NOTE TO USERS

DEVELOPMENT OF A BIO-ASSAY TECHNIQUE FOR EVALUATING PHOSPHORUS AVAILABILITY IN RUMINANT FEEDS

by

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INTRODUCTION

It is well recognized that ruminant animals require a dietary source of phosphorus for maintenance, growth, fattening, milk production and reproduction. Phosphorus is present in the animal body, not only in the skeletal structure and blood, but rather as a constituent of every cell of the body. Its functions are numerous and involve the chemistry of the blood, acid-base balance of the body, skeletal growth, tooth development, muscle metabolism, intermediary metabolism of carbohydrates, fat, and protein, and enzyme activity. In ruminant livestock phosphorus is also required by the microorganisms present in the rumen.

Ruminant feeds in general contain some phosphorus, however, in varying amounts, therefore, under most feeding regimes a supplemental source of phosphorus must be supplied in the ration for optimum results. During the past few years there have been periodic shortages of phosphate carrying materials such as bone meal and defluorinated phosphorus products. To alleviate this situation several other phosphorus sources have appeared on the market and become available for use in rations for poultry and livestock. Considerable research has been conducted with poultry to get some measure as to the relative nutritional value of various phosphate supplements, some with swine, but a limited amount with cattle and sheep. One of the reasons more research has not been conducted with cattle in investigating phosphorus availability of the large number of supplements fed to cattle is due to the time and cost

involved in carrying out long time feeding and metabolism experiments with these animals.

Since rumen microorganisms have been shown to require phosphorus for efficient cellulose digestion, it seemed plausible that an artificial rumen technique could be developed for measuring phosphorus availability which might serve as an acceptable index of availability of phosphorus in supplements fed to ruminant animals. Therefore, it was the purpose of the research herein reported to (1) develop an artificial rumen technique which would be suitable to use as an assay procedure in measuring phosphorus availability of various phosphorus supplements to rumen microorganisms, and (2) to test a number of phosphorus supplements using the procedure developed.

REVIEW OF LITERATURE

Various Phosphorus Sources in Poultry Rations

Following the outbreak of World War II in this country there appeared an extreme shortage of phosphate carrying materials. There wasn't sufficient phosphate available from either defluorinated products or bone meal to meet the needs of the poultry and livestock industries. To relieve this situation several new phosphorus products such as colloidal phosphate, Curacao Island phosphate, and others have appeared on the market and become available for use in livestock and poultry rations.

Since the appearance of these more recent phosphorus carriers a considerable number of feeding trials have been conducted with poultry and some with livestock, with the thought in mind of getting some measure as to the relative availability of the phosphorus present in these various supplements. If, for example, the phosphorus in colloidal clay were as available as that in dicalcium phosphate, a substantial saving could be made in feed cost since colloidal clay costs considerably less than dicalcium phosphate in spite of its lower phosphorus content. However, aside from the availability of the phosphorus itself other factors such as the amount of fluorine present have influenced the value of certain phosphorus sources. Excessive amounts of fluorine in a ration are toxic and this fact alone has been the basis for many feeding trials involving phosphorus sources.

One of the earlier studies concerning the value of defluorinated phosphate and other phosphorus supplements in the diet of the chicks was conducted by Bird et al. (3). These investigators determined the effectiveness of ten different samples of phosphatic materials as sources of phosphorus for bone formation in growing chickens. The samples tested included six of defluorinated superphosphate and one each of defluorinated phosphate rock, phosphate slag, calcium pyrophosphate (beta), and vitreous calcium metaphosphate. The effects of these materials on per cent of bone ash and on growth were compared with the corresponding effects of tricalcium phosphate and of bone meal. Their results for the defluorinated superphosphates showed one of the samples to be almost completely unavailable while the other five were available but less so than bone meal and tricalcium phosphate. Defluorinated phosphate rock, phosphate slag, and vitreous calcium metaphosphate were intermediate in availability between the superphosphates on the one hand and bone meal and tricalcium phosphate on the other. The calcium pyrophosphate was totally unavailable or nearly so. These workers point out that the parallelism between availability and solubility in 0.25 per cent hydrochloric acid at 38° C., was such that determination of solubility could be used as a quick, approximate measure of availability.

Matterson et al. (27) conducted an experiment with growing chicks in which rock phosphates were used as sources of phosphorus. Raw rock phosphate and fused rock phosphate were found to be just as available, if not more so, than tri-calcium phosphate. Calcium meta-phosphate was found to be inferior to both rock and fused rock phosphate.

Gerry et al. (13) studied the problem of fluorine toxicity and the availability of the phosphorus in rock phosphate, superphosphate, and partly defluorinated phosphate to chickens. Raw rock phosphate when fed at the level of one per cent of the ration depressed growth of chicks during their first 8 weeks on experiment. This level of raw rock phosphate supplied .038 per cent fluorine in the ration. Chickens which had received steamed bone meal as their source of phosphorus until sixteen weeks of age and then switched to 2.9 per cent raw rock phosphate were not affected by the change in phosphorus source. Colloidal phosphate and superphosphate, both relatively high in fluorine, were found to be detrimental to growth of young chickens. Melted phosphate, a product low in fluorine, was quite satisfactory as reflected by the growth response of chicks. In general, fluorine was found to accumulate in the bones in proportion to the amount contained in the ration and with the length of the feeding period. Steamed bone meal and treated and melted rock phosphate were the most desirable phosphorus supplements used.

The comparative availability to chicks of a number of common phosphorus supplements and pure phosphate compounds was studied by Gillis et al. (16). The test substances were added singly to a basal chick diet very low in phosphorus. When the test substances were used to raise the phosphorus content of the basal diet to 0.4 per cent, the orthophosphates, pure beta tricalcium phosphate and reagent grades of monocalcium, dicalcium, and tricalcium phosphate were found to be excellent sources of phosphorus and slightly more available than

steamed bone meal. The defluorinated superphosphate and defluorinated phosphate rock products used in these experiments were good sources of phosphorus, but less available than the pure orthophosphates or steamed bone meal. When defluorinated superphosphate and defluorinated phosphate rock, both fused and calcined, were used to supply 0.8 per cent phosphorus in the diet they were of equal value and were nearly as effective as pure beta tricalcium phosphate or steamed bone meal.

Grau and Zweigart (17) found phosphatic clay (soft phosphate with colloidal clay) to be a poorer source of phosphorus than either bone meal or tricalcium phosphate, when fed to chicks in a ration composed of natural ingredients. The criteria used for phosphorus evaluation were percentage bone ash and average weight gain. Although calcification was affected, the most striking effect was on growth rate, which was not benefited by the addition of phosphatic clay to the basal diet. Addition of bone meal to the basal ration which contained 0.45 per cent phosphorus did improve growth rate. These investigators feel that on the basis of their results the fluorine content of phosphatic clay is not responsible for its effect upon growth because they compared tricalcium phosphate with added fluorine and phosphatic clay. They added an amount of sodium fluoride to tricalcium phosphate equivalent to the fluorine supplied by phosphatic clay. This addition of fluorine in the form of sodium fluoride did not depress growth or affect calcification. However, it should be pointed out that the tricalcium phosphate sample prior to the addition of sodium fluoride was one which produced normal calcification but actually depressed growth below that found with the basal diet.

Therefore, it is possible that the added fluorine had no opportunity to exert a further depressing effect.

Miller and Joukovsky (28) compared availability of five different inorganic phosphorus sources to growing chicks. Each source was fed to supply 0.2 and 0.4 per cent phosphorus, respectively, in the ration with a 2:1 calcium to phosphorus ratio. The criteria used in evaluating availability were five week weights, per cent bone ash and per cent mortality. Curacao Island phosphate, bone meal and defluorinated phosphate were all found to be good sources of readily available phosphate. Colloidal phosphate was found to be a poor source of phosphorus and according to the criteria used in evaluation seemed to have less than 50 per cent of the value of the other minerals tested. The feeding of Curacao Island phosphate to supply 0.4 per cent phosphorus resulted in a fluorine level in the bone that was about seven times as high as that resulting from the feeding of bone meal. The chicks receiving colloidal phosphate had a fluorine level in their bones which was about 24 times as high as that resulting from the feeding of bone meal.

In another study involving utilization of soft phosphate with colloidal clay Johnson et al. (21) observed satisfactory chick growth and ash content of the toes at four weeks of age when chicks were fed a 4 per cent level of this phosphorus source. A similar growth response occurred with a 2 per cent level of steamed bone meal as was observed with 4 per cent colloidal clay but the ash content of the toes of chicks receiving bone meal was slightly higher. The 2 per cent level of either steamed bone meal or colloidal clay did not promote maximum chick growth. Combinations of the colloidal clay and steamed bone meal were satisfactory

in promoting chick growth. Rations containing 0.5 and 1.0 per cent of steamed bone meal, respectively, were improved when the colloidal phosphate level was raised from 1.0 to 2.0 or 3.0 per cent. In view of these results the authors conclude that soft phosphate with colloidal clay may be used to supply a part of the phosphorus needs of young chicks but should not make up more than 2.0 per cent of the total ration.

Gillis et al. (15) in a later rather extensive study employing an improved and more critical technique compared availability of phosphorus from different sources using a purified type of diet and also a practical type diet made up of natural ingredients. Their assay procedure involved feeding graded increments of phosphates in diets very deficient in phosphorus and determining the resulting increases in bone ash after 4 weeks on experiment. In studying compounds of moderate or high availability a purified type of diet very low in phosphorus was used. For studying poorly available compounds a ration of satisfactory low phosphorus content was formulated from ingredients of a practical type. Pure beta tricalcium phosphate was used as a standard of comparison and arbitrarily assigned a biological value of 100. Chemically pure orthophosphates, monocalcium, dicalcium, and tricalcium phosphate and sodium and potassium acid phosphate were found to be highly available. The acid salts, monocalcium and potassium acid phosphate were better utilized than the other pure orthophosphates. Feed grade materials of excellent availability were average samples of dicalcium phosphate, defluorinated phosphate and domestic steamed bone meal. Other bone products of slightly lower availability were spent bone char, bone ash and imported bone

meal. None of the pyrophosphates or metaphosphates were satisfactory although significant amounts of phosphorus were utilized from calcium acid pyrophosphate and vitreous metaphosphate. Among the untreated rock products only Curacao Island phosphate showed a satisfactory degree of availability. Excessive mortality was noted on the purified diet when colloidal phosphate was the test compound. In the practical diet colloidal phosphate was approximately 25 per cent as effective as an equivalent amount of the standard in increasing the ash content of bones; however, it had no significant beneficial effect on growth.

Titus et al. (32) compared the availability to young growing chickens of the phosphorus in several defluorinated phosphates with that of the phosphorus in analytical reagent grade dicalcium phosphate. All sources of phosphorus were included in a corn-soybean oil meal diet at levels that supplied 0.2 and 0.4 per cent of phosphorus, respectively. Criteria used in evaluation of availability were average weight, feed efficiency, tibia ash and per cent mortality. The phosphorus in the defluorinated phosphates was found to be as available as that in the analytical reagent grade dicalcium phosphate at both the 0.2 and 0.4 per cent levels.

Several reports appear in the literature where poults have been used in experimental work to determine phosphorus availability. Because of their more rapid growth rate they appear to be more sensitive to source or composition of phosphate than do chicks (33).

Wilcox et al. (33) in a poult study used a purified diet supplemented with dried buttermilk, dried brewers yeast and forage juice. This ration was supplemented with each of 14 different phosphates.

Criteria used to evaluate availability were body weight, bone ash in the tibias and per cent mortality. These workers noted considerable variation in performance among the defluorinated phosphates and among the dicalcium phosphates but in general they all were intermediate or excellent in availability. The authors speculate that this variation within types of phosphates may result from the original source of the material or the processing of the phosphate or both. Imported rock phosphates and colloidal phosphates were found to be relatively unavailable to poults.

In a later study using poults again Wilcox et al. (34) used a practical rather than purified ration. Most of the defluorinated phosphates and the commercial dicalcium phosphates were found to have satisfactory availability in terms of body growth to 4 weeks of age. Some of the phosphates, however, were not satisfactory in terms of bone calcification as evidenced by the percentage of bone ash. Monobasic calcium phosphate appeared to have excellent availability. Steamed bone meal, several dicalcium phosphates and one defluorinated product showed evidence of containing highly available phosphorus. The phosphorus in an imported rock product, a dicalcium phosphate and a defluorinated product indicated low availability. "Colloidal" phosphates showed unsatisfactory availability to turkey poults.

Various Phosphorus Sources in Swine Rations

A number of studies involving a comparison of different phosphate supplements for swine have been conducted. Shrewsbury and Vestal (31) compared steamed bone meal, defluorinated phosphate, rock phosphate and

superphosphate as phosphorus sources for growing and fattening swine and also for gilts and sows. Criteria used in evaluating the phosphorus supplements for growing pigs were rate of gain, feed efficiency, and bone studies involving percentage of fluorine in bone, percentage of ash, length, diameter, wall thickness, weight and breaking strength. In the case of the reproduction studies with gilts and sows birth weight of pigs and percentage of strong pigs were the criteria used. In the rations for growing pigs steamed bone meal was superior to either defluorinated phosphate or rock phosphate while the superphosphate compared favorably with steamed bone meal. The results were usually not related to the fluorine content of the ration as one might expect. In rations for bred sows and gilts steamed bone meal was only slightly superior to defluorinated or rock phosphate.

Gobble and Miller (14) conducted a trial in which they fed soft phosphate with colloidal clay to growing and fattening swine. These workers observed no harmful effects on an all plant ration which contained 2 per cent soft phosphate with colloidal clay; however, a non-significant tendency for a slower rate of gain was noted as compared with pigs receiving an equivalent amount of supplemental phosphorus from dicalcium phosphate. The state of phosphorus nutrition of the pigs receiving colloidal clay as measured by the inorganic phosphorus content of the blood plasma, and the calcium, phosphorus and total ash content of the bones was normal. No gross symptoms of fluorosis were observed in any of the pigs.

Chapman et al. (8) studied the relative value of soft phosphate with colloidal clay, dicalcium phosphate and steamed bone meal as inorganic

phosphorus supplements for growing and finishing swine. The criteria used in evaluating response were rate of gain, feed efficiency, daily feed intake and blood serum phosphorus when the pigs weighed 100 pounds. In addition to the aforementioned criteria, breaking strength, ash, phosphorus and fluorine content of the femurs were also used when the pigs reached the terminating weight of 200 pounds. The use of colloidal clay as compared with either steamed bone meal or dicalcium phosphate resulted in a significant decrease in rate of gain, feed efficiency and breaking strength of the femurs accompanied by a significant increase in ash content and an increase in the fluorine content of the femurs. No significant differences were observed in the phosphorus content of bone ash or blood serum due to differences in source of phosphorus used.

Plumlee and associates (29) investigated the utilization of phosphorus from six different sources using weanling pigs. The phosphorus supplements compared were dicalcium phosphate, steamed bone meal, defluorinated rock phosphate, commercial monocalcium phosphate, imported rock phosphate and soft phosphate with colloidal clay. At the 0.3 per cent dietary phosphorus level growth rate and feed efficiency were similar for all 6 supplements; however, crooked bones and skeletal weakness were more common, appeared earlier and were more severe in the "soft phosphate" than in any other group. Monocalcium phosphate and imported rock phosphate gave the best results of the 6 supplements tested simultaneously at the 0.30 per cent level. "Soft phosphate" at either the 0.45 or 0.60 per cent level markedly decreased growth rate during the first 6 weeks of the feeding period. The 0.60 per cent level of "soft phosphate" pro-

duced excessive pitting and decay of the molars. The serum inorganic phosphorus values were similar for the six supplements at the same phosphorus level, however, they increased with increase in dietary phosphorus.

Combs (10) using baby pigs and a semi-synthetic ration investigated the feasibility of using blood serum alkaline phosphatase, skeletal and soft tissue composition, bone opacity and growth rate as response criteria for evaluating biological availability of the phosphorus contained in various phosphate supplements. Growth rate, serum phosphatase activity, rib and femur ash, and degree of femur X-ray opacity appeared to be satisfactory response criteria to use. The ash content of the soft tissues and the phosphorus content of both skeletal and soft tissues proved unsatisfactory as criteria of response. Using the above listed criteria, the phosphorus in steamed bone meal was observed to be less available than that present in monocalcium phosphate. Colloidal phosphate was found to be highly unavailable to baby pigs. It was also noted that per cent bone ash and growth rate were more precise measures of phosphorus availability than was phosphatase activity.

Various Phosphorus Sources in Ruminant Rations

The literature pertaining to availability or utilization of phosphorus from various sources for ruminants is rather limited, however, during the past few years several papers have appeared. Ammerman et al. (2) using the balance technique on yearling steers evaluated the phos-

phorus present in various inorganic phosphates. The sources tested included two different dicalcium phosphates, bone meal, defluorinated rock phosphate, imported rock phosphate and colloidal phosphate. Approximately one half of the total phosphorus fed was supplied by the supplement being tested and one half by the basal ration. No significant differences were noted in the per cent phosphorus retained, between phosphorus supplements tested, between steers nor between collection periods.

In a later study Ammerman et al. (1) used weanling lambs on a semi-purified basal ration which contained about 0.03 per cent phosphorus to determine utilization of phosphorus from various inorganic sources. The balance technique was again employed as well as determination of the blood serum phosphorus levels. The phosphate sources used in this study included dicalcium phosphate, Curacao Island phosphate, soft phosphate with colloidal clay and defluorinated rock phosphate and were fed at a level to supply at least 75 per cent of the total phosphorus in the ration. The blood serum phosphorus levels revealed no significant differences after depletion and upon feeding of the phosphorus supplements; however, the blood serum levels of the dicalcium phosphate and Curacao Island phosphate fed lambs were higher than those of colloidal and defluorinated rock phosphate but not statistically so. Complete results on the balance data at this time are unavailable; however, preliminary analysis of part of the data have indicated significant differences in per cent phosphorus retained between phosphorus supplements at about the one per cent level.

Richardson et al. (30) fed phosphoric acid and steamed bone meal to beef heifer calves as a source of phosphorus. Heifers receiving 8 grams added phosphorus daily from either steamed bone meal or phosphoric acid

showed normal blood serum phosphorus levels while those receiving no additional phosphorus did not.

Long et al. (22) investigated availability of soft phosphate with colloidal clay and dicalcium phosphate as phosphorus sources for beef heifers. Criteria used in evaluation of these phosphorus sources were rate of gain, feed consumption, plasma phosphorus values, ash content of bones and general appearance. The basal ration contained 0.094 per cent phosphorus. The amount of phosphorus supplied by the test sources was 0.05 per cent phosphorus in the total ration. Heifers receiving dicalcium phosphate as compared with colloidal clay gained faster, ate more feed and had higher plasma phosphorus values. Upon slaughter the ash content of the moisture and fat-free metacarpal bones of the right fore leg was higher for the heifers receiving dicalcium phosphate. Also, the ash content of the jawbones was higher for the heifers which had received dicalcium phosphate. A considerable difference in general appearance was also noted. Those fed dicalcium phosphate appeared very healthy and thrifty. The heifers fed colloidal clay appeared to be less thrifty. Their hair coats were rough and two were lame, walked with difficulty, had enlarged joints, and were much thinner or tended to be emaciated. Chewing on wood, wire or other foreign material was common among those fed colloidal clay.

Phosphorus Requirement of Rumen Microorganisms

The nutrient needs of rumen microorganisms have been classified into three groups. For maximum activity the microorganisms should have

available 1) a source of nitrogen, 2) minerals, both major and minor, and 3) carbohydrates, to supply a readily available source of energy. Of the major elements phosphorus is one which has been shown to be necessary in *in vitro* studies for maximum cellulose digestion.

Burroughs *et al.* (6) in a study of mineral influences upon urea utilization and cellulose digestion by rumen microorganisms, using the artificial rumen technique, found that phosphorus aided cellulose digestion. The basal medium used was a mineral mixture resembling the mineral content of sheep saliva. Additions of iron also were found to aid cellulose digestion and urea utilization. The additions of magnesium, potassium and calcium failed to aid either cellulose digestion or urea utilization. The addition of iron, phosphorus or combinations of these two elements with and without molasses further showed stimulating influences. The authors postulated that the iron and phosphorus were probably responsible for a part but not all of the favorable influence which they observed from the addition of molasses ash.

