. The coefficients of variation are less than ten percent for all samples except cellulose. In all cases, both treatment effect and the linear component of time are highly significant. The linear component due to amount of sample was significant for only alfalfa, cellulose, shrimpshells, and chitin. The high degree of water solubility (Table 4) inherent in cockroaches, grasshoppers, and crabs could mask any possible effect due to amount of sample. Therefore, the water solubility of all chitinous samples examined was compared to the rumen solubility (Table 4).

Rumen solubility of crabmeal was 27% in water and increased to 42% in the rumen. The crude protein fraction of crabmeal showed a substantial increase in solubility due to the rumen environment (from 37.5 to 50.2%), but ether extract and ash components did not.

Shrimpmeal was predominantly shells, giving rise to a crude fiber component which was insoluble in water but almost 20% soluble in the rumen. Shrimpmeal crude protein was 13% soluble in water and 27.5% soluble in the rumen.

Table 4. The solubility[1] of the proximate components of ten gram samples of chitinous material incubated *in vitro* in water or in rumen environment for 48 hr.

Chitinous Source	Component	% Composition	% Solubility in H_2O	% Solubility in Rumen	Difference Due to Rumen
Crabmeal	H_2O	10.50			
	C.F.[2]	10.35	5.5	8.0	2.5
	C.P.[2]	29.44	37.5	50.2	12.5
	ASH	34.65	19.3	14.1	-5.2
	E.E.[2]	2.24	66.2	66.5	0
	Weight		27.0	42.0	15.0
Shrimp-meal	H_2O	9.00			
	C.F.	28.92	0	19.6	19.6
	C.P.	49.54	13.0	27.5	14.5
	ASH	23.86	4.9	0	-4.9
	E.E.	.49	36.3	100	63.7
	Weight		12.4	20.7	8.3
Cock-roaches	H_2O	6.68			
	C.F.	21.24	53.2	67.7	14.5
	C.P.	87.33	61.8	67.9	6.1
	ASH	5.13	92.3	86.5	-5.8
	E.E.	13.82	60.8	68.6	7.7
	Weight		58.8	63.8	5.0
Grass-hoppers	H_2O	10.00			
	C.F.	11.89	4.3	11.0	6.7
	C.P.	59.67	23.3	31.2	7.9
	ASH	3.78	41.5	66.0	24.5
	E.E.	3.49	15.0	67.3	42.3
	Weight		29.1	32.3	3.2
Chitin (purified)	C.F.	90+			
	C.P.	40.72[3]			
	ASH	.65			
	Weight		8.5	21.4	12.9

[1]Solubility = $100 \times \frac{AB - CD}{AB}$; where

A = dry weight of chitinous material put into rumen
B = % of particular component in sample put in rumen
C = dry weight of chitinous material taken out of rumen
D = % of same particular component in sample taken out of rumen

[2]C.F. = Crude fiber; C.P. = Crude protein, N x 6.25; E.E. = Ether extract

[3]Chitin is 6.89%N; 6.89 x 6.25 = 43.06C.P. 40.72 is observed.

The high water solubility of cockroaches, along with the protein content, emphasizes the potential value of this insect not only as a feed for ruminants but also for monogastrics as well.

Grasshoppers were not outstandingly soluble in crude protein, compared to cockroaches, but this was most likely due to the method of preservation mentioned above wherein formaldehyde was used.

Chitin was 8.5% soluble in water, which must be attributed to loss of fine particles through the pores of the sack. The 21.4% solubility in the rumen was quite possibly the sum of at least 12% true digestion plus losses due to water hydrolysis.

The crude fiber breakdown observed in the sample listed in Table 4 is an approximation of chitin digestion (1, 20, 21). The loss of weight from the crude fiber portion of shrimpmeal, grasshoppers, and chitin is of sufficient magnitude to warrant speculation of true chitin digestion.

Feeding Trials

In the first feeding trial, there was no significant difference between treatments for the nine parameters measured (Table 5 and 6). Average daily gain, feed intake, and feed efficiency showed no difference between treatments. In each case, the trend was in favor of the 20% crabmeal diet. Furthermore, there was no difference between treatments for dry matter, crude fiber, or nitrogen digestibility, and, here

Table 5. Feeding Trial Performance Data, Trial I

Performance Trait	Diet (% Crabmeal)		
	0	10	20
Average daily gain (kg/hd/day)	.650	.662	.686
Intake (kg/100kg/hd/day)	3.52	3.52	3.69
Feed Efficiency (kg gain/kg feed)	.184	.186	.210
Urine Output (kg/hd/day)	3.24	3.74	3.98
% Nitrogen Digestion (Absorption)	68.8	72.5	73.4
% Nitrogen Retention	36.5	48.5	43.6
% Dry Matter Digestion	69.9	68.3	64.05
% Crude Fiber Digestion	61.1	59.3	54.5
% Calcium Digestion	48.0	26.6	39.6

Table 6. Trial I Statistics

Predicted Response Y	Regression Coefficients[1] b_0	b_1	C.V.[2] %	% Composition of Total S.S. Treatment[3]	Linear	Lack of Fit
Average Daily Gain (kg/calf/day)	.649	0.002	29.44	0.47	0.43	0.04
Intake (kg/100kg body weight/head/day)	3.486	0.008	8.42	5.75	4.16	1.58
Feed Efficiency (kg gain/kg feed)	.180	0.001	32.78	4.41	3.54	0.85
Urine Output (kg / head/day)	3.390	0.037	22.28	8.02	7.55	0.46
Nitrogen Digestion (% of intake)	69.330	0.228	10.05	6.01	5.39	0.62
Nitrogen Retention (% of digested)	39.380	0.350	50.76	3.76	1.26	2.50
% Dry Matter Digestibility	70.415	-.297	8.39	13.85	7.25	6.46
% Crude Fiber Digestibility	61.600	-.327	15.39	6.25	5.83	.42
% Calcium Digestibility	42.630	-.455	49.26	14.48	2.58	11.89

[1]Model $Y = b_0 + b_1x$; where Y=predicted response and x=amount of crabmeal in ration.

[2]C.V. $= \frac{\sqrt{EMS}}{\bar{X}} \times 100$

[3]All treatment effects were nonsignificant ($P > .05$).

again, nitrogen digestibility was somewhat higher for the 20% crabmeal diet. Nitrogen retention was not effected by diet but tended to be greater for animals on crabmeal. The portion of crabmeal that is digestible apparently equals the nutritive value of the portion of the ration displaced by crabmeal. As seen in Table 6, which statistically summarizes the results of Trial I, the treatment sum of squares was a small percentage of the total sum of squares but was greatly reduced in most cases by the linear component.

Trial II was executed with more animals that were larger, 205 kg mid-trial weight for Trial II compared to 102 kg mid-trial in Trial I. The performance data seen in Table 7 confirms the results obtained in Trial I, indicating that 20% crabmeal would replace a comparable amount of basal ration. Average daily gain, feed intake, and feed efficiency were not effected by diet. Likewise, dry matter, crude fiber, and nitrogen digestion showed no adverse response to crabmeal in the diet. The regression plots of percent dry matter and percent crude fiber digestibility, as a function of dietary crabmeal, were slightly negative but these treatment effects were nonsifnificant, as in Trial I. Total dry matter intake showed a negatively sloped regression as a function of crabmeal, but again, there was not a significant treatment effect (Table 8).