Hall (18) in an "*in vitro*" study of rumen microorganism mineral requirements, using a washed cell suspension technique, reported the optimum range for phosphorus as being from 50-200 parts per million in the medium. The technique employed in arriving at this requirement was to leave phosphorus out of the basal medium and add back various levels using cellulose digestion as the response criterion.

Hubbert *et al.* (19) in some more recent studies using essentially the same technique have reported the phosphorus requirement for rumen microorganisms as being somewhere between 0-75 micrograms per milliliter. The basal medium used in this study was somewhat different from that used

by Hall (18) and may account for the variation in the quantitative requirement given.

Various Artificial Rumen Techniques

Several different techniques for studying in vitro cellulose digestion by rumen microorganisms appear in the literature. Marston (26) published a paper in 1948 in which he used a technique which simulated closely the conditions in the live animal. Since the publication of this paper several investigators have published modifications of his procedure.

Louw et al. (23) described a method for in vitro cellulose digestion which involves the use of a semi-permeable bag which is suspended in a large volume of aqueous growth medium. These workers call attention to the fact that Marston's technique (26) provides no way for removal of non-gaseous fermentation products which, as they accumulate, might be expected to slow the rate and eventually inhibit digestion.

Huhtanen et al. (20) point out that the artificial rumen designed by Louw et al. (23) is rather large and cumbersome for use in routine work. The need for continuous neutralization of the medium was also considered objectionable. These workers simplified the artificial rumen to a miniature form so as to consist essentially of a small cellophane sac into which the substrate is placed and the sac then suspended in a jar containing the bacterial medium.

Burroughs et al. (4) described an artificial rumen technique which involved a fermentation period of from 5 to 10 days. Two of the major features of their method involved the use of continuous 36-hour fermenta-

tion periods and splitting the fermentation material in half at the end of each 36-hour period for dilution purposes. Cellulose digestion was determined on the half portions not used as inoculum. Gram stains were also made at the beginning and end of each fermentation period. Limitations of this technique are the time element involved and the fact that microorganisms under artificial conditions for this length of time might possibly become so modified as to not be representative of the normal rumen flora.

Cheng et al. (9) developed a technique involving the use of washed suspensions of rumen microorganisms and a relatively short fermentation period. One of the advantages of using a washed microbial suspension over the unwashed rumen fluid is that unknown factors are removed that might be present in the rumen fluid. Also, the washed suspension is comparatively free from any metabolic end-products that were produced by the microorganisms while they were in the rumen. Another advantage is the relatively short fermentation period required as compared with the technique of Burroughs et al. (4).

EXPERIMENTAL

Use of a Washed Cell Suspension Technique

In view of the favorable results obtained by Hall et al. (18) using a washed cell suspension technique in mineral studies, it was felt that this was a good starting point with respect to the development of a technique which would be suitable for phosphorus assay purposes. The method reported by Hall et al. (18) has several advantages over the one used by Burroughs et al. (4). The fermentation period is much shorter, the inoculum contains fewer metabolites and unidentified factors, and since smaller fermentation vessels are used more treatments or comparisons may be run simultaneously. Replication is easier too, since 75 ml. centrifuge tubes are employed as the fermentation containers rather than 500 ml. Erlenmeyer flasks which are rather space consuming in a water bath of limited capacity.

The purpose of this first phase of the study was to determine the effect upon cellulose digestion from omission and addition of phosphorus to the basal medium using a washed cell suspension technique. The specific factors studied were the amount of rumen liquor to use, the effect of washing the bacterial inoculum and the effect of varying the digestion or fermentation time.

Materials and methods

Rumen contents were obtained from a 1500-pound fistulated Shorthorn

steer receiving a ration composed of 70 per cent ground corn cobs, 20 per cent ground corn and 10 per cent protein supplement. The supplement consisted of soybean oilmeal, urea, dried molasses, bone meal and a dry form of vitamin A and D. This steer consumed approximately 30 pounds daily of this ration. Later in the experiment this 30 pounds of feed was supplemented with one additional pound of soybean oilmeal daily. During the early stages of the experiment the steer was fed and watered twice daily; however, later in the study he was fed all the feed for a 24 hour period at one feeding, in the morning, in an attempt to help standardize the conditions under which rumen samples were obtained.

After obtaining the rumen contents the liquid portion was strained through four layers of number 50 grade cheesecloth and collected in thermal-neutral containers. This procedure constituted the first step in obtaining rumen inoculum in liquid form. Microorganisms from 40, 80, and 120 ml. of rumen liquid were used per tube in the beginning experiments. The strained rumen fluid was next brought to the laboratory and centrifuged in a Servall angle centrifuge at a speed of approximately 1000 r.p.m. for 2 minutes. This centrifugation sedimented partially digested feed particles and protozoa which were not removed by straining the rumen liquid through cheesecloth. The sedimented material was discarded and the supernatant centrifuged in a Sharples supercentrifuge at a speed of about 25,000 r.p.m. for about 10 minutes. The resulting sediment was collected on a cellophane sheet which was placed inside the centrifuge bowl prior to centrifugation. This sediment, consisting principally of rumen bacteria, was suspended in a liter of distilled water saturated with

carbon dioxide gas. A Waring Blendor was used to suspend the cells in water so that clumps of cells would be dispersed. This bacterial suspension was centrifuged again in the Sharples supercentrifuge at a speed of about 25,000 r.p.m. for about 10 minutes. The procedure as described constituted inoculum resulting from one washing of the bacterial cells. Several experiments were conducted where the bacterial cells were washed two or three times.

The only difference in the preparation of inoculum that was washed 2 or 3 times from that which had been washed once was in the washing process itself. To obtain inoculum which had been washed twice involved taking the sediment which had collected in the centrifuge bowl after one washing and suspending it in one liter of distilled water saturated with carbon dioxide gas and centrifuging in the Sharples supercentrifuge again. To obtain inoculum which had been washed three times involved taking inoculum which had been washed twice and suspending the resulting sediment in one liter of distilled water and once again putting it through the Sharples supercentrifuge. The final sediment obtained from either one, two or three washings of the bacterial cells was suspended in a nutrient solution prepared according to a formula modified from Burroughs et al. (5) and shown in Table 1.

A purified wood cellulose, Solka-floc, was added to the bacterial suspension in an amount to make the concentration of this insoluble cellulose 0.5 per cent. A stream of carbon dioxide gas was directed through the suspension for 10 minutes after which time the pH was adjusted to 7.0 by the addition of a saturated solution of sodium carbonate. Aliquots of 20 milliliters each were pipetted into 75 milliliter pyrex

centrifuge tubes. Usually all the treatments, including the control, were triplicated. Each of the fermentation tubes was fitted with a rubber stopper and inlet and outlet glass tubing. A rather slow constant flowing stream of carbon dioxide gas was passed through the tubes to establish and maintain anaerobic condition and to agitate the fermenting suspension. The tubes were incubated in a constant temperature water

Table 1. Composition of phosphorus deficient basal medium

Constituent	Amount (gm./liter)
$NaHCO_3$	1.750
KCl	0.375
NaCl	0.375
$MgSO_4$	0.075
$CuSO_4 \cdot 5H_2O$	0.001
$MnSO_4$	0.0002
$ZnSO_4$	0.00004
$FeSO_4 \cdot 7H_2O$	0.0375
$CoCl_2 \cdot 6H_2O$	0.001
Urea	1.000

bath which was thermostatically controlled at approximately 39° C. At the end of 24 to 32 hours the fermentations were terminated and cellulose was determined on the entire contents of each tube using the procedure of Crampton and Maynard (11) with slight modification.

The technique employed at the beginning of this investigation was to use a phosphorus deficient basal medium and to add to it graded

increments of phosphorus to determine the response phosphorus would have on the digestion of cellulose by the rumen microorganisms. The phosphorus additions were made directly into the respective tubes using a mixture of 2 parts sodium phosphate, $Na_2HPO_4.H_2O$, and one part potassium phosphate, KH_2PO_4. These two inorganic phosphorus sources are present in the complete basal medium used in artificial rumen studies and in the same proportion as indicated.

Results and discussion

Since previous work by Hall (18) and Cheng (9) had showed that bacteria obtained from 40 ml. of rumen liquid provided a sufficient amount of inoculum for optimum results, when using the washed cell suspension technique, this amount was initially used. Table 2 shows the results of two experiments conducted on different days using microorganisms obtained from 40 ml. of rumen liquid per tube. The bacterial cells were washed once according to the procedure previously described.

Table 2. Effect of phosphorus upon cellulose digestion in vitro, using microorganisms obtained from 40 ml. of rumen liquid per tube and washed once

Treatment	% Cellulose digested	
	Experiment I	Experiment II
Basal	34.2	30.4
Basal + 2 mcg. P/ml. medium	34.3	35.1
Basal + 10 mcg. P/ml. medium	43.6	33.5
Basal + 20 mcg. P/ml. medium	33.1	29.2
Basal + 100 mcg. P/ml. medium	35.0	33.6
Basal + 200 mcg. P/ml. medium	33.4	37.5

In experiment I the maximum response was noted from the addition of 10 mcg. phosphorus per ml. of medium where cellulose digestion was increased 27.5 per cent. In experiment II the maximum response was obtained from the addition of 200 mcg. phosphorus which increased cellulose digestion 23.3 per cent. Experiment II tended to indicate that perhaps the levels of phosphorus added were not high enough since maximum cellulose digestion was observed at the highest level added.

Table 3 shows the effect of using double the normal amount of inoculum, that is, microorganisms from 80 ml. rumen liquid, with higher additions of phosphorus than were used in the first two experiments.

Table 3. Effect of phosphorus upon cellulose digestion in vitro, using microorganisms obtained from 80 ml. of rumen liquid per tube and washed once

Treatment	% Cellulose digested	
	Experiment I	Experiment II
Basal	46.0	49.0
Basal + 100 mcg. P/ml. medium	59.1	54.4
Basal + 200 mcg. P/ml. medium	59.3	56.7
Basal + 400 mcg. P/ml. medium	60.3	54.3
Basal + 600 mcg. P/ml. medium	63.2	53.5
Basal + 700 mcg. P/ml. medium	61.0	52.3
Basal + 800 mcg. P/ml. medium	58.3	54.7
Basal + 900 mcg. P/ml. medium	57.0	55.4
Basal +1000 mcg. P/ml. medium	53.5	51.3

The addition of 600 mcg. phosphorus per ml. of medium in experiment I, Table 3, increased cellulose digestion 37.4 per cent, however, in attempting to repeat these results on a different day, experiment II, Table 3, less than half this increase was noted. Also this increase occurred at the 200 mcg. phosphorus addition level rather than at 600 mcg.

Triple the normal amount of inoculum was next used in an attempt to get a greater response from phosphorus. Table 4 shows the results from the use of microorganisms from 120 ml. of rumen liquid per tube.

Table 4. Effect of phosphorus upon cellulose digestion in vitro, using microorganisms obtained from 120 ml. of rumen liquid per tube and washed once

Treatment	% Cellulose digested
Basal	39.9
Basal + 100 mcg. P/ml. medium	47.8
Basal + 200 mcg. P/ml. medium	45.2
Basal + 400 mcg. P/ml. medium	48.2
Basal + 600 mcg. P/ml. medium	48.6
Basal + 700 mcg. P/ml. medium	46.2
Basal + 800 mcg. P/ml. medium	46.4
Basal + 900 mcg. P/ml. medium	44.9
Basal +1000 mcg. P/ml. medium	47.3

One hundred mcg. phosphorus per ml. medium added to the phosphorus deficient basal seemed to be as effective in stimulating cellulose digestion as were the higher levels. The response, however, was rather small, being only 22.0 per cent greater at the optimum phosphorus level of 600 mcg. per ml. of medium.

Since varying the number of bacteria used per tube as inoculum did not produce widely differing results it was felt that several washings of the bacterial cells might next be tried in an attempt to increase the spread in cellulose digestion between the phosphorus deficient basal and the optimum addition of phosphorus. Repeated washings, it was reasoned, should do a more thorough job of removing phosphorus from the inoculum and from the bacterial cells themselves. Table 5 shows the effect of using as inoculum bacterial cells which had been washed two times.

Table 5. Effect of phosphorus upon cellulose digestion *in vitro*, using microorganism obtained from 40 ml. of rumen liquid per tube and washed twice

Treatment	% Cellulose digested
Basal	42.5
Basal + 2 mcg. P/ml. medium	42.5
Basal + 10 mcg. P/ml. medium	41.1
Basal + 20 mcg. P/ml. medium	41.6
Basal + 100 mcg. P/ml. medium	45.2
Basal + 200 mcg. P/ml. medium	48.0
Basal + 400 mcg. P/ml. medium	51.2

Washing the bacterial cells twice did not seem to improve the response from phosphorus over that observed by one washing. This experiment tended to indicate that higher levels of phosphorus additions perhaps should have been used since the highest percentage of cellulose digested occurred in the tubes where 400 mcg. phosphorus per ml. medium were added; however, the stimulating effect of this phosphorus addition was no greater than in cases where the inoculum was washed one time. An increase in cellulose digestion of 20.5 per cent was noted from the addition of 400 mcg. phosphorus.

Table 6 shows the effect of using as inoculum bacterial cells which had been washed three times.

Table 6. Effect of phosphorus upon cellulose digestion in vitro, using microorganisms obtained from 120 ml. of rumen liquid per tube and washed three times

Treatment	% Cellulose digested
Basal	41.2
Basal + 10 mcg. P/ml. medium	31.9
Basal + 100 mcg. P/ml. medium	38.4
Basal + 200 mcg. P/ml. medium	36.5
Basal + 1000 mcg. P/ml. medium	53.1

The digestion percentages obtained when using inoculum which had been washed three times indicate that it is no better and possibly poorer as an inoculum source than that washed just once. Only at the 1000 mcg. level was cellulose digestion increased over the basal and by only 28.9 per cent. It should be noted that at the 10, 100, and 200 mcg. phosphorus

addition levels, three washings of the bacterial cells had a depressing effect on cellulose digestion. Many of the bacteria perhaps died during the processing procedure since they were exposed to aerobic conditions for a longer period of time and subjected to more rigorous treatment than were bacteria washed just once or twice.

Since varying the amount of rumen liquid used per tube and the number of washings of the inoculum did not appear to help spread the difference in cellulose digestion between the basal and at optimum phosphorus addition it was decided to vary the length of the digestion period. In the preceding experiments all were terminated at the end of 24 hours.

Table 7 shows the effect of varying the digestion time using microorganisms from 40 ml. of rumen liquid per tube.

Table 7. Effect of phosphorus upon cellulose digestion in vitro, using microorganisms obtained from 40 ml. of rumen liquid per tube when digestion time was varied from 24 to 32 hours.

Treatment	% Cellulose digested		
	24 hours	28 hours	32 hours
Basal	27.1	33.2	45.0
Basal + 10 mcg. P/ml. medium	26.7	34.4	48.8
Basal + 200 mcg. P/ml. medium	28.6	41.0	43.0

A 28 hour fermentation time appeared to be more desirable than either the 24 or 32 hour time in this experiment. Cellulose digestion was increased 23.5 per cent at the optimum phosphorus level at 28 hours fermentation as compared with increases of 5.5 and 8.4 per cent at the 24 and 32 hour fermentation times, respectively.

Table 8 shows the effect of varying the digestion time using microorganisms from 80 ml. of rumen liquid per tube.

Table 8. Effect of phosphorus upon cellulose digestion in vitro, using microorganisms obtained from 80 ml. of rumen liquid per tube at digestion times of 28 and 32 hours

Treatment	% Cellulose digested	
	28 hours	32 hours
Basal	52.5	58.1
Basal + 100 mcg. P/ml. medium	57.1	65.5
Basal + 500 mcg. P/ml. medium	59.4	64.9
Basal + 1000 mcg. P/ml. medium	58.3	62.4
Basal + 2000 mcg. P/ml. medium	----	22.6

When using microorganisms from 80 ml. of rumen liquid per tube no benefit was observed in using a 28 or 32 hour fermentation over the normal 24 hour results previously discussed. The addition of 2000 mcg. phosphorus per ml. medium showed a very marked depressing effect on cellulose digestion in the 32 hour fermentation group. Excessive quantities of phosphorus appear to be toxic to the rumen microorganisms.

Summary

A series of experiments,using a washed cell suspension technique failed to consistently demonstrate a significant increase in cellulose digestion from the addition of phosphorus to a phosphorus deficient basal medium. Varying the number of rumen microorganisms used per tube as inoculum, the number of times the inoculum was washed and the length of the fermentation period failed to consistently show wide differences between the per cent cellulose digested on the phosphorus deficient basal and at the optimum level of added phosphorus.

Microorganisms obtained from 40 ml. of rumen liquid appeared to be as effective as those obtained from 80 or 120 ml. when comparing the difference in cellulose digestion on a phosphorus deficient medium and a medium containing phosphorus. Inoculum which had been washed one time appeared to be as effective as that washed two or three times in demonstrating the omission and addition of phosphorus to the medium. No advantage was noted from the use of a fermentation period longer than 24 hours. A 28 and 32 hour period were tried and the per cent cellulose digested on all treatments increased; however, the difference between cellulose digested on the phosphorus deficient medium as compared with the phosphorus repleted medium was not increased.

Use of Phosphorus Depleted Inoculum and the Washed Cell Suspension Technique

Since satisfactory results were not obtained when using non-depleted inoculum and the washed cell suspension technique,it was decided to try

using a phosphorus depleted source of inoculum. It seemed possible that the bacterial cells contained some intracellular phosphorus. If this source of phosphorus could be exhausted, it was postulated that a greater response could be expected from the addition of phosphorus when using this type of inoculum. Therefore, the purpose of this phase of the study was to determine whether or not phosphorus depleted rumen microorganisms were more sensitive to phosphorus additions than the non-depleted microorganisms previously used.

Materials and methods

The inoculum used in this phase of the study was obtained as previously described. Microorganisms obtained from 40 ml. of rumen liquid were originally collected for each 20 ml. of basal medium used during the depletion process. The bacterial cells were washed once and then placed in a large 2 liter Erlenmeyer flask containing the phosphorus deficient basal medium plus cellulose. The microorganisms were grown for 24 hours in this 2 liter flask prior to being used for inoculum in the tubes for phosphorus assay purposes.

At the end of 24 hours the material in the large flask was either used directly as inoculum without dilution or was diluted, as was usually done, by splitting in half and adding an equivalent amount of phosphorus deficient basal medium plus additional cellulose. The half portion not used as inoculum was in some instances depleted for longer periods; that is, 48 and 72 hours. However, at the end of each 24 hour period the material present in the flask was split in half and an equivalent amount

of fresh phosphorus deficient basal medium plus cellulose added. The half portion not being depleted further was used as inoculum for the tubes. The pH of the depleting material was adjusted to 7.0 at the end of each 24 hour period.

Results and discussion

Table 9 presents the results from the use of 24 hour phosphorus depleted undiluted inoculum.

Table 9. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro when using 24 hour phosphorus depleted undiluted inoculum

Treatment	% cellulose digested
Basal	63.3
Basal + 200 mcg. P/ml. medium	81.5
Basal + 400 mcg. P/ml. medium	79.7
Basal + 600 mcg. P/ml. medium	81.0
Basal + 700 mcg. P/ml. medium	80.2
Basal + 800 mcg. P/ml. medium	75.8
Basal + 900 mcg. P/ml. medium	76.9
Basal + 1000 mcg. P/ml. medium	78.3

The source of inoculum for the data presented in Table 9 was microorganisms which had been grown 24 hours on a phosphorus deficient basal medium. At the end of this period of time additional cellulose was added so that approximately 100 mg. would be present in each 20 ml.

aliquot and the pH adjusted to 7.0. No attempt was made in this experiment to dilute the inoculum by adding additional basal medium. The lowest level of phosphorus added was 200 mcg. per ml. medium. At this level 81.5 per cent of the cellulose was digested as compared with 63.3 per cent on the phosphorus deficient basal. Additions of phosphorus above the 200 mcg. level failed to show a corresponding increase in cellulose digestion.

This experiment was repeated at a later date using lower levels of phosphorus. Table 10 shows the results of the second experiment where 24 hour phosphorus depleted undiluted inoculum was used.

Table 10. Effect of low levels of phosphorus upon cellulose digestion by rumen microorganisms in vitro when using 24 hour phosphorus depleted undiluted inoculum

Treatment	% cellulose digested
Basal	41.3
Basal + 10 mcg. P/ml. medium	40.4
Basal + 20 mcg. P/ml. medium	44.8
Basal + 50 mcg. P/ml. medium	44.2
Basal + 70 mcg. P/ml. medium	42.1
Basal + 100 mcg. P/ml. medium	42.8
Basal + 200 mcg. P/ml. medium	42.0
Basal + 500 mcg. P/ml. medium	43.1
Basal + 1000 mcg. P/ml. medium	43.4

No beneficial effect was noted from the use of depleted, undiluted, inoculum in this experiment and was not consistent with results noted in Table 9. In view of this it was felt that inoculum which had been depleted for more than 24 hours should be tested in which the inoculum was diluted by adding an equivalent quantity of fresh basal medium. This procedure, it was reasoned, would tend to dilute out metabolites formed by the microorganisms during the depletion period and thus make the microorganisms more sensitive to the addition of phosphorus.

Table 11 shows the effect from the use of 24, 48 and 72 hour phosphorus depleted and diluted inoculum.

Table 11. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro when using 24, 48 and 72 hour phosphorus depleted and diluted inoculum

Treatment	% Cellulose digested		
	24 hours	48 hours	72 hours
Basal	27.9	13.6	9.1
Basal + 10 mcg. P/ml. medium	47.0	17.0	9.9
Basal + 20 mcg. P/ml. medium	49.4	19.3	12.4
Basal + 50 mcg. P/ml. medium	48.6	17.7	14.3
Basal + 70 mcg. P/ml. medium	47.5	15.9	14.7
Basal + 100 mcg. P/ml. medium	47.1	21.4	12.1
Basal + 200 mcg. P/ml. medium	47.3	18.7	18.1
Basal + 500 mcg. P/ml. medium	56.8	13.9	17.6
Basal + 1000 mcg. P/ml. medium	46.5	10.4	14.0

The use of 24 hour depleted and diluted inoculum caused 27.9 per cent of the cellulose to be digested in the basal as compared with 56.8 per cent where 500 mcg. phosphorus were added. This represents an increase in cellulose digestion of 104 per cent. The addition of the lowest level of phosphorus, 10 mcg. per ml., increased cellulose digestion from 27.9 to 47.0 per cent or an increase of 68 per cent. The use of 48 and 72 hour phosphorus depleted and diluted inoculum failed to show a similar response and the per cent digested at all levels at these two depletion times was greatly reduced.

Since a rather favorable response was noted in the preceding experiment it was felt that this experiment should be repeated using several levels of phosphorus lower than 10 mcg. per ml. medium. Two attempts were made to repeat the results shown in Table 11 using 24 hour depleted inoculum. Table 12 shows the results of these two repeat experiments.

Table 12. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro when using 24 hour phosphorus depleted and diluted inoculum

Treatment	% cellulose digested	
	Experiment I	Experiment II
Basal	37.3	33.2
Basal + 1 mcg. P/ml. medium	34.1	33.2
Basal + 3 mcg. P/ml. medium	34.4	34.6
Basal + 5 mcg. P/ml. medium	36.6	38.6
Basal + 7 mcg. P/ml. medium	34.9	39.4
Basal + 10 mcg. P/ml. medium	34.1	37.1
Basal + 100 mcg. P/ml. medium	34.3	40.9
Basal + 500 mcg. P/ml. medium	32.4	40.9
Basal + 1000 mcg. P/ml. medium	34.1	38.9

The same favorable response noted in Table 11 could not be repeated in two subsequent trials as may be seen from the data presented in Table 12. The second repeat experiment tended to show a progressive increase in cellulose digestion from the addition of graded increments of phosphorus; however, the optimum response was far below that noted in Table 11. Apparently the inoculum itself varied enough from day to day to cause this difference in response.

Summary

Two different types of phosphorus depleted inoculum were used for phosphorus assay purposes using cellulose digestion as the criterion of response. In one case the inoculum was not diluted but used directly at the end of the depletion period. In the other the inoculum was diluted by adding an equivalent amount of the fresh basal medium prior to using as inoculum in the assay tubes. The diluted inoculum in one experiment appeared to have some advantage over the undiluted in promoting cellulose digestion in the presence of optimum phosphorus.

Depletion times of 24, 48 and 72 hours were tried. The 24 hour depleted inoculum appeared to be more desirable than that depleted either 48 or 72 hours. The per cent cellulose digested at both the 48 and 72 hour depletion times was considerably lower than when 24 hour depleted inoculum was used and the difference in response from addition of phosphorus was also considerably lower.

Use of an Artificial Rumen Technique Employing 500 ml. Erlenmeyer Flasks as Fermentation Vessels

The use of the washed cell suspension technique up to this time had not proved very hopeful in securing consistent and large responses from the addition of phosphorus to a phosphorus deficient basal medium; therefore, a change of technique seemed in order. Since Burroughs et al. (4) had used an artificial rumen technique which had showed phosphorus to be required for optimum cellulose digestion, it was decided to try a similar technique.

Materials and methods

The technique of Burroughs et al. (4) which was next tried employed 500 ml. Erlenmeyer flasks as the fermentation vessels rather than 75 ml. centrifuge tubes. Strained rumen liquid was used as inoculum by these investigators; however, in the following experiments several types of inoculum resulting after centrifugation were used.

The basal medium used was the same as previously described for the washed cell suspension technique. The cellulose source was likewise the same. At the end of each 24 hour fermentation period the material in each flask was split in half after three 20 ml. aliquots had been taken from each flask for cellulose determination. Three control samples of 20 ml. each were taken at the start of each fermentation period to determine the original starting cellulose concentration. After the material in each flask was split in half, one portion was discarded and the other

built up to the original starting concentration by adding an equivalent amount of fresh basal medium plus cellulose and fermented further.

Hall (18) while studying unidentified factors influencing cellulose digestion by rumen microorganisms had observed a stimulatory effect from the addition of either a vitamin-free casein or a feather meal hydrolyzate to the basal medium. Since the hydrolyzates contained only a very small amount of phosphorus it was decided to include this growth stimulant in all flasks. If the hydrolyzates speeded up the metabolic activity of the microorganisms, then by its use, the need for phosphorus should be demonstrated sooner, than if it were not included.

The feather meal hydrolyzate was prepared by partially hydrolyzing a commercial feather meal with hydrochloric acid. A five per cent solution was prepared by adding 25 gm. of feather meal to 500 ml. of 3 N hydrochloric acid. The mixture was autoclaved at 15 lb. for four hours. The pH of the acid hydrolyzate was adjusted to between 6.5 and 6.8 by adding solid sodium hydroxide. The insoluble material was removed by centrifugation and filtration.

Hall (18) had observed that the optimum level of hydrolyzate to use in stimulating cellulose digestion was about 0.3 ml. of the five per cent solution per 20 ml. of basal medium. This amount of hydrolyzate added approximately 2 mcg. phosphorus per ml. of the basal medium.

Results and discussion

The first experiment using this technique covered a four day period and the results are shown in Table 13. Inoculum was obtained from two sources. One source was the Shorthorn steer used in earlier experiments employing the washed cell suspension technique. The other source was a Brown Swiss steer receiving a ration consisting of ground corn, soybean

oilmeal and alfalfa hay. The inoculum was obtained under conditions similar to those described previously and brought to the laboratory and processed in the same manner except that the washing of the inoculum was omitted in all but one experiment. The inoculum used differed also in that both the sediment remaining in the bowl of the Sharples centrifuge plus that which drained out into cup, upon stopping, was used.

Table 13. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro using feather meal hydrolyzate as a bacterial growth stimulant and two sources of inoculum

Treatment	Flask No.	Inoculum source	Time and % cellulose digested			
			24 hours	48 hours	72 hours	96 hours
Basal + F.M.H.[a]	I	Shorthorn steer	42.8	39.0	18.1	11.5
Basal + F.M.H. + P[b]	II	Shorthorn steer	43.0	54.4	41.6	40.6
Basal + F.M.H.	III	Brown Swiss steer	50.3	48.2	23.3	13.3
Basal + F.M.H. + P	IV	Brown Swiss steer	55.7	50.4	34.7	40.4

[a]Addition of 0.3 ml. of a five per cent feather meal hydrolyzate solution per 20 ml. of medium in all flasks.

[b]100 mcg. P/ml. medium.

At the end of 48 hours a difference in cellulose digestion was noted between the basal containing feather meal hydrolyzate and the same medium plus 100 mcg. of added phosphorus per ml. where inoculum was obtained from the Shorthorn steer on the poorer ration. This same response was not noted

until 72 hours where inoculum was obtained from the Brown Swiss steer receiving the better ration. At 96 hours the response was very similar for the two sources of inoculum. Cellulose digestion was increased approximately three times over the basal when 100 mcg. phosphorus were added per ml. of medium.

Table 14 shows the results when using three levels of phosphorus. Cellulose determinations were made only at the 72 and 96 hour fermentation times. Inoculum was from the Shorthorn steer receiving the poorer ration.

Table 14. Effect of three levels of phosphorus upon cellulose digestion by rumen microorganisms *in vitro*

Treatment	Flask No.	Time and % cellulose digested	
		72 hours	96 hours
Basal + F.M.H.[a]	I	32.7	10.8
Basal + F.M.H. + 10 mcg. P/ml. medium	II	46.8	25.3
Basal + F.M.H. + 100 mcg. P/ml. medium	III	43.2	30.8
Basal + F.M.H. + 1000 mcg. P/ml. medium	IV	43.0	27.6

[a]0.3 ml. five per cent feather meal hydrolyzate added per 20 ml. medium in all flasks.

A similar response was noted at 96 hours in this experiment as was observed at 72 hours in the preceding one. Apparently the variation in the inoculum itself is responsible since the other conditions were the same. The medium level of phosphorus added, 100 mcg., produced optimum cellulose digestion. It should be noted, however, that the addition of

10 and 1000 mcg. were nearly as effective and that the microorganisms are quite tolerant to high levels of phosphorus.

In the preceding two experiments using this technique the source of inoculum was the sediment collecting in the bowl of the Sharples centrifuge plus the liquid which drains into the cup upon stopping without any washing of the inoculum. The next experiment was set up with the thought of comparing different types of inoculum. Table 15 shows the results when using bowl inoculum only, bowl plus cup inoculum and bowl inoculum which was washed once.

The bowl sediment plus cup liquid inoculum did not prove to be as sensitive to phosphorus addition as either the washed or unwashed bowl sediment alone. The difference in cellulose digestion from phosphorus addition at 24, 48, 72 and 96 hours was 17.9, 47.8, 34.3 and 29.2 per cent, respectively, when unwashed bowl sediment was the inoculum source. When washed bowl sediment was the inoculum source at the same time periods the difference in cellulose digestion was 17.1, 53.5, 12.0 and 11.3 per cent, respectively. When unwashed bowl sediment plus cup liquid was used the difference in cellulose digestion at the four time periods was 8.1, 0, 4.6 and 15.6 per cent, respectively. At the 48 hour fermentation time the washed bowl material showed a slightly greater difference in cellulose digestion over the unwashed material; however, at the 72 and 96 hour fermentation times the advantage was in favor of the unwashed bowl inoculum.

Table 15. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when using three types of inoculum

Flask No.	Type inoculum	Treatment	Time and % cellulose digested			
			24 hours	48 hours	72 hours	96 hours
I	Bowl inoculum unwashed	Basal + F.M.H.[a]	23.8	22.6	1.7	4.7
II	Bowl inoculum unwashed	Basal + F.M H. + P[b]	41.7	70.4	36.0	33.9
III	Bowl and cup inoculum unwashed	Basal + F.M.H.	58.3	63.9	48.3	20.2
IV	Bowl and cup inoculum unwashed	Basal + F.M.H. + P	66.4	63.6	52.9	35.8
V	Bowl inoculum washed once	Basal + F.M.H.	18.3	17.1	3.2	0.0
VI	Bowl inoculum washed once	Basal + F.M.H. + P	35.4	70.6	15.2	11.3

[a]0.3 ml. five per cent feather meal hydrolyzate per 20 ml. medium.

[b]100 mcg. phosphorus per ml. medium.

Summary

An artificial rumen technique modified somewhat from that used by Burroughs et al. (4) was used to determine the effect of omission or addition of phosphorus on cellulose digestion by rumen microorganisms. A feather meal hydrolyzate was used in the basal medium as a source of unidentified factors.

Three experiments each covering a period of four days were conducted using this technique. Differences in cellulose digestion between the basal and the basal plus phosphorus usually began showing up at the 48 hour fermentation time and got progressively greater as the fermentation time was extended when washed bowl sediment and cup liquid was used as the inoculum source.

Three different types of inoculum, unwashed bowl sediment, unwashed bowl sediment and cup liquid, and washed bowl sediment were compared. The bowl sediment, either washed or unwashed, proved to be very sensitive to the addition of phosphorus.

Marked differences in cellulose digestion between the basal and basal plus phosphorus began showing up at the end of the first 24 hour fermentation period. However, when unwashed bowl sediment, including cup liquid, was used as the inoculum source this same difference in cellulose digestion was not noted until the end of the fourth 24 hour period or at the end of 96 hours fermentation.

Use of Factors Stimulatory to Cellulose Digestion Using the Washed Cell Suspension Technique

The use of the artificial rumen technique employing 500 ml. Erlenmeyer flasks previously described, using feather meal hydrolyzate as a bacterial growth stimulant in the basal medium, demonstrated the importance and need for phosphorus by the rumen microorganisms for optimum cellulose digestion. However, this technique did not lend itself well for use as an assay procedure. The shorter fermentation time and the fact that more treatments could be compared using tubes rather than flasks as fermentation containers were factors in favor of the washed cell suspension technique, assuming that the same response could be obtained.

Hall (18) had also noted a stimulatory effect on cellulose digestion by rumen microorganisms in vitro from the inclusion of such factors as biotin, vitamin B_{12} and dextrose as well as from vitamin-free casein and feather meal hydrolyzates when using the washed cell suspension technique. MacLeod and Brumwell (25) likewise found a mixture of amino acids to be stimulatory to microorganisms in cellulose degradation in vitro. Therefore, it was decided to go back to the washed cell suspension technique originally used, and by the use of these bacterial growth stimulants and phosphorus depleted inoculum try to duplicate the favorable results observed when using the flask technique with feather meal hydrolyzate in the basal medium.

Materials and methods

Inoculum for this phase of the study was collected and brought to the laboratory as previously described for the washed cell suspension technique. For the most part the microorganisms were washed once and 24 and 48 hour phosphorus depleted microorganisms used for inoculum in the tubes for assay purposes. Sediment collecting in the bowl of the Sharples centrifuge after one washing of the microorganisms was used as the original starting inoculum in the depletion flasks. At the end of 24 hours the material in the flask which had undergone depletion was split in half with one half portion being depleted further.

A mixture of amino acids, vitamin B_{12}, biotin, dextrose, casein hydrolyzate and feather meal hydrolyzate were the bacterial growth stimulants added to the basal medium. These bacterial growth factors were added either singly or in combinations and either to the depleting material or to the assay tubes or both. The amino acid mixture used was the same as that described by MacLeod (24).

Results and discussion

Several experiments were conducted where the bacterial growth stimulants dextrose, biotin, vitamin B_{12} and an amino acid mixture were added to the basal medium during the depletion period and also during the assay period in the tubes. Different levels of the amino acid mixture were tried; however, constant amounts of the other factors were added since Hall (18) had observed that 5 mcg. vitamin B_{12}, 1 mcg. biotin and 0.5 mg. dextrose per 20 ml. basal medium produced optimum cellulose digestion.

Table 16 shows typical results observed when using two levels of the amino acid mixture both with 24 and 48 hour phosphorus depleted inoculum. These bacterial growth stimulants were added during the depletion period and also during the assay period in the tubes.

Table 16. Effect of phosphorus upon cellulose digestion in vitro by rumen microorganisms when vitamin B_{12}, biotin, dextrose and an amino acid mixture were added to basal medium

Treatment	% cellulose digested	
	24 hour depleted inoculum	48 hour depleted inoculum
Basal[a]	52.9	13.3
Basal + 0.1 ml. A.A.[b] mixture	50.0	12.3
Basal + 0.1 ml. A.A. mixture + 10 mcg. P/ml. medium	52.2	16.8
Basal + 0.1 ml. A.A. mixture + 100 mcg. P/ml. medium	52.2	13.6
Basal + 0.1 ml. A.A. mixture + 1000 mcg. P/ml. medium	53.6	18.1
Basal + 0.5 ml. A.A. mixture	47.5	22.8
Basal + 0.5 ml. A.A. mixture + 10 mcg. P/ml. medium	47.8	22.1
Basal + 0.5 ml. A.A. mixture + 100 mcg. P/ml. medium	49.7	23.2
Basal + 0.5 ml. A.A. mixture + 1000 mcg. P/ml. medium	50.4	16.8

[a]Phosphorus deficient with addition of 5 mcg. vitamin B_{12}, 1 mcg. biotin and 0.5 mg. dextrose/20 ml. medium.

[b]Amino acid.

The results shown in Table 16 indicate little or no benefit from the inclusion of the amino acid mixture in the medium in so far as helping to get a large difference in response between cellulose digestion on the phosphorus deficient basal and at optimum phosphorus addition. These results were typical of other experiments where varying levels of the amino acid mixtures were used; therefore, the use of these bacterial growth stimulants was abandoned.

Since vitamin-free casein hydrolyzate had been shown by Hall (18) to be stimulatory to cellulose digesting microorganisms and since it was thought to be more definable and standardized in composition than feather meal this material was next used. The casein hydrolyzate was prepared by the procedure indicated earlier for feather meal hydrolyzate except that it was a one per cent solution rather than five per cent.

Table 17 shows the results of phosphorus additions without and with the addition of casein hydrolyzate to the basal medium. Twenty-four and 48 hour phosphorus depleted inoculum was used and the casein hydrolyzate was added to the tubes only.

The data in Table 17 indicate that very little, if any, benefit resulted from the addition of casein hydrolyzate to the medium. When 24 hour depleted inoculum was used the difference in cellulose digestion between the basal and basal plus optimum phosphorus was 11.3 per cent; however, the spread between basal plus casein hydrolyzate and optimum phosphorus was 14.7 per cent. When 48 hour depleted inoculum was used the difference between the basal and basal plus optimum phosphorus was 14.3 per cent and when casein hydrolyzate was used this same comparison showed a 15.7 per cent difference.

Table 17. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro when casein hydrolyzate was added to the basal medium

Treatment	% cellulose digested	
	24 hour depleted inoculum	48 hour depleted inoculum
Basal	33.1	17.5
Basal + 10 mcg. P/ml. medium	32.9	31.2
Basal + 50 mcg. P/ml. medium	21.1	31.8
Basal + 100 mcg. P/ml. medium	35.7	29.3
Basal + 200 mcg. P/ml. medium	35.5	25.8
Basal + 500 mcg. P/ml. medium	44.4	28.0
Basal + 0.5 ml. C.H.[a]	47.4	18.8
Basal + 0.5 ml. C.H. + 10 mcg. P/ml. medium	61.2	34.5
Basal + 0.5 ml. C.H. + 50 mcg. P/ml. medium	62.1	30.9
Basal + 0.5 ml. C.H. + 100 mcg. P/ml. medium	61.2	33.8
Basal + 0.5 ml. C.H. + 200 mcg. P/ml. medium	58.3	33.4
Basal + 0.5 ml. C.H. + 500 mcg. P/ml. medium	60.8	26.1

[a]A one per cent casein hydrolyzate solution.

Two experiments were conducted with casein hydrolyzate in the basal medium using non-depleted inoculum. The inoculum was processed the same as described in the beginning of this study. Microorganisms resulting from 120 ml. of rumen liquid per tube were used and were washed twice in a mixture of basal medium and distilled water. Table 18 shows the results of these experiments.