Ten animals were involved in Trial II, but only four, chosen on the basis of nearness of weight to the group average, were used to collect metabolic data. Consequently, nitrogen

Table 7. Feeding Trial Performance Data, Trial II

Performance Trail	% Dietary 0	Crabmeal 20
ADG[1] (kg/calf/day)	1.30	1.27
Intake (kg/100kg/hd/day)	3.17	3.06
Feed Efficiency (gain/feed)	.179	.169
Urine Output (kg/hd/day)	6.7*	8.8
Nitrogen Digestion (% of intake)	66.3	67.8
Nitrogen Retention (% of digestion)	49.9*	23.9
% Dry Matter Digestion	71.1	69.2
% Crude Fiber Digestion	63.3	64.2

[1]ADG = average daily gain

*$P < .05$

Table 8. Trial II Statistics

Predicted Response Y	Regression Coefficients[1]		C.V.[2]	S.E. of the Mean	Treatment Sum of Squares as % of Total Sum of Squares
	b_0	b_1			
Average Daily Gain (kg)	1.309	-.002	18.26	.105	0.70
Intake (kg/100kg body weight/head/day)	3.21	-.009	7.50	.105	5.83
Feed Efficiency (kg gain/kg feed)	.179	-.0005	10.40	.008	6.8
Urine Output (kg/head/day)	6.70	.11	10.75	2.96	58.3*
Nitrogen Digestion (Adsorption) (% of N intake)	66.612	.076	8.84	1.82	6.9
Nitrogen Retention (% of N digested)	49.385	-1.301	28.62	7.47	65.4*
% Dry Matter Digestibility	71.120	-.095	2.95	1.46	23.7
% Crude Fiber Digestibility	63.362	-.043	3.77	1.70	19.3

[1]Model $Y = b_0 + b_1x$; where Y=predicted response and x=amount of crabmeal in ration.
[2]$C.V. = \sqrt{\frac{EMS}{\bar{x}}} \times 100$
*$p < .025$

balance, dry matter, crude fiber digestibility, and urine volume were based on observation from four animals. Average daily gain, feed intake, and feed efficiency were based on observations of ten animals. The only treatments affected by crabmeal addition significantly (P .05) were nitrogen retention and urine volume (P .05). Regressions of nitrogen retention and urine output on crabmeal are presented in Figure 10. It is conceivable that the decrease in nitrogen retention of the animals on the 20% crabmeal diet could be the result of the increased urine output observed in these same animals. But such is not entirely the case, as evidenced by the fact that the percent nitrogen in the urine of 20% crabmeal animals was .956%, while for the control animals it was .785%.

A situation of increasing urine volume and decreasing nitrogen retention, as a function of increasing crabmeal, leads one to speculate about a contaminant in crabmeal. An examination of the trace elements found in crabmeal, to the extent that neutron activation analysis could detect, is seen in Table 9. No element seems to be present in toxic amounts. However, sodium and chlorine are very high and these elements, along with other ions, could cause a salt imbalance, especially when it is considered that the base ration was adequate in salt content from the start. Excess salt introduced along with the crabmeal could explain the increased urine output seen in Trial I (Table 5) and Trial II (Table 7).

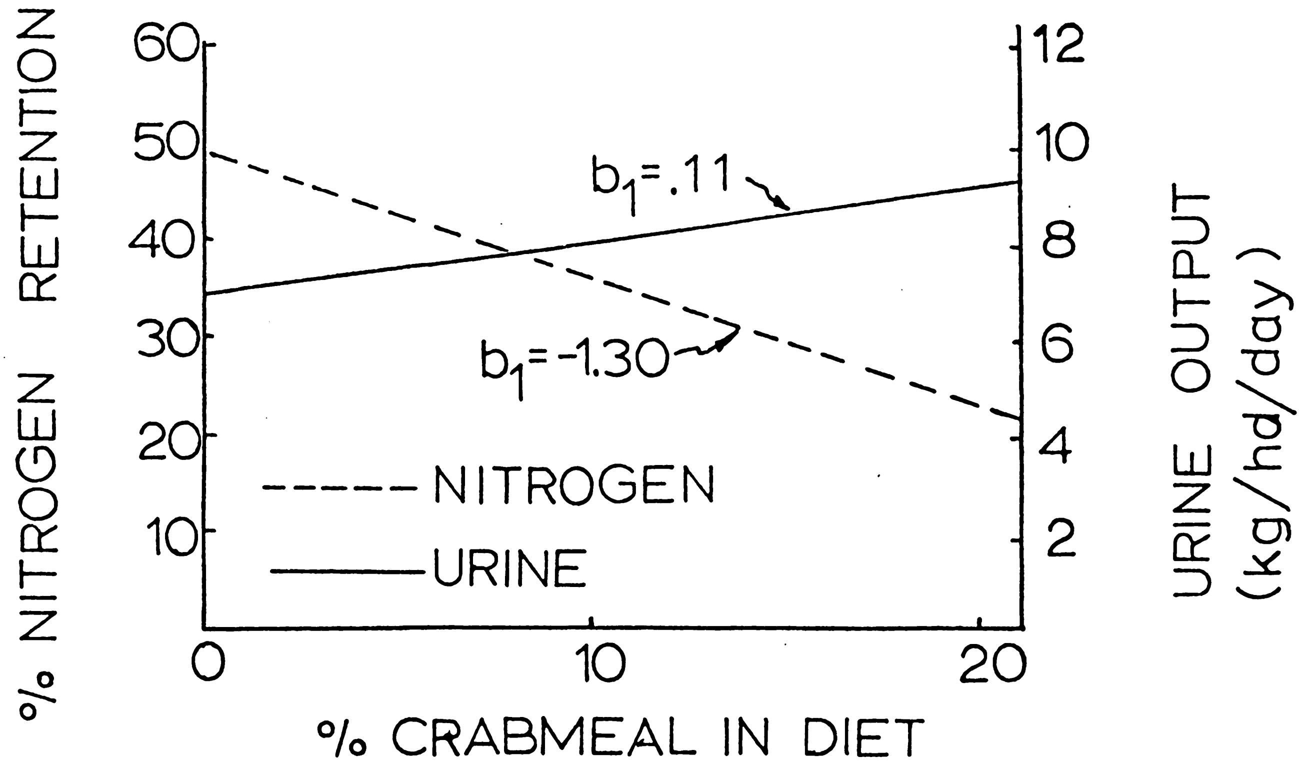


Figure 10. Regression of nitrogen retention and urine output on dietary crabmeal for Trial II animals.

Table 9. Neutron Activation Analysis of Minerals in Crabmeal and Crabmeal Rations.

Element	Crabmeal	Experimental Ration[1]		
		Base	10%	20%
Micro-elements (PPM)				
Bromine	300	12	52	56
Copper	60	2	1	2
Iodine	26	2	1	1
Macro-elements (%)				
Calcium	22.00	.75	1.40	1.80
Sodium	1.10	.37	.62	.60
Chlorine	1.45	1.05	1.40	1.28
Potassium	.55	.79	.35	.48
Magnesium	1.45	.20	.27	.34

[1]Base ration formulated to meet 100% of NRC requirements. Substitution of 10 and 20% crabmeal, by weight, gave rise to 10 and 20% diets.