Table 18. Effect of phosphorus upon cellulose digestion in vitro by rumen microorganisms using non-depleted inoculum plus addition of casein hydrolyzate to basal medium

Treatment	% cellulose digested	
	Experiment I	Experiment II
Basal	14.6	47.9
Basal + 1.0 ml. C.H.[a]	32.7	66.8
Basal + 1.0 ml. C.H. + 1 mcg. P/ml. medium	----	69.7
Basal + 1.0 ml. C.H. + 5 mcg. P/ml. medium	----	71.6
Basal + 1.0 ml. C.H. + 10 mcg. P/ml. medium	33.6	71.5
Basal + 1.0 ml. C.H + 100 mcg. P/ml. medium	38.9	72.4
Basal + 1.0 ml. C.H. + 1000 mcg. P/ml. medium	20.9	----

[a]A one per cent casein hydrolyzate solution.

The experimental conditions of the two experiments reported in Table 18 are essentially the same except for levels of phosphorus added. It is quite apparent from the difference in per cent cellulose digested that the source of inoculum varies from day to day; however, in neither case did the use of casein hydrolyzate show any beneficial advantage for purposes of phosphorus assay. The use of the hydrolyzate caused a higher percentage of cellulose to be digested; however, the further addition of phosphorus did not cause more cellulose to be digested.

Since the casein hydrolyzate did not seem to be helpful when added to the basal medium for the purpose of supplying unidentified factors, it was decided to use feather meal hydrolyzate since this had proved helpful

when using the flask technique.

Two experiments were first conducted using non-depleted inoculum. The results of these two experiments are shown in Table 19.

Table 19. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when using non-depleted inoculum plus addition of feather meal hydrolyzate to the basal medium

Treatment	% cellulose digested	
	Experiment I[a]	Experiment II[b]
Basal	0.0	0.0
Basal + 0.25 ml. F.M.H.[c]	9.4	42.9
Basal + 0.25 ml. F.M.H. + 1 mcg. P/ml. medium	7.7	39.0
Basal + 0.25 ml. F.M.H. + 10 mcg. P/ml. medium	16.6	38.7
Basal + 0.25 ml. F.M.H. + 100 mcg. P/ml. medium	23.3	47.7
Basal + 0.25 ml. F.M.H. + 1000 mcg. P/ml. medium	8.5	38.2

[a]Microorganisms from 40 ml. rumen liquid/tube.

[b]Microorganisms from 80 ml. rumen liquid/tube.

[c]A five per cent feather meal hydrolyzate solution.

In both experiments no digestion was observed on the basal medium without phosphorus. In experiment I, microorganisms were used from 40 ml. of rumen liquid per tube. Cellulose digestion was low for all treatments; however, there was a rather marked increase from addition of 10 and 100

mcg. phosphorus per ml. medium over the basal plus 0.25 ml. feather meal hydrolyzate. Approximately two and one half times as much cellulose was digested where 100 mcg. phosphorus were added. However, in experiment II, where microorganisms from 80 ml. of rumen liquid per tube were used, and where cellulose digestion was higher for all treatments except the basal, similar results were not noted. An increase in cellulose digestion of only 11.2 per cent resulted when 100 mcg. phosphorus were added.

Twenty-four hour phosphorus depleted inoculum was next tried with the addition of 0.25 ml. feather meal hydrolyzate per 20 ml. medium during the depletion period and also during the assay period in the tubes. Table 20 shows the data and Figure 1 graphically illustrates the results of this experiment.

Table 20. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro using phosphorus depleted inoculum plus addition of feather meal hydrolyzate to the basal medium

Treatment	% cellulose digested
Basal	13.2
Basal + 0.25 ml. F.M.H.[a]	14.6
Basal + 0.25 ml. F.M.H. + 1 mcg. P/ml. medium	17.0
Basal + 0.25 ml. F.M.H. + 10 mcg. P/ml. medium	32.0
Basal + 0.25 ml. F.M H. + 100 mcg. P/ml. medium	55.5
Basal + 0.25 ml. F.M H. +1000 mcg. P/ml. medium	48.6

[a]A five per cent feather meal hydrolyzate solution.

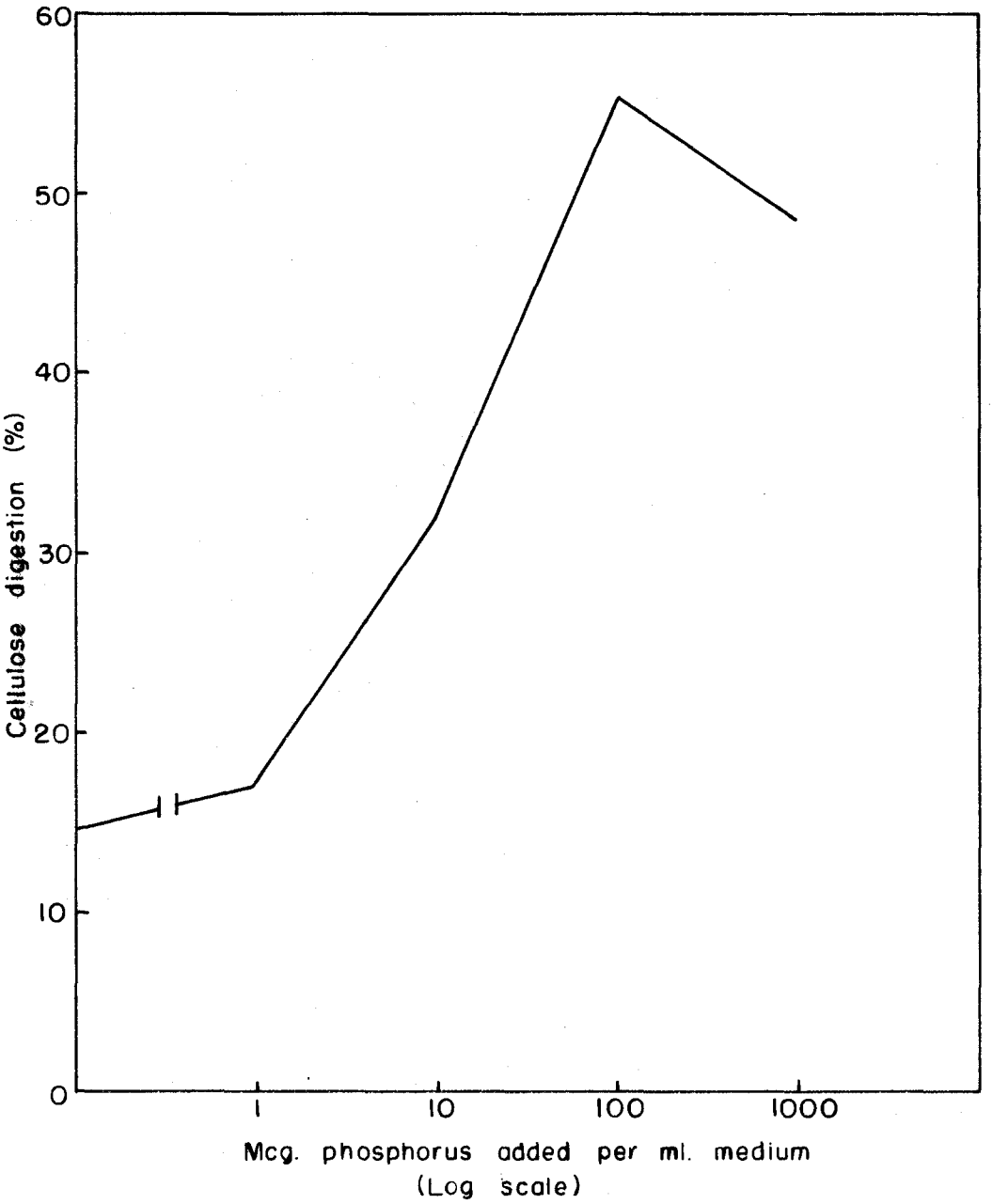

Figure 1. Effect of phosphorus upon cellulose digestion when feather meal hydrolyzate was included in basal medium

Cellulose digestion progressively increased from the addition of 1, 10 and 100 mcg. phosphorus per ml. medium and when these values are plotted on the log scale a linear relationship is noted. The addition of 1000 mcg. phosphorus per ml. of medium was apparently so high as to cause a depressing effect on cellulose digestion by the rumen microorganisms.

As mentioned earlier in this section microorganisms which had been washed once were used as the original source of inoculum for the depletion flasks. During centrifugation of the rumen liquid some dark colored material of a rather sticky nature was noted to collect on the walls of the centrifuge bowl and extend upward for about a half inch. This material was discarded as it was assumed that this material was largely food particles. However, one experiment was conducted in this series where it was included for comparison purposes. Table 21 shows the results of this comparison.

When the dark colored material was included as inoculum cellulose digestion was higher at all treatments. The addition of 100 mcg. phosphorus per ml. of medium increased cellulose digestion from 65.2 to 73.4 per cent or a difference of 8.2 per cent. When the dark colored material was excluded cellulose digestion was lower at all treatments. Addition of 100 mcg. phosphorus increased cellulose digestion from 36.2 to 55.1 per cent or a difference of 18.9 per cent. Discarding the dark colored material apparently made the inoculum more sensitive to phosphorus.

Since 24 hour depleted inoculum appeared to be giving satisfactory responses upon phosphorus addition it was decided to attempt using a

Table 21. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when dark colored material collecting in bottom of centrifuge bowl was included as inoculum

Treatment	% cellulose digested	
	I[a]	II[b]
Basal	51.8	30.0
Basal + 0.3 ml. F.M.H.[c]	65.2	36.2
Basal + 0.3 ml. F.M H. + 1 mcg. P/ml. medium	61.2	26.8
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	68.9	36.5
Basal + 0.3 ml. F.M H. + 100 mcg. P/ml. medium	73.4	55.1
Basal + 0.3 ml. F.M.H. + 1000 mcg. P/ml. medium	19.1	29.6

[a]Dark colored material included in original inoculum.

[b]Dark colored material excluded from original inoculum.

[c]A five per cent feather meal hydrolyzate solution.

shorter depletion time such as 12 hours rather than 24. Table 22 and Figure 2 shows the results of this trial.

Figure 2 shows the response curves using the two sources of inoculum. It is very apparent from the graph that the 24 hour depleted inoculum is much more suitable for assay purposes than the 12 hour depleted material. Cellulose digestion increased from 20.0 to 33.9 or a difference of 13.9 per cent from the addition of 160 mcg. phosphorus when 12-hour depleted inoculum was used. When 24 hour depleted inoculum was used cellulose digestion increased from 21.2 to 59.2 or a difference of 38.0 per cent from the addition of 40 mcg. phosphorus.

Table 22. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* using 12 and 24 hour phosphorus depleted inoculum

Treatment	% cellulose digested	
	12 hour depleted inoculum	24 hour depleted inoculum
Basal	21.3	21.4
Basal + 0.3 ml. F.M.H.[a]	20.0	21.2
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	28.7	33.1
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	28.9	38.1
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	31.6	56.3
Basal + 0.3 ml. F.M H. + 40 mcg. P/ml. medium	31.5	59.2
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	32.9	58.6
Basal + 0.3 ml. F.M.H. + 160 mcg. P/ml. medium	33.9	56.0

[a]A five per cent feather meal hydrolyzate solution.

The use of 24 hour phosphorus depleted inoculum with addition of feather meal hydrolyzate to the basal medium was beginning to show progressive increases in cellulose digestion from the addition of graded increments of phosphorus. These results likewise could be repeated. Therefore, it seemed advisable to investigate other variables in an attempt to make the procedure more sensitive to phosphorus. The variables investigated were digestion time, washed versus unwashed inoculum, diluted depleted versus undiluted depleted inoculum, and levels of feather meal hydrolyzate.

Table 23 shows the results of using depleted undiluted as compared

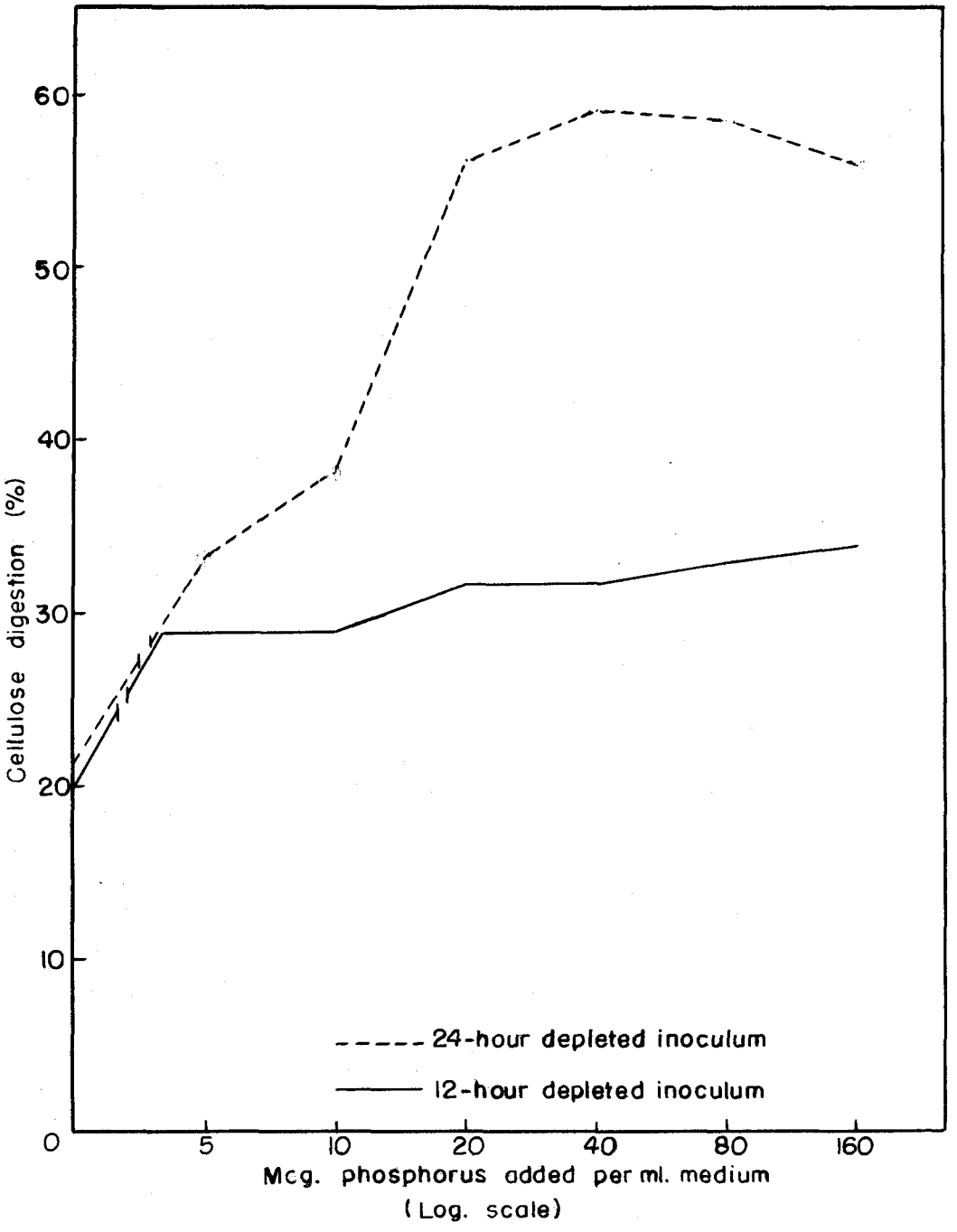

Figure 2. Twelve hour versus 24 hour phosphorus depleted inoculum

Table 23. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro using diluted depleted versus undiluted depleted inoculum at three digestion times

Treatment	% cellulose digested and time		
	20 hour	24 hour	28 hour
24 hour depleted inoculum - undiluted			
Basal	40.5	48.0	53.0
Basal + 0.3 ml. F.M.H.[a]	34.2	40.7	44.8
Basal + 0.3 ml. F.M H. + 100 mcg. P/ml. medium	64.3	65.8	73.5
24 hour depleted inoculum - diluted			
Basal	22.0	27.2	31.4
Basal + 0.3 ml. F.M H.	26.1	30.3	28.9
Basal + 0.3 ml. F.M.H. + 100 mcg. P/ml. medium	46.5	57.1	63.2

[a]A five per cent solution feather meal hydrolyzate.

with depleted diluted inoculum at three digestion times.

When depleted undiluted inoculum was used the increase in cellulose digestion from the addition of 100 mcg. phosphorus at 20, 24 and 28 hour fermentation times was 88.0, 61.7 and 64.0 per cent, respectively. When using the depleted diluted inoculum the increases were 78.2, 88.4 and 118.7 per cent, respectively. The 20 hour fermentation time appeared most desirable when using undiluted inoculum and the 28 hour time most desirable when using diluted inoculum.

Washed inoculum was next compared with unwashed. Table 24 and Figure 3 show the results of this experiment.

Table 24. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* using washed versus unwashed phosphorus depleted inoculum

Treatment	% cellulose digested	
	I[a]	II[b]
Basal	21.0	13.3
Basal + 0.3 ml. F.M.H.[c]	24.5	15.0
Basal + 0.3 ml. F.M.H. + 1 mcg. P/ml. medium	25.8	16.3
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	31.3	29.4
Basal + 0.3 ml. F.M H. + 100 mcg. P/ml. medium	22.6	43.6
Basal + 0.3 ml. F.M.H. + 1000 mcg. P/ml. medium	15.2	17.1

[a]Unwashed inoculum.

[b]Washed inoculum.

[c]A five per cent solution feather meal hydrolyzate.

The washed microorganisms proved to be a much more effective source of inoculum than the unwashed. Figure 3 graphically illustrates the straight line response from the addition of 1, 10 and 100 mcg. phosphorus per ml. medium. The addition of 1000 mcg. phosphorus caused a very marked depression on cellulose digestion with both sources of inoculum.

Different levels of feather meal hydrolyzate were next tried using both diluted and undiluted phosphorus depleted microorganisms as sources of inoculum. Table 25 shows the results of this experiment.

When undiluted inoculum was used the increase in cellulose digestion

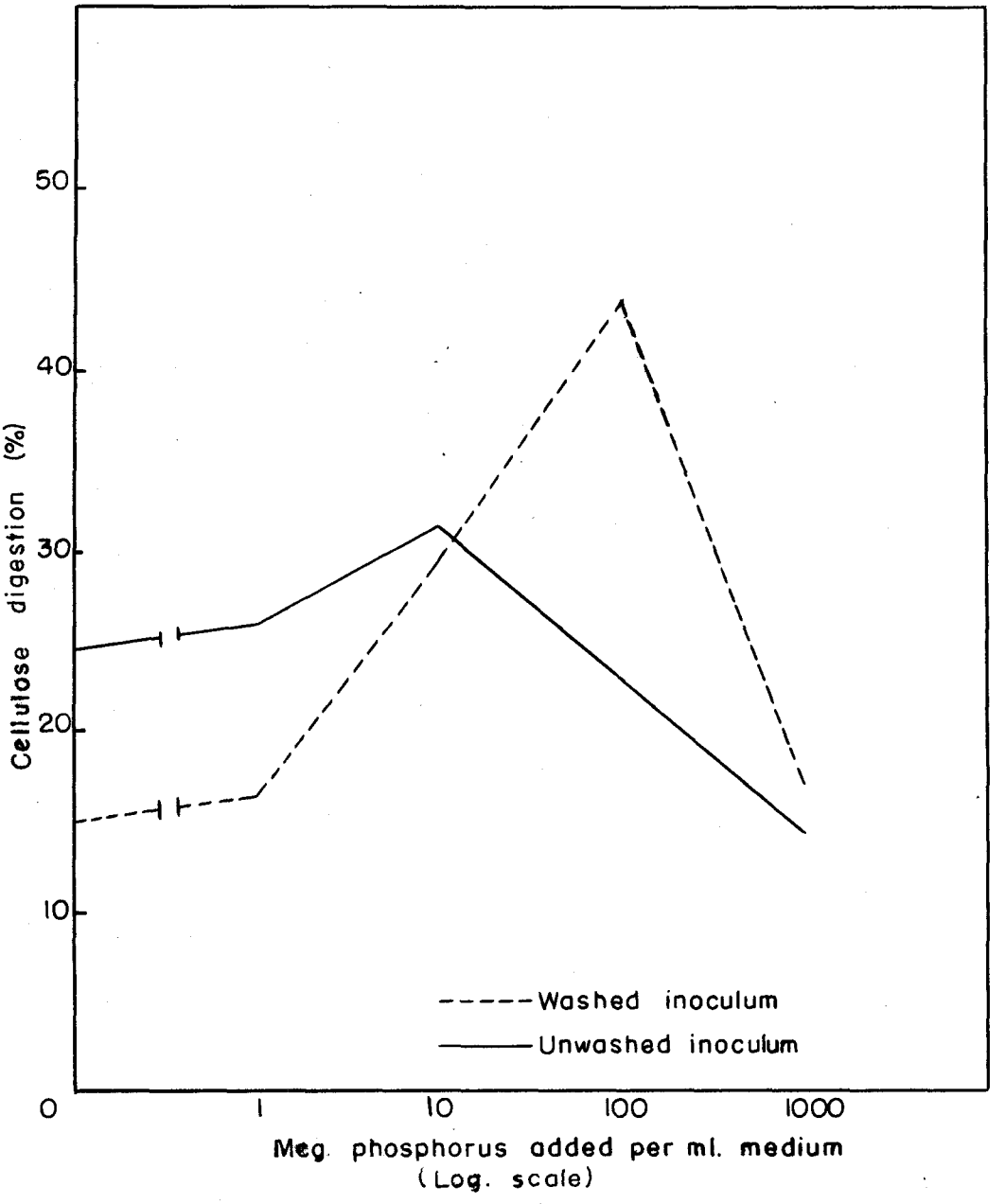

Figure 3. Washed versus unwashed phosphorus depleted inoculum

Table 25. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro using different levels of feather meal hydrolyzate and diluted versus undiluted phosphorus depleted inoculum

Treatment	% cellulose digested	
	Undiluted inoculum	Diluted inoculum
Basal	40.0	30.1
Basal + 0.2 ml. F.M.H.[a]	40.8	31.9
Basal + 0.2 ml. F.M H. + 100 mcg. P/ml. medium	62.1	56.3
Basal	38.7	27.7
Basal + 0.25 ml. F.M.H.	36.0	32.7
Basal + 0.25 ml. F.M.H. + 100 mcg. P/ml. medium	64.4	57.1
Basal	37.5	15.3
Basal + 0.30 ml. F.M.H.	37.3	23.4
Basal + 0.30 ml. F.M.H. + 100 mcg. P/ml. medium	64.2	58.4
Basal	33.4	13.3
Basal + 0.35 ml. F.M.H.	29.5	24.4
Basal + 0.35 ml. F.M.H. + 100 mcg. P/ml. medium	54.4	48.4

[a]A five per cent solution feather meal hydrolyzate.

from addition of 100 mcg. of phosphorus over the basal including 0.2, 0.25, 0.30 and 0.35 ml. feather meal hydrolyzate was 52.2, 78.9, 72.1, and 84.4 per cent, respectively. When diluted inoculum was used the increase in cellulose digestion from addition of 100 mcg. of phosphorus over the basal including 0.2, 0.25, 0.30 and 0.35 ml. feather meal hydrolyzate was 76.5,

74.6, 149.6 and 98.4 per cent, respectively. Diluted inoculum with 0.3 ml. feather meal hydrolyzate per 20 ml. medium gave the greatest response from phosphorus addition.

Summary

Factors stimulatory to rumen microorganisms in cellulose degradation in vitro were added to the basal medium using the washed cell, suspension technique and phosphorus depleted and non-depleted inoculum. The following factors were used: biotin, vitamin B_{12}, dextrose, and amino acid mixture, casein hydrolyzate and feather meal hydrolyzate.

The use of a combination of biotin, vitamin B_{12}, dextrose and various levels of an amino acid mixture in the basal medium did not show a larger spread in cellulose digestion between the phosphorus deficient basal and basal plus phosphorus than was observed without its inclusion. Vitamin-free casein hydrolyzate likewise did not prove beneficial, at the level used when both phosphorus depleted and non-depleted microorganisms were the sources of inoculum.

The addition of feather meal hydrolyzate did prove to be effective in showing a large difference in cellulose digestion between the basal including feather meal hydrolyzate and this same basal plus additions of phosphorus. Twelve-hour phosphorus depleted inoculum was not nearly as effective as a 24 hour depletion period in bringing about this response.

Phosphorus depleted and diluted inoculum, in general, was more effective than phosphorus depleted inoculum which had not been diluted.

Washed inoculum likewise was more effective than the unwashed material. Varying the digestion time from 20 to 28 hours had little effect in altering the response between the basal and basal plus phosphorus. The use of 0.20, 0.25, 0.30 and 0.35 ml. of feather meal hydrolyzate per 20 ml. of medium did not produce a markedly different response in cellulose digestion upon phosphorus addition except in the case of the diluted inoculum and 0.3 ml. feather meal hydrolyzate. Discarding the dark colored material which collected at the bottom of the Sharples bowl appeared to make the microorganisms more sensitive to phosphorus addition.

Comparison of Effect from Addition of Feather Meal Hydrolyzate, Casein Hydrolyzate, Rumen Liquor or Additional Sodium Chloride to the Basal Medium

The addition of feather meal hydrolyzate to the basal medium to supply a source of unidentified factors stimulatory to rumen microorganisms in cellulose degradation had proved very beneficial in previous experiments. Hall (18) had noted vitamin-free casein hydrolyzate to be almost equally as effective; however, in several previous experiments this was not found to be true in this study. Hubbert (19) had observed that the use of additional sodium chloride in the basal medium had likewise stimulated the microorganisms to digest more cellulose. It was reasoned, therefore, that the beneficial effect noted from the use of hydrolyzates might possibly be from the salt formation resulting during neutralization after the feather meal or casein had been partially hydrolyzed. Therefore, the purpose of this portion of the study was to compare the results

from the addition of feather meal hydrolyzate to those observed when additional sodium chloride was used, to compare feather meal and casein hydrolyzate, and to compare rumen liquor with feather meal and casein hydrolyzate as a source of unidentified factors.

Materials and methods

The washed cell suspension technique with the use of 24 hour phosphorus depleted inoculum was employed as described previously. The material collecting in the bowl of the Sharples centrifuge, after being washed once, was used as the original source of inoculum prior to depletion. About one fourth inch of the dark colored material collecting at the bottom of the bowl was discarded.

Microorganisms resulting from 40 ml. of rumen liquid per 20 ml. of basal medium were originally used as the starting inoculum in the depletion flasks. A 24 hour depletion time and a 24 hour digestion time in the tubes were used. Hydrolyzate additions were 0.3 ml. of a five per cent solution per 20 ml. basal medium of either the vitamin-free casein or feather meal during depletion and also during the phosphorus assay period in the tubes. In the experiment where rumen liquid was added, two ml. were added per 20 ml. of basal medium.

Results and discussion

Table 26 shows the results of adding additional salt to the basal medium.

The addition of 10 mcg. phosphorus to the basal medium containing

Table 26. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when adding additional sodium chloride to the basal medium

Treatment	% cellulose digested		
	I[a]	II[b]	III[c]
Basal	23.8	19.5	23.4
Basal + 5 mcg. P/ml. medium	25.5	19.6	22.1
Basal + 10 mcg. P/ml. medium	26.2	24.9	25.3
Basal + 20 mcg. P/ml. medium	20.9	26.9	27.4
Basal + 40 mcg. P/ml. medium	23.5	23.8	23.8
Basal + 80 mcg. P/ml. medium	22.9	----	----

[a]No additional salt during depletion or during tube assay.

[b]Addition of 30 mg. sodium chloride during tube assay period only.

[c]Addition of 60 mg. sodium chloride during tube assay period only.

no additional salt increased cellulose digestion ten per cent. When 30 and 60 mg. salt were added per 20 ml. basal medium during the phosphorus assay period in the tubes only, cellulose digestion was increased by 37.9 and 12.8 per cent, respectively, from the addition of 20 mcg. phosphorus. These increases are considerably lower than those noted when feather meal hydrolyzate was included in the basal medium.

The addition of feather meal hydrolyzate and sodium chloride were next compared when each was added during the depletion period and also during the phosphorus assay period in the tubes. Table 27 and Figure 4 show the results of this experiment.

Table 27. A comparison of the effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when either feather meal hydrolyzate or sodium chloride were added to the basal medium

Treatment	% cellulose digested
F.M.H.[a] Addition	
Basal	30.9
Basal + 0.3 ml. F.M.H.	33.2
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	52.7
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	60.9
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	65.7
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	65.5
NaCl[b] Addition	
Basal	18.1
Basal + 50 mg. NaCl	24.3
Basal + 50 mg. NaCl + 10 mcg. P/ml. medium	30.5
Basal + 50 mg. NaCl + 20 mcg. P/ml. medium	29.0
Basal + 50 mg. NaCl + 40 mcg. P/ml. medium	29.8
Basal + 50 mg. NaCl + 80 mcg. P/ml. medium	31.5

[a]A five per cent solution feather meal hydrolyzate.

[b]Sodium chloride.

When feather meal hydrolyzate was used the addition of 40 mcg. phosphorus per ml. medium increased cellulose digestion by 97.9 per cent. The use of sodium chloride increased cellulose digestion from 22.6 per cent when 40 mcg. phosphorus were added to 29.6 per cent when 80 mcg. were

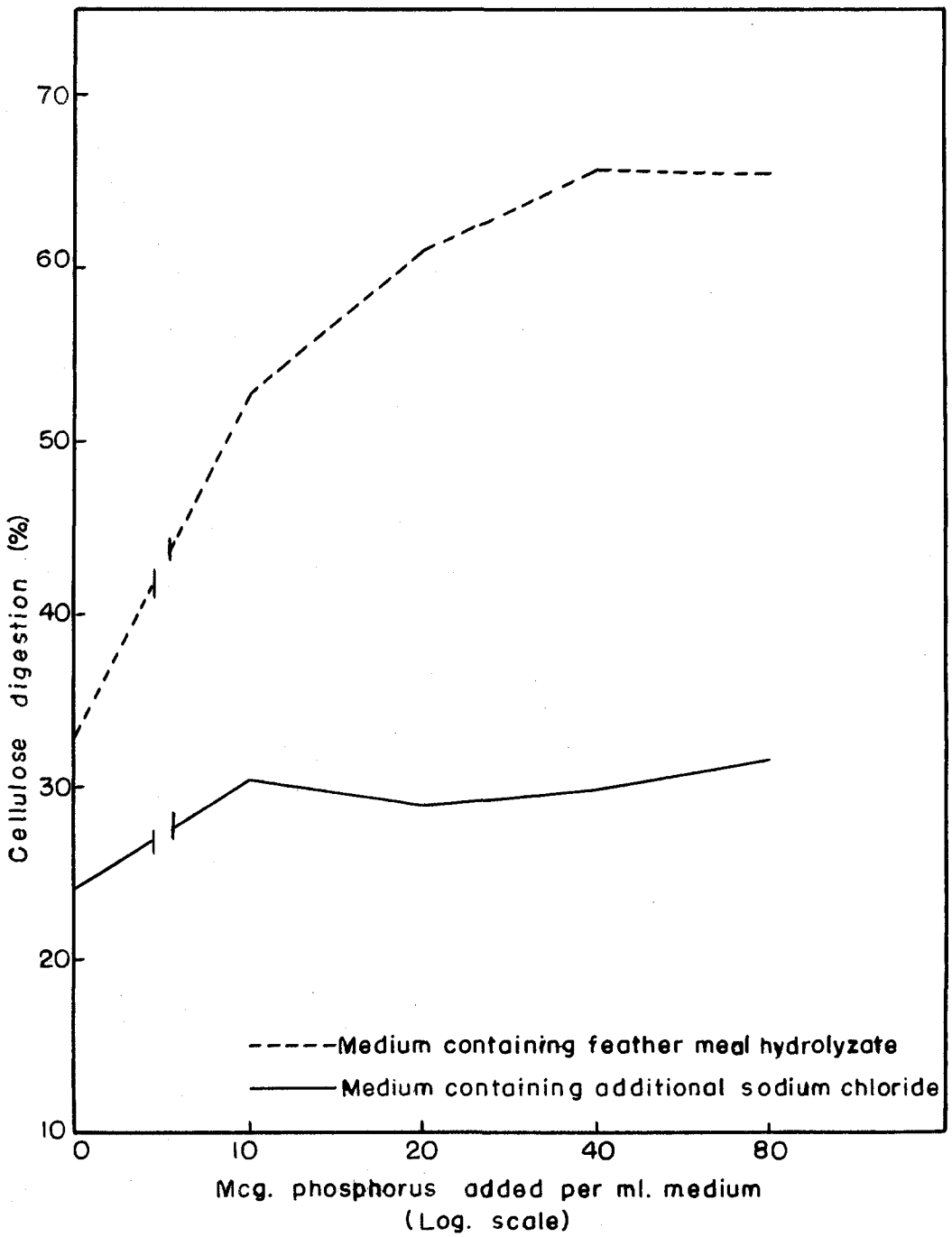

Figure 4. Comparison of addition of feather meal hydrolyzate and additional sodium chloride to basal medium

added. The feather meal hydrolyzate as indicated in Figure 4 was much more effective in promoting cellulose digestion upon phosphorus addition than was the sodium chloride.

A second experiment was conducted comparing feather meal hydrolyzate with sodium chloride. Table 28 shows the results of this experiment. Sixty mg. sodium chloride or 0.3 ml. of a five per cent feather meal hydrolyzate solution were added per 20 ml. basal medium during depletion and also during the phosphorus assay period in the tubes. Numerous levels of phosphorus were used with the feather meal hydrolyzate in this experiment and higher levels of phosphorus were used with the sodium chloride than in the previous experiment.

Table 28. A comparison of the effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro when either feather meal hydrolyzate or sodium chloride were added to the basal medium

Treatment	% cellulose digested
F.M H.[a] Addition	
Basal	25.5
Basal + 0.3 ml. F.M.H.	25.5
Basal + 0.3 ml. F.M.H. + 3 mcg. P/ml. medium	32.8
Basal + 0.3 ml. F.M.H. + 6 mcg. P/ml. medium	34.9
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	41.7
Basal + 0.3 ml. F.M.H. + 15 mcg. P/ml. medium	54.2
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	66.1

[a]A five per cent solution feather meal hydrolyzate.

Table 28 (continued)

Treatment	% cellulose digested
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	78.9
Basal + 0.3 ml. F.M.H. + 60 mcg. P/ml. medium	78.9
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	79.3
Basal + 0.3 ml. F.M.H. + 100 mcg. P/ml. medium	76.0
Basal + 0.3 ml. F.M.H. + 150 mcg. P/ml. medium	78.1
Basal + 0.3 ml. F.M.H. + 200 mcg. P/ml. medium	77.5
Basal + 0.3 ml. F.M.H. + 300 mcg. P/ml. medium	80.2
Basal + 0.3 ml. F.M.H. + 400 mcg. P/ml. medium	77.6
Basal + 0.3 ml. F.M.H. + 500 mcg. P/ml. medium	72.8
Basal + 0.3 ml. F.M.H. + 1000 mcg. P/ml. medium	63.6
Basal + 0.3 ml. F.M.H. + 2000 mcg. P/ml. medium	28.4
NaCl[b] Addition	
Basal + 60 mg. NaCl	25.0
Basal + 60 mg. NaCl + 10 mcg. P/ml. medium	36.4
Basal + 60 mg. NaCl + 100 mcg. P/ml. medium	40.9
Basal + 60 mg. NaCl + 200 mcg. P/ml. medium	35.9
Basal + 60 mg. NaCl + 1000 mcg. P/ml. medium	18.2

[b]Sodium chloride.

Cellulose digestion increased from 25.5 per cent on the basal to 80.2 per cent when 300 mcg. phosphorus per ml. medium were added or an increase of 214.5 per cent when feather meal hydrolyzate was included in the basal medium. Cellulose digestion increased from 25.0 per cent

to 40.9 per cent when 100 mcg. phosphorus per ml. medium were added or an increase of 61.6 per cent when sodium chloride was included in the basal medium. This was the optimum response in both cases. It is interesting to note that when using feather meal hydrolyzate the addition of 40 mcg. phosphorus was about as effective in promoting cellulose as higher levels up to 400. Beyond 400 mcg. there was a progressive decrease for the three levels used. This illustrates the wide tolerance which microorganisms apparently have with respect to phosphorus concentration in the medium. These results also indicate that the feather meal hydrolyzate apparently contains something other than salt.

Feather meal hydrolyzate was next compared with vitamin-free casein hydrolyzate. Table 29 shows the results of this experiment.

The addition of 100 mcg. phosphorus per ml. medium to the basal medium containing casein hydrolyzate increased cellulose digestion 55.0 per cent. The addition of 50 mcg. phosphorus to the basal medium containing feather meal hydrolyzate increased cellulose digestion 100.7 per cent.

This was the optimum response in both cases. Since the basal cellulose digestion percentage was higher for the medium containing casein hydrolyzate one might suspect that it contained more phosphorus. The casein hydrolyzate was a five per cent solution rather than a one per cent as used previously and thereby possibly accounts for the more favorable response noted in this experiment.

One experiment was conducted in which feather meal hydrolyzate, vitamin-free casein hydrolyzate and rumen liquor were compared as sources

Table 29. Effect of phosphorus upon cellulose digestion by rumen microorganisms in vitro when either feather meal hydrolyzate or vitamin-free casein hydrolyzate were added to the basal medium

Treatment	% cellulose digested	
	I[a]	II[b]
Basal	32.4	27.5
Basal + 0.3 ml. hydrolyzate	38.7	29.9
Basal + 0.3 ml. hydrolyzate + 10 mcg. P/ml. medium	51.2	45.8
Basal + 0.3 ml. hydrolyzate + 50 mcg. P/ml. medium	58.5	60.0
Basal + 0.3 ml. hydrolyzate + 100 mcg. P/ml. medium	60.0	56.5
Basal + 0.3 ml. hydrolyzate + 200 mcg. P/ml. medium	57.5	56.0
Basal + 0.3 ml. hydrolyzate + 300 mcg. P/ml. medium	54.9	52.1
Basal + 0.3 ml. hydrolyzate + 500 mcg. P/ml. medium	52.9	50.4
Basal + 0.3 ml. hydrolyzate + 1000 mcg. P/ml. medium	43.3	40.9

[a]Five per cent vitamin-free casein hydrolyzate.

[b]Five per cent feather meal hydrolyzate.

of unidentified factors. Table 30 shows the results of this experiment.

Casein hydrolyzate compared very favorably with feather meal hydrolyzate in this experiment. In the medium containing feather meal hydrolyzate the addition of 100 mcg. phosphorus per ml. medium increased cellulose digestion 93.6 per cent. The addition of 100 mcg. phosphorus in the medium containing casein hydrolyzate increased cellulose digestion 73.9 per cent. The addition of phosphorus to the medium containing rumen liquid did not increase cellulose digestion further.

Table 30. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when feather meal hydrolyzate, casein hydrolyzate or rumen liquid were added to the basal medium

Treatment	% cellulose digested
Basal	26.6
Basal + 0.3 ml. F.M.H.[a]	29.6
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	43.7
Basal + 0.3 ml. F.M.H. + 100 mcg. P/ml. medium	57.3
Basal	24.9
Basal + 0.3 ml. C.H.[b]	31.1
Basal + 0.3 ml. C.H. + 10 mcg. P/ml. medium	39.6
Basal + 0.3 ml. C.H. + 100 mcg. P/ml. medium	54.1
Basal	48.2
Basal + 2.0 ml. r.l.[c]	62.4
Basal + 2.0 ml. r.l. + 10 mcg. P/ml. medium	60.0
Basal + 2.0 ml. r.l. + 100 mcg. P/ml. medium	54.5

[a]A five per cent feather meal hydrolyzate solution.

[b]A five per cent casein hydrolyzate solution.

[c]Rumen liquid.

Apparently there was a considerable amount of phosphorus present in the rumen liquid.

Summary

A series of experiments were conducted to compare the effect of

phosphorus upon cellulose digestion when either feather meal hydrolyzate, casein hydrolyzate, additional sodium chloride or rumen liquid was added to the basal medium. Twenty-four hour phosphorus depleted microorganisms were used as the inoculum source. The above listed materials in most cases were added to the basal medium in the depletion flask and also during the assay period in the tubes.