Purified Chitin Assay

In keeping with the outline introduced in the Materials and Methods section, a pilot study was performed to determine if a dose-response relationship could be established between the amount of purified chitin assayed and the level of acetate obtained as an endpoint. These results were positive (Figure 11) so a standard curve, using more points, was undertaken. The results were again positive (Figure 11). In this experiment, a blank (sample containing no known acetate source) and a standard were run. The standard contained the same amount of acetate (211 mg) as 1.0 g of chitin. Background acetate, as determined by the blank, seemed to approximate the amount seen in 0.1 g of samples. Efficiency, expressed as % recovery compared to the standard, was 31%.

The length of time of exposure of chitin to 14N KOH was a suspected variable. In an effort to determine the optimum length of heating time in KOH, samples of 1.0 g chitin were exposed to 25 ml of KOH at 160C for various times (Figure 12). The length of heating time chosen for subsequent work was 30 min.

Insect Chitin Assay

A dose-response curve having been established for purified chitin, an attempt to obtain similar results using insect skins (cockroaches, *P. americana* or *P. germanica*) was undertaken. Figure 13 shows that a dose-response (yield of

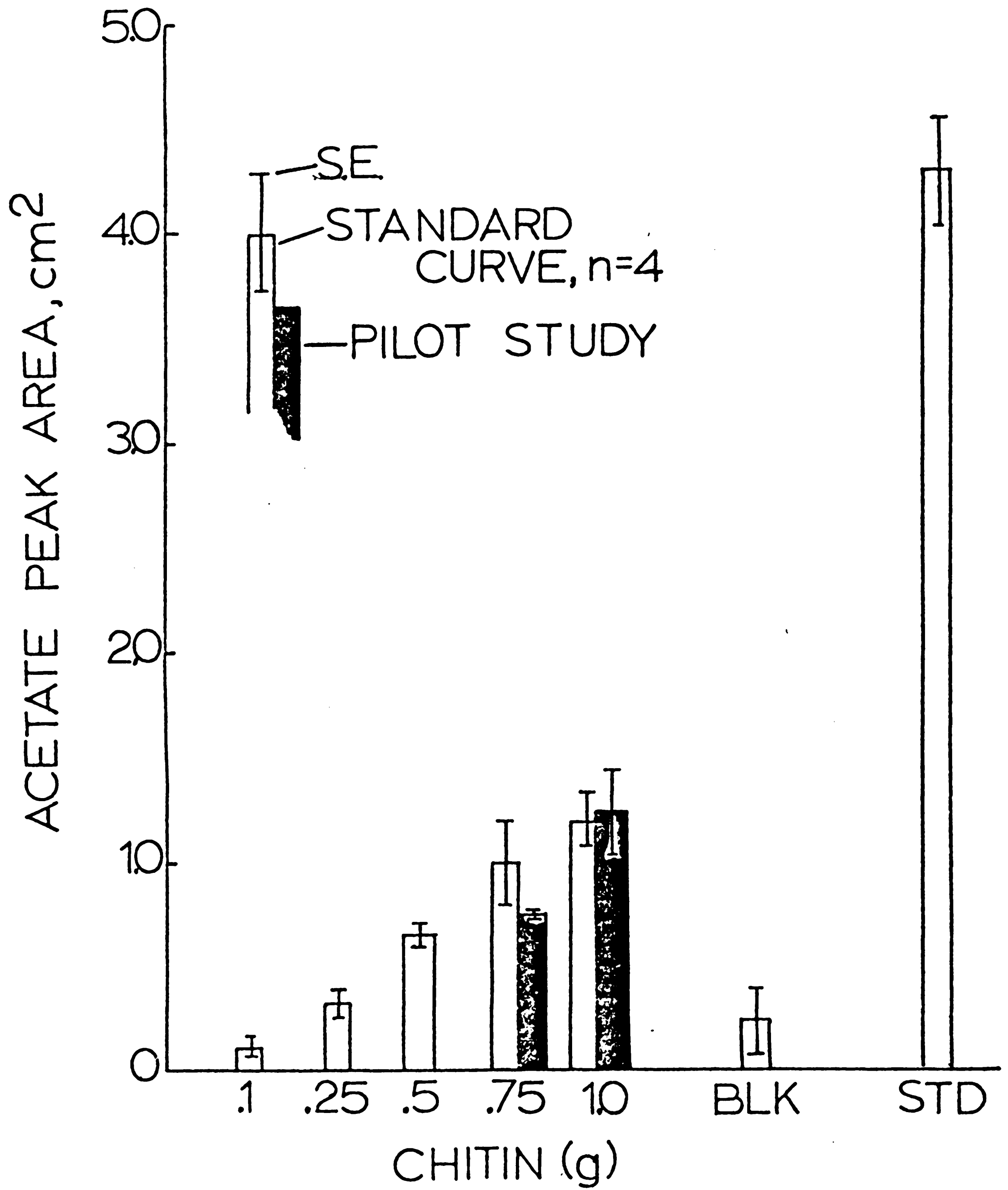


Figure 11. Pilot study and standard curve establishing a dose-response relationship between purified chitin and acetate.