Feather meal hydrolyzate was the most effective of the materials tried in increasing cellulose digestion upon phosphorus addition, followed by vitamin-free casein hydrolyzate and sodium chloride. The use of rumen liquid did not prove beneficial since upon phosphorus addition cellulose digestion was decreased.

Use of Inoculum from Different Sources and From Steers on Different Rations

Inoculum for most of the experiments to date had been obtained from the Shorthorn steer receiving the high corn cob ration. Since the use of phosphorus depleted inoculum plus feather meal hydrolyzate in the basal medium was showing consistent and relatively large increases in cellulose digestion from phosphorus additions it was decided to try several other sources of inoculum to determine if the same results could be repeated.

Materials and methods

Inoculum was obtained from three sources other than the Shorthorn which had been used prior to the present time. One source was the Brown

Swiss steer which had been used several times earlier in this study. This steer received a ration consisting of ground corn, soybean oilmeal and good quality alfalfa hay. The other two sources of inoculum were two fistulated Holstein steers which were located at the College Dairy Farm. These steers weighed approximately 800 pounds and were receiving a grain mixture of equal parts of ground corn, ground oats and wheat bran. Their roughage consisted of a rather poor quality mixed hay.

The inoculum was collected in the same manner as described earlier for the Shorthorn steer. Microorganisms from 40 ml. of rumen liquid were collected for each 20 ml. of basal medium. The microorganisms were depleted for 24 hours on the basal medium including feather meal hydrolyzate. The 24 hour depleted material was split in half and made up to the starting nutrient concentration prior to being used as inoculum in the tubes. Phosphorus additions were made directly into the assay tubes.

Results and discussion

Inoculum from the Brown Swiss and Shorthorn steers was collected the same day and the depletion periods and phosphorus assays run simultaneously. Table 31 shows the results of this comparison.

The addition of 100 mcg. phosphorus per ml. medium increased cellulose digestion 67.0 per cent when using inoculum from the Brown Swiss steer and 137.9 per cent when using inoculum from the Shorthorn steer. Progressive increases in cellulose digestion are noted from the addition of 1, 10 and 100 mcg. phosphorus with both sources of inoculum. A depression

Table 31. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when using two different sources of inoculum

Treatment	% cellulose digested	
	Brown Swiss inoculum	Shorthorn inoculum
Basal	27.8	33.4
Basal + 0.3 ml. F.M.H.[a]	25.5	32.2
Basal + 0.3 ml. F.M.H. + 1 mcg. P/ml. medium	29.7	35.6
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	36.0	51.2
Basal + 0.3 ml. F.M.H. + 100 mcg. P/ml. medium	42.6	76.6
Basal + 0.3 ml. F.M.H. + 1000 mcg. P/ml. medium	20.9	61.0

[a]A five per cent solution feather meal hydrolyzate.

is also noted from the addition of 1000 mcg. phosphorus with both sources of inoculum. The microorganisms from the Shorthorn steer appeared to be more sensitive to phosphorus.

Table 32 and Figure 5 show the results from the use of inoculum from the two Holstein steers at the dairy farm and also a repeat trial using inoculum from the Brown Swiss. These three experiments were run on different days.

The use of inoculum from the two Holstein steers gave results which compared very favorably with those of the Shorthorn steer. Figure 5 shows what appears to be straight line responses from the addition of 0, 5, 10, 20 and 40 mcg. phosphorus per ml. medium. The addition of 80 mcg. phosphorus caused a slight decrease in cellulose digestion. The use of inoculum from the Brown Swiss steer did not show a similar picture.

Table 32. Effect of phosphorus upon cellulose digestion by rumen microorganisms *in vitro* when using three different sources of inoculum

Treatment	% cellulose digested		
	I[a]	II[b]	III[c]
Basal	17.7	31.9	23.9
Basal + 0.3 ml. F.M.H.[d]	20.6	39.9	25.4
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	31.4	52.5	49.0
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	36.3	64.2	48.9
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	46.2	73.2	51.0
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	49.8	80.1	46.4
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	49.1	77.5	52.8

[a]Inoculum from Holstein steer No. 1.

[b]Inoculum from Holstein steer No. 2.

[c]Inoculum from Brown Swiss steer.

[d]Five per cent feather meal hydrolyzate solution.

The addition of 5 mcg. phosphorus increased cellulose digestion from 25.4 to 49.0 per cent or an increase of 92.9 per cent; however, the addition of higher increments of phosphorus did not give a straight line response as was observed for the other two steers. Apparently the ration being fed is a factor to consider in experiments of this kind since the Brown Swiss steer was receiving a higher quality ration than the other two steers.

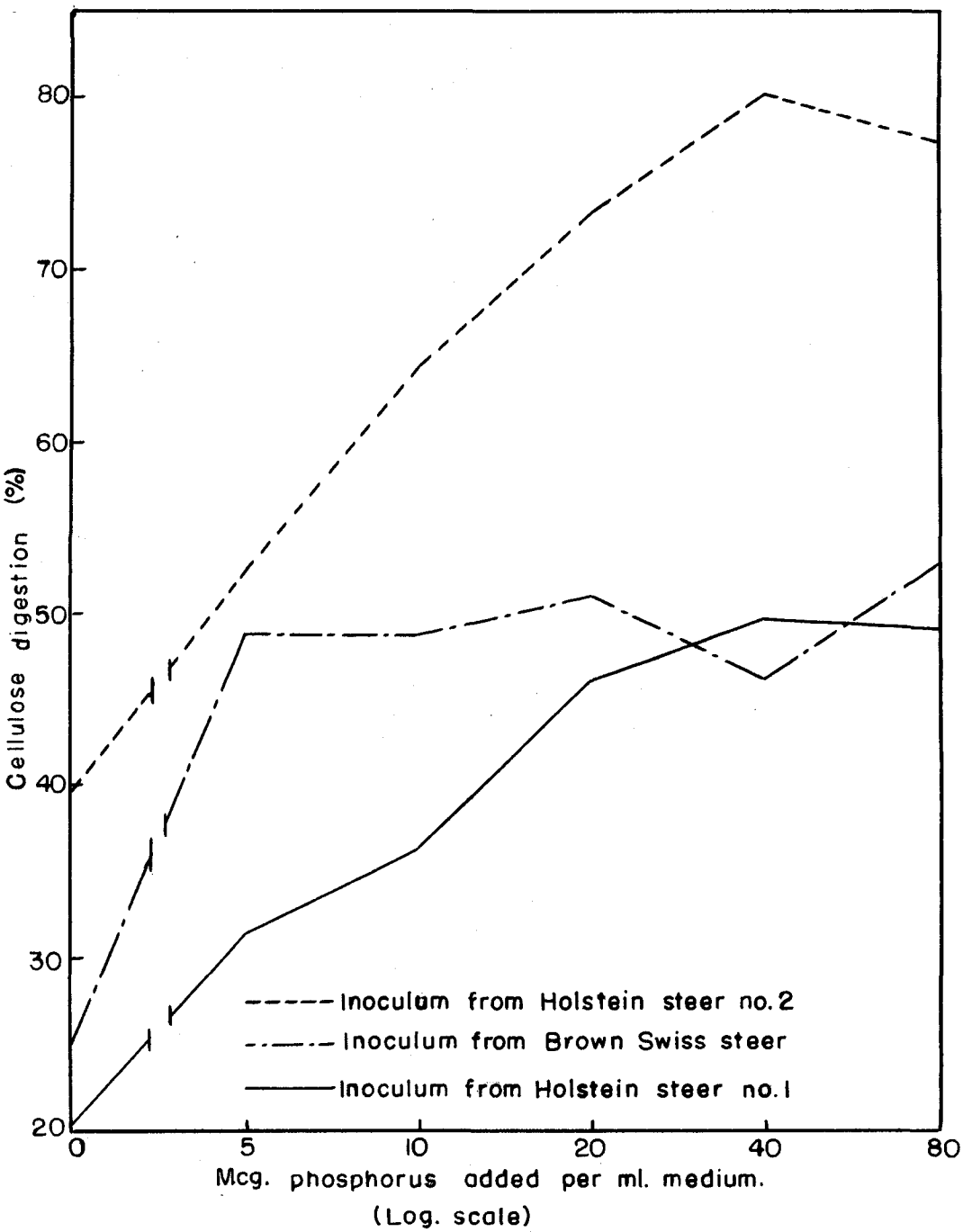

Figure 5. Effect of inoculum from different sources on cellulose digestion

Summary

Inoculum from four different sources was used to determine if similar responses in cellulose digestion would be noted from addition of phosphorus to the basal medium. The four sources of inoculum represented three different rations. The Shorthorn steer received a rather low quality ration, the Brown Swiss a high quality ration, and the two Holstein steers a ration intermediate in quality between the Shorthorn and Brown Swiss.

Similar responses were noted in cellulose digestion from addition of phosphorus when inoculum was from either the Shorthorn steer on the low quality ration or from the two Holstein steers on the medium quality ration. When inoculum from the Brown Swiss was used the response pattern was somewhat different. Approximately the same overall increase in cellulose digestion was noted from the addition of 5 mcg. phosphorus per ml. medium when Brown Swiss inoculum was used as was noted from 40 mcg. phosphorus when the other sources of inoculum were used.

Tentative Technique Proposed for Assay of Phosphorus in Supplemental Feed Sources

Previous experiments indicated that consistent increases in cellulose digestion *in vitro* could be obtained from the addition of phosphorus to a phosphorus deficient basal medium. Certain conditions were necessary, however, before these consistent responses were noted. It is the purpose of this section to describe the tentative technique proposed for assay of phosphorus in supplemental feed sources.

Materials and methods

The technique which finally proved satisfactory after considerable experimentation consisted of measuring cellulose digestion in a series of small fermentation tubes to which graded amounts of a standard phosphorus solution were added at the beginning of a 24 hour fermentation period. In order to obtain the greatest response from phosphorus upon cellulose digestion it was necessary to first carry out a 24 hour preliminary fermentation making use of a washed suspension of rumen microorganisms in a phosphorus deficient medium. This preliminary fermentation was carried out in a large flask in which special precautions were taken to supply all nutrients required by rumen microorganisms, except phosphorus, for efficient cellulose digestion. In addition to supplying the known nutrient requirements of energy, nitrogen and minerals, the technique was greatly facilitated by supplying unknown nutrients in the medium such as specially hydrolyzed casein or feather meal.

In a typical experiment approximately two liters of rumen fluid were first obtained from a fistulated steer by straining rumen ingesta through four layers of number 50 cheesecloth into previously warmed thermos bottles. The strained rumen fluid was next centrifuged in a Servall angle centrifuge at a speed of about 1000 r.p.m. for two minutes. This process sedimented partially digested feed particles and protozoa which were not completely removed by the cheesecloth. The supernatant was next centrifuged in a Sharples centrifuge at a speed of about 25,000 r.p.m. The bacteria in the bowl of the centrifuge with the exception of about one fourth inch at the bottom were collected on a cellophane sheet placed

inside the bowl, removed, and suspended in one liter of distilled water saturated with carbon dioxide gas.

This bacterial suspension was again put through the Sharples centrifuge to sediment the bacteria and get rid of food nutrients that might conceivably be clinging to the surface of bacterial cells. The resulting sediment was added to one liter of phosphorus deficient medium as described in Table 1 along with 5 gms. of finely divided cellulose and 15 ml. of a special five per cent feather meal hydrolyzate or vitamin-free casein hydrolyzate.

The preliminary 24 hour fermentation of the suspension was carried out in a 2-liter Erlenmeyer flask suspended in a water bath maintained at 39° C. This phosphorus-depletion fermentation was carried out under anaerobic conditions maintained by passing a constant stream of carbon dioxide gas through the suspension.

At the end of the 24 hour period, half of the contents of the flask were discarded and the flask made up to one liter by adding phosphorus deficient medium and approximately 5 gms. of cellulose. Aliquots of 20 ml. of the suspension were pipetted into 75 ml. centrifuge tubes which also served as fermentation tubes. To each tube was added 0.3 ml. of either feather meal or casein hydrolyzate plus the concentration of the standard phosphorus which was wanted. The standard phosphorus was made up into different dilutions containing several levels of inorganic phosphorus per ml. One ml. of each solution was added to respective tubes and all determinations were made in triplicate. The standard phosphorus solution used consisted of two parts disodium hydrogen phosphate, Na_2HPO_4, and one part potassium acid phosphate, KH_2PO_4.

The fermentation tubes were each fitted with a stopper with inlet and outlet glass tubings for bubbling a slow constant flow of carbon dioxide gas for purposes of agitation and maintaining anaerobic conditions. The tubes were fermented at 39° C. in a water bath. At the end of 24 hours fermentation, cellulose digestion was determined on the entire tube contents as previously described by Cheng et al. (9).

The vitamin-free casein and feather meal hydrolyzates were prepared as described earlier.

Results and discussion

The technique employed produced an approximate linear relationship within limits, between the amount of phosphorus added to the deficient medium and the amount of cellulose digested by rumen microorganisms. This relationship could be regularly produced with inoculum obtained from different steers and from steers fed different rations.

The slopes of the lines obtained were noted to vary somewhat from one determination to another. This is to be expected since the starting inoculum varies somewhat as to numbers and kinds of organisms present from day to day even if approximate standardized conditions are employed in sampling rumen ingesta. Since this situation exists it is necessary therefore to run a standard response curve at the same time a given supplement is being tested.

Summary

An in vitro fermentation with rumen microorganisms is described for

measuring phosphorus availability using a standard phosphorus source. Cellulose digestion in a series of fermentation tubes was related to graded amounts of standard phosphorus added at the beginning of a 24 hour fermentation period. A preliminary fermentation making use of a washed suspension of rumen microorganisms in a phosphorus-deficient media greatly facilitated the assay fermentation. Also, the use of specially hydrolyzed casein or feather meal added to the media proved helpful in obtaining the greatest response of phosphorus additions upon cellulose digestion.

Assay on the Availability of Phosphorus from Feed Supplements

The purpose of this last phase of the study was to assay the availability of phosphorus from various feed supplements commonly used in ruminant rations by the in vitro technique developed for this purpose.

Materials and methods

The artificial rumen technique employed was as described in the preceding section. All determinations were made in duplicate. The source of inoculum was the Shorthorn steer described earlier receiving the high corn cob ration.

Five sources of phosphorus were tested. These supplements were composite dicalcium phosphate, an acidulated product of phosphate, steamed bone meal, Curacao rock phosphate and soft phosphate with colloidal clay. These supplements were supplied by the National Mineral Feeds Association and are believed to be representative of the phosphorus feeding supple-

ments currently being marketed in the United States. Table 33 shows the phosphorus content of the various supplements tested. Samples of the above listed phosphorus supplements were ground into as fine particles as possible using a mortar and pestle and were either suspended into water solutions or weighed directly into the fermentation tubes. At the same time a series of tubes were set up using the standard phosphorus solution for the purpose of obtaining a standard response curve. The levels of phosphorus used were calculated in terms of micrograms of phosphorus per ml. of medium and were added in logarithmic amounts.

Table 33. Phosphorus content of supplements tested

Supplement	% phosphorus
Composite dicalcium phosphate	18.5
Acidulated product	20.0
Steamed bone meal	12.5
Curacao rock phosphate	14.0
Soft phosphate with colloidal clay	9.0

Results and discussion

Figures 6 through 10 graphically illustrate the effect of the various test compounds upon cellulose digestion as compared with their respective standards. The data from which these curves were plotted may be found in the Appendix, Tables 36 through 40. The points plotted for dicalcium phosphate, the acidulated product, steamed bone meal and Curacao rock

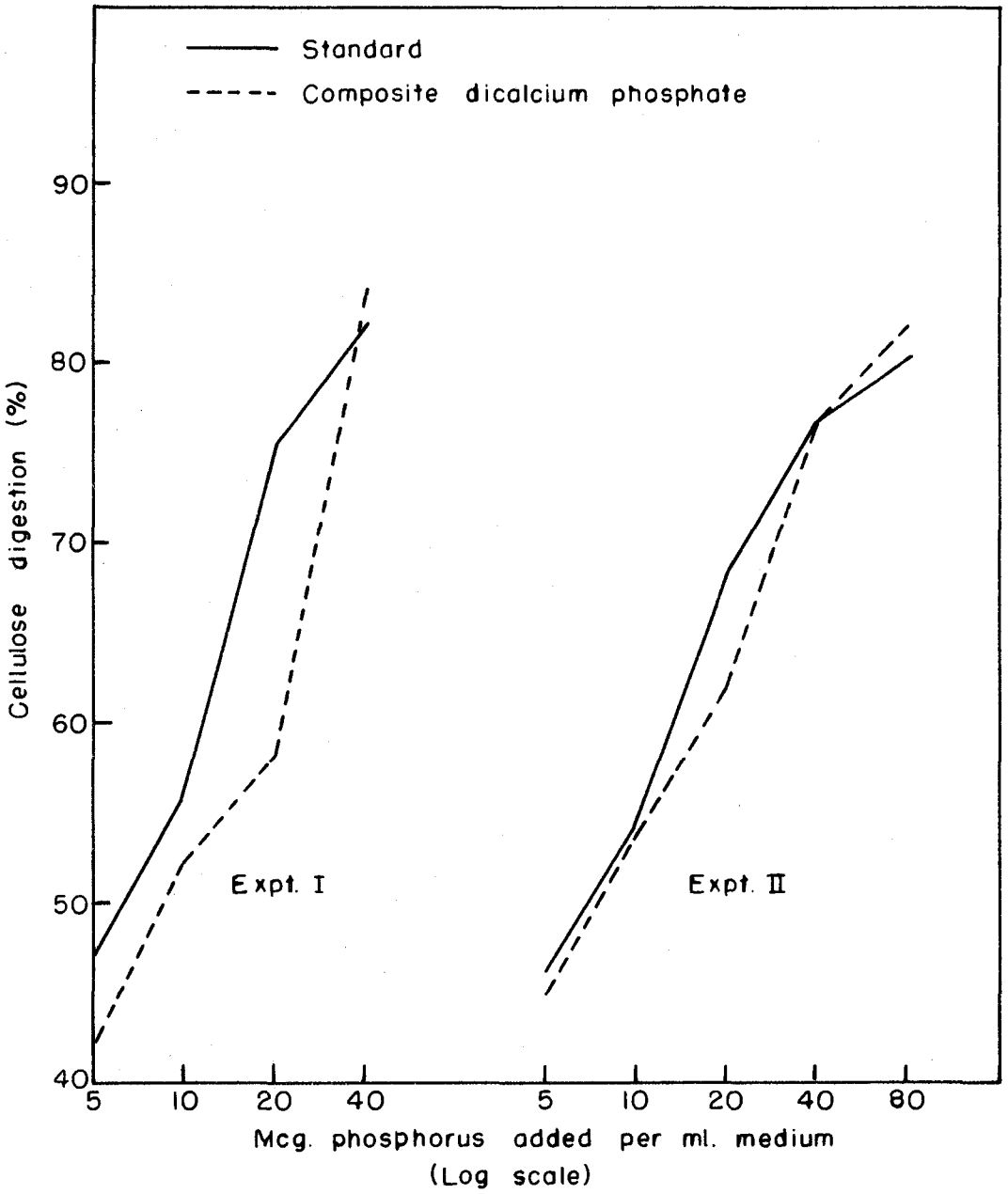

Figure 6. Coefficients of digestion for cellulose in the presence of composite dicalcium phosphate

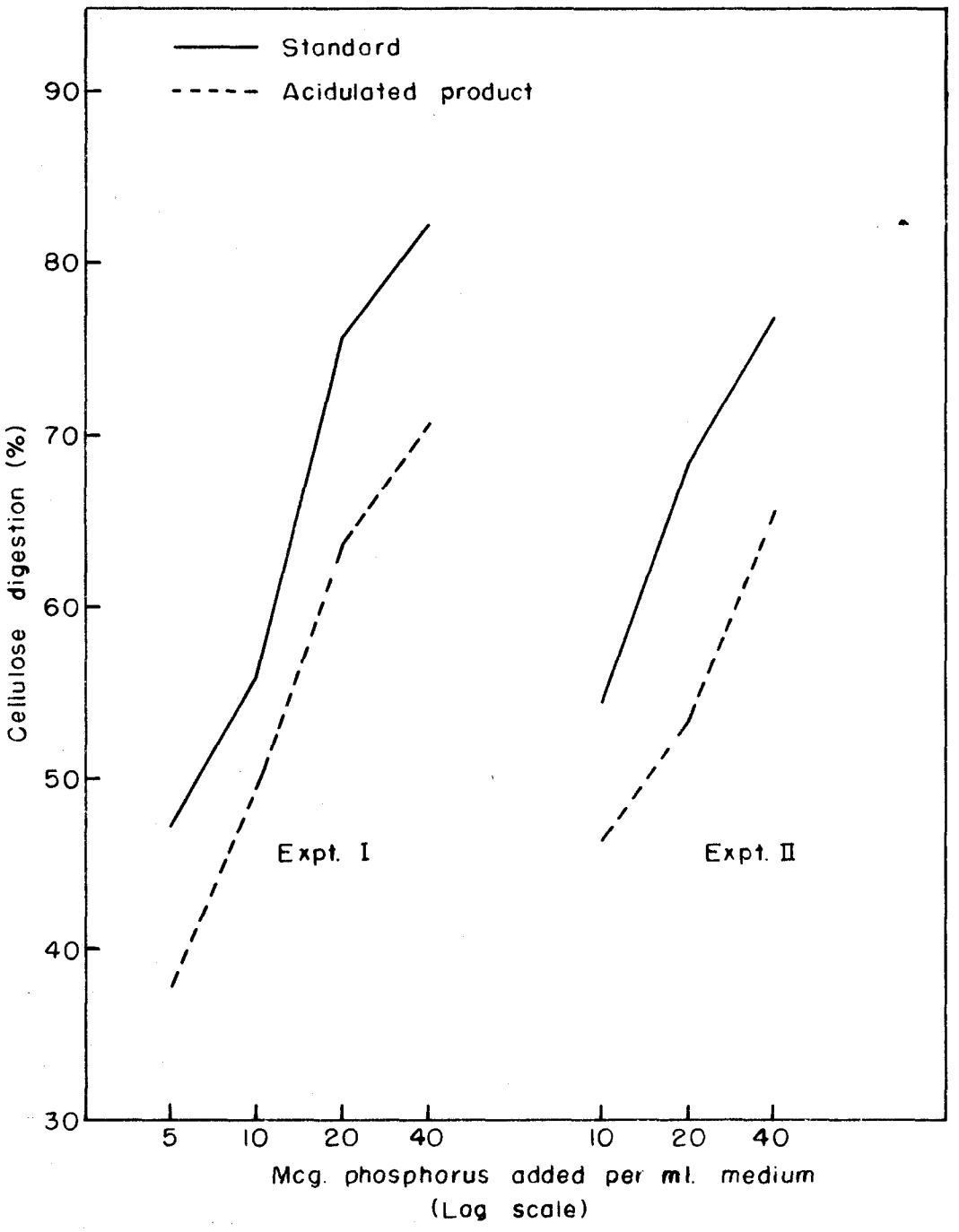

Figure 7. Coefficients of digestion for cellulose in the presence of the acidulated product

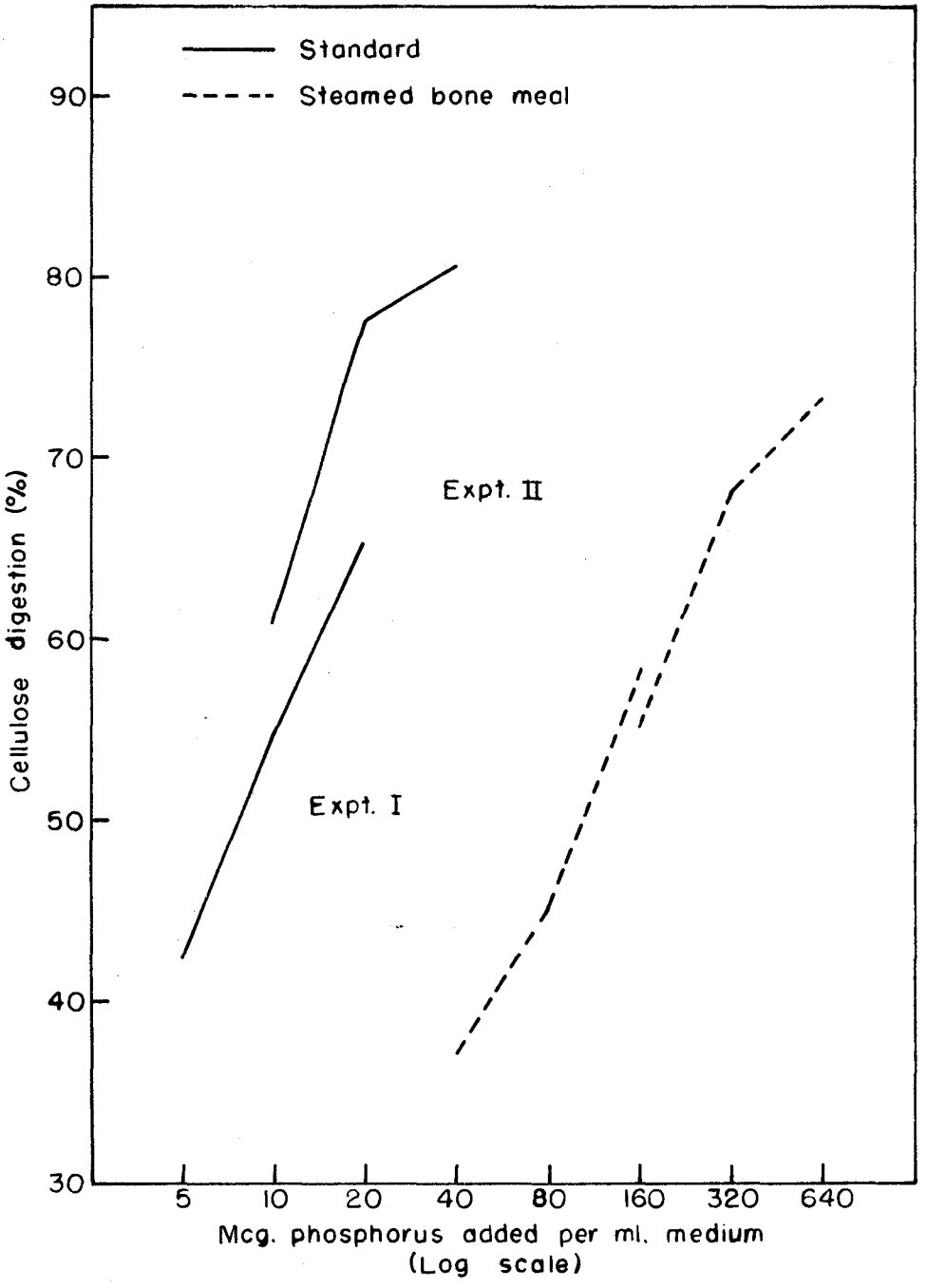

Figure 8. Coefficients of digestion for cellulose in the presence of steamed bone meal

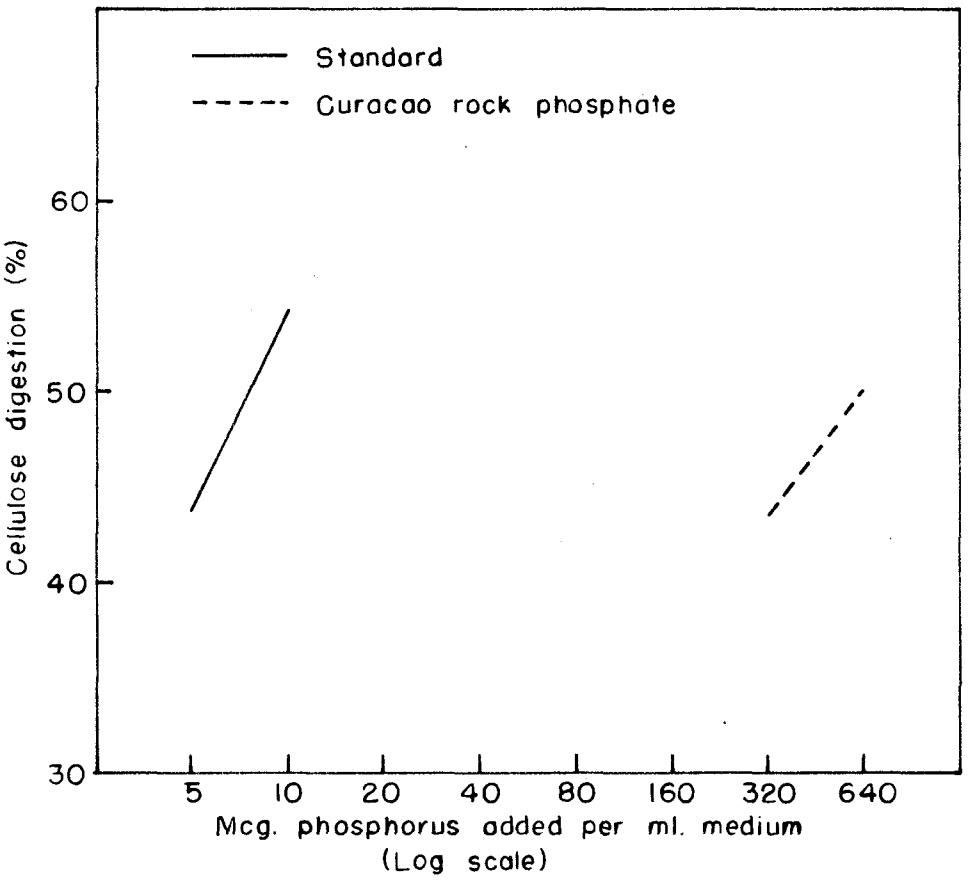

Figure 9. Coefficients of digestion for cellulose in the presence of Curacao rock phosphate

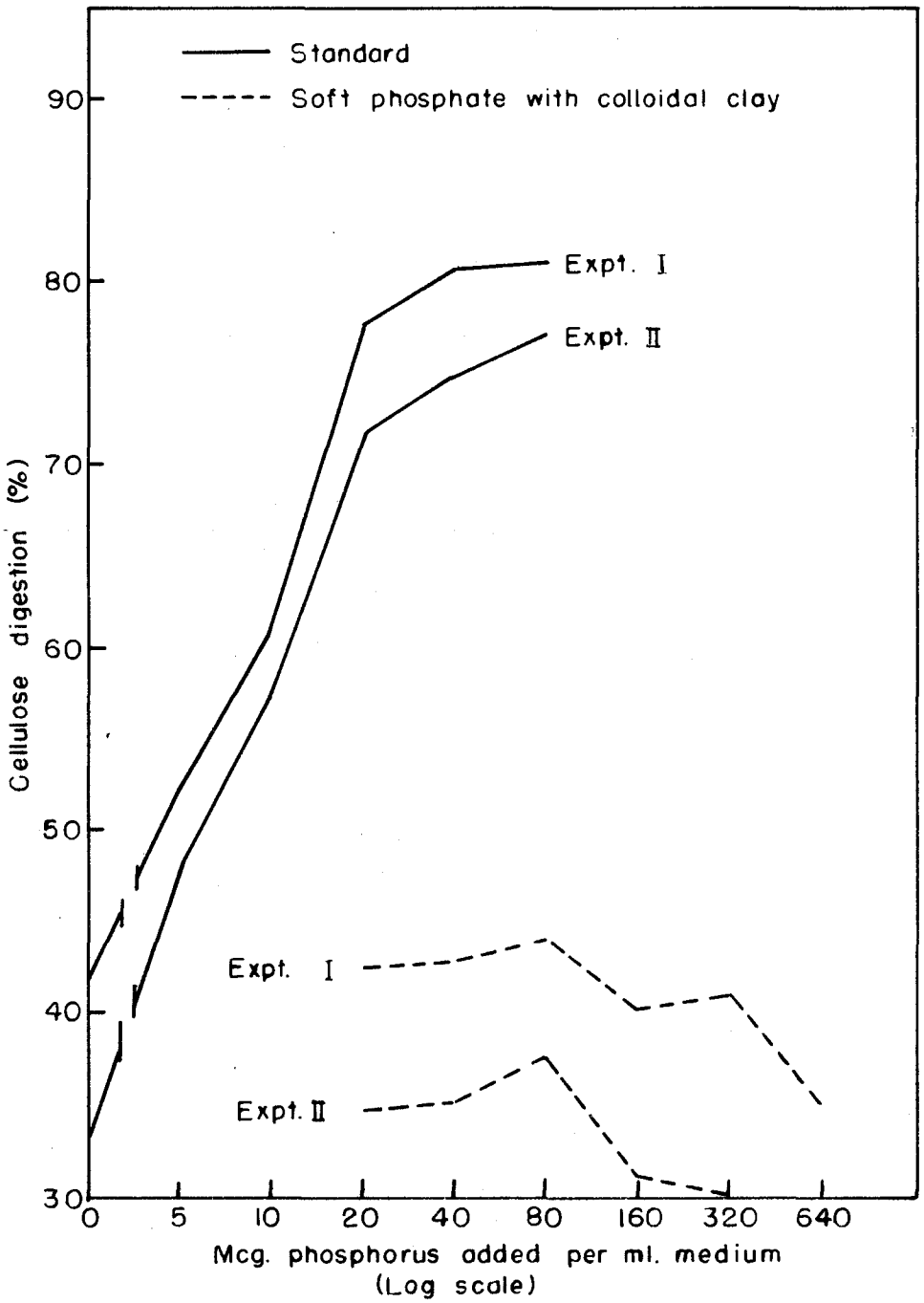

Figure 10. Coefficients of digestion for cellulose in the presence of soft phosphate with colloidal clay

phosphate are those actually used in statistical analysis of the data. Cellulose digestion coefficients resulting from the use of all levels of soft phosphate with colloidal clay have been plotted since a response parallel to the standard was not obtained and statistical analysis therefore not possible.

Figure 11 graphically summarizes the effects of the various test compounds. All logarithmic levels used are presented and the plotted values are average values where more than one experiment was conducted.

The relative efficiency of different phosphorus sources in aiding in cellulose digestion as measured in the artificial rumen was determined through the medium of the parallel line assay technique given in sections 4.11 and 41.2 of "Statistical Method of Biology Assay," 1952, by D. J. Finney (12). Preliminary trials showed that when the standard phosphorus supplement was added to the rumen medium in increasing amounts so that phosphorus was assumed to be added at equal intervals on the logarithmic scale, the increase in digestibility of cellulose was essentially linear. Further, when different phosphorus carriers were compared, the digestibilities of cellulose gave essentially parallel responses.

The necessary tests of statistical validity of parallel line assays were made for dicalcium phosphate, the acidulated product, steamed bone meal and Curacao rock phosphate. Since a parallel line response was not obtained from the use of soft phosphate with colloidal clay statistical analysis was not possible. The results of these tests are summarized in Table 34.

The estimate of the relative effectiveness of the several sources with respect to the standard phosphorus was determined as the antilog of

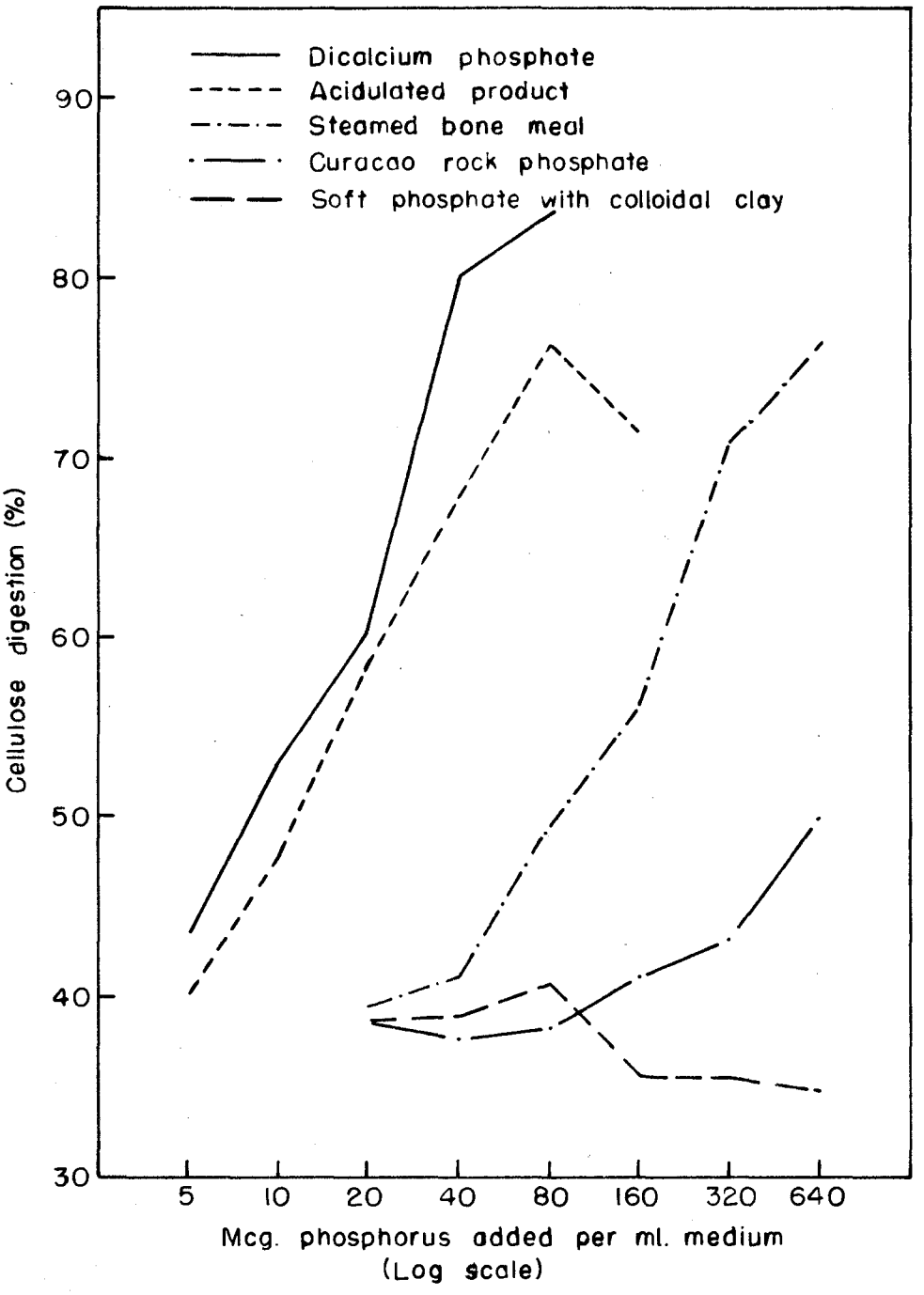

Figure 11. Summary of digestion coefficients for cellulose in the presence of various test compounds

Table 34. Summaries of mean squares for coefficients of digestion for cellulose when different materials were tested against the standard material

Sources of variation	Test substances													
	Dicalcium phosphate				Acidulated product				Steamed bone meal				Curacao rock phosphate	
	I[a]		II[b]		I		II		I		II		I	
	D.F.[c]	M.S.[d]	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
Levels of phosphorus	1	525.34	9	405.54	7	739.61	5	259.64	5	227.40	5	195.26	3	53.58
Materials	1	144.00	1	618.27	1	373.45	1	388.74	1	154.08	1	171.76	1	10.12
Slope	1	3294.74	1	3543.80	1	2823.80	1	882.00	1	970.20	1	720.10	1	144.50
Parallelism	1	2.88	1	4.29	1	6.55	1	6.12	1	1.53	1	0.91	1	6.12
Remainder	4		6		4		2		2		2			
Between tubes within levels	8	16.82	10	16.05	8	20.64	6	5.06	6	9.16	6	9.16	4	.71
Total	15		19		15		11		11		11		7	

[a]Assay No. I.

[b]Assay No. II.

[c]Degrees of freedom.

[d]Mean squares.

the horizontal distance between the parallel lines of response shown in the example graph, dose-response diagram, in Figure 12.

The horizontal distance between the two response lines, M, was determined from the equation:

$$M = \frac{\bar{Y}u - \bar{Y}s}{b}$$

where $\bar{Y}u$ = the average of the digestion coefficients for all observations for the unknown or test material.

$\bar{Y}s$ = the average of the digestion coefficients for all observations obtained for the standard material.

b = the combined regression coefficient for the test and standard response lines.