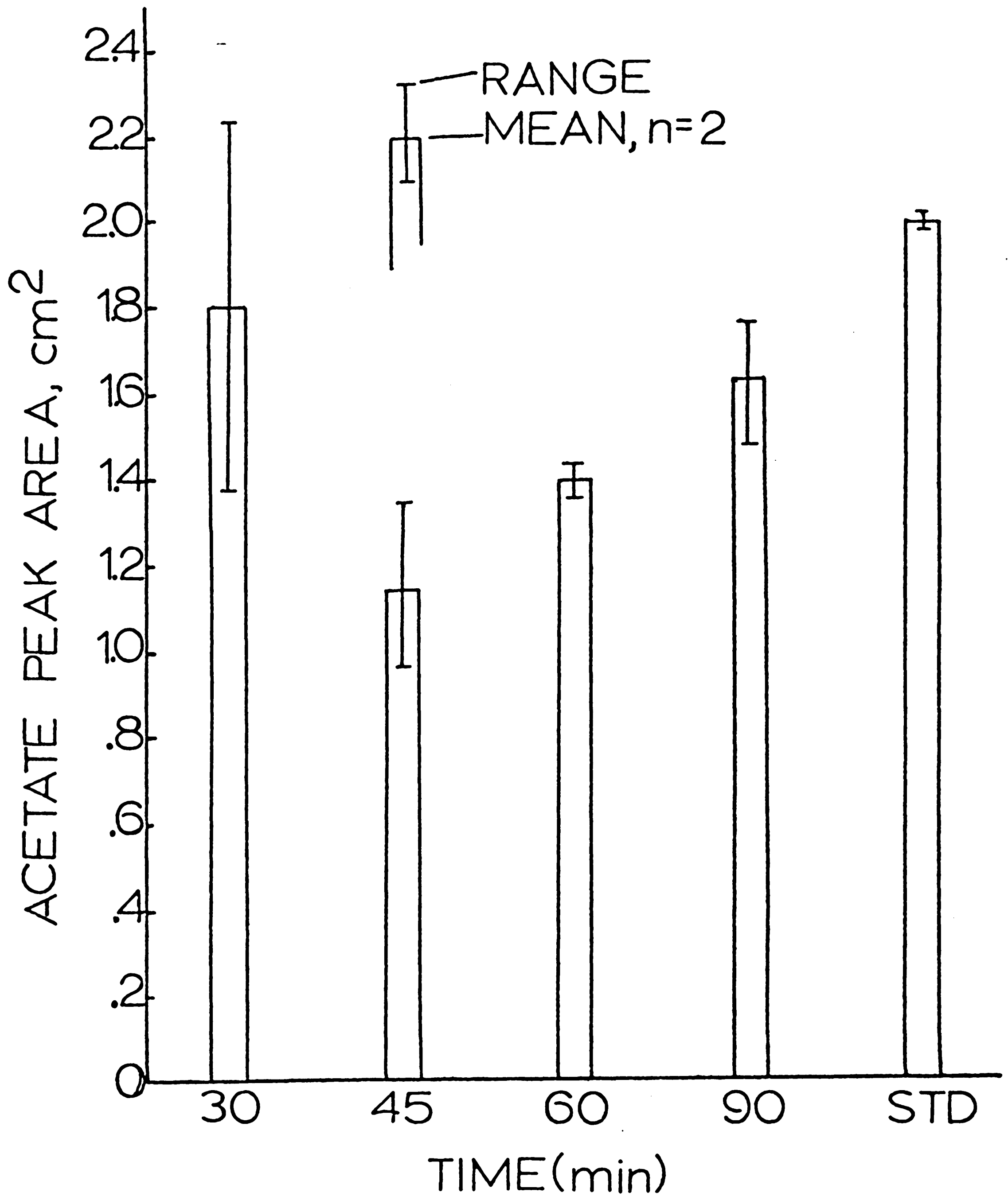


Figure 12. Effect of length of time of heating in 14N KOH at 160C on yield of acetate from purified chitin.

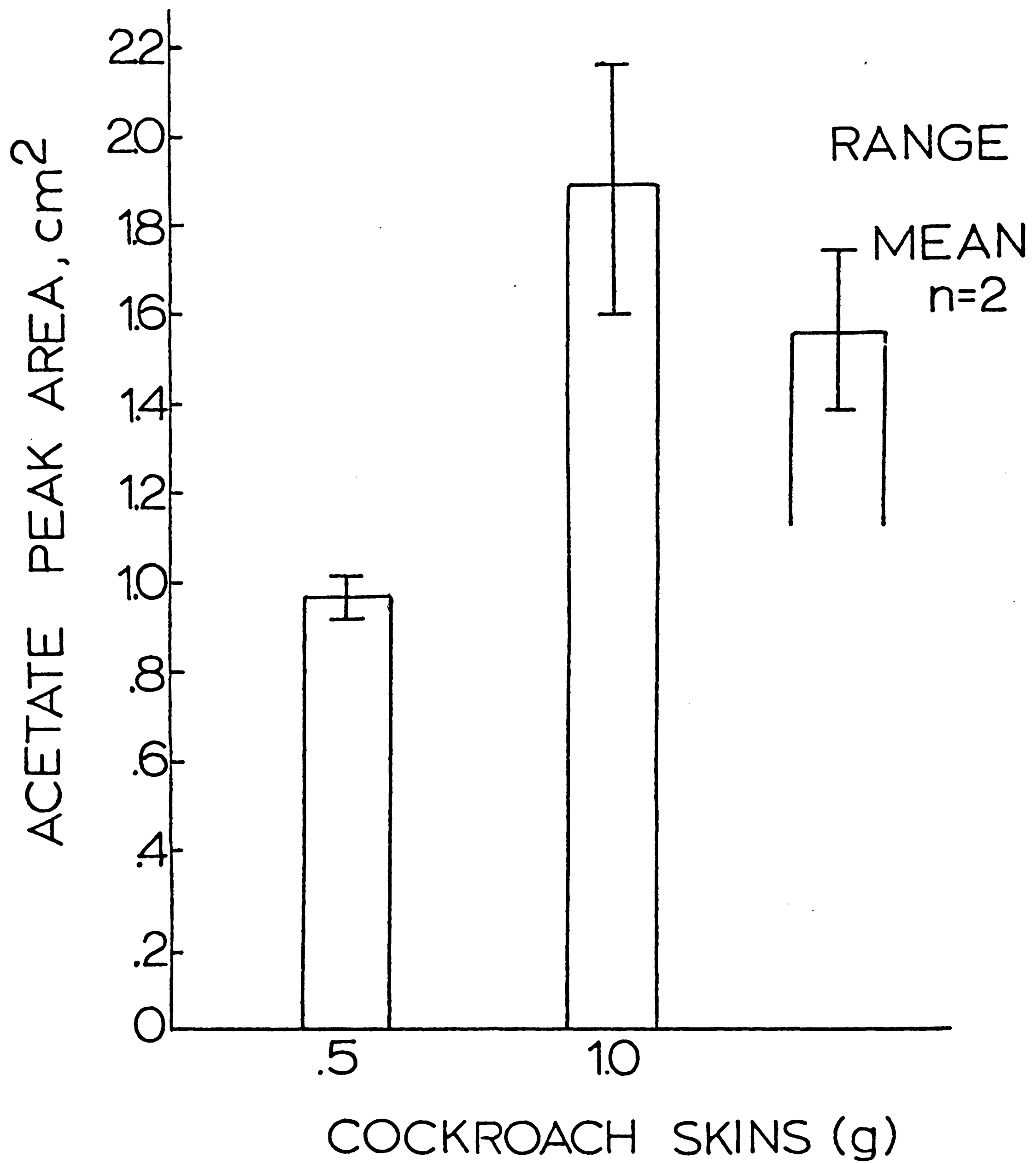


Figure 13. Pilot study to determine dose-response relationship between insect chitin and acetate.

acetate proportional to amount of insect skins) was possible using insect skins.

In an effort to better understand the low recovery (31% efficient) of acetate obtained in earlier experiments, a serial collection (10 ml at a time) of the steam distillate of 1 g of cockroach skins was chromatographed. It was found that acetate was recovered in measurable amounts in up to 90 ml of distillate. Consequently, a procedural change was introduced whereby 100 ml of distillate were collected in all assays undertaken thereafter.

A standard curve was run using insect skins in 250, 500, 750, and 1000 mg amounts, and the following additional samples: A standard (a known amount of acetate injected directly onto the gas chromatograph); a blank (a sample containing no known acetate and taken through the entire procedure); a sodium acetate blank (a sample containing the same amount of acetate as in the standard and taken through the entire procedure); and an unknown (a sample containing insect skins in an amount unknown to the investigator until after completion of the experiment). The results are seen in Figure 14. Percent recovery of sodium acetate was increased to 83.3% due to more distillate collection. Prediction of the amount of sample weight in the unknown as determined from the standard curve was within 88% of the actual amount. The unknown was at the lower end of the standard curve, which may have effected accuracy.

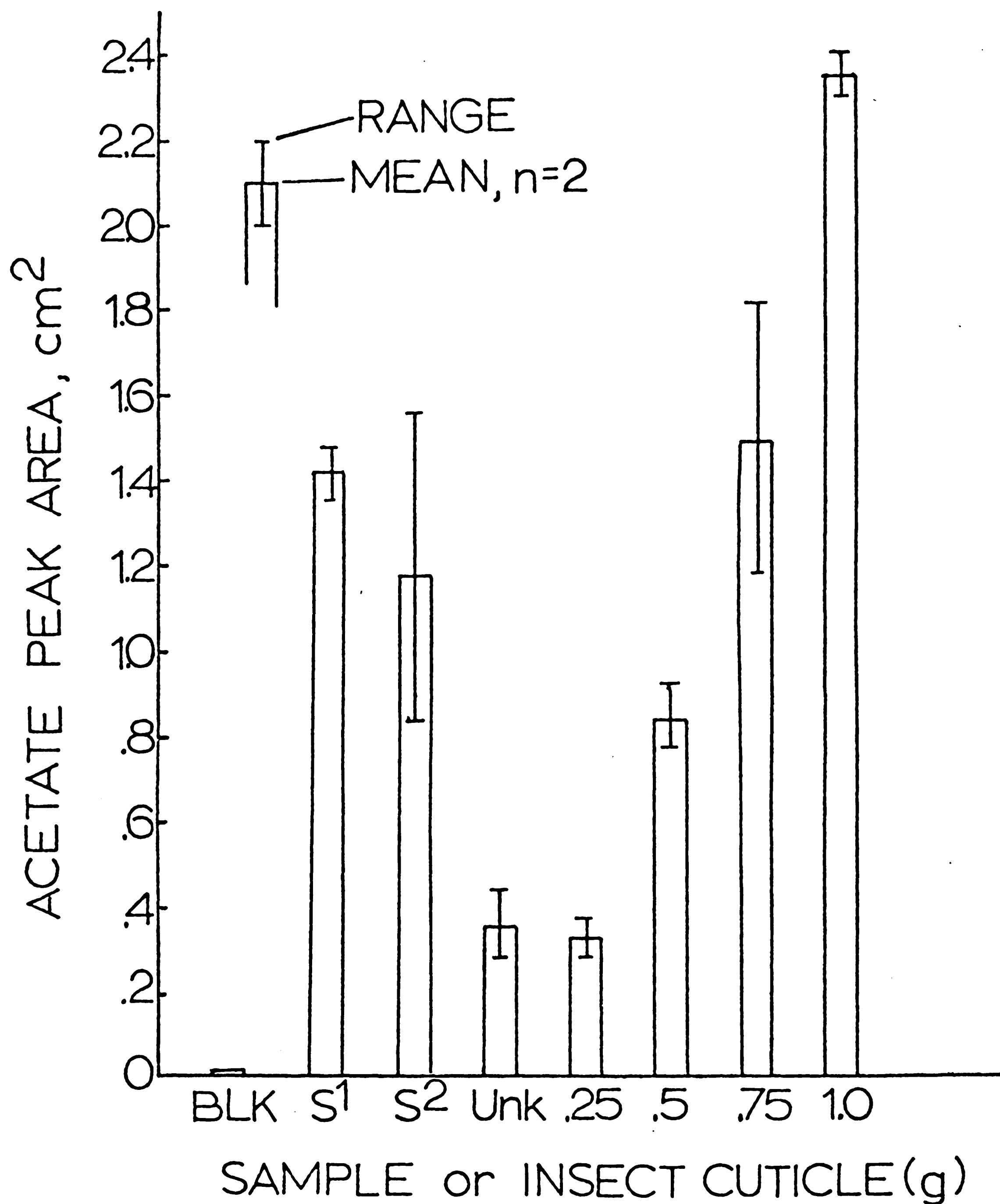


Figure 14. Standard curve establishing dose-response relationship between insect chitin and acetate and the efficiency of the assay.

BLK = blank, sample containing no known acetate
S^1 = standard injected directly into gas chromatograph
S^2 = sample containing acetate in equal concentration to S^1
UNK = unknown amount of insect chitin

Resolving Power (Insect Chitin Assay)

The degree of resolution that could be obtained with the insect chitin assay was investigated. Due to the difficulty of obtaining cast skins, samples of 350, 400, and 450 mg of cast skin could only be assayed in duplicate. (Each duplicate was chromatographed in duplicate.) To circumvent suspected gas chromatograph erratic response, each acetate peak area value run adjacent to it in the chromatograph. These results can be seen in Figure 15. It would appear, with the materials and methods used, that resolving amounts of insect chitin that vary by 50 mg would represent the lower limit of the assay for critical quantitation.

Chitin Digestion in Feeding Trials

With an assay now developed for determination of chitin from purified sources and insect skin, efforts were directed to determine the best manner of isolating chitin from crabmeal in feces or feed. All possible combinations of acid-detergent fiber analysis (44) and decalcification (39) were employed on chitin and crabmeal. These treatments and the resulting acetate peak areas are seen in Table 10. Treatment 5 (or 6) of this table was the method of choice owing to the high value obtained on both crabmeal and chitin. This method, decalcification followed by acid detergent fiber treatment and subsequent heating in KOH, was used on the feed and fecal samples obtained in feeding Trials I and II. The percent chitin digestion of these feeding trials is shown in Table 11. The Trial I animals