The limits of M were determined as

$$M \pm \frac{ts}{b}\left(\frac{1}{Ns} + \frac{1}{Nt} + \frac{M^2}{S_x^2}\right)^{1/2}$$

where t = student's t.

s = the experimental error standard deviation.

b = the combined regression coefficient.

Ns = the total number of tubes for standard.

Nt = the total number of tubes for test material.

S_x^2 = the experimental error sum of squares for level of phosphorus.

Table 35 summarizes and shows the relative efficiency of four phosphorus sources as compared with the standard or control. Dicalcium phosphate proved to be the most effective phosphorus carrier tested followed by the acidulated product, steamed bone meal and Curacao rock phosphate. Colloidal clay was noted to have a depressing effect on cellulose digestion

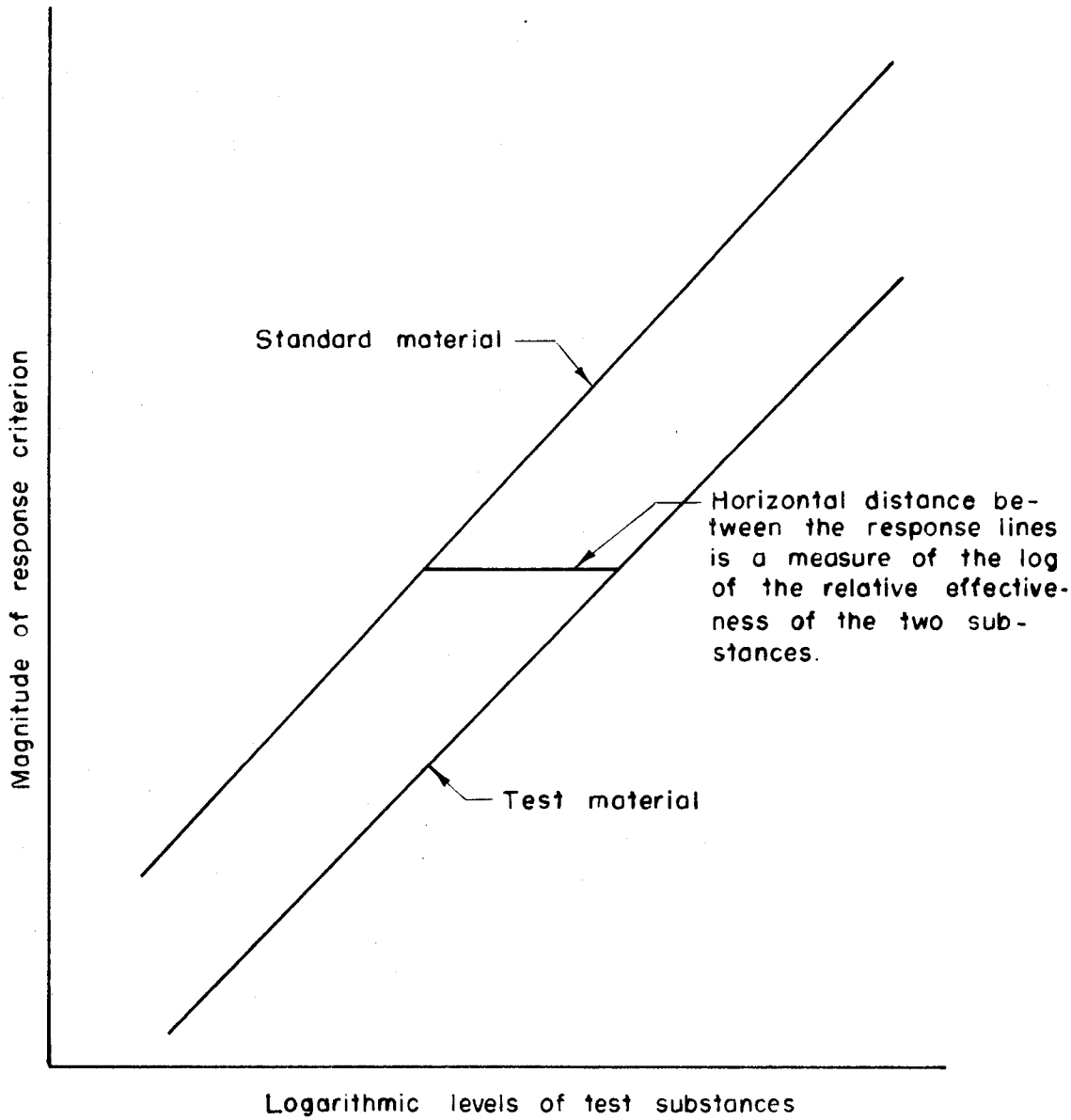

Figure 12. Dose-response diagram

Table 35. Summary of the relative efficiencies of several different phosphorus carriers

Experiment No.	Material	Phosphorus levels, PPM	b	Lambda[a]	Relative effectiveness, %	Limits, % Lower	Limits, % Upper
I	Control	5,10,20,40					
	Dicalium phosphate	5,10,20,40	42.64	0.10	75.1	69.3	79.6
II	Control	5,10,20,40,80					
	Dicalium phosphate	5,10,20,40,80	31.27	0.13	92.5	84.6	101.2
I	Control	5,10,20,40					
	Acidulated product	5,10,20,40	39.48	0.11	58.2	53.2	65.7
II	Control	10,20,40					
	Acidulated product	10,20,40	34.88	0.06	47.1	40.4	54.9
I	Control	5,10,20					
	Steamed bone meal	40,80,160	36.59	0.08	8.0	7.0	9.1
II	Control	10,20,40					
	Steamed bone meal	160,320,640	31.52	0.10	3.6	3.4	3.8
I	Control	5,10					
	Curacao rock phosphate	320,640	120.01	0.007	1.1	1.1	1.1

[a]Lambda = the ratio of the standard deviation divided by the regression coefficient, s/b.

when added in excess of 80 mcg. per ml. medium; possibly this could be due to its fluorine content.

It will be noted from the figures in Table 35 for per cent relative effectiveness that considerable variation exists between experiments involving the same phosphorus carrier. Values calculated for the two experiments with dicalcium phosphate showed it to be 75.1 and 92.5 per cent as effective in promoting cellulose digestion as was the standard. The acidulated product on the basis of two experiments was 47.1 and 58.2 per cent as effective and the steamed bone meal 8.0 and 3.6 per cent as effective as the standard. Curacao rock phosphate on the basis of one experiment was only about one per cent as effective as the standard, whereas in two other experiments a response parallel to the standard was not obtained and statistical analysis not possible. This discrepancy perhaps can be explained in part by the fact that none of the phosphorus carriers tested were soluble in water; therefore, they were added to the fermentation tubes either in the form of a water suspension or weighed directly into the tubes. In either case some error in the amount of phosphorus added may have resulted. Some of this variation could possibly occur during the fermentation period. Since the phosphorus carriers were not water soluble a special effort was made to keep them from settling to the bottom of the tubes by regulating the carbon dioxide flow. However, in spite of efforts to prevent settling it undoubtedly occurred in some tubes. Also, when the flow of carbon dioxide became too great, some sticking of the phosphorus carriers on the upper part of the fermentation tubes may have resulted and consequently was unavailable to the rumen microorganisms.

Summary

Five phosphorus compounds commonly used as supplemental phosphorus sources in livestock rations were tested in an artificial rumen to determine the effect each would have in promoting cellulose digestion. On the basis of a limited number of experiments dicalcium phosphate appeared to be most available followed by the acidulated product, steamed bone meal, Curacao rock phosphate and soft phosphate with colloidal clay. Colloidal clay actually showed a depressing effect upon cellulose digestion when added in excess of 80 mcg. per ml. medium. It is postulated that the fluorine content of the colloidal possibly may be responsible for the depression.

Statistical methods involved in the treatment of the data as well as some possible reasons for differences in response from repeat experiments using the same phosphorus test source likewise are discussed.

GENERAL DISCUSSION

The results of the experiments reported in this thesis show that the rumen microorganisms requirement for phosphorus for maximum cellulose digestion *in vitro* can possibly be used as a criterion in evaluating phosphorus availability in various phosphorus sources used in livestock rations. However, it should be pointed out that certain conditions were necessary before significant responses from phosphorus additions could be observed *in vitro* and that these results might not apply directly to the live animal.

In order to obtain a linear response in cellulose digestion *in vitro* from the addition of various levels of phosphorus to a phosphorus deficient basal medium it was necessary to modify the washed cell suspension technique described by Cheng (9). Two major changes were necessary before consistent and significant responses from phosphorus additions were observed. First, it was necessary to deplete the microorganisms of their phosphorus reserves by incubating them for 24 hours on a phosphorus deficient basal medium and secondly, to supply a source of unidentified factors such as feather meal hydrolyzate or vitamin-free casein hydrolyzate. The possibility exists that rumen microorganisms may undergo some alteration during the depletion period and therefore might not be strictly representative of those found in the rumen. If this occurs then caution must be observed in interpreting results obtained by this artificial rumen technique.

It is interesting, however, to note that the results obtained *in vitro* by this technique parallel quite closely to those obtained by other workers in *in vivo* experiments when using some of the same phosphorus test compounds. Grau and Zweigart (17) found soft phosphate with colloidal clay to be a poorer source of phosphorus than either bone meal or tricalcium phosphate for chicks. Miller and Joukovsky (28) likewise found colloidal clay to be a very poor source of phosphorus. They did, however, find Curacao Island phosphate to be a good source for growing chicks. Johnson *et al.* (21) reported a similar growth response from a 2 per cent level of steamed bone meal as was observed with 4 per cent colloidal clay. Gillis *et al.* (15) found dicalcium phosphate and steamed bone meal to have excellent availability, Curacao Island phosphate satisfactory availability, and colloidal phosphate to have poor availability for chicks. Titus *et al.* (32) found dicalcium phosphate to have excellent availability for young growing chickens. Wilcox *et al.* (34) in a poult study found colloidal phosphate to have unsatisfactory availability. Steamed bone meal and several dicalcium phosphates were highly available.

In swine experiments Shrewsbury and Vestal (31) found steamed bone meal superior to rock phosphate in rations for growing pigs but in rations for bred sows it was only slightly superior to rock phosphate. Gobble and Miller (14) found colloidal clay a poorer phosphorus source for growing and fattening pigs than dicalcium phosphate. Chapman *et al.* (8) likewise found colloidal clay to be a poorer source of phosphorus than either steamed bone meal or dicalcium phosphate for growing pigs. Plumlee and associates (29) also found soft phosphate with colloidal clay to be

a poor source of phosphorus for weanling pigs. Combs et al. (10) in a baby pig study observed colloidal phosphate to be highly unavailable and the phosphorus in steamed bone meal to be less available than that in monocalcium phosphate.

Ammerman et al. (1) in a study involving weanling lambs noted higher phosphorus blood serum levels in lambs receiving dicalcium and Curacao Island phosphate than in those receiving colloidal clay and defluorinated rock phosphate. Long et al. (22) noted dicalcium phosphate to be superior to colloidal clay for beef heifers.

In all of the in vivo experiments cited dicalcium phosphate has been noted to have excellent availability and colloidal clay very poor availability. This compares very favorably with the in vitro results with rumen microorganisms using cellulose digestion as the criteria of response. At the present time at this laboratory an in vivo study with steers is being carried on to compare with the in vitro results noted. Preliminary results indicate the response from dicalcium phosphate and colloidal clay to parallel quite closely to the in vitro results with rumen microorganisms obtained in this study.

Some variation is noted in the present in vitro technique with respect to the relative per cent effectiveness of the various phosphorus sources where experiments were conducted on different days using the same phosphorus source. It would appear that some further work may refine the technique in eliminating some of this variation. Perhaps finding a way to get the phosphorus sources into solution would be helpful.

At present it would seem that this in vitro technique for evaluating

phosphorus availability could best be used as a screening device to separate those compounds which show promise in ruminant feeding from those that do not. While results of *in vitro* studies with rumen microorganisms will need to be verified by more live animal experimentation this method can save considerable time and money if utilized as a screening tool.

SUMMARY

A series of artificial rumen experiments were conducted in an attempt to develop an *in vitro* technique which would be satisfactory as an assay procedure in evaluating phosphorus availability in ruminant feeds. Variables studied were the number of rumen microorganisms used as inoculum, washing of the rumen microorganisms, length of the fermentation periods, types and sources of inoculum, phosphorus depleted versus non-depleted inoculum and the addition of factors to the basal medium known to be stimulatory to rumen microorganisms for the digestion of cellulose.

The technique which finally proved satisfactory consisted of measuring cellulose digestion in a series of small fermentation tubes to which graded amounts of phosphorus were added at the beginning of a 24 hour fermentation period. Phosphorus depleted inoculum was used and was obtained by incubating the microorganisms for 24 hours on a phosphorus deficient basal medium. Feather meal hydrolyzate was added to the basal medium during the depletion period in the flask and also during the assay period in the tubes. This technique produced an approximate linear relationship, within limits, between the amounts of phosphorus added to the deficient medium and the amounts of cellulose digested by the rumen microorganisms.

The technique developed can be briefly summarized as follows: in a typical experiment rumen fluid was first obtained from a fistulated

steer by straining rumen ingesta through four layers of number 50 cheesecloth into previously warmed thermos bottles. The strained rumen fluid was next centrifuged at a speed of about 1000 r.p.m. for two minutes. This process sedimented partially digested feed particles and protozoa which were not completely removed by the cheesecloth. The supernatant was next centrifuged in a Sharples centrifuge at a speed of 25,000 r.p.m. The bacteria in the bowl of the centrifuge with the exception of about one fourth inch at the bottom were collected and suspended in 1 liter of distilled water saturated with carbon dioxide gas.

This bacterial suspension was again put through the Sharples centrifuge to sediment the bacteria and remove food nutrients that might be clinging to the surface of bacterial cells. The resulting sediment was added to 1 liter of phosphorus deficient medium along with 5 grams of finely divided cellulose and 15 ml. of a special five per cent hydrolyzate of feather meal or vitamin-free casein. The preliminary 24 hour fermentation of this suspension was carried out in a 2 liter Erlenmeyer flask which was immersed in a water bath maintained at 39° C. This phosphorus-depletion fermentation was carried out under anaerobic conditions maintained by passing a constant stream of carbon dioxide gas through the suspension.

At the end of the 24 hour period half of the contents of the flask was discarded and the flask made up to 1 liter by adding phosphorus deficient medium and 5 grams of cellulose. Aliquots of 20 ml. of the suspension were pipetted into 75 ml. centrifuge tubes which also served as fermentation tubes. To each tube was added 0.3 ml. of hydrolyzate of feather meal or casein. A standard curve was obtained by adding graded

amounts of the standard phosphorus solution to the tubes. Graded levels of phosphorus feeding supplements were added to other series of tubes in determining phosphorus availability. These tubes were each fitted with a stopper with inlet and outlet glass tubing for bubbling a slow constant flow of carbon dioxide gas for purposes of agitation and maintaining anaerobic conditions. The tubes were fermented at 39° C. in a water bath and at the end of the 24 hour fermentation, cellulose digestion was determined on the entire tube contents.

The availability of phosphorus in five phosphorus supplements was determined by the above procedure. These supplements were composite dicalcium phosphate, an acidulated product of phosphate, steamed bone meal, Curacao rock phosphate and soft phosphate with colloidal clay. Dicalcium phosphate appeared to be the most available source of phosphorus of the five compounds tested in promoting cellulose digestion in vitro by rumen microorganisms followed by the acidulated product, steamed bone meal, Curacao rock phosphate and soft phosphate with colloidal clay. The merits of this laboratory technique in measuring phosphorus availability were discussed with respect to their transposition to feeding practice with cattle and sheep.

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APPENDIX

Table 36. Assay results of composite dicalcium phosphate

Treatment	% cellulose digested	
Standard	I[a]	II[b]
Basal	37.2	32.1
Basal + 0.3 ml. F.M.H.[c]	36.7	34.0
Basal + 0.3 ml. F.M H. + 5 mcg. P/ml. medium	47.2	46.2
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	55.7	54.2
Basal + 0.3 ml. F.M H. + 20 mcg. P/ml. medium	75.5	68.5
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	82.1	76.9
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	85.2	80.2
Composite dicalcium phosphate		
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	42.0	44.9
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	52.3	53.8
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	58.4	62.3
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	84.0	76.9
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	85.1	82.1

[a]Experiment I - 7-23-55.

[b]Experiment II - 8- 3-55.

[c]Five per cent feather meal hydrolyzate solution.

Table 37. Assay results of the acidulated product

Treatment	% cellulose digested	
	I[a]	II[b]
Standard		
Basal	37.2	32.1
Basal + 0.3 ml. F.M.H.[c]	36.7	34.0
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	47.2	46.2
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	55.7	54.2
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	75.5	68.5
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	82.1	76.9
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	85.2	80.2
Acidulated product		
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	37.7	42.5
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	49.8	46.4
Basal + 0.3 ml. F.M H. + 20 mcg. P/ml. medium	63.7	53.3
Basal + 0.3 ml. F.M H. + 40 mcg. P/ml. medium	70.7	65.7
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	84.0	68.8
Basal + 0.3 ml. F.M.H. + 160 mcg. P/ml. medium	----	71.5

[a]Experiment I - 7-23-55.

[b]Experiment II - 8- 3-55.

[c]Five per cent feather meal hydrolyzate solution.

Table 38. Assay results of steamed bone meal

Treatment	% cellulose digested	
	I[a]	II[b]
Standard		
Basal	31.2	43.8
Basal + 0.3 ml. F.M.H.[c]	29.7	41.9
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	42.2	52.5
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	54.6	60.9
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	65.1	77.6
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	65.8	80.6
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	67.2	81.0
Steamed bone meal		
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	34.7	44.4
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	37.1	45.6
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	45.0	54.2
Basal + 0.3 ml. F.M.H. + 160 mcg. P/ml. medium	58.3	55.0
Basal + 0.3 ml. F.M.H. + 320 mcg. P/ml. medium	73.6	68.1
Basal + 0.3 ml. F.M.H. + 640 mcg. P/ml. medium	80.0	73.3

[a]Experiment I - 7-15-55.

[b]Experiment II - 7-19-55.

[c]Five per cent feather meal hydrolyzate solution.

Table 39. Assay results of Curacao rock phosphate, 7-11-55

Treatment	% cellulose digested
Standard	
Basal	32.6
Basal + 0.3 ml. F.M.H.[a]	33.6
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	43.7
Basal + 0.3 ml. F.M H. + 10 mcg. P/ml. medium	54.0
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	62.8
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	61.4
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	62.1
Curacao rock phosphate	
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	38.6
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	37.6
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	38.4
Basal + 0.3 ml. F.M H. + 160 mcg. P/ml. medium	41.1
Basal + 0.3 ml. F.M.H. + 320 mcg. P/ml. medium	43.2
Basal + 0.3 ml. F.M.H. + 640 mcg. P/ml. medium	50.0

[a]Five per cent feather meal hydrolyzate solution.

Table 40. Assay results of soft phosphate with colloidal clay

Treatment	% cellulose digested	
Standard	I[a]	II[b]
Basal	43.8	34.5
Basal + 0.3 ml. F.M.H.[c]	41.9	33.5
Basal + 0.3 ml. F.M.H. + 5 mcg. P/ml. medium	52.5	48.2
Basal + 0.3 ml. F.M.H. + 10 mcg. P/ml. medium	60.9	57.4
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	77.6	71.7
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	80.6	74.7
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	81.0	77.1
Soft phosphate with colloidal clay		
Basal + 0.3 ml. F.M.H. + 20 mcg. P/ml. medium	42.5	34.7
Basal + 0.3 ml. F.M.H. + 40 mcg. P/ml. medium	42.9	35.2
Basal + 0.3 ml. F.M.H. + 80 mcg. P/ml. medium	44.1	37.7
Basal + 0.3 ml. F.M H. + 160 mcg. P/ml. medium	40.3	31.0
Basal + 0.3 ml. F.M.H. + 320 mcg. P/ml. medium	41.0	30.1
Basal + 0.3 ml. F.M.H. + 640 mcg. P/ml. medium	34.9	----

[a]Experiment I - 7-19-55.

[b]Experiment II - 8- 8-55.

[c]Five per cent feather meal hydrolyzate solution.