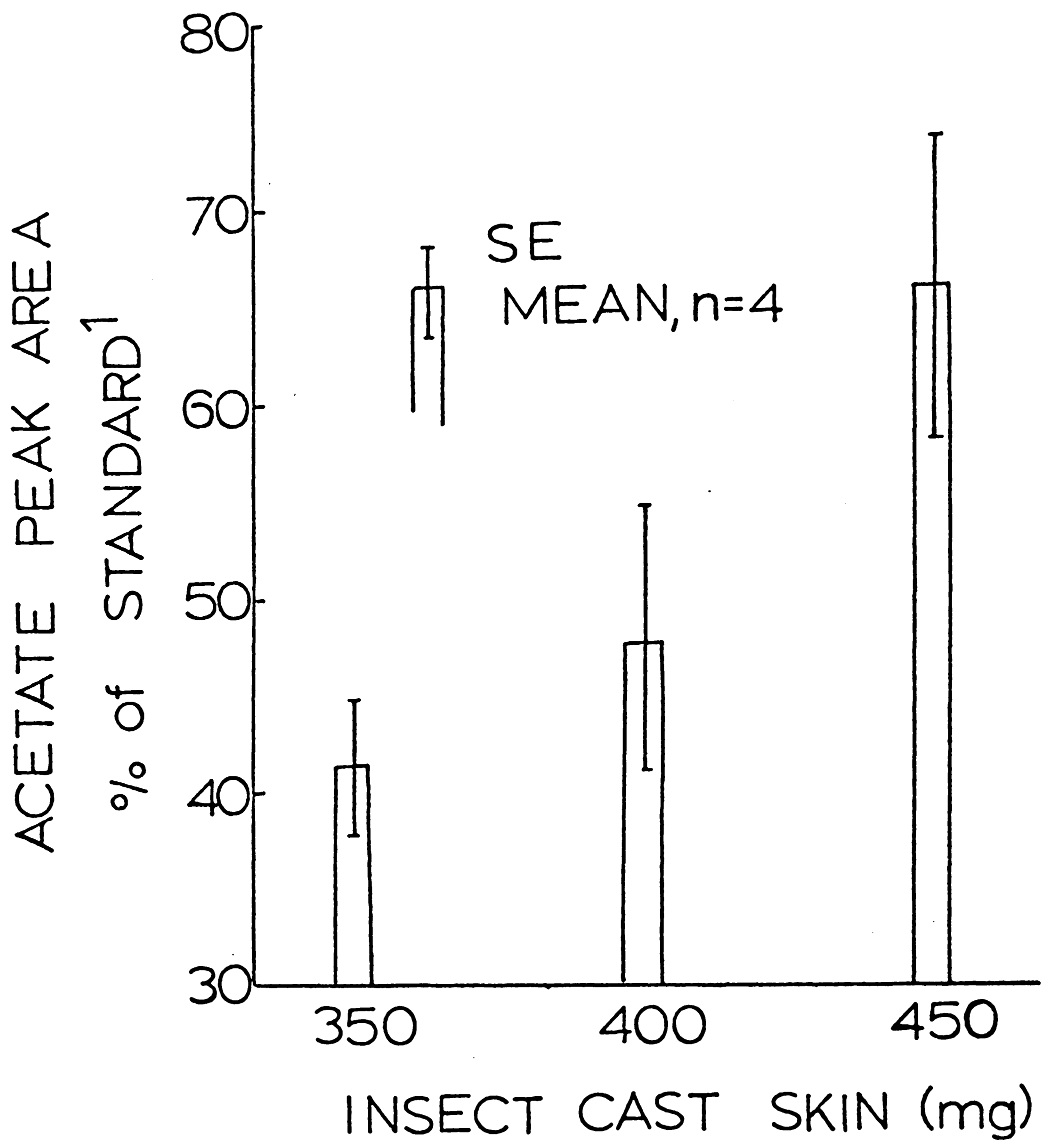


Figure 15. Determination of the degree of resolution of the insect chitin assay.

Table 10. Acetate peak areas resulting from varying treatments of chitin and crabmeal in 10 g amounts.

Treatment	Acetate Peak Area (cm^2)
1. ADF[1] on CM[2]	3.42
2. ADF on Chitin	3.25
3. Decal[3] on Chitin	4.19
4. Decal on CM	1.48
5. ADF on Decal CM	3.53
6. ADF on Decal Chitin	4.59
7. Decal on ADF Chitin	3.85
8. Decal on ADF CM	3.22

[1]ADF = Acid Detergent Fiber Analysis
[2]CM = Crabmeal
[3]Decalcification Procedure

Table 11. % Chitin Digestion in Feeding Trials I and II[1]

Animal Feces	% Dietary Crabmeal	mg Chitin[4] / g Feces	g Chitin[5] Excreted	g Chitin[6] Consumed	% Chitin[7] Digestion
[2] 1615	10	7.700	28.77	60.67	52
	20	6.302	27.41	153.41	82
1617	10	5.809	19.46	69.29	72
	20	15.543	136.78	186.08	26
1618	10	8.442	64.33	91.05	29
	20	1.756	15.45	118.08	87
1620	10	8.690	44.32	81.52	46
	20	9.109	18.04	110.08	83
1625	10	4.395	9.98	45.47	78
	20	11.714	54.94	167.89	67
1628	10	7.152	22.38	79.62	72
	20	7.526	46.76	159.51	70
[3] 5	20	11.867	84.37	197.98	57
53	20	5.100	34.68	191.12	82
78	20	3.252	112.309	350.949	68
90	20	1.364	64.476	350.949	81

[1]Trial I: 10% AVE=58 ± 19, 20% AVE=69 ± 22.5; Trial II: AVE= 72 ± 11.8

[2]Trial I Animals

[3]Trial II Animals

[4]$\frac{\text{mg chitin}}{\text{g feces}} = \frac{x/s \times A}{B \times C}$; where

x = peak area of acetate from sample
s = acetate standard peak area, conc = 1 mg Ac/ml
A = 100, ml of distillate containing sample
B = .21, the amount of acetate in chitin
C = 10, the grams feces originally assayed for chitin

[5]g chitin excreted = total g dry feces $\times \frac{\text{mg chitin}}{\text{g feces}} \times \frac{\text{g}}{1000\text{ mg}}$

[6]g chitin consumed = $\frac{\text{g chitin}}{\text{g feed}}$ x g feed consumed; where
10% diet = .004762 g chitin/g
20% diet = .009523 g chitin/g

[7]% digestion = $\frac{\text{retained}}{\text{consumed}} \times 100$

on 10% crabmeal averaged 59 ± 19% chitin digestion. Trial I animals on 20% crabmeal averaged 69 ± 22.5%, while Trial II animals, on the same diet, averaged 72 ± 11.8% chitin digestion. The wide range of values seen within animals in Table 11 is a function of when the animals went on the diets in the course of execution of the Latin square. It seems that the first chitinous diet an animal was exposed to, the chitin was poorly digested; the second chitinous diet was well digested. In the determination of chitin in feces and feed samples, the sample error was 16.7%. This relatively close duplication adds further creditability to the values seen in Table 11.

In Vitro Incubation

Short term artificial rumen incubation of purified chitin, as outlined in Materials and Methods, indicated that under the conditions of study, chitin was not degraded *in vitro* (Figure 16) and that the system as used would not suffice as a vehicle for further study. Some endogenous acetate was found in the positive and negative control treatments, but the incubated rumen fluid, Treatment 3, showed a lower level than the unincubated rumen fluid, Treatment 4. This same result was observed in a subsequent short term rumen incubation trial reported below.

Rumen incubation of insect chitin was performed as per Materials and Methods, and the results are shown in Figure 17. A certain amount of acetate was endogenous to the rumen system and was not completely removed by the assay procedure. There was no apparent effect when respective samples were corrected

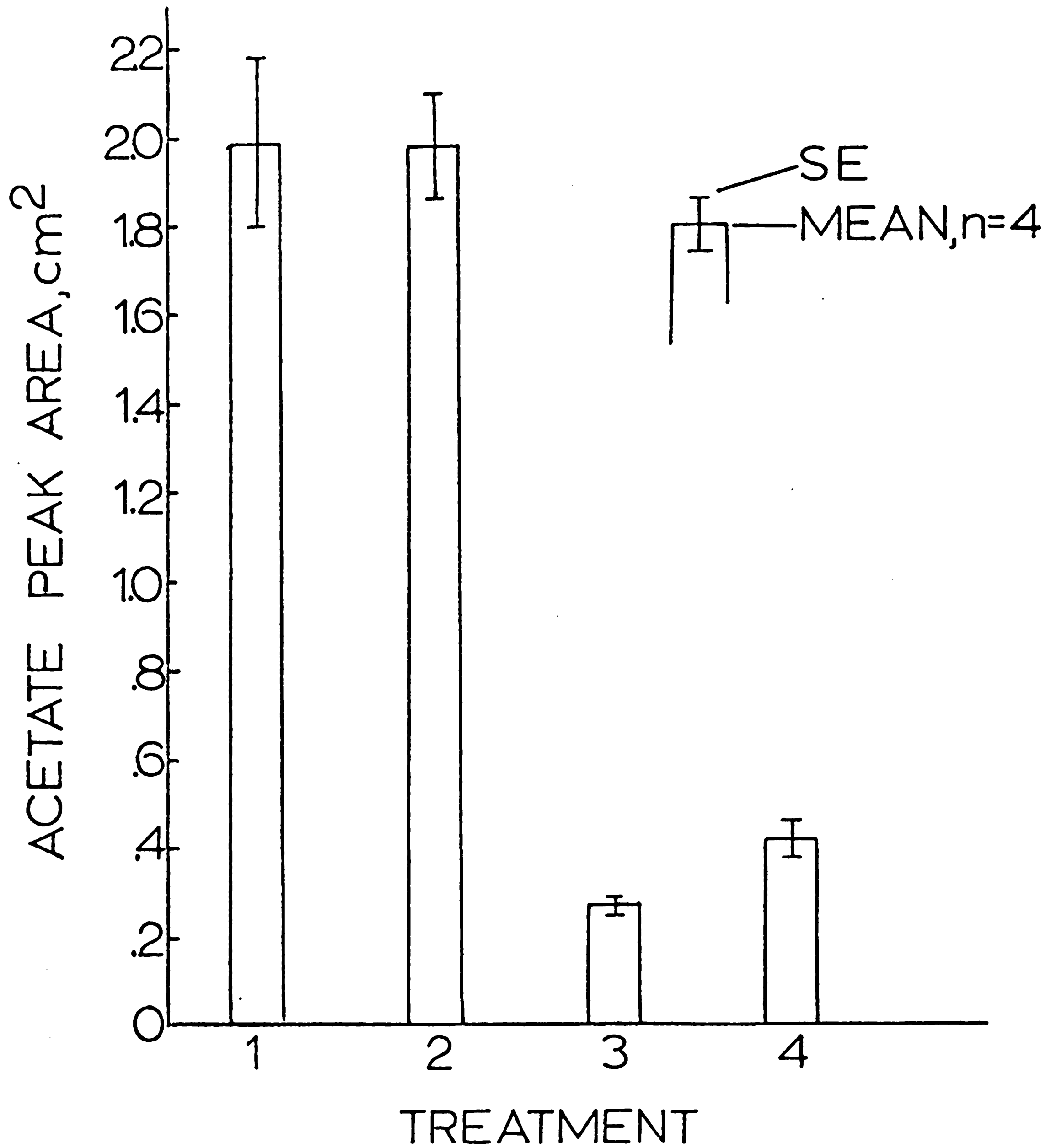


Figure 16. In vitro incubation of purified chitin.

Treatment 1: Chitin (400 mg), 3 hr incubation in rumen fluid.
2: Chitin (400 mg), in rumen fluid, no incubation.
3: Rumen fluid, no chitin, 3 hr incubation.
4: Rumen fluid, no chitin, no incubation.

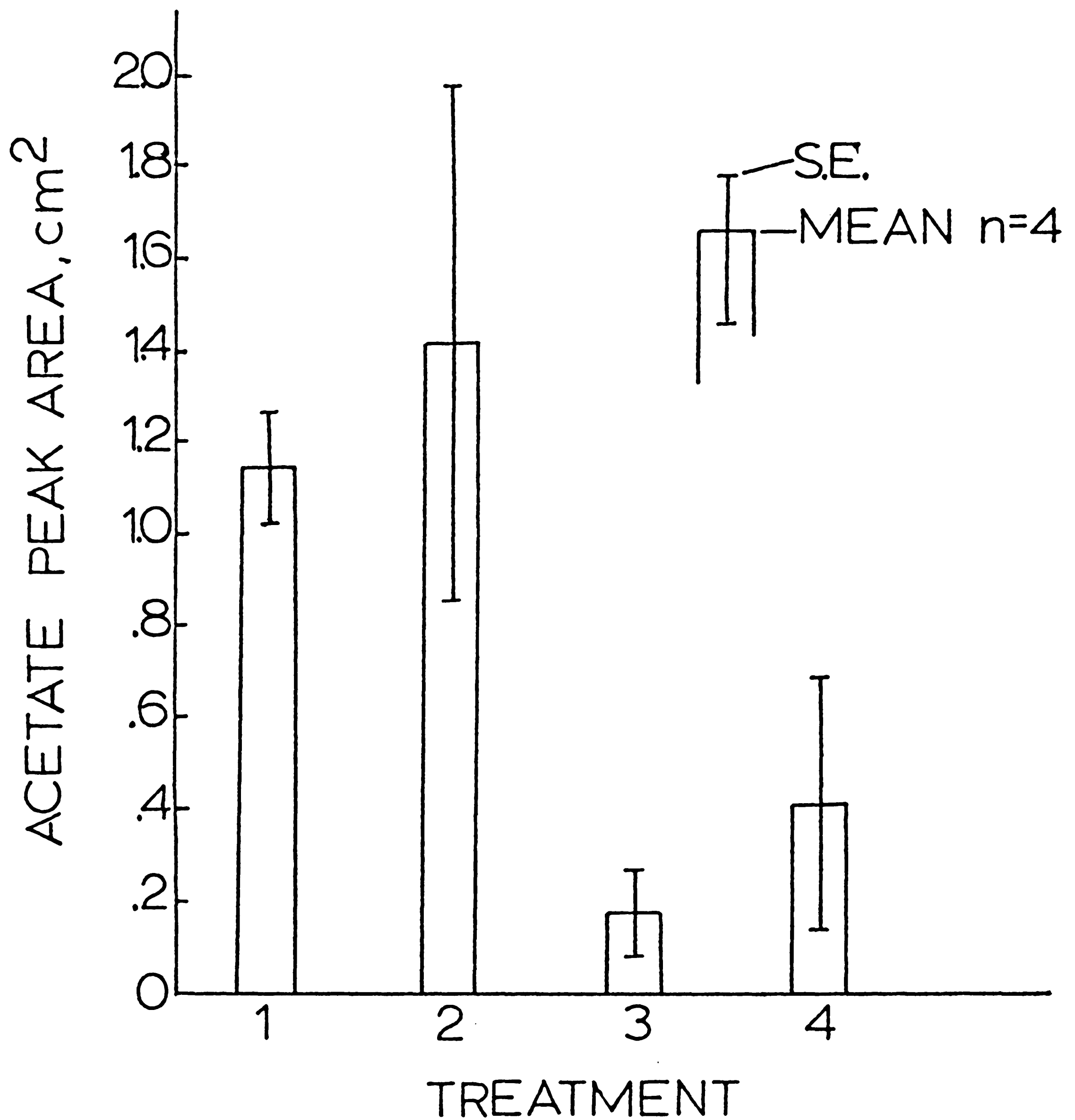


Figure 17. In vitro incubation of cockroach cast skins.

Treatment 1: Chitin (400 mg), in rumen fluid, 3 hr incubation.
2: Chitin (400 mg), in rumen fluid, no incubation.
3: Rumen fluid, no chitin, 3 hr incubation.
4: Rumen fluid, no chitin, no incubation.

for endogenous acetate levels. When the incubated rumen fluid acetate level was subtracted from the incubated insect cast skins plus rumen fluid acetate level, Treatments 1 and 2 showed no difference. This fact, along with the overlap of standard errors seen in Figure 17, leads one to conclude that in the system studied, insect chitin deacetylation did not occur at detectable levels.

The lack of detection of chitin digestion in the *in vitro* incubations is in contrast to the results of the feeding trials and *in vivo* solubility studies where chitin digestion was observed. These results have several possible explanations: lack of microbial adaptation, either via enzyme induction or population stimulation, with resultant lack of chitinase availability; failure to detect deacetylation due to insensitiveness of assay; and for low level of chitin digestion because of poor microbe to substrate ratios.

Despite a functioning artificial rumen system, as evidenced by gas production and expansion of the balloon stoppers, it must be concluded that the system, as described, would not suffice for further studies of chitin digestion. It is believed that use of rumen fluid from adapted animals and longer times of incubation would have produced positive results.

SUMMARY AND CONCLUSIONS

Three methods were employed to investigate the value of chitinous materials for ruminants: In vivo solubility trials, feeding trials, and in vitro incubations.

In vivo solubility was determined by calculating the % dry matter weight loss of a sample exposed to the rumen environment. The samples investigated were: cellulose, alfalfa, crabmeal, shrimpmeal, cockroaches, grasshoppers, and purified chitin. Nylon bags containing these samples in varying amounts were suspended in the rumen of fistulated steers for varying times. Water solubility of these samples was also investigated by exposing nylon bags containing these samples to water hydrolysis for 48 hrs at 37C. Average rumen solubility of cellulose, alfalfa, crabmeal, shrimpmeal, cockroaches, grasshoppers, and purified chitin was 34.9, 49.3, 35.9, 17.3, 65.4, 32.1, and 21.4, respectively. Rumen solubility minus water solubility for crabmeal, shrimpmeal, cockroaches, grasshoppers, and chitin was 15, 8.3, 5.0, 3.2, and 12.9%, respectively.

Proximate analysis of these samples before and after rumen exposure and water hydrolysis revealed that the crude protein fractions of crabmeal, shrimpmeal, and cockroaches were 37.5, 13.0, and 61.8% soluble in water and 50.2, 27.5, and 67.9% soluble in the rumen, respectively. Crude fiber content of these samples showed a water solubility of 5.5,

0, and 53.2% and a rumen solubility of 8.0, 19.6, and 67.7%, respectively.

Two feeding trials were performed using crabmeal as the source of chitinous material. The first feeding trial involved six young Holstein bulls in a 3 x 3 Latin square designed experiment. Three diets were fed: a base ration, formulated to meet 100% of N. R. C. requirements, a 10% (by weight) crabmeal ration, and a 20% crabmeal ration. After an adjustment period, the animals were fed each diet for four weeks. The third week of each time period the animals were placed in metabolism crates for determination of digestibility parameters. In the first feeding trial, there was no difference between treatments for average daily gain, feed intake, feed efficiency, nitrogen digestion or retention, dry matter or crude fiber digestibility, or urine volume. Chitin digestion averaged 58 and 69% for 10 and 20% diets, respectively.

The second feeding trial involved ten young Holstein males in a 2 x 2 Latin square designed experiment. Four animals were placed in metabolism crates for digestibility determinations. The two diets fed were identical to the base and 20% rations of Trial I. The results, after five weeks on each diet, indicated there was no difference between treatments for average daily gain, feed intake, feed efficiency, nitrogen digestion, dry matter digestibility, or crude fiber digestibility. Urine output and nitrogen

retention were significantly different (P .05) with more urine being produced by animals on the crabmeal ration, while less nitrogen was retained. Chitin digestion averaged 22 ± 19%.

Chitin quantitation of samples was achieved by assaying for acetate. Experiments substantiated the fact that the amount of acetate derived from a given amount of chitin (via KOH hydrolysis) was directly proportional to the amount of chitin.

The third phase of this investigation involved in vitro rumen incubation of purified chitin and insect skins. Samples were placed in rumen fluid and incubated for 3 hr. In addition, positive and negative control samples were run; samples plus rumen fluid, not incubated, and rumen fluid, both incubated and unincubated, were assayed for base acetate levels. Neither purified chitin nor insect cast skins underwent any apparent deacetylation during the 3 hr of incubation.

In conclusion, of the several chitinous materials investigated it can be stated that cockroaches specifically and insects in general have outstanding potential as a nutrient for ruminants, and that crabmeal can substitute for up to 20% of a young bovine's diet.

Crabmeal is presently valued at $.055/kg ($50.00 per ton). In competition with corn, which is 80% TDN, currently priced at $.066/kg ($60.00 per ton), crabmeal must exceed 95% TDN, which is highly unlikely. To compete with soybean meal

presently valued at $.132/kg ($120.00 per ton) and 42% crude protein, crabmeal, being 30% crude protein, need only cost less than $.099/kg ($900.00 per ton). This is contingent upon the biological value of crabmeal protein being equal to that of soybean protein, which is questionable considering the high non-protein nitrogen content of crabmeal.

It can be argued that at the present time, economics make it risky to harvest and process insects for ruminant consumption or to transport and feed crabmeal. However, there can be no argument with the fact that an exploding world population creates ever-increasing pressure on the world food supply. Now is the time to investigate untapped food reserves. It is the author's opinion that chitinous materials do represent a large unused energy and protein reserve and young ruminants can grow normally on up to 20% crabmeal diets. Further work is in order to determine adult ruminant utilization of the feeds.

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EVALUATION OF CHITINOUS MATERIALS

AS A FEED FOR RUMINANTS

Richard S. Patton

Abstract

Chitinous products were investigated by *in vivo* rumen fermentation. Samples of cockroaches, grasshoppers, shrimp-shells, crabmeal, and purified chitin were placed in the rumen of fistulated steers. Average rumen solubility (weight loss from a nylon bag) of 66.5, 32.0, 17.4, 35.7, and 21.5%, respectively, were observed. Measurements of water solubility in a non-rumen system indicated that the rumen system was solubilizing 8, 7, and 12% of cockroaches, grasshoppers, and crabmeal, respectively, over that in water. A feeding trial was conducted where crabmeal was evaluated at 0, 10, and 20% of a basal diet. The experiment was conducted by using six ruminating calves in two replications of a 3 x 3 Latin square design. In each 4-week period, nitrogen balance data was collected during the third week. Crabmeal nutritive value was established. It substituted equally for base ration at the levels studied. No treatment differences were detected for gain, feed intake, absorbed and retained nitrogen, dry matter, or crude fiber digestibility ($P < .05$).

In a second feeding trial using a 2 x 2 Latin square design, a zero and 20% diet were fed to two groups of five ruminating calves for two time periods. Nitrogen balance

data collected as in Trial I on two animals of each group, further established crabmeal nutritive value. There was no difference between groups for all performance parameters measured except that animals fed crabmeal had a lowered nitrogen retention ($P < .05$).

Chitin digestion in Trial I averaged 58 ± 19% and 69 ± 22% for 10 and 20% crabmeal diets, respectively; for Trial II, chitin digestion averaged 72 ± 12%.

In *in vitro* incubations of purified chitin and insect skins in strained rumen fluid for three hours, there was no apparent deacetylation of chitin beyond that observed in positive and negative control samples.